To the Graduate Council:

I am submitting herewith a thesis written by Scott C. Sensenig entitled "Energy Metabolite and Glucoregulatory Hormone Concentrations and Net Fluxes Across Portal-Drained Viscera, Hepatic, Total Splanchnic and Peripheral Tissues in Gestating, Lactating and Weaning Ewes." I have examined the final copy of this thesis for form and content and recommend that if be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

R. N. Heitmann, Major Professor

We have read this thesis
and recommend its acceptance:

James K. Miller

Accepted for the Council:

Vice Provost
and Dean of the Graduate School

ENERGY METABOLITE AND GLUCOREGULATORY HORMONE CONCENTRATIONS AND NET FLUXES ACROSS PORTAL-DRAINED VISCERA, HEPATIC, TOTAL SPLANCHNIC AND PERIPHERAL TISSUES IN GESTATING, LACTATING AND WEANING EWES

A Thesis

Presented for the

Master of Science Degree

The University of Tennessee

Knoxville, Tennessee

Scott Charles Sensenig

June 1987

Dedicated

in loving memory of

Charles A. Ostrom and Norman Sensenig

ACKNOWLEDGEMENTS

The author wishes to extend his gratitude and appreciation to the following individuals who have assisted in some way towards the achievement of his Master of Science degree:

Dr. Richard N. Heitmann, for your valuable friendship, encouragement, patience, guidance, team spirit, advice, cosmopolitanism, wit and wisdom throughout the entire course of this program;

Dianne J. Dawes-Torre ("Bub" II), for your cherished friendship, enthusiasm, humor, exuberant participation in practical jokes and persistence throughout your successful completion of "boot camp" training;

Jessica A. Butler, for your unparalleled experience as an anesthetist as well as your invaluable assistance and preparation for the numerous surgeries and experiments but more importantly, for your lightheartedness, free spiritedness and for the priviledge of being your friend;

Kenneth P. Zanzalari and Gregory A. Kiess (the other "Valley Boys"), for your friendships and countless wild and crazy moments together;

Leona Jane Batory and Kelly J. Diehl, for your enjoyable company, for your friendships and for dishing out as well as being the brunt of endless pranks;

Joseph W. Covington ("Jo Jo"), for your hearty laughter, fun, ludicrous times and your friendship;

Dr. James K. Miller and Dr. Thomas T. Chen, for your service as members of his graduate committee, valuable advice, scientific expertise and for your careful review of this manuscript;

Nancy Ramsey, for your valuable advice in review of this manuscript, your listening ear and true friendship;

Kristen Moy, for your inspiration and your enjoyable, unforgettable company, many fond memories and especially for your lasting love and endearing friendship;

Also, the author would like to express his sincere gratitude to the following people who have brightened and enriched and his life during the past several years;

Wendy J. Pacheco, Pamela J. Pecon ("Pambo"), Patrick M. Torre and Patricia D. Whaley;

And last but not by any means least, the writer extends many thanks to Dad & Barbara, Mom, his brothers Mike, Jay and Todd, as well as Dolly, Mom Mom, and Gramma, for all your love and continued support throughout the years of his academic career;

The author extends a special note of appreciation to all the following girls (ewes) who so generouly donated their blood so as to make this research possible:

Big Momma, Ewe 2, Big Horn, Stomper a.k.a. Honey, Pooch, Tiny, Chevy, Heinz, Jessie's Girl, Asshole, Pearl, Fannie, P ewe, Yellow Jacket and Cupid.

ABSTRACT

Open, mid- (78-109d) and late- (126-147d) gestating, lactating (7-35d post partum) and weaning (42-56d post partum) ewes were fitted with catheters in the femoral artery and femoral, portal, hepatic and mesenteric veins to measure net fluxes of glucose, ketones, free fatty acids (FFA), insulin and glucagon across portal-drained viscera (PDV), liver (HEP) and hindquarter tissues. All gestating ewes were singleparous and subsequently, all lactating ewes nursed a single lamb. Blood flow across splanchnic tissues was calculated by measuring downstream dilution of para-aminohippurate (PAH). Hindquarter flow was measured by infusing PAH into the femoral artery and measuring jugular-femoral vein differences. Net fluxes were found by multiplying venoarterial differences by tissue blood flows. Arterial concentrations of glucose decreased from 55 in open ewes to 45, 46 and 46 mg/dl in mid- and late-gestation and lactation, respectively, then returned to control values in weaning ewes without changes in tissue flux. Arterial concentrations of FFA increased from 173 in open ewes to 313, 384 and 223 μM in mid- and late-gestation and lactation undoubtedly due to increased PDV release from 3 in open ewes to 28, 31 and 14 μmol/min and hindquarter release from 7 in open ewes to 44, 29 and 20 μmol/min and despite increased HEP uptake from 36 in open ewes to 93, 102 and 53 μmol/min, respectively. FFA concentrations in the artery decreased from 173 in open ewes to 101 μM in weaning ewes due to decreased PDV release from 3 in open ewes to -8 μmol/min and despite decreased HEP uptake from

