SOME DEVELOPMENTAL ASPECTS OF GLUCOSE AND VOLATILE FATTY ACID METABOLISM IN THE CONVENTIONAL AND GERMFREE RUMINANT

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KIM H. PONTO

ABSTRACT

SOME DEVELOPMENTAL ASPECTS OF GLUCOSE AND VOLATILE FATTY ACID METABOLISM IN THE CONVENTIONAL AND GERMFREE RUMINANT

By

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Plasma glucose levels and glucose and acetate half-lives, hepatic glutamic-pyruvate transaminase, glucose-6-phosphatase, pyruvate kinase and glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities plus hepatic concentrations of glucose-6-phosphate and phosphoenolpyruvate were investigated in conventional and germfree pygmy goats fed either milk, milk plus glucose or milk plus volatile fatty acids. A number of conventional animals fed a pelleted grain ration served as controls. Plasma glucose concentrations decreased from 98.4 to 71.6 mg/100 ml, glucose half-lives increased from 16.8 to 30.3 minutes and acetate half-lives increased from 2.58 to 4.50 minutes between the ages of 60 and 180 days. In all cases, differences due to age were highly significant. In general, the activities of glutamic-pyruvic transaminase and glucose-6-phosphatase increased with age while pyruvate kinase activity was quite variable. A correlation coefficient of -0.70 ($P < 0.05$) was obtained for the relationship between pyruvate kinase activity and glucose half-life. Relationships between hepatic concentrations of glucose-6-phosphate and phosphoenolpyruvate and overall patterns of metabolic activity were inconclusive. Effects due to dietary treatment and germfree versus conventional status of the animal were not observed.

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This manuscript is dedicated
to the memory of my father
Hilton A. Ponto

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I INTRODUCTION

At different stages from fetal to adult life, the ruminant utilizes various substrates for energy metabolism. Carbohydrate for the developing fetus is provided by placental transfer of glucose. At birth, this energy source is replaced by a milk diet which supplies both carbohydrate and lipid. In the ruminant, rumen fermentation begins at weaning and provides volatile fatty acids (VFA) as the major energy source. As the ruminant adapts first from high carbohydrate utilization prenatally to a high carbohydrate and lipid diet postnatally and finally to high VFA utilization at the adult stage, there are many metabolic changes which must take place.

Adult patterns of VFA metabolism in the ruminant have been investigated to a much greater extent than the metabolism of carbohydrate by the prenatal and postnatal animal. Because the carbohydrate metabolism of the young ruminant resembles that of the non-ruminant animal (Ballard *et al.*, 1969) and then adapts to the metabolism typical of the adult ruminant, enzyme activities and metabolite concentrations in the tissues of the ruminant must be altered with successive changes of the substrates for energy metabolism. Metabolic activity may be altered by substrate composition and availability or age dependent, constitutive changes in tissue enzyme levels.

Most research published to date would support the view, that changes in enzyme activities are substrate dependent whereas this conclusion has been questioned by Purser and Bergen (1969). This study was initiated to determine whether changes in ruminant metabolism are age or substrate dependent. Germfree goats, which should retain a carbohydrate (glucose) dependent energy metabolism because of the absence of ruminal microbiota, were used to test the above hypotheses. To this end, metabolic changes, as monitored by glucose and acetate tolerance tests, metabolic intermediates, and key enzyme activities were studied employing germfree and conventional animals.

II REVIEW OF LITERATURE

A. Glycolytic and Gluconeogenic Enzyme Alterations During Development

1. Patterns of glycolysis

Hepatic glucose phosphorylating activity has been found to be high in adult guinea pigs, rats and rabbits but marginal in fetal and newborn livers of these same animals (Walker, 1962). Walker (1965) showed that glucokinase [E.C.2.7.1.2] activity was absent in the fetal liver of the guinea pig but activity developed immediately after birth. Hepatic glucokinase activity in the rat however, appeared only after 16 days of life and reached adult levels 10-12 days later (Ballard and Oliver, 1964a). This increase in rat glucokinase activity was accounted for by de novo synthesis of the enzyme (Walker, 1965).

Ballard and Oliver (1964b) showed that the activity of the high K_m, insulin dependent hexokinase (glucokinase) was absent in fetal and adult sheep liver. It has also been shown that the activity of the low K_m, insulin independent hexokinase [E.C.2.7.1.1] was much lower in the liver of postnatal sheep than in rat liver (Ballard and Oliver, 1965). These authors concluded that negligible glucokinase activity was a metabolic consequence of maternal and placental regulation of fetal glucose concentrations and that lower hexokinase activity in the adult ruminant was due to the animal's own gluconeogenic regulatory systems (Ballard _et al_, 1969).

In the liver of the developing rat and sheep, total nicotinamide nucleotide coenzymes increased steadily and reached a maximum when growth ceased (Craiger _et al_., 1962). The ratio of oxidized and reduced forms of nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP),

$$([NAD] + [NADH]) : ([NADP] + [NADPH_2])$$

in the adult liver of the rat and sheep has been determined and a species difference existed. In the young rat and sheep the ratio was 2.4 and 1.6 respectively. At maturity this ratio was 1.3 for the rat and 2.5 for the sheep, demonstrating a relative increase in NADP during the development of the rat and NAD during the development of the sheep. Increased NADP levels may reflect the importance of the oxidative pentose phosphate pathway for reductive synthesis in rat liver, while increased NAD levels in the liver of sheep may reflect the level of acetate oxidation via the tricarboxylic acid cycle. That is, greater emphasis is placed on the oxidative metabolism of acetate by the sheep in comparison to the rat (Craiger _et al_., 1962). Filsell _et al_. (1963) found slowing rates of reduction of NADP in the presence of glucose-6-phosphate, 6-phosphogluconate and glucose plus ATP (adenosine triphosphate) in lamb liver homogenates and concluded that the lower coenzyme ratio observed in the adult sheep was consistant with changing patterns of carbohydrate metabolism with age, i.e., less glycolytic activity in the maturing ruminant.

Other investigators have discussed similar decreases in the glycolytic metabolism of other species. Nemeth and Dickerman (1960) showed decreased glucose-6-phosphate dehydrogenase [E.C.1.1.1.49] levels in the liver of the developing guinea pig and Howarth et al. (1968) found a decreased activity in this same enzyme plus fructose-1, 6-diphosphate aldolase [E.C.4.1.2.6] and glyceraldehyde-3-phosphate dehydrogenase [E.C.1.2.1.12] when hepatic activity of finished steers was compared to the activity exhibited by calves. Goetsch (1966) found a general decrease in the glycolytic enzymes of the adult ruminant and implied that these changes were due to developing rumen function with less carbohydrate (substrate) available to the glycolytic pathway.

2. Patterns of gluconeogenesis and glycogen release from the liver

While developmental changes are occurring in the glycolytic pathway, concurrent changes are also taking place in hepatic enzymes concerned with gluconeogenesis and glycogen release. Shelley (1960 and 1961) determined that in the liver of the fetal sheep, approximately 2 mg of glycogen per gram wet liver weight were laid down daily over the last half of gestation, which resulted in a total store of approximately 90 mg glycogen per gram wet liver weight at birth. This onset of glycogen accumulation late in gestation was also observed in the guinea pig by Nemeth (1954). Immediately after birth, about 90 percent of the liver glycogen stores were mobilized. This rapid decline takes from two to three hours and even occurs in animals which suckled one-half hour after birth (Shelley, 1960). Adult glycogen levels of approximately 40 mg/gm wet liver weight are reached by two to three weeks <u>post</u> <u>partum</u>.

Kornfeld and Brown (1963) examined the enzymes related to glycogen synthesis in the fetal and neonatal guinea pig. The activity of UDP glucose-glycogen-glucosyltransferase [E.C.2.4.1.11] and UDP-glucose pyrophosphorylase [E.C.2.7.7.9] increased from approximately 50 percent of the adult level in the 45 to 60 day old fetus to the full adult level at term. α-glucan phosphorylase [E.C.2.4.1.1] activity increased from 75 percent of the adult level in the 45 to 60 day fetus to twice the adult level at term. Normal activity was reached on the sixth day of life. Glycogen however was only found to increase after the 67th day of the 68 day gestation period. The characteristic decrease in liver glycogen was also noted at birth in the guinea pig (Kornfeld and Brown, 1963). Enzymes involved in the degradation of glycogen appeared late in gestation only after glycogen synthesizing enzymes had been established in the rat liver (Jacquot, 1964). Ballard and Oliver (1965) found that during the development of the sheep, UDP glucose-glycogen-glucosyltransferase, UDP-glucose-pyrophosphorylase and glycogen content were highest in two week old lambs, while considerably lower levels were found in the adult sheep liver.

When compared to the adult rat, the activity of hexokinase and the rate of incorporation of $\left[{}^{14}C\right]$ -glucose into glycogen were much lower in liver of postnatal sheep (Ballard and Oliver, 1965). These workers found that the activity of hexosediphosphatase [E.C.3.1.3.11], glucose-6-phosphatase [E.C.3.1.3.9] and the incorporation rates of $\left[{}^{14}C\right]$ -pyruvate and $\left[{}^{14}C\right]$ -propionate into glycogen increased from low levels in the liver of fetal sheep and were at the highest levels a few weeks after birth. The corresponding rates of these enzymes were slightly lower in adult sheep.

Krebs (1954) first recognized that gluconeogenesis was not a simple reversal of glycolysis but that certain distinct enzymes existed to accomplish the synthesis of glucose from non-hexose precursors. Enzymes which are considered rate-limiting in gluconeogenesis are pyruvate carboxylase [E.C.6.4.1.1], phosphoenolpyruvate carboxykinase [E.C.4.1.1.32], fructose diphosphatase [E.C.3.1.3.11] and glucose-6-phosphatase. In addition for the ruminant, propionyl carboxylase [E.C.6.4.1.3] should also be included as a gluconeogenic control enzyme (Baird _et_ _al_., 1968). During conditions which stimulate gluconeogenesis, such as fasting and diabetes, these enzymes increased in activity and when glucose was available their activities declined (Shargo _et_ _al_., 1963; Weber _et_ _al_., 1964). These key enzymes of gluconeogenesis metabolically oppose the glycolytic enzymes pyruvate kinase [E.C.2.7.1.40] phosphofructokinase [E.C.2.7.1.11] and glucokinase (Krebs, 1963; Nordlie and Lardy, 1963; Seubert and Huth, 1965; and Utter and Keech, 1963).

Ballard and Oliver (1963) showed that in the fetal rat liver, synthesis of phosphoenolpyruvate from malate could not be detected and that $[^{14}C]$ -glucose and $[^{14}C]$ -pyruvate could be gluconeogenic only after the birth of the animal. It was also shown that gluconeogenesis from pyruvate was absent from the liver of fetal rats and appeared first in neonatal rats (Ballard and Oliver, 1965). Vernon *et al.* (1968) using L-lactate, casein hydrolysate, L-serine, L-threonine, L-alanine and glycerol as substrates showed that gluconeogenesis occurred only after birth in rats. Other work with neonatal rat liver (Yeung and Oliver, 1967) has shown that utilization of aspartate, glutamate and alanine for gluconeogenesis was initiated only during post natal life, reaching a maximum at the fifth day. It was further shown that overall gluconeogenic activity followed the activity pattern of aspartate transaminase.

When comparing sheep with rats a different gluconeogeneic pattern is found. Ballard and Oliver (1965) showed that liver of fetal sheep in the fourth month of gestation, converted pyruvate to glucose at 20 times the rate of the liver of a fetal rat in its 19th day of gestation. The rat showed rapid increases in this pathway at birth, while concomitant gluconeogenic increases in the sheep were not as dramatic. Enzymatic work (Ballard and Hanson, 1967) has shown that the activity of pyruvate carboxylase and phosphoenolpyruvate carboxykinase have reached adult levels by the time of birth in the rat. Wagle (1966) compared gluconeogenic activities of the adult rat and sheep by determining the activity of key gluconeogenic enzymes. He showed that the enzymes pyruvate carboxylase, phosphoenolpyruvate carboxykinase, glucose-6-phosphatase and fructose-1, 6-diphosphatase had twice the activity of the same enzyme in rat liver. It was concluded that this greater gluconeogenic activity exhibited by the ruminant was reasonable since the ruminant must synthesize the major portion of its own glucose.

The gluconeogenic enzyme glucose-6-phosphatase has been shown to develop before birth in the guinea pig (Lea and Walker, 1964; Nemeth, 1954) and Dawkins (1961) noted increases in the activity of this enzyme in the liver and kidney of the lamb, rat and guinea pig only 24 hours after birth. Hepatic glucose-6-phosphatase activity was found to be greater in steers than in calves. Calves reached adult enzymatic activity 8-12 weeks _post partum_ (Bartley _et al_., 1966). These metabolic changes in gluconeogenesis coincided with the development of a functional rumen (Bartley _et al_., 1966).

Intracellular distribution of the gluconeogenic enzymes pyruvate carboxylase and phosphoenolpyruvate carboxykinase differs between species. In most animals, pyruvate carboxylase was found to be located within liver mitochondria, while phosphoenolpyruvate carboxykinase occurs in the cytosol of liver cells (Ballard and Hanson, 1967; Freedman and Kohn, 1964; Keech and Utter, 1963). Oxalacetate formed by carboxylation of pyruvate by the mitochondrial pyruvate carboxylase must migrate into the cellular cytosol to be converted to phosphoenolpyruvate and finally glucose. Studies on the rates of gluconeogenesis have shown that oxalacetate cannot pass out of the mitochondria and into the cytosol fast enough to be a main precursor of glucose (Lardy et al., 1965). Thus it has been suggested that oxalacetate must be converted to aspartate and/or malate in the mitochondrion. These metabolites can then pass into the cytosol and be reconverted to oxalacetate, which is then transformed to phosphoenolpyruvate (Haynes, 1965; Shargo and Lardy,1966).

Ballard et al. (1969) however cautioned that the distinction between intra- and extramitochondrial enzymes are not as distinct in the bovine as in the rat. They found pyruvate carboxylase activity in the cytosol and this activity increased in response to physiological stress i.e., lactation, ketosis, and starvation. Phosphoenolpyruvate carboxykinase activity appeared in significant amounts in the mitochrondrion of bovine liver but did not exhibit the same adaptive properties as pyruvate carboxylase (Ballard et al., 1969).

Extramitochondrial pyruvate carboxylase enzyme found in the ruminant may participate in the control of gluconeogenesis from pyruvate. Pyruvate carboxylase has been found to be activated by acetyl CoA and propionyl CoA, respectively. It has more recently been found that purified calf liver pyruvate carboxylase is activated (cooperativity) by butyryl CoA (Ballard *et al.*, 1969), the first product of butyrate metabolism. These volatile fatty acids arising from rumen fermentation can be converted to the corresponding coenzyme A derivatives in the cytosol (Hanson and Ballard, 1967) and activate the extramitochondrial pyruvate carboxylase. Thus at a time when volatile fatty acids are plentiful and can be used for energy purposes, pyruvate and gluconeogenic precursors arising from the rumen are used for gluconeogenesis. As further evidence for this control, Black (1963) has shown *in vivo* that during spontaneous ketosis, when acyl CoA concentrations may be elevated, the pathway for the carboxylation of pyruvate to oxalacetate was favored over decarboxylation of pyruvate to acetyl CoA. Enzymes associated with glycolysis may also be inhibited by increased concentrations of acetyl CoA (Randle *et al.*, 1966).

B. Dietary Glucose and Gluconeogenic Substrates For The Ruminant

Absorption of glucose from the gastrointestinal tract of the ruminant fed high-roughage diets is minimal (Heald, 1951; Annison *et al.*, 1957; Bensadoun *et al.*, 1962; and Roe *et al.*, 1966). Fries and Conner (1960) estimated from portal-carotid blood glucose differences that glucose could provide roughly 10 percent of the available energy for a young bull calf when fed a diet of alfalfa hay and corn.