36 in open ewes to 13 μmol/min. Arterial concentration of aceteacetate increased in lactation and weaning from 29 in open ewes to 47 and 37 μM perhaps due to increased PDV production from 44 in open ewes to 61 and 62 μmol/min, respectively. Arterial concentration of 3-hydroxybutyrate increased from 563 in open ewes to 739, 836, 963 and 1118 μM in mid- and late-gestation, lactation and weaning, respectively, due to increased HEP release in mid- and late-gestation from 226 in open ewes to 362 and 557 μmol/min and increased PDV release in lactation and weaning from 270 in open ewes to 343 and 412 μmol/min and despite increased hindquarter uptake in lactation and weaning from -110 in open ewes to -268 and -197 μmol/min, respectively. Arterial concentration of insulin decreased from 72 in open ewes to 45, 32, 19 and 33 μU/ml in mid- and late-gestation, lactation and weaning due to decreased PDV production from 29 in open ewes to 15, 16, 7 and 22 μU/min, respectively. Arterial concentration of glucagon increased from 195 in open ewes to 260 pg/ml in late gestation due to increased PDV production from 4.2 in open ewes to 5.3 μg\h. Insulin to glucagon ratio decreased from 9.4 in open ewes to 6.5, 2.7, 2.1 and 3.5 in mid- and late-gestation, lactation and weaning primarily due to decreased insulin concentrations concomitant with relatively unchanged glucagon concentrations. Glucose and insulin decreased concomitantly in mid- and late-gestation and lactation favoring increased PDV and hindquarter FFA release and hepatic uptake and subsequent increased hepatic ketogenesis resulting in increased ketone concentrations. Therefore, FFA and ketones may be used as alternate energy fuels sparing glucose for increased utero-placental-fetal and mammary tissue utilization.

"...we must recognize that our divisions into sciences are not a part of nature; they exist only in the mind which, by reason of its infirmity, is forced to create categories of bodies and of phenomena, so as to understand them better by studying their characteristics or properties from special points of view. It follows that the same body may be studied chemically or physically, but in nature there is really neither chemistry or physics, nor zoology, nor physiology, nor pathology; there are only bodies to be classified or phenomena to be known and mastered."

Claude Bernard 1856

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER I

INTRODUCTION

Homeostasis and Homeorhesis

"La vie est une fonction chimique," was first suggested during the eighteenth century by Lavoisier, the famous French chemist and founder of the science of nutrition (111). To a nutritionist, the chemical processes of life involve various metabolic reactions which convert ingested foodstuffs into nutrients which are absorbed and partitioned for use in maintenance and growth of body tissue as well as formation of body reserves (17).

The partitioning of absorbed nutrients can be categorized into two types of hormonal regulation, homeostasis and homeorhesis (17). Homeostatic regulation refers to body metabolism needed to maintain physiological equilibrium. Examples of homeostasis include the maintenance of constant body temperature as well as post-prandial partitioning of nutrients. Homeorhetic regulation refers to changes in metabolism of various tissues in order to support a new physiological state. Examples of homeorhesis include metabolic changes which occur during gestation and lactation.

Since both gestation and lactation are necessary for the continuation of the species, the body must partition an increased amount of nutrients toward the utero-placental-fetal and mammary tissues to meet increased metabolic demands. However, if the body cannot adapt to

the new changes in metabolism, metabolic disorders may develop. An important, as well as deleterious, metabolic disorder, known as ketosis can occur, especially in ruminants.

Ketosis refers to an abnormal increase in ketone bodies, i.e. acetone, acetoacetate and 3-hydroxybutyrate, in the blood. Domestic ruminants are particularly susceptible to ketosis undoubtedly due to a dependence upon hepatic gluconeogenesis for a source of glucose as well as the high degree of genetic selection based upon increased productivity, i.e. milk production in the dairy cow and number of offspring in the ewe. (8). Therefore, a prolonged energy deficit concomitant with an elevated hepatic influx of ketone precursors and subsequent overproduction of ketone bodies (25) results in impaired hepatic gluconeogenesis (116,170) and is believed to be the major cause of ruminant ketosis. Ketosis in sheep is also referred to as ovine pregnancy toxemia and occurs peri-parturient in multiparous ewes. In cows the condition is known as acetonemia or spontaneous bovine ketosis and occurs during peak lactation. The incidence of ketosis in both species is believed to be between 2-15 % (7).

Both conditions are characterized by hypoglycemia, hyperketonemia, and inappetence (8,14,19,89). Other biochemical changes include elevated plasma concentrations of free fatty acids (12,15,60,140), decreased Kreb's cycle intermediates (12) and lowered plasma concentrations of insulin (48,140). Successful treatment of ketotic animals can be accomplished in several ways such as intravenous injection of glucose (58), oral drenching with propylene glycol (2,58) as well as intramuscular injection of glucocorticoids (9,58).

Homeostatic and homeorhetic regulation affect not only the partitioning of nutrients but also the formation and utilization of body reserves. Body reserves include lipid, stored in adipose tissue, and glycogen, stored in liver and skeletal muscle, from which the metabolic fuels, i.e. free fatty acids, ketone bodies and glucose, are derived and utilized to fire the chemical processes of life. The release of metabolites from body reserves is regulated by interactions of the pancreatic hormones, insulin and glucagon, upon hepatic and adipose tissue. As a result of either homeostasis or homeorhesis, insulin and glucagon concentrations, and perhaps more importantly the insulin to glucagon ratio (32), vary and therefore finely regulate body metabolism.