For diets high in concentrates, investigations have shown that appreciable amounts of starch escape rumen fermentation, reach the small intestine and may be absorbed as glucose. Approximately 331 g of starch disappeared from the small intestine of steers consuming 1002 g of starch from a 20 percent ground corn ration and 624 g of starch disappeared from the small intestine of these same steers consuming 2684 g of starch from an 80 percent corn ration (Karr et al., 1966). Experiments with sheep have provided similar results. About 16 g of starch disappeared posterior to the rumen of sheep fed 172 g of starch from 20 percent ground corn ration while 101 g of starch disappeared in the small intestine of these sheep consuming 576 g of starch from an 80 percent ground corn ration (Tucker et al., 1968). MacRae and Armstrong (1966 and 1969) have reported intermediate values (to those of Tucker et al.) for amounts of α-linked glucose polymer presented to the duodenum when high barley diets were fed to sheep. Thus, when quantatively assessing the requirement of gluconeogenic precursors for the ruminant, account must be taken of diets which are high in grain, since an appreciable amount of glucose is passed to the duodenum for absorption.

Since the extent to which dietary glucose escapes rumen fermentation depends on the diet itself, the diet influences the importance of gluconeogenic precursors required by the ruminant. Under dietary conditions which increase the importance of the gluconeogenic capacity of the ruminant, propionic acid and gluconeogenic amino acids become the important precursors for this process.

Acetic, propionic and butyric acids which are the major end products of rumen fermentation account for 70-80 percent of the ruminant's energy requirements (Bergman _et_ _al_., 1965 and Warner, 1964). Of these three acids, only propionic acid can be gluconeogenic (Annison _et_ _al_., 1963 and Leng and Annison, 1963).

The first direct quantitative measurements of the role of propionate in gluconeogenesis was made by Bergman _et_ _al_. (1966). In sheep fed a maintenance ration of 800 g of alfalfa per day, it was determined by infusion of propionate-2-^{14}C into the rumen vein, that propionate accounted for about 27 percent (range for four animals 24-33) of the glucose turnover and this contribution could be raised to 50 percent by carrier propionate infusion. Metabolized protein accounted for 63 percent of the glucose turnover and the remaining 10 percent probably came from dietary glucose and other carbohydrate precursors i.e., lactate. The contribution of protein for gluconeogenesis was calculated from urinary excretion of nitrogen by methods which strictly apply to the non-ruminant (West and Todd, 1961). Bergman _et_ _al_.(1966) overlooked the fact that urea produced by the liver from rumen ammonia can pass into the urine of the ruminant. Such a large contribution from protein for gluconeogenesis was obviously an overestimate.

Recently Wolff and Bergman (1972) reevaluated the amount of blood glucose derived from plasma amino acids using U -^{14}C - labeled alanine, aspartate, glutamate, glycine and serine. In sheep fed a near-maintenance ration, at least 11 percent of the blood glucose was derived from amino acids. A maximum of 30 percent of the blood glucose could be derived from amino acids based on net hepatic uptake of these amino acids but part of this uptake was undoubtedly diverted into synthesis of plasma proteins and other amino acid metabolism pathways. It was concluded that only between 11 and 30 percent of the blood glucose in the fed sheep was derived from plasma amino acids.

Leng et al. (1967) found by the intraruminal infusion of $\left[^{14}C\right]$-propionate into sheep on a diet of 800 g lucerne per day, that 54 percent of both glucose and lactate arose from propionate carbon. The intraruminal infusion of labelled $\left[^{14}C\right]$-propionate provided evidence that there was appreciable conversion of propionate to lactate at the rumen wall. This phenomenon had not been allowed for by Bergman et al. (1966) because they had measured only the gluconeogenic contribution of absorbed propionate.

Judson et al. (1968) have examined the effect of increased dietary crushed maize on the contribution of propionate for gluconeogenesis. In sheep fed 400 g of crushed maize per day, only 27 percent of the animals' glucose was derived from propionate. They explained their unaltered levels of propionate production from such a ration (compared to all roughage diets) by increased amounts of starch escaping rumen fermentation. They suggested that glucose absorption from the gastrointestinal tract may have reduced gluconeogenesis from propionate.

Other evidence has shown that the gluconeogenic needs of sheep may be entirely met by propionate alone. Armstrong (1965) has calculated that the requirements for energy absorbed as propionate to meet glucose needs for the non-pregnant sheep and pregnant sheep to be 18 and 28 kcal/ $kg^{0.75}$/day. Annison and Lindsay (1962) found that sheep fed a diet of 400 g lucerne and 500 g maize absorbed 64 kcal/ $kg^{0.75}$/day as propionate. If 50 percent of this propionate is converted to glucose, the glucose requirements of these animals would be adequately met.

The once great emphasis placed on the role of amino acids as precursors of the process of gluconeogenesis, a process of more importance to the ruminant than the non-ruminant, has lessened. Purser (1970) pointed out the paradox that the ruminant which apparently is in great need of amino acids for protein synthesis would have at the same time a great requirement for these amino acids as precursors of gluconeogenesis. In the non-pregnant ruminant with only maintenance requirements, gluconeogenesis from amino acids may be minimal but may become increasingly more important to the animal under the stress of growth, pregnancy or lactation.

C. Glucose Tolerance, Blood Glucose Levels And Dietary Effects On These Parameters

Previous work involving the intravenous glucose tolerance test in man showed that blood glucose disappearence followed an exponential decrease with time and this decrease has been mathematically defined. The relationship between log blood glucose increment and time after injection is linear (Greville, 1943; Amatuzio et al., 1953; Duncan, 1956; and Hlad et al., 1956). The constant rate of removal of injected glucose indicated that glucose utilization follows first order kinetics and is a linear function of the log arterial blood glucose concentration. This relationship was found to be constant over a wide range of blood glucose concentrations in the dog (Lang et al., 1954; Soskin and Levine, 1937).

The glucose tolerance test, applied to the adult ruminant, has shown that the rate of removal of injected glucose by ruminant tissues is much slower than that observed in the non-ruminant (McCandless et al., 1948; McCandless and Dye, 1950; Reid, 1952; Jarrett and Potter, 1957; and Reid, 1958). Fasted calves and calves made diabetic either by pancreatectomy or alloxan treatment had lowered glucose tolerance (Cook et al., 1949; and McCandless and Dye, 1949). Six week old calves were found to clear blood glucose as rapidly as the normal dog or human since blood glucose levels of 200 to 300 mg% returned to normal values in one to two hours. This same glucose load required four to six hours to reach normal levels in the adult sheep (McCandless and Dye, 1949; McCandless and Dye, 1950; Jarrett and Potter, 1957). Equine glucose tolerance rates fell between those of the ruminant and non-ruminant animal (Mehring and Tyznik, 1970).

Fasting blood glucose level of the young calf was found to be about 97 mg% or about twice the level of the adult animal (McCandless and Dye, 1950). Young lambs had about the same fasting blood glucose levels as the calf and these levels declined steadily until at five to six weeks of age, when rumination was observed, they fell to 50-65 mg% (McCandless and Dye, 1950). This general trend in decreasing blood glucose has been observed in other ruminants by many workers (Lambert *et al.*, 1955; McCarthy and Kesler, 1956; Wing *et al.*, 1955; Attebery and Colvin, 1963; Hodgson *et al.*, 1932; and Murley *et al.*, 1952).

Factors causing the decreased glucose tolerance and plasma glucose levels have been investigated with conflicting results. McCarthy and Kesler (1956) fed calves either whole milk or a milk replacer during which time hay was fed *ad libitum*. By eight weeks, blood glucose levels fell from 90 mg% to 50 mg% and a concomitant increase in blood VFA due to increased rumen cellulose fermentation was observed. They concluded that the diet caused the decreasing blood glucose levels. Attebery and Colvin (1963) using calves over a 13 week feeding trial period were able to maintain a constant fasting blood glucose level by feeding a diet of milk solids, but the inclusion of grain and hay resulted in gradual decline in fasting blood glucose. The effect of diet on glucose tolerance was determined by Harry et al. (1967) by feeding calves essentially an all milk and milk solid diet or a diet of milk and milk solids plus grain and/or hay. They found that the glucose tolerance, performed after a 12-14 hour fast, was constant for the calves fed the milk diet while decreased glucose tolerance was observed in the treatments which included hay and/or grain. They stated, "extrinsic stimuli many be more important than intrinsic stimuli in the transition of the ruminant energy metabolism from one involving glucose primarily to one which uses predominantly volatile fatty acids." Others (McCandless and Dye, 1950; Hale and King, 1958; and Omari and Nakajima, 1961) have concluded that glucose tolerance is dependent on diet and nature of rumen fermentation.

Reid (1958) also showed that the diet of the mature ruminant had a pronounced effect on glucose tolerance. Adult sheep, fed a diet containing 50 percent cracked maize ad libitum, showed the shortest glucose half-life; those on a restricted maize diet, an intermediate half-life and the longest glucose half-life was recorded for sheep on a restricted roughage diet. Thus he showed that the shortest half-life or fastest rate of glucose utilization occurred on diets which supplied the greatest amount of propionate for gluconeogenesis. This contradicts McCandless and Dye (1950) who postulated that decreased glucose tolerance reflected quantitative differences in metabolism coincident to the fermentation of carbohydrate in the rumen. Low glucose tolerance characteristic of the adult ruminant was believed to be due to increased rates of gluconeogenesis.

Just the opposite dietary effects have also been observed. Murley et al. (1952) and Wing et al. (1955) employing calves and feeding reconstituted skimmed milk diets found that at four days of age fasting blood glucose levels were approximately 85 mg% and fell to approximately 55 mg% by the fifth week. The fasting blood glucose decreased even though composition of the diet was unchanged, i.e. only skimmed milk. Jacobson et al. (1951) compared calves fed milk diets with calves on varying roughage diets and found similar trends in blood glucose levels regardless of treatment. Other workers (Hodgson et al., 1932 and Lambert, 1955) have found that diet has no effect on fasting blood glucose in maturing dairy cattle.

In an interesting study by Reid (1953), the major portion of the decrease in blood glucose during the first four to five weeks of life of the lamb was due to a decrease in corpuscle glucose. Post-absorptive blood glucose levels fell steadily with advancing age from levels of 85 - 90 mg% in the first week of life and stable adult levels were reached at the age of six to nine weeks. Since 50 percent of the total decrease in blood glucose occurred before rumination had started to any appreciable amount, diet was not the factor influencing blood glucose levels.

Few intravenous acetate tolerance tests have been reported in the literature. Ciaranfi and Fonnesu (1945) found that a load of 0.25 g acetic acid per kg body weight was rapidly removed from the blood of the dog and disappearance occurred at a constant rate depending on the initial blood concentration. Disappearance was markedly reduced in the diabetic dog (Ciaranfi and Fonnesu, 1945). Acetate tolerance in the cat was found to be similar to that of the dog (Smyth, 1947) whereas the rate of disappearance in the adult sheep was considerably slower (Jarrett _et al._, 1952; Reid, 1958). Jarrett and Filsell (1960) showed that after the injection of acetate at the rate of 5 m moles per kg body weight, blood acetate levels returned to pre-injection levels within 30 minutes in lambs under four months of age. Lambs over four months of age required 60 minutes, the same time required by adult sheep. This observation supported the hypothesis that the young lamb metabolically behaves much like the non-ruminant and gradually develops the metabolic characteristics of the adult ruminant.

Reid (1958) showed that the diet of the adult sheep had a marked effect on acetate tolerance. An acetate half-life of approximately 10 minutes was observed for sheep consuming a diet containing 50 percent cracked maize and increased to approximately 25 minutes for those animals consuming a restricted high-roughage diet. Reid suggested that the faster rate of acetate utilization in those sheep fed the 50 percent maize ration was related to an increased rate of entry of acetate into the pathways of oxidation and fat synthesis.

Support for this suggestion stemmed from blood acetate levels which were considerably lower in relation to rumen levels on diets containing increased amounts of wheat or maize when compared to relative blood acetate levels of sheep fed a roughage ration.

D. Gross Dietary Effects on the Gluconeogenic and Glycolytic Pathways as Assessed by Activities of Key Enzymes

Alterations of major metabolic pathways (gluconeogenesis and glycolysis) in response to dietary and hormonal manipulations have been extensively investigated. These investigations, however, have been made employing the rat and, to a lesser extent, other nonruminants, while the ruminant has been largely neglected. Because of this lack of knowledge concerning changes in the carbohydrate metabolism of the ruminant due to the effects of dietary regime, most current information as to the assessment of major metabolic pathways by key enzyme activities proceeds from the nonruminant.

Changes in the activity of the key enzymes associated with gluconeogenesis have been observed in response to dietary and hormonal treatments. Starvation, diabetes, high protein-low carbohydrate diets, and adrenocortical hormones have all been shown to increase the activity of hepatic glucose-6-phosphatase (Langdon and Weakley, 1955; Freedland and Harper, 1957; Freedland and Harper, 1958a and b; Kvam and Parks,1960; Weber _et_ _al_., 1961; Weber _et_ _al_.,1963; Weber _et_ _al_., 1965a,b and c; Freedland _et_ _al_., 1966; Freedland and Harper, 1966; Szepesi and Freedland, 1967; and Szepesi and Freedland, 1968c), fructose-1, 6-diphosphatase (Mokrasch et al., 1956; Kvam and Parks, 1960; Weber et al., 1961; Weber et al., 1965a,b and c; Szepesi and Freedland, 1967; Szepesi and Freedland, 1968c), pyruvate carboxylase and phosphoenolpyruvate carboxykinase (Henning et al., 1963; Shrago et al., 1963; Weber et al., 1965a; and Tepperman et al., 1970). Decreased activity due to starvation, diabetes and low carbohydrate diets have been shown for the glycolytic enzymes glucokinase (Salas et al., 1963; Vinuela et al., 1963; Blumenthal et al., 1964; Oliver and Cooke, 1964; Sharma et al., 1964; and Sillero et al., 1969) and pyruvate kinase (Krebs and Eggleston, 1965; Weber et al., 1965d; Yudkin and Krauss, 1967; Sillero et al., 1969; Szepesi and Freedland, 1968a; and Szepesi and Freedland, 1969a). Activity of glutamic-pyruvic transaminase [E.C.2.6.1.2], an enzyme closely related to gluconeogenesis, has been shown to increase in response to the same factors which increase the gluconeogenic capacity of the rat. The factors have been found to be starvation, diabetes, high protein-low carbohydrate diets and glucocorticoids (Rosen et al., 1958; Rosen et al., 1959; Segal et al., 1962; Szepesi and Freedland, 1968b and c; Szepesi and Freedland, 1969b). Glucose-6-phosphate dehydrogenase [E.C.1.1.1.49], the initial enzyme of the pentose phosphate cycle, also has been shown to be influenced by the dietary regime of the rat and to be an indicator of capacity of the pathway as starvation, diabetes, low carbohydrate and high fat diets all have been shown to depress the activity of this enzyme (Niemeyer et al., 1962;

Ono et al., 1963; Vaughn and Winders, 1964; Benevenga et al., 1964; McDonald and Johnson, 1965; Peraino, 1967; and Szepesi and Freedland, 1968c). Fitch and Chaikoff (1960) showed a three-to-ten fold increase in glucose-6-phosphate dehydrogenase activity in rats fed a 60 percent hexose diet and Peraino (1967) showed that protein was required in the diet for the induction of the enzyme by carbohydrate to be expressed.

The effects of diet on overall metabolic pathways of the ruminant have been reported. Alterations in gluconeogenic or glycolytic metabolism have been assessed to a limited extent in the ruminant by enzymatic studies but whether the changes have been adaptions to diets or were constitutive effects has not been clearly demonstrated.

To study the effect of diet on the rate of glucose utilization, Ford (1965) fed rations with increasing amounts of crude protein to adult sheep and observed increasing rates of glucose utilization and percentages of exhaled carbon dioxide derived from glucose. Also (Ford and Reilly, 1969), increased glucose entry rates of 2.25 mg/min/kg were observed for sheep fed a pelleted grass diet as compared to 1.2 mg/min/kg observed for animals consuming a hay diet. Concurrent increased rates of free amino acids utilization were also reported. Ulyatt et al. (1970) presented work concerning the effect of diet on glucose entry rates in sheep which were largely at variance with Ford (1965) and Ford and Reilly (1970). Feeding an 85 percent barley, dried grass or hay diet which supplied greatly differing amounts of α-linked glucose polymer and crude protein, no differences in glucose entry rate, plasma glucose concentration or size of the total body glucose pool was observed. It would be expected that the diets high in starch may produce a higher proportion of propionate during rumen fermentation and considerable amounts of dietary starch may escape fermentation and become available for absorption in the lower gastrointestinal tract. Both factors would then be expected to alter the rate of glucose utilization. The effect of diet on glucose entry remains unsolved.