Insulin and glucagon serve as the primary hormonal regulators of intermediary energy metabolism, particularly during gestation and lactation. Insulin promotes lipogenesis, glycolysis and glycogenesis but inhibits hepatic ketogenesis (33,96). On the other hand, glucagon promotes lipolysis, gluconeogenesis, glycogenolysis and hepatic ketogenesis. The primary target tissues of insulin are skeletal muscle and adipose whereas those of glucagon are liver (36). Therefore, changes in the insulin to glucagon ratio determine the degree of anabolism or catabolism predominating in the animal. For example, an elevated insulin to glucagon ratio would favor an anabolic state while a lowered insulin to glucagon ratio would favor a catabolic state. Since insulin favors growth promotion (109,150), a high insulin to glucagon ratio would be desirable in growing animals. Low insulin to glucagon ratio are evident during late pregnancy (143), lactation (71), fasting

(70) and clinical ketosis (31). Arterial insulin concentrations were found to be lower during late pregnancy (30,55,56,73,143,166,168) and lactation (37,39,79,105,162,168) concomitant with relatively no significant changes in glucagon concentrations (71,143). Circulating concentrations of insulin were found to be higher in low producing than in high producing dairy cows (63).

Since insulin concentrations exhibit greater changes during homeorhetic conditions compared to glucagon, the hypothesis for developing this study is that the changes in circulating insulin followed by changes in the insulin to glucagon ratio will result in an alteration of glucose, FFA and ketone body metabolism in such a way as to favor nutrient partitioning, i.e. glucose, toward the fetus and mammary gland during gestation and lactation, respectively.

CHAPTER II

LITERATURE REVIEW

Glucose Metabolism during Pregnancy and Lactation

Exogenous insulin infused into the uterine artery of gestating sheep increased umbilical as well as fetal uptake and utilization of glucose (47,125,150). Also, the secretion of insulin by the fetus is suggested to be partly responsible for umbilical glucose uptake (130). However, placental uptake of glucose is believed to be insensitive to variations in maternal insulin concentrations (66) despite specific binding of insulin to placental membranes (132).

During lactation, the nonruminant mammary gland becomes highly sensitive to insulin and relatively insensitive to glucagon (136) thus enhancing extraction of glucose from the circulation by the mammary gland (79). This does not appear to be true during lactation in the ruminant. Insulin does not influence glucose extraction by the bovine mammary gland (95) nor does it alter the metabolism of acetate, 3-hydroxybutyrate or triacylglycerides by direct effects on the mammary gland (16,94). Therefore, the low insulin concentrations evident during late gestation and early lactation and thus a low insulin to glucagon ratio, enhance mobilization of body reserves, particularly glucose, free fatty acids and ketone bodies needed to supplement the increased energy needs of the utero-placental-fetal and mammary tissues (162,166).

Ruminants absorb little or no carbohydrate from the

gastrointestinal tract and depend largely upon hepatic gluconeogenesis for provision of glucose (102). Also, the kidneys can function to provide additional glucose for the animal. In the normal fed open and pregnant sheep, renal gluconeogenesis can provide 10 % of the whole-body glucose, while in fasted and hypoglycemic sheep, the kidneys can produce up to 15 % (83,84). Propionate and amino acids serve as the main precursors of glucose. The splanchnic tissue uses glucose for maintenance and energy but no significant hepatic glucose utilization has been reported in ruminants (26). Approximately 46-50 % of the propionate absorbed via the portal system is converted to glucose (27,155) and this accounts for 20-40 % of the ruminant's total glucose production (27). Also amino acid precursors of glucose account for an additional 20-40 % of the glucose production via gluconeogenesis in adult ruminants (27).

In the dairy cow, carbohydrate metabolism is more or less precarious during both physiological states (10). Although a greater percentage of ruminal propionate is diverted toward hepatic gluconeogenesis (155,176) and hepatic glucose production (39), the activities of several hepatic gluconeogenic enzymes increase (107,152) and blood glucose concentrations decrease (22,25,30,41,119,143,158,166) during gestation.

Therefore, during gestation, less glucose is available to the dam to meet increased energy needs, perhaps due to the increased glucose utilization by the utero-placental-fetal tissues. Although no significant uptake of fructose or pyruvate by the pregnant uterus occurs, the uptake of glucose by the uterus does increase during

gestation (119). Of the total glucose production rate of pregnant ewes, 32 % was taken up by the uterus (67). Of the total uterine glucose uptake, 28-31 % was utilized by the fetus while 64-72 % was utilized by the utero-placental tissues (41,67). Uterine glucose uptake increases even further depending on the number of viable fetuses carried by the dam (133). By day 125 of gestation in multiparous ewes, only 51 % of the uterine glucose uptake was utilized by the utero-placental tissue (133). In pregnant cows, 33 % of the uterine glucose uptake was transferred directly to the fetus while 50 % appeared to be metabolized anaerobically by the utero-placental tissue to lactate, the majority of which was utilized by the fetus (44). Fetal glucose uptake was reported to be directly related to the glucose concentration between maternal arterial blood and fetal umbilical arterial blood (77) while the involvement of insulin in glucose extraction by the utero-placental-fetal tissue appears somewhat controversial (47,66,125,130,150).

Although glucose production and uterine glucose uptake increased during late gestation, the uterine glucose/oxygen quotient was only 0.76 during the last three weeks of gestation suggesting that substrates other than glucose were used for uterine oxidative metabolism (119).

During lactation, sheep must provide more glucose than during gestation (23). Glucose is the primary metabolite utilized by the mammary gland (3) and hypoglycemia is the primary cause of hypogalactia (92). Of the total glucose entering the circulation, 60-85 % is utilized by the mammary gland of lactating goats (3). Gluconeogenesis from propionate increases during early lactation in both sheep (176) and dairy cows (1). Glucose turnover increases three-fold in peak

lactating ewes (23) while glucose synthesis increases two-fold (39) and mammary gland oxidation of glucose increases four-fold in lactating goats (3). Lactating rats also have an elevated hepatic glucose production (37).

Seventeen percent of the glucose taken up by sheep (122) and 25 % of the glucose taken up by goat (3) mammary gland was oxidized which contributed to 20 and 39 % of the mammary CO2, respectively. Extraction by the mammary gland of glucose entering the circulation is approximately 100 % in high producing dairy cows but only 40 % in low producing dairy cows (91). The remaining carbon taken up via the mammary gland may be metabolized to form lactose (40,57,86,122), citrate (40) and triacylglycerides (40,57,86). Also, glucose can enter the pentose phosphate shunt (PPS) and form reducing equivalents needed for fatty acid synthesis (57). In the goat mammary gland, the flux of glucose-6-phosphate through the PPS was sufficient to account for 34 % of the reduced NADP required for lipogenesis (40). However, glucose taken up by the mammary gland is used primarily to form milk lactose. Glucose can provide 71-85 % of the carbon needed for mammary gland lactose formation (86,122).

Although hepatic glucose production is elevated during lactation, arterial glucose concentrations are lower during peak lactation than either late lactation (54) or in nonlactating (79) animals. Since blood glucose concentrations are lowered during lactation, less glucose is available to nonlactating tissue due to the partitioning of glucose toward the lactating mammary gland. The peripheral tissue of lactating sheep utilized less glucose due to a fall in activity of key glycolytic

enzymes of skeletal muscle (167). Therefore alternate energy metabolites, i.e. FFA and ketone bodies, might play a significant role during lactation and possibly gestation.

FFA and Acetate Metabolism during Pregnancy and Lactation

Free fatty acids comprise less than 5 % of the total fatty acids present in plasma yet are known to be metabolically the most active of the plasma lipids. Plasma FFA arise primarily from hormone sensitive lipase activated lipolysis of adipose tissue triacylglycerides and to a certain extent from lipoprotein lipase action upon chylomicrons and very low density lipoproteins during tissue FFA uptake and subsequent re-esterification (108).

Free fatty acids can serve as oxidizable substrates for the skeletal muscle (128), heart, liver and kidney cortex (59) but apparently not for the gravid uterus (128). In ruminants, FFA are not a major source of energy for the fed pregnant ewe but contribute significantly to muscle respiratory CO2 in fasted pregnant ewes (128). The adipose tissue of ruminants is the predominant site of fatty acid synthesis with the perirenal and omental fat pads exhibiting the highest amount of activity (75).

During the last third of gestation there is a switch from net lipid accumulation to net lipid mobilization accompanied by a decline in capacity for lipid synthesis (168). Hormonal conditions favoring lipid mobilization (157), i.e. low insulin to glucagon ratio, are evident during late gestation (143). Plasma levels of FFA are elevated during gestation with respect to nonpregnant animals (39,134,143,168). Since

FFA are not utilized significantly by either skeletal muscle or gravid uterus, perhaps fetal utilization might explain the elevated plasma FFA levels during gestation. Placental transfer of FFA occurs in primates (129) as well as in rabbits (50) however, the sheep placenta is relatively impermeable to transfer of FFA to the fetus (76,121). Plasma FFA concentrations of sheep were ten times greater in the maternal circulation relative to that found in the fetal circulation. However, despite this concentration gradient, the ovine placenta is permeable to palmitic acid but not linoleic acid (121). Fetal plasma is comparatively low in linoleic acid compared to maternal plasma (121,159). Of the total FFA found in the plasma of late gestating ewes, palmitic acid comprises 20 % (173) while linoleic acid accounts for only 2.4 % (120). The placenta can synthesize arachidonic acid from the small amount of maternal linoleic acid for subsequent transfer to the fetus (43,50,148).

A portion of the circulating FFA can be metabolized by the liver to form acetate (145,173). Maternal acetate transfer to the fetal circulation occurs, depending upon the maternal acetate concentration and the placental concentration gradient for acetate, a could conceivably account for 7 % of the daily total carbon transferred to the ovine fetus (42).

Therefore, during gestation, substantial evidence indicates that only a minute quantity of maternal circulating FFA are utilized directly by the utero-placental-fetal tissues and skeletal muscle. Instead, the majority of FFA are perhaps metabolized by the liver to form acetate and ketone bodies (19,145,173).

The net lipid mobilization observed during late gestation continues through early lactation (115,168). Plasma FFA concentrations are elevated during early lactation in sheep (134) and cows (115). High producing dairy cows have higher plasma FFA concentrations than low producing dairy cows (63). Although neither gestation nor lactation alters the basal rate of adipose tissue lipolysis (163), plasma FFA elevation caused by net lipid release is undoubtedly due to an almost complete disappearance of fatty acid re-esterification in the adipose tissue (115). Lactation results in a loss of lipid from adipocytes as evidenced by their diminished mean cell volume (163). Concomitant with decreased lipogenic activity of adipose tissue during lactation is an increased mammary gland lipogenesis (164,165). Also during lactation, a higher proportion of short and medium chain milk fatty acids are produced by the ruminant mammary gland (165).

Free fatty acid uptake by the mammary gland appears negligible (18) except during energy restriction (4,91), and perhaps the increased supply of FFA is converted to other blood metabolites, i.e. acetate, triacylglycerides and ketone bodies, from which the mammary gland can synthesize milk fatty acids (45,62,87,98,104,108,145,173). Mammary gland uptake of acetate, triacylglycerides and FFA accounts for 25, 60 and 4 % of milk fatty acid carbon, respectively (85). Acetate is utilized for synthesis of milk fatty acids of chain length up to C-16 (156). Lactogenesis results in increased activity of enzymes associated with acetate incorporation into milk fatty acids (114). In the fed, nongestating, nonlactating ewe, circulating acetate is largely a product of rumen fermentation and is primarily oxidized to CO_2 in the gut and

muscle, perhaps accounting for 30-40 % of their oxidative metabolism (129). In lactating animals, muscle extraction of acetate is depressed thus sparing acetate for mammary gland utilization (126).