Work has shown that the factors which increased the gluconeogenic capacity of the adult sheep, i.e., fasting and diabetes, have been reflected by increased activities of key gluconeogenic enzymes (Filsell et al., 1969). It was shown that after a six to seven day fast, hepatic activities of glucose-6-phosphatase, fructose-1, 6-diposphatase and pyruvate carboxylase increased with pyruvate carboxylase exhibiting a four-fold increase. Phosphoenolpyruvate carboxykinase showed about a two-fold increase only in sheep which were made diabetic by pancreatectomy or alloxan treatment. Wagle and Nelson (1966) compared the activities of gluconeogenic enzymes of adult sheep to those of the rat. The activity of glucose-6-phosphatase, fructose-1, 6-diphosphatase, phosphoenolpyruvate carboxykinase and pyruvate carboxylase in sheep was twice that observed in rats and they attributed the high enzyme activity to the greater gluconeogenic capacity of the adult ruminant. Unfortunately, neither the diet of the sheep nor the rat were stated in the report.

Goetsch (1966) observed a gradual decrease in activity enzymes associated with glycolysis as calves matured over an eight week period. The decrease was stated to be due to decreased rates of glucose absorption from the lower tract as a consequence of rumen development.

Bartley _et al_. (1966) found that the activities of phosphorylase and glucose-6-phosphatase increased in calves between eight to twelve weeks of age to levels found in the adult cow. The increased activities were cited as evidence that metabolic adaptations involving carbohydrate metabolism coincide with development of rumen function (a response to a decrease in availability of glucose from the intestinal tract). Also, the intraduodenal infusion of massive doses of glucose into fifteen week old calves or mature cows decreased the activity of glucose-6-phosphatase and phosphorylase to the level of that observed in calves with nonfunctional rumens. Therefore, the enzymatic responses observed with maturity were related to the availability of glucose. Bartley _et al_. (1966) thus provided evidence for the adaptability of metabolic processes of ruminants to substrate availability.

Purser and Bergen (1969) compared the metabolic processes of gnotobiotic goats, and conventional goats and rats, fed a diet containing high levels of easily digestible carbohydrates. It was assumed that gluconeogenic activity was assessed by glucose-6-phosphatase activity and glycolytic activity by pyruvate kinase. Glucose-6-phosphatase activity was found to be greater in the gnotobiotic and conventional goat than the rat while pyruvate kinase activity was comparable in all three. The glucose-6-phosphatase activity of the gnotobiotic and conventional goat was not different. This was contrary to the expectation that the gnotobiotic goat, with the absence of normal rumen development, would have exhibited lower glucose-6-phosphatase activity. No differences in pyruvate kinase activity were noted for the gnotobiotic or conventional goat, indicating no greater glycolytic activity in the gnotobiotic goat. This was also contraty to expectation. Purser and Bergen (1969) were able to show that a gnotobiotic animal fed the same diet only lower in protein, had an apparent reduction in the rate of glycolysis as measured by a substantial decrease in pyruvate kinase activity. Decreased plasma glucose concentration and increased glucose half-life with increasing age were reported for both the gnotobiotic and conventional animal. The evidence indicated that in contrast to Bartley _et al_. (1966) and Goetsch (1966) changes in metabolic processes of the ruminant may not be limited to the inductive role of various nutrients.

III EXPERIMENTAL PROCEDURE

A. Introduction

Experimentation consisted of two trials which were conducted over a period of two years, 1969 and 1970. Pygmy goats were obtained by hysterotomy and maintained as germfree or conventional animals. Dietary treatments were imposed, consisting of a "glucose", "volatile fatty acid" or a commercial pelleted germfree animal diet. Changes in patterns of carbohydrate metabolism as a function of maturity of the animal were assessed by glucose and acetate tolerance tests, liver metabolite levels and activities of key gluconeogenic or glycolytic enzymes.

B. General Conduct of Experiment

1. Procurement and maintenance of the pygmy goat.

Because of their size and temperment, pygmy goats were the animal of choice for these germfree experiments. Male pygmy goats were obtained from the Primate Research Center, Oregon State University and bred to Toggenburg does. Offspring of this cross were only part pygmy although they will be referred to as pygmy goats throughout this manuscript.

The preparative, surgical, and *post partum* technique of the hysterotomy have been fully described (Oxender *et al.*, 1971). Germfree preparative procedures, sterility control and isolator equipment also have been described (Meyer *et al*., 1964). Kids taken by hysterotomy into germfree isolators will be referred to as germfree and those taken by this procedure but kept in nonsterile isolators will be called conventional goats.

2. Experimental diets and feeding regimes

Composition of the basal diet is shown in Table 1. This diet was the major constituent of the glucose and volatile fatty acid diets which were made by adding isocaloric amounts of glucose or volatile fatty acid salts in the amounts shown in Table 2 to this basal diet. These diets were sterilized and fed on a dry matter basis at the rate of 3 percent on the animal's body weight. Feeding of this liquid diet was accomplished by using a regular human infant's baby bottle and nipple. The pelleted germfree diet was a commercial preparation and was fed *ad libitum*. The composition of this diet appears in Table 3.

3. Experimental Protocol - 1969

Six germfree and three conventional animals were all maintained from birth on the basal diet with two of the conventional goat's diets supplemented (*ad libitum*) with the pelleted germfree animal diet. Glucose and acetate tolerance tests were performed *in situ* twenty-four hours before the sacrifice of the animal. At the time of sacrifice, the kid was quickly removed from the isolator, electrocuted and its liver was excised and chilled for enzyme analysis. Activities of the hepatic enzymes pyruvate

TABLE 1. Composition of Basal Diet

Constituents	Amount
Cow's milk	19.0 L
Glucose	227.0 g
Solka Floc[a]	213.0 g
Magnesium sulfate $(M_gSO_4\ 7H_2O)$	11.4 g
Vitamin mix[b]	22.7 g

[a] Solka Floc, Brown Company, Chicago, Ill.

[b] General Biochemicals, Chagrin Falls, Ohio. Composition of the vitamin mixture in grams per 45.5 kg: Thiamine 4.36, riboflavin 10.90, niacin 72.64, Ca pantothenate 43.58, choline chloride 3,632.00, 0.1% B_{12} in manitol 72.64, folic acid 0.91, PABA 181.60, biotin 0.36, pyridoxine 3.63, and dextrose filler.

TABLE 2. Composition of Experimental Diets

Constituents	Glucose Diet	VFA Diet
Basal diet	1.5 L	1.5 L
Glucose	32.2 g	
Sodium acetate		30.0 g
Sodium propionate		11.1 g
Sodium butyrate		2.1 g

TABLE 3. Composition of Pelleted Germfree Animal Diet[1]

Constituents	Amount (g/kg)
L-lysine HCl	5.000
DL-methionine	5.000
Soybean meal (50%)	300.000
Alfalfa meal (17%)	35.000
Corn, whole yellow ground	589.625
Oil, corn	30.000
Salt mix (see below)	25.250
BHT (butylated hydroxytoluene)	0.125
Vitamin mix (see below)	10.000

Salt Mix	g/kg
Calcium carbonate ($CaCO_3$)	5.00000
Cobalt chloride ($CoCl_2 \cdot 6H_2O$)	0.00266
Cupric sulfate ($CuSO_4 \cdot 5H_2O$)	0.00553
Calcium phosphate dibasic (Ca_2HPO_4)	10.00000
Ferric citrate ($FeC_6H_7O_5 \cdot 5H_2O$)	0.13213
Manganese sulfate ($MnSO_4 \cdot 4H_2O$)	0.20329
Potassium iodate (KIO_3)	0.00222
Sodium chloride (NaCl)	10.00000
Zind sulfate ($ZnSO_4 \cdot 7H_2O$)	0.06587

Vitamin Mix	g/kg
PABA	0.0506
B_{12} vitamin	0.0044
Calcium pantothenate	0.2860
Choline chloride	1.9800
Folic acid	0.0110
Menadione	0.8800
Niacin	0.0660
Pyridoxine HCl	0.0220
Riboflavin	0.0308
Thiamine HCl	0.0660
	IU/kg
Vitamin A	26,400.0
Vitamin D_3	1,200.0
Vitamin E acetate	100.0

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[1] General Biochemicals, Chagrin Falls, Ohio.

kinase, glutamic-pyruvic transaminase and glucose-6-phosphatase were determined.

Age of the animal at sacrifice varied. Two germfree animals were sacrificed at 49 days of age, four germfree animals at approximately 124 days and the conventional animals at approximately 130 days.

4. Experimental Protocal - 1970

Experimental protocol used in 1970 was slightly more detailed. Animals were procured and maintained by exactly the same techniques as those employed in 1969, however the number of animals used, the diets imposed, the number of tolerance tests given and the liver parameters investigated were altered.

Six germfree goats were used three of which were fed the glucose diet (Table 2) and the rest were fed the VFA diet (Table 2). There were five conventional animals, two consumed the glucose diet, two the VFA diet and one, the pelleted germfree animal diet. Two tolerance tests per animal were performed _in situ_; the first at two months and the second at six months of age.

Twenty-four hours after the last tolerance test, each animal was removed from its germfree or conventional isolator and immobilized on its left side upon a surgical table. A 10 ml blood sample from the jugular vein was taken for plasma amino acids. Hair was carefully clipped from the rib cage area directly posterior to the axilla. After removal of the hair, the area between the third and fourth rib from the

the caudal section of the rib cage was anesthetized to permit a three centimeter incision, running dorsal to ventral between the two ribs and extending into the abdominal cavity. After this incision was made, the lobe of the liver closest to this opening was exteriorized. Approximately three grams of liver were excised with surgical scissors and quickly plunged into liquid nitrogen. This area of liver was sampled each time and the time required for removing the hepatic tissue until plunging it into liquid nitrogen took consistently under 10 seconds. These samples were stored at -70°C until the determination of the concentration of liver metabolites i.e., glucose-6-phosphate and phosphoenolpyruvate, was conducted. After the excision of the liver tissue, the animal was immediately electrocuted and the remaining liver tissue was removed and quickly chilled. One 5-10 gram sample of the liver portion was frozen for liver free amino acid determination and the rest was used immediately for enzyme analysis. Activity of the hepatic enzymes, pyruvate kinase, glucose-6-phosphatase, glucose-6-phosphate and 6-phosphogluconate dehydrogenase was determined.

C. Procedures and Analytical Determinations

1. Performance of the tolerance tests

All glucose and acetate tolerance tests were performed at eight o'clock in the morning on twenty-four fasted animals. Tolerance tests on germfree animals were performed under sterile conditions. The goat was physically restrained and the hair was clipped from the area over the external jugular veins. An 18 gauge bleeding needle was inserted into the jugular vein and an initial 4 ml (T_0) blood sample was withdrawn. Teflon tubing of a diameter small enough to pass through the needle was then inserted into the vein and the needle was subsequently removed.

Simultaneous infusion of glucose and acetate was accomplished via this catheter by mixing these solutions in the syringe and then injecting the mixture over the period of one minute. A glucose solution (30% w/v) and acetate solution (25% w/v) were injected at the rate of 0.30 g/kg and 0.25 g/kg respectively. Both solutions were sterile. These methods and dosages are those of Reid (1958).

At 5, 10, 15 and 20 minutes after infusion of the glucose-acetate solution, blood samples were taken. Four ml aliquots were withdrawn using a 10 ml syringe and immediately transferred the heparinized test tubes. Frequent flushing of the catheter with heparinized saline was required to prevent clots in the teflon tube.

2. Determination of blood glucose and acetate concentrations and calculation of half-life

The 4 ml blood samples were centrifuged at 10,000 x g for 10 minutes and 2 ml of the plasma was pipetted into another centrifuge tube. Then 0.4 ml of 25% 5-sulfosalicylic acid was added and the mixture was allowed to remain on ice for 30 minutes while precipitation of plasma proteins ensued. After complete precipitation of proteins had been effected, the protein-free supernatant was stored frozen until analyzed for glucose and acetate.

Glucose analysis was achieved by a modification of the glucose oxidase method of Hugget and Nixon (1957). A 0.2 ml aliquot of the protein-free supernatant was neutralized with 0.02 N sodium hydroxide solution which gives a final volume of 3.3 ml. One ml of sample, standard glucose solutions or blank was used in duplicate in the assay applying the prepared Glucostat[1] reagents and procedure.

Blood acetate was determined by gas-liquid chromatography using the protein free supernatant just described. Approximately 5 μ l aliquots were injected onto a 6.0 foot by 0.25 inch glass column packed with Chromosorb 101[2] in a Packard Gas Chromatograph. Inlet, column and detector temperatures were 188, 195 and 215°C, respectively. Carrier gas was nitrogen, flowing at the rate of 40 ml per minute and detection was accomplished using a hydrogen flame ionization detector. The concentration of acetate in these samples was determined by employing appropriate acetate standard solutions and comparing peak heights.

Calculation of the half-life of glucose and acetate was by means of the method of Reid, 1958. If the relationship between log blood glucose or acetate concentration and time is linear, then the half-life of these metatolites can be represented by:

$$T_{\frac{1}{2}} = \frac{1}{k} \log_e 2$$

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[1] Worthington Biochemical Corporation, Free hold, New Jersey, 07728.

[2] Varian Aerograph, Walnut Creek, California, 94598.

where $T_{\frac{1}{2}}$ is the half-life (min.) and k is the specific rate constant (min.^{-1}), equal to the fraction of the excess glucose or acetate removed per minute. The constant k is more simply the slope of the line which delineates the relationship between log blood glucose or acetate concentration (pre minus post injection conc.) and the sampling time.

3. Liver free amino acids

Thawed samples of liver (taken for liver free amino acid analysis) were prepared essentially according to Clark *et al*., 1966, using norleucine as an internal standard. One ml of 1mM norleucine was added to 1 g of tissue in 4 ml of deionized distilled water and homogenized in a Virtis homogenizer at top speed for 3 minutes. The homogenates were mixed with 5 ml of 10% (w/v) 5-sulfosalicylic acid and centrifuged at 32,000 x g for 10 minutes after precipitation of protein was complete.

Five ml of the protein-free supernatant were pipetted into a round bottom flask and concentrated in a rotary flash evaporator to near dryness. The residue was dissolved in 3 ml of 0.3N lithium-0.05M citrate buffers (pH 1.9). A dual column Technicon TSM amino acid analyzer performed the amino acid analyses on 0.4 ml aliquot of this solution (Bergen and Potter, 1971; Makdani *et al*., 1971).

4. Plasma amino acid determination

The 10 ml blood samples, taken at the time of sacrifice, were collected into heparinized syringes and the plasma separated by centrifugation as described above. To each ml of plasma thus obtained, 0.1 μ mole of norleucine was added as the internal standard. A 0.1 ml aliquot of 50% (w/v) 5-sulfosalicylic acid then was added per ml of plasma and the proteins were allowed to precipitate for 30 minutes on ice. The protein-free supernatant was decanted after centrifugation of the mixture at 20,000 x g. The supernatant was stored frozen until plasma amino acid concentrations were determined with the Technicon Auto Analyzer.

5. Liver metabolite extraction and determination

a. Tissue powdering procedure

The tissue powdering procedure was performed in a cold-room at 4°C to reduce water uptake by the tissue from the air. Three gram samples of tissue were placed in a mortar and pestle, precooled by liquid nitrogen and ground. The samples were not allowed to thaw during this process. After the powdering was complete the ground tissue was added to 6% $HClO_4$ (w/v) to effect precipitation of proteins and extraction of metabolic intermediates. The ratio of the final total liquid volume of the sample to its original weight was 1:6. For example, if 2 g of tissue were used, then 10.5 ml of $HClO_4$ were needed (based on 25% dry matter of liver tissue).