Paradoxically, during late gestation and early lactation there exists an increased supply of FFA yet there is little direct utilization of FFA by the tissues responsible for increasing the body's energy demands, i.e. utero-placental-fetal and mammary tissue. This is resolved by the fact that during gestation and lactation, FFA are converted to acetate and more importantly ketone bodies which function to conserve glucose.

Ketone Body Metabolism during Pregnancy and Lactation

Free fatty acids serve as precursors for production of the ketone bodies, acetoacetate, acetone and 3-hydroxybutyrate, by the liver (53,81,110,115,145). Hepatic extraction of FFA is a nearly constant (10 %) proportion of the FFA transported to the liver regardless of the physiological state (81). In normal fed sheep, hepatic ketogenesis could explain 30 % of the liver FFA uptake while in ketotic sheep, ketone body production by the liver could have been the product of 80 % of hepatic FFA uptake (81). Two pathways have been proposed for the formation of acetoacetate in the liver (Figure 1). The first is a simple deacylation of acetoacetyl-CoA to form acetoacetate. The second involves the condensation of acetoacetyl-CoA with acetyl-CoA to form 3-hydroxy-3-methylglutayrl-CoA (HMG-CoA) followed by lyase action upon HMG-CoA thus forming acetyl-CoA and free acetoacetate (99,108). Acetoacetate continually undergoes spontaneous decarboxylation to yield

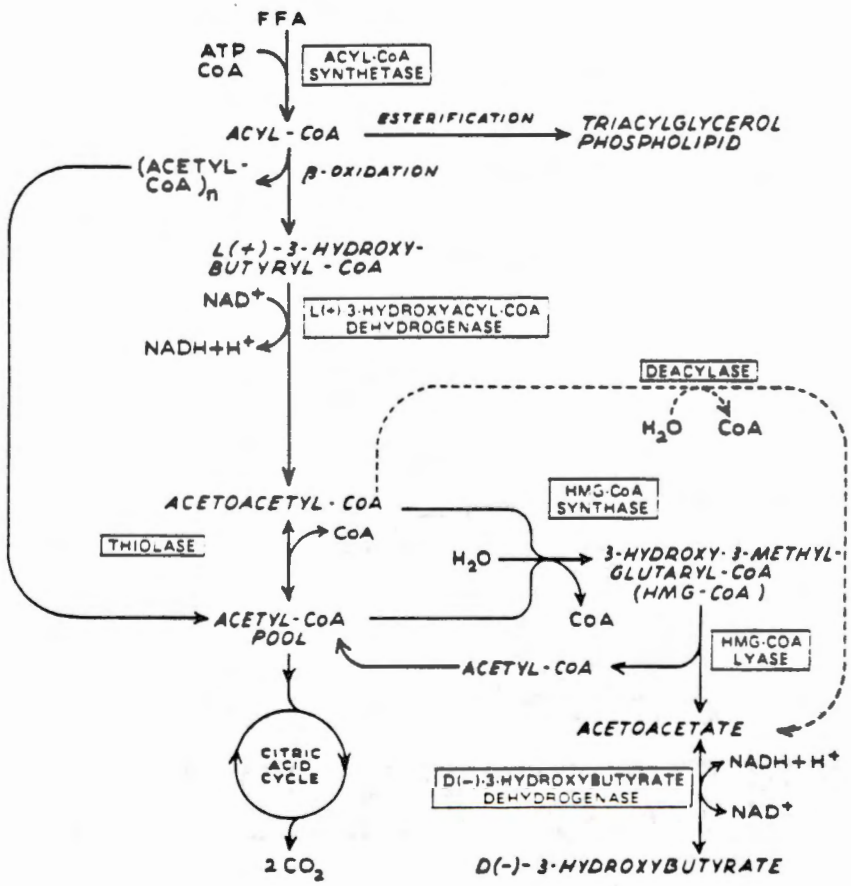

FIGURE 1. Pathways of ketogenesis in the liver (108).

acetone (Figure 2). The body is unable to oxidize acetone, consequently it is eliminated eventually via the lungs and urine. Biochemically, acetoacetate and 3-hydroxybutyrate are the most important ketone bodies due to their presence in the blood and effect upon the body's energy metabolism. In vivo, acetoacetate and 3-hydroxybutyrate are in equilibrium with each other dependent upon the redox potential, i.e. NADH+:NAD (Figure 2). Depending upon the ratio of NADH+:NAD, acetoacetate and 3-hydroxybutyrate can interconvert in the presence of 3-hydroxybutyrate dehydrogenase found in various tissues including the liver. The ratio of 3-hydroxybutyrate:acetoacetate fluctuates between 1:1 in nonruminants and 10:1 in ruminants (108,171).