A 15 ml centrifuge tube containing a glass rod and 5 ml of 6% $HClO_4$ was weighed. The powdered tissue was then added and the centrifuge tube reweighed. From the increase in weight the corresponding amount of 6% $HClO_4$ was calculated from the above ratio and added allowing for the original 5 ml in the tube. The glass rod was used to stir and grind tissue. After an extraction time of 10 minutes, the preparation was centrifuged at 3000 x g and the supernatant was decanted into 50 ml beakers cooled in ice. Titration of this acidic supernatant to pH 6.0 was accomplished using 5 M K_2CO_3 and a Corning pH meter. This pH provides maximum stability of the metabolites determined particularly phosphoenolpyruvate. The amount of K_2CO_3 added was recorded and the dilution due to its addition was calculated into the final dilution factor. After the evolution of CO_2 stopped the perchlorate salts settled, the supernatant was decanted and used immediately for the determination of liver metabolites.

b. Determination of D-glucose-6-phosphate

The determination of D-glucose-6-phosphate was performed by the method of Hohorst in Bergmeyer (1963). The method is based on the following reaction catalyzed by the enzyme, glucose-6-phosphate dehydrogenase:

$$\text{Glucose-6-phosphate+NADP} \leftrightharpoons \text{6-phosphogluconolactone+NADPH}_2$$

Extent of the reaction is measured by the increase in optical density at 340 m µ when NADP is reduced. The blank solution was 0.4 m triethanolamine buffer (pH 7.6) and the final volume of the assay was 1.025 ml. All spectrophotometric measurements were made in duplicate on a Gilford Recording Spectrophotometer, Model 2000.

c. Determination of phosphoenolpyruvate

This determination was performed by the method of Czok and Eckert in Bergmeyer (1963). The concentration of phosphoenolpyruvate is measured by the following coupled reactions:

$$\text{PEP} + \text{ADP} \xrightleftharpoons[M_g^{+2},\ K^{+}]{\text{Pyruvate Kinase}} \text{pyruvate} + \text{ATP}$$

$$\text{Pyruvate} + \text{NADH}_2 \xrightleftharpoons{\text{LDH}} \text{lactate} + \text{NAD}$$

The decrease in optical density due to the oxidation of NADPH_2 is proportional to the amount of phosphoenolpyruvate present. The assay was carried out at 340 mμ to increase the accuracy of analysis and the maximum amount of deproteinized sample used in the assay was 1.50 ml. The assay was blanked against air and measurements were made in duplicate using the Gilford Recording Spectrophotometer, Model 2000.

6. Enzyme preparation and assay

a. Liver preparation

The whole liver was weighed and minced with surgical scissors on ice. This mincing procedure was done to obtain random samples of liver for assay, since enzyme activity for particular enzymes may be localized in hepatic tissue.

One portion of this liver was homogenized with 19 volumes of cold, 0.1 M sodium citrate buffer (pH 6.5) in a Potter-Elvehjem homogenizer, strained through two layers of cheesecloth and used the same day of preparation for the assay of glucose-6-phosphatase activity.

Another sample of tissue was homogenized with 4 volumes of cold 0.14 M KCl in a Potter-Elvehjem homogenizer and was centrifuged for 20 minutes at 10,000 x g for the assay of glutamic-pyruvic transaminase, 30 minutes at 20,000 x g for the assay of glucose-6-phosphate and 6-phosphogluconate dehydrogenase, and 30 minutes at 30,000 x g for the assay of pyruvate kinase. All supernatants were filtered through cheesecloth and used the same day for assay.

b. Glucose-6-phosphatase assay

The method of assay of glucose-6-phosphatase activity was that described by Harper in Bergmeyer (1963). The rate of the enzymatic hydrolysis of glucose-6-phosphate by glucose-6-phosphatase was measured by the increase in inorganic phosphate in a 15 min assay. Absorbance due to the liver homogenate was corrected for and citrate buffer (pH 6.5) was used as the blank. All spectrophotometric measurements were made using a Coleman Junior II. This method has been used in this laboratory in other studies (Purser and Bergen, 1968).

c. Glutamic-pyruvic transaminase assay

The assay of this enzyme activity was performed by the method of Bergmeyer and Bernt in Bergmeyer (1963).

The glutamic-pyruvic transaminase reaction was coupled with the lactic acid dehydrogenase reaction and glutamic-pyruvic transaminase activity was then measured by the decrease in optical density at 340 mμ due to the oxidation of $NADH_2$. The blank used was 0.5 m phosphate buffer (pH 7.6) and spectrophotometric measurements were made using a Gilford Recording Spectrophotometer, Model 2000.

d. Glucose-6-phosphate and 6-phosphogluconate dehydrogenase assay

The assay of these enzyme activities was made following the method of Freedland (1967). The rate of the enzymatic reactions was followed by the increase in optical density at 340 mμ due to the reduction of NADP. The blank solution was 0.017 m glycylglycine (pH 7.6) and all spectrophotometric measurements were made in duplicate using a Gilford Recording Spectrophotometer.

e. Pyruvate kinase assay

This assay for enzymatic activity was performed by the method of Bücher and Pfleiderer in Colowick and Kaplan (1955). The pyruvate kinase reaction was coupled to the lactic acid dehydrogenase reaction and the decrease in optical density at 340 mμ due to the oxidation of $NADH_2$ was used to measure enzymatic rate. The blank solution was 0.05m triethanolamine buffer (pH 7.5) and spectrophotometric measurements were made in duplicate using a Gilford Recording Spectrophotometer. This assay has been used in this laboratory for other metabolic studies (Purser and Bergen, 1968).

f. Protein determination on liver enzyme preparations

Protein determinations on liver homogenates were performed by the Folin-phenol method, modified by Miller, 1959. Bovine serum albumin was the standard reference protein and spectrophotometric measurements were performed on a Coleman Junior 11 spectrophotometer.

D. Statistical Analysis

The data from the 1969 trial were analyzed by a one-way analysis of variance. Least squares analysis of variance was employed to define the significant relationship in the 1970 trial. The inverse relationship between pyruvate kinase and glucose half-life was analyzed for statistical difference by the student's t-test.

IV RESULTS AND DISCUSSION

The experimental design differed between the 1969 and 1970 trials. The 1969 trial consisted of six germfree and three conventional pygmy goats. All six of the germfree goats and one of the conventional animals were fed the basal diet (Table 1) and the two other conventional animals received the germfree pelleted diet (Table 3). These animals were sacrificed at various ages and all experimental parameters were determined at this time. The germfree animals, 20 and 21, were sacrificed at 49 days of age while animals 11, 17, 18 and 19 were sacrificed at 146, 118, 115 and 117 days respectively. Animal 11 was approximately 30 days older than the others, but since it was germfree it was included in this group. Animal 16, a conventional animal, fed the basal diet was sacrificed at 123 days while 14 and 15, conventional animals fed the pelleted diet, were sacrificed at 151 and 121 days.

Statistical analysis of all the data of the 1969 trial was computed by a one-way analysis of variance. The effect of age on the metabolic parameters investigated was analyzed. The three groups by animal number were 20 and 21; 11, 17, 18 and 19; and 14, 15 and 16, representing the ages of 49, approximately 124 and 130 days. The reason for such an arrangement is that there was a lack of control animals if one were to analyze on the basis of dietary regime or germfree status.

The experimental design of the 1970 trial consisted of six germfree pygmy goats, three of which received the glucose diet (Table 2) and the other three the VFA diet (Table 2). There were four conventional animals, two of which received the glucose diet and two the VFA regime. Finally there was one conventional goat which received the pelleted germfree diet (Table 3). Fasting plasma glucose levels and glucose and acetate half-lives were determined on all animals at approximately 60 days of age. At 180 days fasting plasma glucose levels, glucose and acetate half-lives were repeated, the animals sacrificed and enzyme activities and metabolite determinations were made.

These data were analyzed by the method of least squares. The effect of diet, germfree status and diet-germfree status interactions on the metabolic parameters investigated were determined. The one conventional animal receiving the pelleted diet was excluded from the statistical analysis.

As the ruminant matures fasting blood glucose levels have been observed to decrease from approximately 100 mg% to 50-60 mg% (McCandless and Dye, 1950 and Reid, 1953) and glucose and acetate half-lives increase (Jarrett and Filsell, 1960 and Purser and Bergen, 1969). Experimental work with young ruminants has shown that fasting blood glucose level and glucose tolerance could be held constant over extended periods if a diet of milk and milk solids was fed to calves

(Attebery and Colvin, 1963 and Harry et al., 1967). The inclusion of hay and/or grain in the diet (Harry et al., 1967) and subsequently the initiation of rumen fermentation (McCarthy and Kesler, 1956; McCandless and Dye, 1950 and Hale and King, 1958) were postulated to be the cause of decreased blood glucose concentration and increased glucose tolerance. In the adult sheep, Reid (1958) showed that glucose and acetate tolerance could be greatly influenced by diet. Shortest half-lives (fastest rate of utilization) for both glucose and acetate were observed when a 50 percent maize diet was fed. Longest half-lives (slowest rate of utilization) were observed in those sheep consuming a restricted roughage diet. These studies indicate dietary control over blood glucose levels and glucose and acetate tolerance in both the young and adult ruminant.

Others (Murley et al., 1952 and Wing et al., 1955) found that blood glucose levels decreased to adult levels by the fifth week of feeding a skimmed milk diet to calves. Hodgson et al. (1932), Jacobson et al. (1951) and Lambert (1955) also showed that various diets had no effect on fasting blood glucose levels in maturing cattle and Reid (1953) found blood glucose levels to fall in lambs before rumination could be detected. These results advocate some factor other than diet in control of fasting blood glucose.

One of the objectives of the 1969 and 1970 trials was to determine the control of these processes. The 1969 trial employed germfree and conventional pygmy goats, fed a basal diet which contained essentially cows milk with an extra 12 g of glucose per liter or a germfree pelleted diet which was 30 percent ground corn (Tables 1 and 3). In 1970 the dietary regime was changed from the basal diet of 1969 to the "glucose" and "VFA" diet. The glucose diet consisted of the basal diet to which an extra 21.4 g of glucose per liter had been added. The VFA diet consisted of 20 g, 7.4 g and 1.4 g of sodium acetate, propionate and butyrate per liter of basal diet. The glucose and VFA diets of 1970 were used with the expectation of exaggerating differences in certain metabolic patterns.

The high carbohydrate diets of both 1969 and 1970 were expected to maintain the rate of glycolysis (especially in the germfree animal) at the rate observed in the very young ruminant, while the animals consuming the VFA diet of 1970 were expected to develop patterns of metabolism characteristic of the adult ruminant, i.e., decreased rates of glycolysis and increased rates of gluconeogenesis. Control animals were conventional pygmy goats fed either the basal, glucose or VFA diet. Also included in these experiments were conventional goats fed a high grain diet (germfree pelleted diet) in order to have a normal reference ruminant.

The effect of the basal diet in 1969 on fasting plasma glucose concentration and glucose and acetate tolerance, if these parameters are under dietary control in the germfree goat, would be the maintenance of plasma glucose and glucose and acetate half-lives at those levels characteristic of the very young ruminant. Since the absence of rumen fermentation would allow the direct absorption from the lower gut and hence, utilization of the glucose and other carbohydrates of the basal diet, plasma glucose concentration would be expected to be high and the rate of glucose and acetate utilization fast in comparison to those conventional goats fed the pelleted diet. Because rumen fermentation was allowed to develop in these conventional animals, decreased plasma glucose concentration and a decreased rate of glucose and acetate utilization, all characteristic of the normal ruminant, was expected. The conventional animal fed the basal diet was a control to the germfree animals and was used to assess the effect of germfree status on the animal.

Dietary changes of 1970 were made in order to increase differences in the parameters measured, however the same general trends were expected. The glucose diet fed to both germfree and conventional (control) animals was expected to maintain plasma glucose levels and glucose and acetate utilization rates above those levels and rates seen in germfree and conventional animals fed the VFA diet and the animal fed the germfree pelleted diet.

The germfree animals fed the VFA diet, a diet which supplied acetate, propionate and butyrate, were expected to exhibit plasma glucose concentrations and glucose and acetate utilization rates typical of the animal having normal rumen function. Plasma glucose levels were expected to be lowered and glucose and acetate tolerance decreased in comparison to the germfree goats fed the glucose diet and similar to the conventional animals fed the germfree pelleted diet.

Tables 4, 5 and 6 present fasting plasma glucose concentrations (T_0 glucose) and half-lives for plasma glucose and acetate from the 1969 and 1970 trials. In 1969 (Table 4) the two germfree animals at 49 days of age had an average plasma glucose concentration of 132.5 mg/100 ml and the four germfree animals aged 124 days had an average T_0 glucose concentration of 115.6 mg/100 ml. In comparison, the conventional animals at 130 days averaged 94.7 mg/100 ml.

Since these differences approached statistical significance at the $P < 0.05$ level, these data suggest decreased T_0 glucose concentrations with advanced age. Any conclusions as to the effect of age on fasting plasma glucose concentrations should be made by comparing the two groups of germfree animals. If this parameter (T_0 glucose) is under dietary control, one would not expect a decrease. The decrease in plasma glucose levels seems to be due to advancing age and independent of diet, rumen development and volatile fatty acid production.

Animal 16, a conventional animal, had a fasting plasma glucose concentration of 110.4 mg/100 ml which is comparable to those levels observed in germfree animals at 124 days of age. This animal may have been behaving more like a germfree animal since the basal diet fed via a nipple-bottle passed directly to the abomasum since Ørksov _et_ _al_. (1970)

TABLE 4. 1969 T_0 Glucose and Glucose and Acetate Half-lives

Animal Number	Weight (kg)	Status	Age of Animal (days)	T_0 Glucose (mg/100 ml)	Glucose Half-life (min)	Acetate Half-life (min)
20	2.27	GF[a]	49	127.3	32.4	47.5
21	2.77	GF	49	137.6	29.4	23.3
		Mean ± S.E.M.		132.5± 5.2	30.9± 1.5	35.4±[b] 12.1
17	7.26	GF	118	133.9	18.0	6.8
18	6.80	GF	115	106.3	52.0	7.4
19	7.71	GF	117	127.5	35.8	9.7
11	7.71	GF	146	94.9	19.8	12.8
		Mean ± S.E.M.		115.6± 9.1	31.4± 7.9	9.2± 1.4
14	8.16	CON[c,d]	151	83.9	86.0	4.3
15	6.80	CON[d]	121	89.8	18.2	15.3
16	6.35	CON	123	110.4	16.6	16.8
		Mean ± S.E.M.		94.7± 8.0	40.3± 22.8	12.1± 3.9

[a] Animals designated GF were germfree.

[b] Significantly different ($P < 0.025$) from means of other age groups in the acetate half-life trial.

[c] Animals designated CON were conventional.

[d] Fed the pelleted germfree animal diet ad libitum in addition to basal diet.

TABLE 5. 1970 T_0 Glucose and Glucose and Acetate Half-lives at Approximately 60 Days of Age

Animal Number	Weight (kg)	Age of Animal (days)	Status	Diet	T_0 Glucose (mg/100 ml)	Glucose Half-life (min)	Acetate Half-life (min)
22	10.9	111	GF	Glucose	94.6	11.5	3.09
24	6.8	61	GF	Glucose	144.0	6.9	1.20
27	6.8	55	GF	Glucose	45.1	50.2	-
			Mean ± S.E.M.		94.6± 28.5	22.8± 13.7	2.14± 0.94
23	6.8	69	GF	VFA[b]	153.4	-	-
25	6.8	64	GF	VFA	99.0	9.7	-
26	6.8	63	GF	VFA	99.2	18.5	2.67
			Mean ± S.E.M.		117.2± 18.1	14.1± 4.4	
30	9.1	61	CON	Glucose	84.4	15.4	2.73
31	7.7	59	CON	Glucose	85.0	17.3	3.54
			Mean ± S.E.M.		84.7± 0.3	16.4± 0.9	3.13± 0.39
28	9.1	67	CON	VFA	105.6	13.4	1.69
32	7.7	59	CON	VFA	89.0	14.7	3.04
			Mean ± S.E.M.		97.3± 8.3	14.1± 0.7	2.36± 0.67
36	9.1	100	CON	Pellets[c]	86.2	110.3	4.83

[a] Table No. 2.

[b] Table No. 2.

[c] Table No. 3.