In nonruminants, the liver appears to be the only source of ketone bodies found in the blood (108). In ruminants, two major sites of ketogenesis exist, the liver (81) and rumen epithelium (13,38,99). Ketone body production in the liver is referred to as hepatic ketogenesis while ketone body production by rumen epithelium is referred to as alimentary ketogenesis. In a normal fed ruminant, ketone bodies derived from the two ketogenic sites will have an alimentary to hepatic ketogenesis ratio of approximately 3:2 (24,81). Thus in the fed state, the primary source of ketone bodies is from rumen epithelium (81,137). Alimentary ketogenesis is the result of butyrate absorption from rumen microbial fermentation and subsequent metabolism by rumen epithelium (5,6,51,144). Ketone body concentration in portal blood exceeds that of peripheral blood which is indicative of alimentary ketogenesis (5). The rumen epithelium contains the following enzymes involved in ketone body formation: 1) 3-hydroxy-3-methylglutaryl-CoA synthetase and lyase

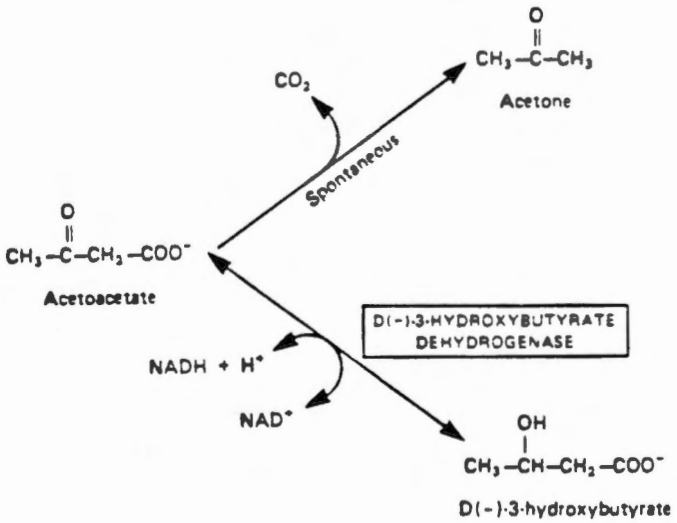

FIGURE 2. Interrelationships of the ketone bodies (108).

(13,38); 2) 3-oxoacid-CoA transferase (38); 3) acetoacetyl-CoA thiolase (13); 4) acetoacetyl-CoA deacylase (13,38); and 5) 3-hydroxybutyrate dehydrogenase (171). Acetoacetate can either be released from rumen epithelium into the blood or converted to and released as 3-hydroxybutyrate by the epithelium due to the presence of 3-hydroxybutyrate dehydrogenase (99).

Hepatic ketogenesis becomes the primary source of ketone bodies during physiological perturbations characterized by FFA mobilization, i.e. pregnancy, lactation and fasting (81,137). During pregnancy and lactation, when carbohydrate shortages occur, ketone bodies become the preferred oxidizable energy metabolites over both FFA and glucose (78,108,139).

Although the liver possesses the enzymes necessary for ketone body formation, it is unable to oxidize ketone bodies due to the absence of essential acetoacetate activating enzymes, i.e. 3-oxoacid-CoA transferase or acetoacetyl-CoA synthetase (108,175).

However, most extrahepatic tissues possess the capability of ketone body utilization since acetoacetate activating enzymes are present (108,175). Ketones can be utilized in the heart (103), kidney (172), skeletal muscle (103,128,138), placenta (49), uterus (127,128) and lactating mammary gland (64) in both ruminants and non-ruminants. Oxidation of ketone bodies occurs in both human (123) and rat (65) brain. In the sheep however, ketone bodies are not oxidized by the brain, which relies primarily upon glucose as as energy source (103). Ketone body oxidation can account for 60 % of the total oxygen consumption of the heart and skeletal muscle (103). In the skeletal

muscle of the sheep hindlimb, ketone body oxidation can account for 18 % of the oxygen utilization and 20 % of the CO2 production (128). During fasting, the sheep hindlimb oxidation of ketone bodies increases approximately two-fold (128), while during ketosis, there is a decreased capacity of the sheep kidney and heart to utilize ketone bodies (161).

Therefore, during times of increased energy demands, i.e. pregnancy and lactation, concomitant with lowered blood glucose and insulin:glucagon ratio and elevated FFA mobilization, overall ketogenesis increases and ketone bodies function to conserve glucose needed for utero-placental-fetal and mammary tissues.

During late gestation, blood ketones become significantly elevated (90,160). The increased blood ketones could perhaps be an alternate energy source for the utero-placental-fetal tissues in times of hypoglycemia. The placenta (49,147) and uterus (127) are capable of oxidizing ketones. Of the 3-hydroxybutyrate taken up by the uterus of pregnant sheep, 64 % was oxidized and accounted for 12 % of the uterine oxygen consumption while no apparent uterine oxidation of acetoacetate occurred (127). The sheep placenta has a relatively low activity of the acetoacetate activating enzyme, 3-oxoacid-CoA transferase (49). During moderate hyperketonemia in the ewe, the net uterine uptake of 3-hydroxybutyrate increases 2.5-fold (41). Between the third and fourth months of ovine gestation, placental 3-hydroxybutyrate dehydrogenase activity increases, perhaps contributing to increased 3-hydroxybutyrate levels in the blood (49).

Both the rat (141) and human (142) placenta are permeable to ketone bodies, especially 3-hydroxybutyrate, suggesting ketone body transfer to

and utilization by the fetus for fatty acid and cholesterol synthesis *in utero*. However, in the sheep, there is no apparent maternal transfer of ketone bodies to the fetus and therefore, ketone bodies cannot be utilized as a prenatal energy source by the ovine fetus (118). During maternal starvation in the rat, ketone bodies may enhance *in utero* and neonatal life by restricting cell replication thus preserving fetal tissue stores of protein and lipid (146).

Following parturition, plasma concentrations of 3-hydroxybutyrate are higher in lactating than in non-lactating animals (39). Concentrations of 3-hydroxybutyrate are greater in high producing dairy cows than in low producing dairy cows (63). In lactating sheep, skeletal muscle oxidation of 3-hydroxybutyrate accounts for 17 % of the muscle oxygen uptake which is similar to that found in nonlactating sheep suggesting no increased utilization of ketone bodies by peripheral tissues during lactation (126). On the other hand, mammary gland uptake of ketone bodies is significant during lactation (28) and is directed primarily for milk fat synthesis (46,104,135). 3-hydroxybutyrate can contribute to all milk fatty acids by contributing the first four carbon atoms but is used primarily for synthesis of C4-C12 milk fatty acids and to a lesser extent C12-C18 acids (151). Thirty and ninety-five percent of the 3-hydroxybutyrate utilized for milk fat synthesis in ruminants and nonruminants, respectively, is first split into two carbon units prior to milk fat incorporation (104,151). The mammary gland of sheep extracts 42-53 % of the circulating 3-hydroxybutyrate (85,126) which can supply 3-13 % of the carbon required for milk fat synthesis (85,106,124). Therefore, a deficiency of 3-hydroxybutyrate would not

appear to be a causative factor in depression of milk fat production (124).

In fasted or ketotic cows, mammary uptake of 3-hydroxybutyrate increases concomitant with a mammary net release of acetoacetate into the circulation. Although the mammary gland utilizes 3-hydroxybutyrate for milk fat synthesis, little oxidation occurs due to the low activity of the acetoacetate activating enzyme, 3-oxoacid-CoA transferase (46). Although theoretically, if all the 3-hydroxybutyrate taken up by the mammary gland were oxidized, it could conceivably explain 42 % of the mammary oxygen consumption (149). Also, ketogenesis is virtually nonexistent in the mammary gland since essentially no activities of the ketone body forming enzymes, i.e. 3-hydroxy-3-methylglutaryl-CoA synthetase or acetoacetyl-CoA deacylase, are present (13).

Consequently, the objectives of this study were to establish base line concentration and net flux values of glucose, FFA, acetoacetate, 3-hydroxybutyrate, insulin and glucagon during gestation, lactation and weaning in singleparous ewes for future comparison in a similar study to be performed on multiparous ewes.

CHAPTER III

MATERIALS AND METHODS

Effects of pregnancy, lactation and weaning upon various metabolite concentrations and net fluxes were studied in ewes of various breeds, i.e. Dorset, Finn cross, Hampshire, Rambouillet and Suffolk, weighing between 50-70 kg. All gestating ewes were singleparous, consequently all lactating ewes were nursing a single lamb. Five treatment groups were studied as follows: 1) control, nongestating, nonlactating ewes; 2) mid-gestating ewes, (78-109 d); 3) late-gestating ewes, (126-147 d); 4) lactating ewes, (7-35 d post-partum); and 5) weaning ewes, (42-56 d post-partum). Gestation was divided into mid- and late- periods due to the difference in tissue growth between these two times. In mid-gestation, the predominant weight gain of the ewe is due to uterine/placental growth (93,168) while during late-gestation, maternal weight gain is due to increased fetal growth (49,168). Ewes were assumed to be pregnant until verified by ultrasound. The animals were housed in individual 1.8 x 3.0 m pens at approximately 19-25 degrees C with constant lighting. The sheep were fed commercially prepared alfalfa pellets according to National Research Council (NRC) recommendations in two aliquots daily at 0800 and 1700 hours. All sheep were accustomed to being handled by laboratory personnel, therefore restraint was not necessary during the experiments. All animals were surgically prepared as described by Katz and Bergman (82)

and Yelverton et al. (178) and allowed to recover at least 10 days prior to any sampling. Chronic indwelling catheters were placed so that their tips resided in the following five locations: 1) caudal vena cava; 2) caudal aorta; 3) common mesenteric vein; 4) hepatic vein; and 5) portal vein. Blood sampled from the caudal vena cava and aorta catheters was used to measure metabolite fluxes across the hindquarters. Also, blood sampled from the aorta catheter was used to determine arterial concentration of various metabolites. Blood sampled from the hepatic and portal veins was used to measure liver and portal-drained viscera metabolite fluxes, respectively. Finally, the common mesenteric vein was used for infusion of para-aminohippuric acid (PAH) (Eastman Kodak Co., Rochester, N. Y.) in order to determine the whole blood flow rate.

On experimental days, PAH was infused (1.5% at 0.764 ml/min) into the common mesenteric vein and allowed to equilibrate for a one hour period prior to taking the initial sample. At the end of the equilibration period, 15 ml blood samples were taken simultaneously from the four remaining catheter sites at six consecutive 30 min intervals. Blood was then analyzed for packed cell volume as well as concentrations of glucose, ketone bodies, (i.e. acetoacetate and 3-hydroxybutyrate), and PAH. Plasma was analyzed for FFA, insulin and glucagon.

Plasma glucose was analyzed enzymatically with a glucose diagnostic kit No. 510 (Sigma Chemical Co., St. Louis, Mo.) which is based on the Somogyi deproteinization method for determination of blood glucose (153). The exact procedure is detailed in Appendix I.

Plasma free fatty acids were analyzed enzymatically with a WAKO

NEFA-C kit (WAKO Pure Chemical Industries, Ltd., Osaka, Japan). The detailed procedure is found in Appendix II.

Ketone bodies, i.e. acetoacetate and 3-hydroxybutyrate, in the blood were enzymatically determined based upon the method described by Williamson, et al. (174) and detailed in Appendix III and IV, respectively.