TABLE 6. 1970 T_0 Glucose and Glucose and Acetate Half-lives at Approximately 180 Days of Age

Animal Number	Weight (kg)	Age of Animal (days)	Status	Diet	T_0 Glucose (mg/100 ml)	Glucose Half-life (min)	Acetate Half-life (min)
22	10.9	180	GF	Glucose[a]	99.9	14.0	4.99
24	6.8	181	GF	Glucose	78.5	14.7	7.11
27	6.8	183	GF	Glucose	44.6	74.1	6.56
			Mean ± S.E.M.		74.3±	34.2±	6.22±
					16.1	19.9	0.64
23	6.8	180	GF	VFA[b]	81.9	17.1	4.73
25	6.8	180	GF	VFA	79.4	37.2	4.86
26	6.8	183	GF	VFA	91.5	11.9	3.74
			Mean ± S.E.M.		84.3±	22.1±	4.44±
					3.7	7.7	0.35
30	9.1	188	CON	Glucose	62.2	-	3.47
31	7.7	178	CON	Glucose	67.6	18.2	3.91
			Mean ± S.E.M.		64.9±	-	3.69±
					2.7	-	0.22
28	9.1	183	CON	VFA	61.3	40.6	3.84
32	7.7	183	CON	VFA	64.8	52.8	3.42
			Mean ± S.E.M.		63.1±	46.7±	3.63±
					1.8	6.1	0.21
36	9.1	182	CON	Pellets[c]	75.7	25.4	5.32

[a] Table No. 2

[b] Table No. 2

[c] Table No. 3

have shown that complete closure of the esophageal groove continued to occur in lambs weaned onto a teat-bottle.

The conventional animals 14 and 15 had T_o glucose levels of 83.9 and 89.8 mg/100 ml respectively. These animals were fed the pelleted germfree animal diet *ad libitum* in addition to the basal diet. This lowered T_o glucose concentration may be a consequence of rumen fermentation and the production of volatile fatty acids thereby hastening the decrease in T_o glucose. Within the limits of the experimental design and large animal variability, it is difficult to draw definite conclusions as to the effect of age or diet on T_o glucose.

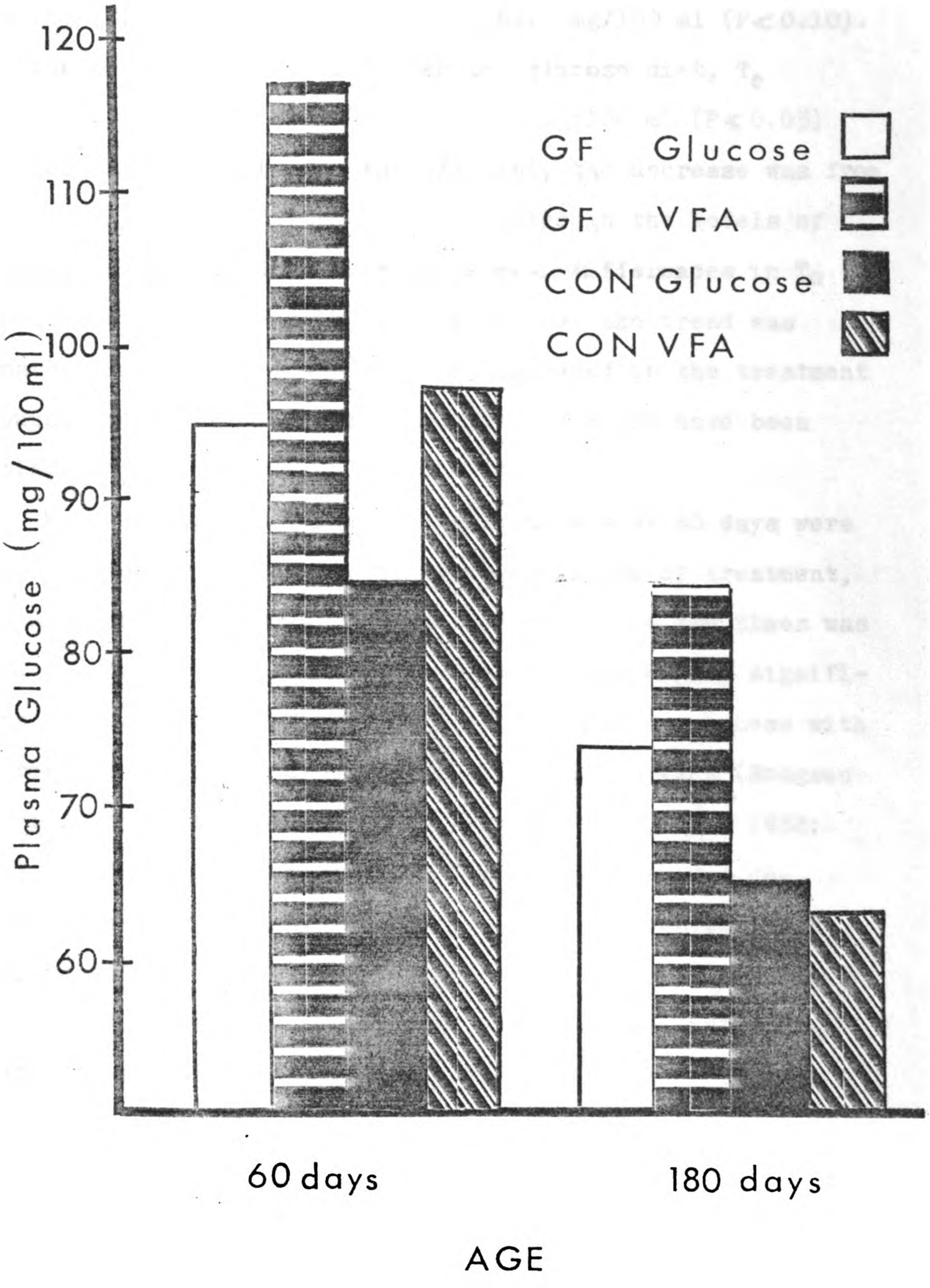
The 1970 trial was designed to differentiate between age and dietary effects on T_o glucose. The T_o glucose results for the 1970 trial at 60 and 180 days are given in Tables 5 and 6 respectively. Analyzed individually at 60 and 180 days, there was no statistical difference demonstrated between treatment means of the four groups of germfree and conventional animals fed the glucose or VFA diet. Animals fed the VFA diet did not exhibit lower T_o glucose concentrations in comparison to the animals fed the glucose diet.

As illustrated by Figure 1, there was a decrease in average T_o glucose for each treatment group between 60 and 180 days and these decreases approached statistical significance. Average fasting plasma glucose concentrations for the germfree animals fed the glucose diet fell from 94.6 to

Fig. 1 1970 Trial Average fasting plasma glucose concentrations at 60 and 180 days.

Abbreviations:
GF = germfree
COM = conventional

Composition of glucose and VFA diets found in Table 2.


120
110
100
90
80
70
60
Plasma Glucose (mg/100 ml)
GF Glucose
GF VFA
CON Glucose
CON VFA
60 days
180 days
AGE

74.3 mg/100 ml and this difference was significant at the $P < 0.25$ level. The decrease observed in the germfree animals fed the VFA diet was from 117.2 to 84.3 mg/100 ml ($P < 0.10$). For the conventional animals fed the glucose diet, T_0 glucose levels fell from 84.7 to 64.9 mg/100 ml ($P < 0.05$) and for those animals fed the VFA diet, the decrease was from 97.3 to 63.1 mg/100 ml ($P < 0.10$). Although the levels of statistical significance for these mean differences in T_0 glucose between 60 and 180 days were low, the trend was apparent and had more animals been included in the treatment groups, the higher level of significance might have been achieved.

When T_0 glucose values for all animals at 60 days were compared to the values at 180 days regardless of treatment, the decrease in average T_0 glucose between the two times was 98.4 to 71.6 mg/100 ml and this decrease was highly significant at the $P < 0.005$ level. The decrease in T_0 glucose with increasing age is in accordance with other workers (Hodgson et al., 1932; Jacobson et al., 1951; Murley et al., 1952; Reid, 1953 and Wing et al., 1955) who have observed decreased fasting glucose levels in ruminants with advanced age, in spite of widely varying dietary treatments. Also Purser and Bergen (1969) using germfree and conventional goats fed a diet similar to the 1969 basal diet, observed decreased fasting plasma glucose concentrations with increased age.

The results of the 1969 and 1970 trials indicate that high carbohydrate diets fed to germfree or conventional ruminants do not maintain fasting plasma glucose concentrations typical of the young animal. Decreased T_0 glucose with advanced age occurred regardless of diet.

Glucose half-lives for the animals in the 1969 trial appear in Table 4. There was no statistical difference between the means of the treatment groups. The ability of the animals to remove an intravenous glucose load did not differ with age between the two groups of germfree animals. Furthermore, changes in glucose half-life due to diet and germfree or conventional status were obscured by animal variability in the conventional animals.

Likewise, there were no statistical differences attributable to type of animal (germfree versus conventional) or diet (glucose versus VFA) at either 60 or 180 days in the 1970 trial (Tables 5 and 6). This is in contrast to the results of Reid (1958) who showed that the glucose half-life of adult sheep was affected by diet. Shortest glucose half-lives occurred in animals fed 1800 g of a 50 percent cracked maize diet while longest half-lives were seen in animals fed 700 g of wheaten chaff. Harry et al. (1967) found that glucose half-lives were constant in calves fed a diet of milk and milk solids while decreased glucose tolerance was observed when this diet included hay and/or grain. Other reports (McCandless and Dye, 1950; Hale and King, 1958 and Omari and Nakajima, 1961) also showed that the diet of the ruminant affects glucose tolerance.

Changes in average glucose half-lives for the four treatment groups between 60 and 180 days are illustrated in Figure 2. In general there was an increase with age in glucose half-life for each treatment group. However, only the increase in this parameter for the conventional animals fed the VFA diet was significant ($P < 0.05$). When the glucose half-lives for all animals at 60 days were compared to the glucose half-lives at 180 days regardless of treatment, the increase in average glucose half-life was from 16.8 to 30.3 minutes and this increase was significant at the $P < 0.01$ level.

Webb *et al*. (1969) found that there was no difference in glucose tolerance among three groups of dairy calves fed either milk plus milk solids, milk plus high-starch concentrate or milk plus high-fiber concentrate over 100 days. Glucose tolerance decreased markedly with advancing age in all treatment groups. Purser and Bergen (1969) found similar trends as glucose half-lives increased with increasing age and differences between germfree and conventional goats were not apparent.

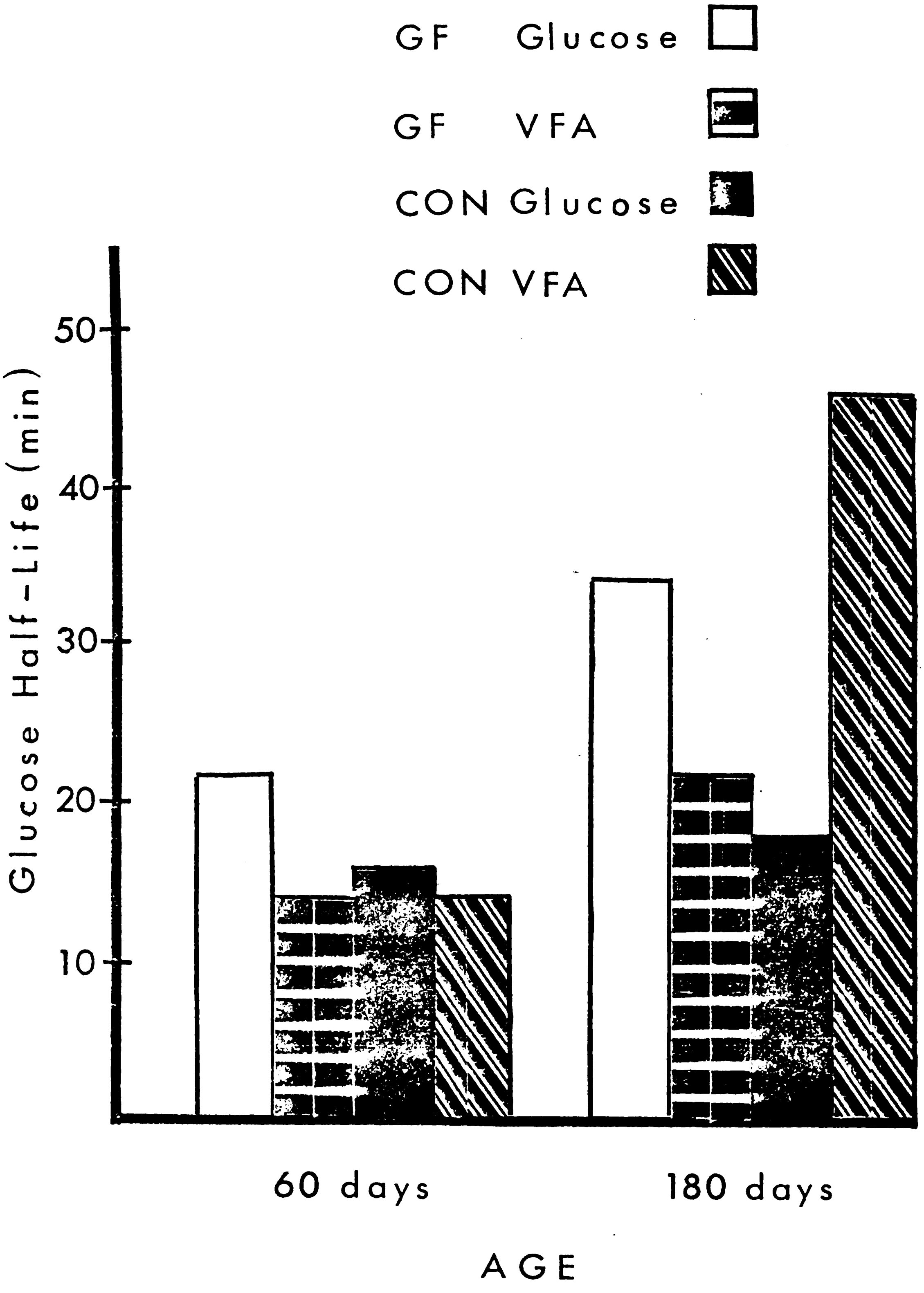
The results of this study indicate that glucose half-life increases with advancing age and may require as long as 180 days for this increase to become apparent. Furthermore, this decreased tolerance to glucose does not depend upon animal type (germfree versus conventional) or diet (glucose versus VFA).

Acetate half-lives for animals in the 1969 trial appear

Fig. 2 1970 Average glucose half-lives at 60 and 180 days.

Abbreviations:
GF = germfree
CON = conventional

Composition of glucose and VFA diets found in Table 2.


GF Glucose
GF VFA
CON Glucose
CON VFA
Glucose Half-Life (min)
50
40
30
20
10
60 days
180 days
AGE

in Table 4. The two youngest (49 days) germfree goats had a mean acetate half-life of 35.4 minutes, which was significantly longer ($P < 0.025$) than the other half-lives. The four germfree animals at 124 days of age had an average acetate half-life of 9.2 minutes while the average half-life for the 130 day old conventional animals was 12.1 minutes. These means were not significantly different.

Between the two groups of germfree animals, there was a large decrease in acetate half-life with advancing age. This decrease in acetate half-life is contrary to the observation of Jarrett and Filsell (1960) who found that lambs under four months of age removed an intravenous acetate load considerably faster than adult sheep. Also, acetate half-lives of this length were not observed in 60 day old goats in the 1970 trial (Table 5). This decrease in acetate half-life with advancing age is difficult to explain and is most likely due to animal variation.

Reid (1958) showed that the diet would affect acetate half-life. When sheep were fed a restricted high-roughage diet, the acetate half-life was approximately 25 minutes, whereas a diet of 50% maize fed ad libitum resulted in a half-life of 10 minutes. The 124 day old germfree goats and the group of conventional animals both had acetate half-lives in the range of 10 minutes. The similarity of acetate half-life values of germfree and conventional animals may be due to diets which were quite similar in their effect on acetate half-life. The basal diet would not be fermented in either the conventional animals, due to rumen bypass, or the germfree animals. Also, the germfree pelleted diet was a high grain diet. These factors decreased the half-life of acetate to those levels observed in adult sheep fed a high grain diet.

Acetate half-lives for the animals in the 1970 trial appear in Tables 5 and 6. At 60 days of age there were no statistically significant differences among treatment means of the four groups of germfree and conventional animals fed the glucose or VFA diets. However, at 180 days of age, the mean acetate half-life for both germfree and conventional animals fed the glucose diet was 4.9 minutes, while the mean for germfree and conventional animals fed the VFA diet was 4.0 minutes and this difference approached statistical significance at the $P < 0.10$ level.

On the basis of the work by Reid (1958) the diet which supplied the VFA should have produced longer acetate half-lives while the glucose diet should have maintained shorter acetate half-lives. In either case, whether fed the glucose or VFA diet, the acetate half-lives observed in these animals were much shorter than those half lives reported by Reid (1958), indicating that the dietary differences in the 1970 trial did not exaggerate differences in acetate half-life. Both diets maintained low acetate half-life values, characteristic of a young ruminant or an adult ruminant fed a high concentrate diet.

Interestingly, there was a statistically significant difference in acetate half-life between germfree and conventional animals at 180 days. The mean acetate half-life for germfree animals was 5.3 minutes while the mean acetate half-life for conventional animals was 3.7 minutes and the difference was significant at the $P < 0.025$ level. Since the germfree and conventional groups of animals contained animals from the two dietary treatments, the effect on acetate half-life appeared to depend upon conventional versus germfree status. However, the reason that the absence of a normal complement of bacteria produced a significantly longer acetate half-life is difficult to explain and may warrant further research.

There was an increase in average acetate half-life for each treatment group between 60 and 180 days. Average acetate half-life for the germfree animals fed the glucose diet increased from 2.14 to 6.22 minutes and this increase was significant at the $P < 0.05$ level. The increase observed in the germfree animals fed the VFA diet was from 2.67 to 4.44 minutes ($P < 0.10$). For the conventional animals fed the glucose diet, acetate half-life increased from 3.13 to 3.69 minutes ($P < 0.10$) and for those animals fed the VFA diet, the increase was from 2.36 to 3.63 minutes but this difference was not statistically significant. The increase in acetate half-life with advancing age became more apparent when the acetate half-lives of all animals at 60 days were statistically compared to the acetate half-lives of all animals at 180 days regardless of treatment. The increase was from 2.58 to 4.50 minutes and this difference was significant at the $P < 0.005$ level.

This increased acetate half-life or decreased ability to metabolize an intravenous acetate load with increased age was similar to the observations of Jarrett and Filsell (1960). These workers observed a sudden decrease in the rate of metabolism of an injected dose of acetate at four months of age in sheep and this decrease was independent of diet and the onset of rumination. Here, acetate half-lives increased with age and acetate half-lives were in general short, comparable to those half-lives observed in sheep fed a high grain diet (Reid, 1958).

Hepatic glutamic-pyruvic transminase activity for the 1969 trial is presented in Table 7. The mean enzyme activity for the two groups of germfree pygmy goats increased approximately two-fold between the ages of 49 and 124 days when enzyme activity was expressed on either a per milligram hepatic protein or a per gram liver basis. Likewise, there was an increase in mean transminase activity with advancing age, although not as pronounced, when activity was expressed on a body weight basis. The conventional animals had a mean glutamic-pyruvic transaminase activity comparable to the mean activity of the older group of germfree animals. There were no statistical differences demonstrated between treatment means for the various methods of calculation of enzyme activity.

In the nonruminant animal, enhanced gluconeogenesis

TABLE 7. 1969 Glutamate-Pyruvate Transaminase Activity

Animal Number	Age of Animal (days)	Status	Glutamic-pyruvate transaminase		
			1[a]	2[b]	3[c]
20	49	GF	7.48	0.568	199.2
21	49	GF	4.31	0.314	86.9
		Mean ± S.E.M.	5.89± 1.58	0.441± 0.127	143.0± 56.2
17	118	GF	7.36	0.699	129.9
18	115	GF	13.27	0.995	203.1
19	117	GF	16.68	1.351	220.5
11	146	GF	3.81	0.305	80.9
		Mean ± S.E.M.	10.28± 2.89	0.837± 0.222	158.6± 32.5
14	151	CON[d]	6.34	0.634	162.9
15	121	CON[d]	6.25	0.663	143.1
16	123	CON	9.47	0.881	201.1
		Mean ± S.E.M.	7.35± 1.06	0.726± 0.080	169.0± 17.0

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[a] Glutamate pyruvate transaminase activity: m μ moles NADH oxidized per minute per milligram hepatic protein.

[b] Glutamate pyruvate transaminase activity: μ moles NADH oxidized per minute per gram liver.

[c] Glutamate pyruvate transaminase activity: μ moles NADH oxidized per minute per 10 kilograms body weight.

[d] Fed pelleted germfree animal diet ad libitum in addition to sterile glucose diet.

from amino acids and parallel increases in glutamic-pyruvic transaminase activity have been found to occur when the utilization of glucose was limited by hormonal and dietary conditions such as diabetes, starvation, or administration of a high protein - low carbohydrate diet (Rosen _et al_., 1958; Rosen _et al_., 1959; Segal _et al_., 1962; Szepesi and Freedland, 1958b and c; Szepesi and Freedland, 1969b). By extension to the ruminant animal, glutamic-pyruvic transaminase activity may indicate the initiation of increased rates of gluconeogenesis in the maturing animal. This enzyme activity would increase at the onset of rumen function when lesser amounts of glucose become available for intestinal absorption and greater rates of gluconeogenesis ensue or the activity of this enzyme may be controlled by constitutive processes independent of substrate alterations. It would appear that since the germfree animals were fed the same diet, the increased glutamic-pyruvic activity exhibited by the older group of animals was due to processes other than the initiation of gluconeogenesis because of substrate alterations. Also, the conventional animals in which ruminal fermentation and consequently substrate alterations were allowed to occur, no greater glutamic-pyruvic transaminase activity was detected.

From a statistical standpoint, definite conclusions as to the constitutive properties of this enzyme in the ruminant animal are indeed difficult to make. Further investigation into the factors controlling glutamic-pyruvic transaminase activity may reveal the processes controlling the maturation of the ruminant animal.

Glucose-6-phosphatase activity for the animals in the 1969 and 1970 trials appear in Tables 8 and 9 respectively. In 1969, there was a slight increase in mean enzyme activity in the germfree animals between the ages of 49 and 124 days of age when enzyme activity was calculated on a per gram hepatic protein or a per gram liver basis. On a body weight basis, however, there was a decrease in glucose-6-phosphatase activity in these animals. Conventional animals on the average showed higher enzyme activity but variability was great within this group. In 1970, these germfree pygmy goats fed the glucose diet had in general greater glucose-6-phosphatase activity than those germfree animals fed the VFA diet regardless of the method of enzyme activity calculation. The single conventional animal had the lowest activity of all the animals in the trial. In general, animals at 180 days of age from the 1970 trial had a higher glucose-6-phosphatase activity than the younger animals in the 1969 trial. There were no statistically significant differences demonstrated between treatment means for either year, 1969 or 1970.

The activity of glucose-6-phosphatase, a key enzyme in the gluconeogenic pathway, has been found to increase in response to starvation, diabetes and high protein-low carbohydrate diets (Langdon and Weakley, 1955; Freedland and Harper, 1957; Freedland and Harper, 1958a and b; Weber *et al*., 1965a,b and c; Freedland and Harper, 1966 and Szepesi and

TABLE 8. 1969 Glucose-6-Phosphatase Activity

Animal Number	Age of Animal (days)	Status	Glucose-6-Phosphatase		
			1[a]	2[b]	3[c]
20	49	GF	0.071	14.16	4967.3
21	49	GF	0.123	24.63	6820.0
		Mean ± S.E.M.	0.097± 0.026	19.39± 5.24	5893.0± 926.3
17	118	GF	0.102	20.53	3816.5
18	115	GF	0.093	18.65	3806.4
19	117	GF	0.110	22.05	3598.6
11	146	GF	0.133	26.55	7049.0
		Mean ± S.E.M.	0.110± 0.008	21.95± 1.68	4567.5± 828.6
14	151	CON[d]	0.434	86.79	22,305.0
15	121	CON[d]	0.054	10.88	2348.9
16	123	CON	0.079	15.81	3609.4
		Mean ± S.E.M.	0.189± 0.123	37.82± 24.52	9421.1± 6452.2

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[a] G-6-Pase activity: μ moles P_i cleaved per minute per milligram hepatic protein.

[b] G-6-Pase activity: μ moles P_i cleaved per minute per gram liver.

[c] G-6-Pase activity: μ moles P_i cleaved per minute per 10 kilogram body weight.

[d] Fed the pelleted germfree diet *ad libitum* in addition to sterile glucose diet.

TABLE 9. 1970 Glucose-6-Phosphatase Activity at Approximately 180 Days of Age

Animal Number	Status	Diet	Glucose-6-Phosphatase 1[a]	2[b]	3[c]
22	GF	Glucose	0.294	12.94	1953.9
24	GF	Glucose	0.357	16.29	3616.4
27	GF	Glucose	0.502	26.49	4662.2
	Mean ± S.E.M.		0.384±	18.57±	3410.8±
			0.062	4.10	788.5
23	GF	VFA	0.348	13.92	3354.7
25	GF	VFA	0.305	14.14	2417.9
26	GF	VFA	0.284	17.03	3255.7
	Mean ± S.E.M.		0.312±	15.03±	3002.7±
			0.018	1.00	294.4
30	CON	Glucose	0.352	19.14	3598.3
31	CON	Glucose	0.334	18.14	3101.9
	Mean ± S.E.M.		0.343±	18.64±	3350.1±
			0.009	0.50	248.2
28	CON	VFA	0.356	22.19	3040.0
32	CON	VFA	0.285	17.81	3223.6
	Mean ± S.E.M.		0.321±	20.00±	3131.8±
			0.035	±.19	91.8
36	CON	Pellets	0.093	13.12	1902.4

[a] G-6-Pase activity: µ moles P_i cleaved per minute per milligram hepatic protein.

[b] G-6-Pase activity: µ moles P_i cleaved per minute per gram liver.

[c] G-6-Pase activity: µ moles P_i cleaved per minute per 10 kilogram body weight.

Freedland, 1968c). Fisell et al. (1969) showed using adult sheep that fasting and diabetes increased the gluconeogenic capacity of the ruminant animal and the increased rate of gluconeogenesis was reflected by increased activity of the key gluconeogenic enzymes, glucose-6-phosphatase, fructose-1, 6-diphosphatase and pyruvate carboxylase. Bartley et al. (1966) showed that glucose-6-phosphatase increased in calves between the ages of 8 and 12 weeks to levels typical of the adult cow. Intraduodenal infusion of glucose into 15 week old calves decreased the activity of glucose-6-phosphatase and provided evidence for the adaptability of metabolic processes in the ruminant by substrate availability (Bartley et al., 1966).

In this study, low statistical significance of treatment means due to low animal numbers and large variability, make the interpretation of the results difficult. However, the slight increase in glucose-6-phosphatase activity with age in germfree animals fed the basal diet and the increased enzyme activity seen in the older animals in the 1970 trial compared to the animals in the 1969 trial suggest that constitutive processes may have operated to control this enzyme activity. Also, the time at which these processes exerted their effect was between 124 and 180 days of age in the pygmy goat. This perhaps explains the reason for the oldest animal in 1969 to also have the greatest glucose-6-phosphatase activity.

Animals, whether germfree or conventional, fed the glucose diet had greater glucose-6-phosphatase activity than those raised on the VFA diet and this was opposite to the effect of intraduodenally infused glucose in 15 week-old calves, Bartley _et al_. (1966). On the basis of this work, the availability of dietary glucose was expected to decrease gluconeogenesis and consequently the activity of glucose-6-phosphatase, while the VFA diet was expected to increase gluconeogenesis if metabolic pathways are influenced by substrate availability. Explanations for the greater activity in those animals fed the glucose diet are difficult to make particularly since Purser and Bergen (1969) found that the glucose-6-phosphatase activity of their germfree and conventional pygmy goats was comparable. As in the case of glutamic-pyruvic transaminase activity, further work with glucose-6-phosphatase may reveal the controlling processes of maturation in the ruminant.

Pyruvate kinase enzyme activity is presented in Tables 10 and 11 for the 1969 and 1970 trials respectively. In 1969, the group of germfree animals at 124 days of age had a greater mean pyruvate kinase enzyme activity compared to those germfree animals at 49 days, when enzyme activity was calculated on a per milligram hepatic protein or a per gram liver basis. The converse was true when activity was calculated on a body weight basis. Mean pyruvate kinase activity in the conventional animals was less than that observed in the older groups of germfree animals when activity was calculated on a per milligram hepatic protein

TABLE 10. 1969 Pyruvate Kinase Activity

Animal Number	Age of Animal (days)	Status	Pyruvate Kinase		
			1[a]	2[b]	3[c]
20	49	GF	0.104	8.00	2806.4
21	49	GF	0.099	6.45	1786.0
		Mean ± S.E.M.	0.102± 0.003	7.23± 0.78	2296.2± 510.2
17	118	GF	0.119	10.19	1894.3
18	115	GF	0.124	8.70	1775.7
19	117	GF	0.099	7.18	1171.7
11	146	GF	0.124	9.33	2477.1
		Mean ± S.E.M.	0.116± 0.006	8.85± 0.63	1829.7± 267.6
14	151	CON[d]	0.116	10.82	2780.7
15	121	CON[d]	0.109	10.46	2258.3
16	123	CON	0.119	9.52	2173.4
		Mean ± S.E.M.	0.112± 0.002	10.26± 0.38	2404.1± 189.8

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[a] Pyruvate kinase activity: µ moles NADH oxidized per minute per milligram hepatic protein.

[b] Pyruvate kinase activity: µ moles NADH oxidized per minute per gram liver.

[c] Pyruvate kinase activity: µ moles NADH oxidized per minute per 10 kilogram body weight.

[d] Fed the pelleted germfree animal diet *ad libitum* in addition to sterile glucose diet.

TABLE 11. 1970 Pyruvate Kinase Activity at Approximately 180 days of age

Animal Number	Status	Diet	Pyruvate Kinase 1[a]	2[b]	3[c]
22	GF	Glucose	0.072	14.4	2174.4
24	GF	Glucose	0.123	24.6	5461.2
27	GF	Glucose	0.143	28.6	5033.6
	Mean ± S.E.M.		0.112± 0.021	22.5± 4.2	4223.1± 1031.8
23	GF	VFA	0.149	29.8	7181.8
25	GF	VFA	0.145	29.0	4959.0
26	GF	VFA	0.137	27.4	5206.0
	Mean ± S.E.M.		0.144± 0.004	28.7± 0.7	5782.2± 703.4
30	CON	Glucose	0.179	35.8	6730.4
31	CON	Glucose	0.136	27.2	4651.2
	Mean ± S.E.M.		0.158± 0.022	31.5± 4.3	5690.8± 1039.6
28	CON	VFA	0.097	19.4	2657.8
32	CON	VFA	0.171	34.2	6190.2
	Mean ± S.E.M.		0.134± 0.037	26.8± 7.4	4424.0± 1766.2
36	CON	Pellets	0.099	19.96	2894.2

[a] Pyruvate kinase activity: µ moles NADH oxidized per minute per milligram hepatic protein.

[b] Pyruvate kinase activity: µ moles NADH oxidized per minute per gram liver.

[c] Pyruvate kinase activity: µ moles NADH oxidized per minute per 10 kilogram body weight.

basis but greater when the other two methods for the calculation of enzyme activity were used. In 1970, there were no recognizable trends in the data in relation to the treatments imposed, i.e. germfree versus conventional status or dietary regime. There were no statistically significant differences demonstrated between treatment means in either the 1969 or 1970 trials.

Pyruvate kinase has been shown to be a control enzyme in the glycolytic pathway as the activity of this enzyme is influenced by certain dietary and hormonal conditions. In the nonruminant animal, decreased enzymatic activity due to starvation, diabetes and low carbohydrate diets has been shown to occur (Krebs and Eggleston, 1965; Weber *et al*., 1965d; Yudkin and Krauss, 1967; Szepesi and Freedland, 1968a; Sillero *et al*., 1969 and Szepesi and Freedland, 1969a). In the ruminant animal, Goetsch (1966) found a gradual decrease in glycolytic activity as calves matured over an eight week period. This decrease was thought to be a consequence of decreased availability of glucose for absorption as a consequence of rumen development.

On the basis of the preceding, it was thought that glycolytic activity in the pygmy goat would be controlled by the availability of dietary glucose. In the 1969 trial, germfree animals at any age would have similar pyruvate kinase enzyme activities and their activity would be greater than the activity determined in conventional animals in which rumen function was allowed to develop. Compared to the younger group of germfree animals, average pyruvate kinase activity was greater in the older group of germfree animals when activity was calculated on a per milligram hepatic protein and per gram liver basis but lesser on a body weight basis. The method of calculation more indicative of true metabolic processes is questionable but more importantly, mean emzyme activity did not appear similar between these two groups fed the same diet. Pyruvate kinase activity in the conventional animals was slightly lesser or even greater than the activity in the older germfree animals, depending on the method of activity calculation. Again, the behavior of this enzyme activity was different than that which was expected. In the 1970 trial, the glucose diet was expected to maintain pyruvate kinase activity above the activity determined in animals fed the VFA diet. Also, the conventional animal was expected to have an activity comparable to the animals fed the VFA diet. The mean pyruvate activity between treatment groups varied but any relationships between the glucose and VFA diet or germfree and conventional status of the animal and the activity of this enzyme were not discernible. The lack of response of this enzyme to imposed treatments is reflected in low statistically significant differences between treatment means for both years and is similar to the findings of Purser and Bergen (1969).

In these animals, pyruvate kinase activity may not be the appropriate measure of glycolytic activity as its response seemed not to be influenced by the availability of dietary glucose. Also, within the limits of this experiment, other processes which act to control pyruvate kinase activity were not identified. Perhaps, a better means of assessing glycolytic activity in these animals must first be found before controlling factors may be determined.

Since pyruvate kinase activity was assayed one day after glucose tolerance tests were given, correlation coefficients between pyruvate kinase activity and glucose half-life were determined individually for the two years. The correlation coefficient between mean pyruvate kinase activity and mean glucose half-life for the three treatment groups in 1969 was -0.690 ($P < 0.05$) and in 1970 the correlation coefficient was -0.680 ($P < 0.01$). The animals with the greatest pyruvate kinase activity had the shortest glucose half-life or fastest rate of glucose utilization. This relationship between pyruvate kinase and glucose utilization in germfree and conventional goats was also reported by Purser and Bergen (1969), however they obtained a higher correlation coefficient of -0.949 ($P < 0.01$). Interestingly, there seems to be a relationship between this enzyme activity and the rate of glucose utilization in these animals, but this enzyme seems to be unaffected by the availability of dietary carbohydrate. It would seem that in the pygmy goat at least, differences in pyruvate kinase activity and therefore, the rate of glucose utilization occur between animals but the factors affecting individual enzyme activity are not clear. Further elucidation of the processes controlling these changes in the ruminant may reveal more major metabolic differences between the ruminant and nonruminant and contribute to the better understanding of ruminant metabolism.

Hepatic glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities for the 1970 trial are presented in Tables 12 and 13. In the case of glucose-6-phosphate dehydrogenase, the mean activity for the animals fed the VFA diet was greater and significantly different from the mean activity of the animals fed the glucose diet. Mean enzyme activity (μ moles/min) for the animals consuming the VFA diet was 2.41, 0.482 and 85.2 (per milligram hepatic protein, per gram liver and per 10 kilograms body weight) as compared to 1.65, 0.329 and 59.2 for the animals fed the glucose diet and these means were statistically different at the $P < 0.025$ level. There were no statistically significant differences between treatment means for 6-phosphogluconate dehydrogenase activity.

Starvation, diabetes, low carbohydrate and high fat diets have been shown to decrease with activity of glucose-6-phosphate dehydrogenase (Niemeyer et al., 1962; Ono et al., 1963; Vaughn and Winders, 1964; Benevenga et al., 1964; McDonald and Johnson, 1965; Peraino, 1967 and Szepesi and Freedland, 1968c). Howarth et al. (1968) showed that the activities of glucose-6-phosphate and 6-phosphogluconate dehydrogenase in adipose tissue were higher in steers than

TABLE 12. 1970 Glucose-6-Phosphate Dehydrogenase Activity to Approximately 180 Days of Age

			Glucose-6-Phosphate Dehydrogenase		
Animal Number	Status	Diet	1[a]	2[b]	3[c]
22	GF	Glucose	1.83	0.366	55.3
24	GF	Glucose	1.53	0.306	67.9
27	GF	Glucose	2.57	0.514	90.5
	Mean ± S.E.M.		1.98± 0.31	0.395± 0.062	71.2± 10.3
23	GF	VFA	1.84	0.368	88.7
25	GF	VFA	2.49	0.498	85.2
26	GF	VFA	2.53	0.506	96.1
	Mean ± S.E.M.		2.28± 0.22	0.457± 0.045	90.0± 3.2
30	CON	Glucose	1.19	0.238	44.7
31	CON	Glucose	1.44	0.288	49.7
	Mean ± S.E.M.		1.32± 0.12	0.263± 0.025	46.9± 2.2
28	CON	VFA	2.58	0.516	70.7
32	CON	VFA	2.49	0.498	90.1
	Mean ± S.E.M.		2.54± .04	0.507± .009	80.4± 9.7
36	CON	Pellets	3.21	0.642	93.1

[a] Glucose-6-phosphate dehydrogenase activity: µ moles NADP reduced per minute per milligram hepatic protein.

[b] Glucose-6-phosphate dehydrogenase activity: µ moles NADP reduced per minute per gram liver.

[c] Glucose-6-phosphate dehydrogenase activity: µ moles NADP reduced per minute per 10 kilogram body weight.

TABLE 13. 1970 6-Phosphogluconate Dehydrogenase Activity at Approximately 180 Days of Age

			6-Phosphogluconate Dehydrogenase		
Animal Number	Status	Diet	1[a]	2[b]	3[c]
22	GF	Glucose	5.29	1.06	159.8
24	GF	Glucose	5.90	1.18	261.9
27	GF	Glucose	5.35	1.07	188.3
	Mean ± S.E.M.		5.51± 0.19	1.10± 0.04	203.3± 30.4
23	GF	VFA	6.84	1.36	329.7
25	GF	VFA	4.96	0.99	169.6
26	GF	VFA	6.77	1.35	257.3
	Mean ± S.E.M.		6.19± 0.61	1.23± 0.12	252.2± 46.3
30	CON	Glucose	5.91	1.18	222.2
31	CON	Glucose	6.53	1.31	223.3
	Mean ± S.E.M.		6.22± 0.31	1.24± .06	222.8± 0.5
28	CON	VFA	5.11	1.02	140.0
32	CON	VFA	7.69	1.54	278.3
	Mean ± S.E.M.		6.40± 1.29	1.28± 0.26	209.2± 69.2
36	CON	Pellets	6.38	1.28	185.0

[a] 6-Phosphogluconate dehydrogenase activity: m μ moles NADP reduced per minute per milligram hepatic protein.

[b] 6-Phosphogluconate dehydrogenase activity: μ moles NADP reduced per minute per gram liver.

[c] 6-Phosphogluconate dehydrogenase activity: μ moles NADP reduced per minute per 10 kilogram body weight.

calves and that there were greater changes in these enzymes in adipose tissue than in liver and muscle tissues. They suggested that this was a reflection of a greater capacity for fat synthesis in the mature ruminant. The activities of these enzymes however were found to be lower in steers than in calves fed a whole milk diet twice daily (Howarth et al., 1968).

In this experiment, animals fed the glucose diet had lower glucose-6-phosphate dehydrogenase activity than the animals fed the VFA diet which is difficult to reconcile as this is contrary to the observations of others. It is attractive to hypothesize that in these goats fed the VFA diet, enzyme changes have taken place which severely limit the amount of glucose that can be used for lipogenesis. This change is brought about by decreased activity of the citrate cleavage pathway (Ballard et al., 1969). Animals which were fed the VFA diet then had a source of acetate and butyrate which are efficient precursors for lipid synthesis and had a correspondingly greater need for $NADPH_2$, greater than the animals fed the glucose diet. Greater glucose-6-phosphate dehydrogenase activity then in the VFA diet-fed animals may reflect a greater need for $NADPH_2$ due to greater rates of lipogenesis. If this is the case, volatile fatty acids may be acting to control which precursor for lipogenesis is used predominantly. This is pure conjecture but may explain at least in part the observed relationship between dietary VFA and increased glucose-6-phosphate dehydrogenase.

Hepatic metabolic substrates for the 1970 trial are presented in Table 14. Liver tissue concentrations of glucose-6-phosphate and phosphoenolpyruvate were determined, since changes in tissue levels of metabolic intermediates may occur which could produce metabolic changes in the absence of enzyme adaptions.

Animals fed the glucose diet had a mean tissue phosphoenolpyruvate concentration of 0.0330 μ mole/g liver which was significantly different at the $P < 0.25$ level from the mean concentration of 0.0229 μ mole/g liver for those animals fed the VFA diet. This possibly indicates a limitation in pyruvate kinase activity of these pygmy goats fed the glucose diet compared to animals consuming the VFA diet and may be due to the greater amount of carbohydrate in the glucose diet. It would appear then at least that greater amounts of dietary glucose may not increase glycolytic activity in these animals. This along with the fact that pyruvate kinase activity did not respond to the dietary treatments suggest that glycolytic activity in pygmy goats at this age (180 days) may become refractory to increased dietary carbohydrate.

The concentrations of glucose-6-phosphate were variable and no consistent pattern was discernible. Increased concentrations of glucose-6-phosphate in those animals with increased amounts of phosphoenolpyruvate would indicate a decreased rate of glycolytic activity and be consistent with the work of Bartley *et al*. (1966). However, in these

TABLE 14. 1970 Hepatic Metabolic Substrates

Animal Number	Status	Diet	Glucose-6 Phosphate (μ moles/g liver)	Phospho-enolpyruvate (μ moles/g liver)
22	GN	Glucose[a]	-	-
24	GN	Glucose	0.250	0.0373
27	GN	Glucose	0.035	0.0257
		Mean ± S.E.M.	0.143± 0.108	0.0315± 0.0058
23	GN	VFA[b]	0.792	0.0167
25	GN	VFA	0.284	0.0244
26	GN	VFA	0.485	0.0271
		Mean ± S.E.M.	0.520± 0.148	0.0227± .0031
30	CON	Glucose	0.284	0.0258
31	CON	Glucose	0.0690	0.0434
		Mean ± S.E.M.	0.177± 0.108	0.0346± 0.0088
28	CON	VFA	0.021	0.0200
32	CON	VFA	0.064	0.0263
		Mean ± S.E.M.	0.042± 0.022	0.0231± 0.0032
36	CON	Pellets[c]	0.023	0.0312

[a] Table No. 2

[b] Table No. 2

[c] Table No. 3

animals such a relationship did not appear. Conventional animals had the lowest level of this metabolite and this could indicate a greater rate of gluconeogenesis. Animals with greater glucose-6-phosphatase activity did not consistently have lesser amounts of glucose-6-phosphate. A better understanding of the overall metabolic processes in these animals would come from knowing the levels of more hepatic intermediates.

Tables 15, 16, 17 and 18 present the essential and non-essential plasma and liver free amino acid patterns of the animals in the 1970 trial. Similar analyses were not performed in 1969. Plasma and liver samples were taken after a 16 hour fast, when the animal was approximately 180 days of age. The data were not analyzed statistically because of their consistent patterns.

The major reason for these analyses stems from the work of Felig *et al*. (1970) which showed that in the post-absorptive human, plasma alanine concentration was 344 μ moles/ liter and that this amino acid accounted for approximately 50 percent of the total net amino acid consumption of liver with glycine, serine and threonine supplying most of the remainder. This amino acid uptake by the liver was determined by arteriohepatic venous (A-HV) difference. After a five to six week fast, the absolute amounts of the A-HV difference for the individual amino acids were greatly reduced but alanine still accounted for approximately 50 percent of the total new amino acid consumption. Under this condition of extended fast, glycine, serine and threonine

TABLE 15. Plasma Essential Amino Acids (μ moles/ml)

Animal Number	Status	Diet	Lys	His	Arg	Thr	Val	Met	Ile	Leu	Phe
22	GF	Glucose	0.076	0.074	0.186	0.047	0.134	0.033	0.037	0.079	0.039
24	GF	Glucose	0.042	0.076	0.175	0.067	0.173	0.044	0.065	0.120	0.042
27	GF	Glucose	0.067	0.044	0.177	0.046	0.161	0.034	0.046	0.095	0.031
	Mean ± S.E.M.		0.062± 0.010	0.065± 0.010	0.179± 0.003	0.053± 0.007	0.156± 0.012	0.036± 0.004	0.049± 0.008	0.098± 0.012	0.038± 0.003
23	GF	VFA	0.028	0.059	0.132	0.023	0.104	0.039	0.077	0.061	0.023
25	GF	VFA	0.072	0.053	0.163	0.034	0.099	0.033	0.036	0.066	0.024
26	GF	VFA	0.065	0.066	0.219	0.042	0.132	0.028	0.043	0.082	0.033
	Mean ± S.E.M.		0.055± 0.014	0.059± 0.004	0.171± 0.025	0.032± 0.006	0.112± 0.009	0.034± 0.003	0.052± 0.012	0.069± 0.006	0.027± 0.003
30	CON	Glucose	0.050	0.064	0.139	0.037	0.106	0.018	0.028	0.054	0.021
31	CON	Glucose	0.041	0.053	0.159	0.048	0.120	0.033	0.049	0.087	0.032
	Mean ± S.E.M.		0.045± 0.005	0.059± 0.006	0.149± 0.010	0.043± 0.005	0.113± 0.007	0.026± 0.008	0.039± 0.009	0.071± 0.016	0.026± 0.005±
28	CON	VFA	0.058	0.044	0.109	0.056	0.174	0.035	0.045	0.105	0.048
32	CON	VFA	0.049	0.043	0.150	0.043	0.100	0.024	0.033	0.063	0.036
	Mean ± S.E.M.		0.054± 0.005	0.044± 0.001	0.130± 0.020	0.049± 0.006	0.137± 0.036	0.029± 0.005	0.039± 0.006	0.084± 0.021	0.042± 0.006
36	CON	Pellets	0.133	0.062	0.232	0.073	0.204	0.035	0.074	0.098	0.031

TABLE 16. Plasma Non-essential Amino Acids (μ moles/ml)

Animal Number	Status	Diet	Orn	NML	Asp	Ser	Asn	Glu	Gln	Pro	Gly	Ala	Cys	Tyr
22	GF	Glucose	0.054	0.202	0.008	0.208	0.023	0.094	0.053	0.137	0.471	0.246	0.019	0.054
24	GF	Glucose	0.064	0.096	0.011	0.195	0.029	0.109	0.062	0.126	0.418	0.260	0.034	0.048
27	GF	Glucose	0.060	0.076	0.009	0.057	0.018	0.075	0.147	0.087	0.496	0.157	0.198	0.038
	Mean ± S.E.M.		0.059±	0.124±	0.009±	0.154±	0.024±	0.092±	0.087±	0.117±	0.462±	0.221±	0.083±	0.046±
			0.003	0.039	0.001	0.048	0.003	0.009	0.029	0.015	0.023	0.032	0.057	0.004
23	GF	VFA	---	0.125	0.011	0.076	0.013	0.092	0.037	0.075	0.473	0.190	0.081	0.025
25	GF	VFA	0.078	0.131	0.071	0.007	0.017	0.091	0.037	0.076	0.447	0.179	0.019	0.022
26	GF	VFA	---	0.088	0.012	0.083	0.025	0.102	0.104	0.120	0.491	0.219	0.185	0.033
	Mean ± S.E.M.		---	0.115±	0.031±	0.055±	0.018±	0.095±	0.059±	0.090±	0.470±	0.196±	0.095±	0.026±
			---	0.014	0.019	0.024	0.004	0.004	0.022	0.015	0.013	0.012	0.048	0.003
30	CON	Glucose	---	0.073	0.008	0.065	0.019	0.075	0.081	0.088	0.445	0.131	0.016	0.025
31	CON	Glucose	---	0.059	0.014	0.074	0.025	0.102	0.073	0.099	0.477	0.154	0.018	0.034
	Mean ± S.E.M.		---	0.066±	0.011±	0.069±	0.022±	0.088±	0.076±	0.093±	0.461±	0.142±	0.017±	0.029±
			---	0.007	0.003	0.004	0.003	0.013	0.004	0.005	0.016	0.012	0.001	0.004
28	CON	VFA	---	0.081	0.005	0.064	0.022	0.086	0.067	0.061	0.263	0.207	0.021	0.052
32	CON	VFA	---	0.079	0.014	0.098	0.022	0.079	0.079	0.109	0.728	0.175	0.019	0.033
	Mean ± S.E.M..		---	0.081±	0.009±	0.081±	0.022±	0.083±	0.073±	0.085±	0.496±	0.191±	0.020±	0.043±
			---	0.001	0.004	0.017	0.000	0.003	0.006	0.024	0.232	0.016	0.001	0.009
36	CON	Pellets	0.035	0.059	0.017	0.038	0.024	0.107	0.037	0.070	0.371	0.184	0.044	0.041

TABLE 17. Liver Free Essential Amino Acids (μ moles/g)

Animal Number	Status	Diet	Lys	His	Arg	Thr	Val	Met	Ile	Leu	Phe
22	GF	Glucose	0.049	0.074	0.021	0.044	0.032	0.026	0.017	0.048	0.016
24	GF	Glucose	0.066	0.081	0.030	0.032	0.042	0.034	0.017	0.057	0.013
27	GF	Glucose	0.065	0.061	0.029	0.035	0.041	0.029	0.020	0.055	0.015
	Mean ± S.E.M.		0.061± 0.005	0.072± 0.006	0.027± 0.003	0.037± 0.004	0.038± 0.003	0.029± 0.002	0.018± 0.001	0.053± 0.002	0.014± 0.001
23	GF	VFA	0.049	0.085	0.024	0.029	0.032	0.026	0.016	0.044	0.011
25	GF	VFA	0.065	0.059	0.031	0.041	0.036	0.025	0.021	0.055	0.014
26	GF	VFA	0.046	0.092	0.028	0.035	0.041	0.027	0.021	0.059	0.015
	Mean ± S.E.M.		0.053± 0.006	0.078± 0.009	0.027± 0.019	0.036± 0.003	0.036± 0.003	0.026± 0.000	0.019± 0.002	0.052± 0.004	0.013± 0.001
30	CON	Glucose	0.068	0.069	0.024	0.040	0.046	0.034	0.022	0.068	0.016
31	CON	Glucose	0.063	0.050	0.026	0.057	0.048	0.036	0.024	0.069	0.023
	Mean ± S.E.M.		0.066± 0.003	0.059± 0.009	0.025± 0.002	0.048± 0.008	0.047± 0.001	0.035± 0.001	0.023± 0.001	0.068± 0.000	0.019± 0.004
28	CON	VFA	0.121	0.066	0.032	0.054	0.052	0.035	0.024	0.068	0.020
32	CON	VFA	0.059	0.065	0.032	0.034	0.039	0.032	0.019	0.027	0.014
	Mean ± S.E.M.		0.090± 0.031	0.065± 0.000	0.032± 0.000	0.044± 0.010	0.045± 0.006	0.033± 0.001	0.022± 0.002	0.048± 0.021	0.017± 0.003
36	CON	Pellets	0.061	0.073	0.027	0.035	0.041	0.029	0.018	0.047	0.013

TABLE 18. Liver Free Non-essential Amino Acids (μ moles/g)

Animal Number	Status	Diet	Orn	NML	Asp	Ser	Asn	Glu	Pro	Gly	Ala	Cys	Tyr
22	GF	Glucose	0.479	0.057	0.171	0.577	0.025	1.346	0.060	0.785	0.332	0.043	0.012
24	GF	Glucose	0.348	0.042	0.176	0.534	0.040	1.178	0.069	0.632	0.372	0.062	0.022
27	GF	Glucose	0.214	0.048	0.149	0.221	0.030	0.821	0.057	1.005	0.358	0.031	0.199
	Mean ± S.E.M.		0.347± 0.076	0.049± 0.004	0.165± 0.008	0.444± 0.112	0.032± 0.004	1.115± 0.154	0.062± 0.004	0.807± 0.108	0.353± 0.012	0.045± 0.009	0.078± 0.061
23	GF	VFA	0.320	0.046	0.087	0.267	0.034	0.507	0.030	0.404	0.309	0.027	0.224
25	GF	VFA	0.401	0.059	0.223	0.747	0.046	0.985	0.043	0.934	0.268	0.095	0.019
26	GF	VFA	0.385	0.040	0.163	0.293	0.042	1.586	0.056	0.884	0.467	0.093	0.186
	Mean ± S.E.M.		0.368± 0.025	0.049± 0.006	0.157± 0.039	0.435± 0.156	0.040± 0.004	1.025± 0.312	0.043± 0.007	0.740± 0.169	0.347± 0.061	0.075± 0.019	0.143± 0.063
30	CON	Glucose	0.253	0.025	0.149	0.193	0.048	1.034	0.071	0.788	0.239	0.067	0.031
31	CON	Glucose	0.281	0.036	0.298	0.169	0.042	1.570	0.173	1.509	0.227	0.235	0.033
	Mean ± S.E.M.		0.266± 0.014	0.030± 0.005	0.223± 0.074	0.181± 0.012	0.045± 0.003	1.302± 0.268	0.122± 0.051	1.148± 0.361	0.233± 0.006	0.151± 0.084	0.031± 0.001
28	CON	VFA	0.189	0.069	0.302	0.422	0.039	0.795	0.128	0.718	0.583	0.028	0.029
32	CON	VFA	0.363	0.046	0.144	0.227	0.034	1.132	0.053	0.937	0.254	0.066	0.019
	Mean ± S.E.M.		0.276± 0.086	0.578± 0.012	0.223± 0.079	0.325± 0.098	0.037± 0.003	0.964± 0.168	0.090± 0.037	0.828± 0.109	0.418± 0.164	0.048± 0.019	0.024± 0.004
36	CON	Pellets	0.205	0.021	0.130	0.114	0.263	0.747	0.052	0.695	0.283	0.108	0.016

were variable in their contribution to net amino acid consumption.

The source of increased plasma alanine levels in the post-absorptive human was determined to be increased mobilization of peripheral muscle amino acids. This was shown by arterial-venous (A-V) difference across the forearm. Over a four to six week fast the amounts of all plasma acids tested by A-V difference were greatly reduced, indicating decreased mobilization of amino acids from muscle with duration of the stress.

The results from the 1970 trial show that there are no large, apparent increases in plasma or liver free alanine, glycine, serine or threonine concentration nor are there increases for any other amino acid in the various treatment groups (Tables 15, 16, 17 and 18). It was expected that if the ruminant behaved like the human under conditions which increase the rate of gluconeogenesis, noticeable increases in certain plasma amino acids would have appeared particularly in the germfree goats fed the "volatile fatty acid" diet and to a lesser extent in the conventional animals fed the "glucose" or "volatile fatty acid" diet and the conventional goat fed the pelleted diet.

Since Felig <u>et</u> <u>al</u>. (1970) showed that, with time in the severely fasted human, the plasma concentrations of gluconeogenic amino acids decreased until almost normal patterns were reattained, differences in amino acid patterns of these goats due to treatments which increase the rate of gluconeogenesis would be greatly lessened after 180 days duration. Plasma amino acid concentrations would certainly be given enough time to readjust under the prolonged stress. If the ruminant reacts like the human to gluconeogenic stress, the increases in alanine or other gluconeogenic amino acids due to their mobilization from muscle would have been most apparent just after the initiation of the treatments expected to increase gluconeogenesis. Because of the type of liquid diet that was fed to the animals in this experiment and the fact that they were fed by nipple-bottle, differences between the post absorptive state of the germfree or conventional goat and the human due to previous diet may be minimal.

An explanation of the differences in plasma and liver amino acid patterns could be different rates of amino acid mobilization and utilization between the two species. Apparently in the post absorptive state, amino acid mobilization from muscle tissue in the human is faster than the rate of their utilization as gluconeogenic precursors and this accounts for the transitory elevation of plasma amino acids. In the post-absorptive ruminant, if the rate of mobilization of muscle tissue amino acids was faster or slower than the rate of their utilization for gluconeogenesis, then increased or decreased levels of plasma amino acids would have been observed, respectively. Also, if the rate of utilization of amino acids was in equilibrium with the rate of amino acid mobilization no change would be observed.

Unfortunately, to make this kind of comparison of plasma amino acid levels the amino acid pattern of fed animals should have been determined but this was not done in this experiment.

Comparisons can be made between groups of animals in which the rate of gluconeogenesis was expected to be greater than those groups where more normal metabolic patterns were expected. Since these comparisons show no difference in plasma amino acid levels, the lack of sufficient numbers of animals per group or diets which do not exaggerate differences in overall patterns of metabolism are at fault.

Another reason for the lack of apparent differences among the groups of animals may come from the supposed constitutive control of gluconeogenesis in the ruminant (Purser and Bergen, 1969). If there is an inherent rate of gluconeogenesis in the ruminant and if the choice of diets or germfree status in this experiment were not able to exaggerate the rate of utilization of amino acids as gluconeogenic precursors, differences in the amino acid levels of the animals in the post-absorptive state between groups will not be apparent. These circumstances best explain the observed results.

The plasma non-essential to essential amino acid ratios (NEAA/EAA) for each treatment group of the 1970 trial are shown in Table 19. These ratios were calculated from the mean amino acid concentrations of each treatment group. The samples upon which the plasma amino acid determinations were

TABLE 19. Plasma Non-essential to Essential Amino Acid Ratio[a]

Status	Diet	NEAA[b]/EAA[c]
GF	Glucose	1.93
GF	VFA	2.05
CON	Glucose	1.89
CON	VFA	1.95
CON	Pellets	1.09

[a] Calculated from the mean amino acid concentrations of each treatment group.

[b] Non-essential amino acid.

[c] Essential amino acid.

made, were taken from the animals after a 16 hour fast at approximately 180 days of age.

The NEAA/EAA ratio has been used clinically as an indicator of kwashiorkor or protein-calorie malnutrition in humans (Waterlow, 1969). These ratios were examined in this set of experiments to generally assess the effect of the various diets on the protein-calorie nutrition of the animal and to determine whether the overall results of the experiment must be discussed in terms of an additional dietary effect.

The discussion of the results proceeds from the work of Oltjen _et_ _al_. (1969). In their experiments, heifer and bull calves were maintained before and after weaning on three experimental diets fed _ad_ _libitum_. These diets were a natural diet, a purified diet containing urea and a purified diet containing protein. The natural diet consisted essentially of cracked corn, alfalfa and timothy hay and soybean meal. The purified diets contained in common wood pulp, corn starch and cerelose in comparable amounts but differed in that one purified diet contained 4.7 percent urea and the other 14.9 percent isolated soy protein. Besides studying blood protein patterns and blood glucose levels at 42 and 84 days of age and hair amino acid content at 210 days of age, blood plasma free amino acid patterns were determined at 42, 84 and 210 days.

Although Oltjen _et_ _al_. (1969) did not calculate the NEAA /EAA ratios from their plasma amino acid data for the treatment groups, this calculation yields interesting results. The NEAA/EAA ratios for the heifers consuming the natural diet were very consistent with age. These data were 1.68, 1.88 and 1.65 at 42 , 84 and 210 days of age (all subsequent ratios were calculated at these ages). NEAA/EAA ratios for those heifers consuming the soy diet (2.18, 2.36 and 1.88) were more variable but comparable to the above ratios. The ratios were consistently higher and increased steadily for those heifers consuming the urea diet (2.12, 2.61 and 3.29). Similar results were obtained from the data on bull calves, however, no bull calves consumed the natural diet. The NEAA/EAA ratios for bull calves consuming the soy diet were fairly consistent and comparable to those ratios for heifers on the same diet. They were 2.21, 2.34 and 1.24, while for those bull calves on the urea diet they were 2.42, 3.50 and 4.39. Again, as for the heifers on the urea diet, ratios were consistently higher and steadily increased with age.

Since the natural diet contained only 10% more gross energy than the urea diet and only 5% more gross energy than the soy diet, these ratios were affected by differences in the amount of protein presented to the animal for utilization The NEAA/EAA ratios which were consistently higher and steadily increased with age for both those heifers and bull calves consuming the urea diet reflected decreased amounts of microbial protein for utilization by the animal. These results showed that for the ruminant the NEAA/EAA ratio is an indicator of the state or protein-calorie nutrition.

Normal ratios were approximately 2.0 as it is in the human (Waterlow, 1969) and as protein-calorie malnutrition ensued, these values increased.

The NEAA/EAA ratios in Table 19 range from 1.09 to 2.05 over the treatment groups of the 1970 trial. Interpretation of these ratios from the work of Oltjen *et al*. would indicate that the animals in this experiment were normal with respect to protein-calorie nutrition. The importance of this conclusion is that results of this experiment were not confounded by diets which did not meet the protein-calorie requirements of the animal and complicate the interpretation of the results by an additional dietary stress.

V GENERAL DISCUSSION

The cause of the decrease in blood glucose and increase in glucose half-life in the ruminant animal has been investigated many times in the past. In general, these phenomenon have been attributed to either changes in ration and therefore substrates at the onset of rumen development or processes which proceed independent of ration and rumen function. The specific cause or causes for these changes are yet unknown.

It was realized that as the ruminant adapted first from carbohydrate utilization prenatally to a high carbohydrate and lipid diet postnatally and finally to the utilization of VFA at maturity, many metabolic changes must take place. The increased dependence on gluconeogenesis in the mature animal compared to the immature ruminant must be made manifest in alterations in the activities of key gluconeogenic and glycolytic enzymes. Enzyme studies with the ruminant animal have in general shown that gluconeogenesis increased and glycolytic activity decreased as the ruminant matured and the changes were related to decreased availability of glucose for intestinal absorption. Most research published would support this view that alterations in enzyme activity were substrate dependent. Preliminary work with germfree goats questioned this conclusion (Purser and Bergen, 1969) and this present work was initiated to further define the factors which effect the observed changes in ruminant metabolism.

In this study with germfree and conventional pygmy goats, plasma glucose decreased and glucose and acetate half-lives increased significantly with advancing age of the animal and these changes were unrelated to dietary glucose or VFA. Glutamic-pyruvic transaminase and glucose-6-phosphatase activities increased with age while pyruvate kinase activity was variable. The activities of these enzymes were also unaffected by the imposed dietary treatments. In addition, an inverse relationship between pyruvate kinase activity and glucose half-life was found. In conclusion, the metabolic values which were studied in this experiment underwent changes characteristic of the maturing ruminant, but these changes were constitutive and, unrelated to direct dietary effects.

VI CONCLUSIONS

1. In germfree and conventional pygmy goats, fasting plasma glucose concentrations decreased from 98.4 to 71.6 mg% ($P < 0.005$) between the ages of 60 and 180 days.

2. Dietary glucose did not maintain fasting plasma glucose levels in germfree or conventional animals with time.

3. Glucose half-life increased from 16.8 to 30.3 minutes in germfree and conventional animals between the ages of 60 and 180 days and the increase was significant at the $P < 0.01$ level.

4. Decreased tolerance to glucose did not depend on animal type (germfree versus conventional) or diet (glucose versus VFA).

5. Acetate half-life increased from 2.58 to 4.50 minutes between the ages of 60 and 180 days in germfree and conventional animals and this increase was significant at the $P < 0.005$ level.

6. Decreased tolerance to acetate did not depend on animal type or diet.

7. Hepatic glutamic-pyruvic transaminase activity increased

with age in germfree animals fed identical diets.

8. Hepatic glucose-6-phosphatase activity increased with age in germfree and conventional animals and there were no significant dietary interactions.

9. Hepatic pyruvate kinase activity was unaffected by dietary treatments.

10. A correlation coefficient of -0.690 ($P < 0.05$) in 1969 and -0.680 ($P < 0.01$) in 1970 was obtained for the relationship between pyruvate kinase activity and glucose half-life values.

11. Relationships between hepatic glucose-6-phosphate and phosphoenolpyruvate and overall patterns of glucose utilization were inconclusive.

12. The NEAA/EAA ratios in these animals from plasma amino acid studies ranged from 1.09 to 2.05 indicating normal animals with respect to protein-calorie nutrition.

13. In all the parameters studied, there were no mean value differences due to the germfree or conventional status of this animal (pygmy goat).

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