Insulin and glucagon were determined using a double antibody radioimmunoassay (RIA) technique (177). Intra-assay errors of greater than 5 % were not accepted. Interassay errors, measured by a pooled ewe sample, were always less than 10 % and usually less than 7 %. So as to distinguish between pancreatic and gut glucagon, rabbit anti-glucagon antibody (R. H. Unger, University of Texas Health Science Center, Dallas, Tx.) was used as the first-antibody in the glucagon RIA due to its high specificity for pancreatic glucagon. Detailed steps for both insulin and glucagon RIA's are found in Appendix V and VI, respectively.

PAH was determined as described by Katz and Bergman (80) and the detailed procedure is found in Appendix VII.

Calculations

Whole blood flow rates through the portal-drained viscera and liver were calculated by the indicator-dilution method using the equation:

$$F = \frac{I^{PAH}}{Ca^{PAH} - Cv^{PAH}}$$

where F equals the whole blood flow through the tissue in l/min, I^{PAH} is

the infusion rate of PAH optical density (OD) units/min and Ca^{PAH} and Cv^{PAH} are the PAH OD units/l in the arterial input and venous drain. Portal and hepatic vein flows were measured directly and hepatic arterial flow calculated from the difference between the two. Plasma flows were obtained by subtracting that portion of the flow represented by the packed cell volume. Net fluxes of glucose, FFA, acetoacetate, 3-hydroxybutyrate, insulin and glucagon across the portal-drained viscera, liver and hindquarters were calculated using the following equations:

$$\text{Portal-drained viscera flux} = PF \times (Cp - Ca)$$

$$\text{Hepatic flux} = PF \times (Ch - Cp) + AF \times (Ch - Ca)$$

$$\text{Rump flux} = RF \times (Ca - Cv)$$

where PF, AF and RF are the whole blood or plasma flow rates (l/min) in the portal vein, hepatic artery and the caudal aorta, respectively. Also, Cp, Ca, Ch and Cv are the concentrations of either glucose (mg/dl), FFA (μm/l), insulin (μU/ml) or glucagon (pg/ml) in the plasma or acetoacetate (μM/l) or 3-hydroxybutyrate (μM/l) in whole blood. A positive flux indicates net release and a negative flux indicates net uptake of a metabolite by a specific tissue.

Extraction ratios for each metabolite and each tissue were also calculated. Extraction ratios are that fraction of the metabolite presented to the tissue, i.e. tissue flow multiplied by the concentration in the arterial input, that is taken up by the tissue, i.e. net flux.

Statistical Analysis

Data was analyzed by a two-way analysis of variance to determine the significance of difference of the aforementioned various energy metabolite and glucoregulatory hormone concentrations and net fluxes between control ewes and either gestating, lactating or weaning ewes.

CHAPTER IV

RESULTS

Whole Blood Flow

Whole blood flow rates for the portal and hepatic veins and rump as well as portal to hepatic vein blood flow ratio are shown in Table 1. Portal vein blood flow ranged from 1.8 to 2.2 l/min while hepatic vein blood flow ranged from 2.4 to 2.7 l/min. No significant differences in portal and hepatic blood flows were noticed between open and either gestating, lactating or weaning ewes. Caudal vena cava whole blood, which represents hindquarter or rump flow, ranged from 1.4 to 1.8 l/min and is in agreement with previously published data (70,143). Only during lactation did rump blood flow tend to increase ($P<.1$) by 22 % compared with controls. Portal to hepatic vein blood flow ratio ranged from 76 to 83 % and is in close aggreement with previous research done on fed open (69,80) and pregnant (143) as well as fasted (70) animals. Portal to hepatic vein ratio did tend to show a statistically significant increase ($P>.05$ but $P<.1$) during lactation and weaned states by 7 and 9 %, respectively, however, physiological significance appears rather doubtful since these values are comparable to previously reported normal physiological ranges (11,26,69,70).

Glucose

Arterial plasma concentration of glucose in controls was 55 mg/dl and decreased significantly by 18, 16 and 16 % during mid- and late-

TABLE 1. Whole blood flow rates in open, gestating, lactating and weaning ewes.[1,2]

Physiological state	Exp	Obs	Portal vein	Hepatic vein	Caudal vena cava	Portal hepatic ratio
			----------1/min----------			
Open	9	44	2.03 ± 0.230	2.64 ± 0.258	1.44 ± 0.179	0.76 ± 0.02
Mid Gestation	7	40	1.85 ± 0.103	2.38 ± 0.116	1.57 ± 0.085	0.78 ± 0.02
Late Gestation	8	48	1.99 ± 0.179	2.49 ± 0.219	1.77 ± 0.093	0.80 ± 0.01
Lactation	9	54	2.13 ± 0.130	2.62 ± 0.142	1.81 ± 0.080†	0.81 ± 0.01†
Weaned	4	24	2.24 ± 0.092	2.69 ± 0.082	1.49 ± 0.321	0.83 ± 0.01†

[1]Mean ± SEM.

[2]Statistically different from open ewes; †P<.1.

gestation and lactation, respectively (Table 2). Arterial concentration returned to control values during the weaned state. Portal-drained viscera (PDV) net fluxes remained unchanged in controls and during gestation and lactation indicating a net release of glucose. However, during weaned condition, PDV net flux decreased significantly by 148 % indicating a state of net uptake. Liver net flux, an indication of hepatic gluconeogenesis, remained unchanged during gestation, lactation and weaned states compared to controls. Glucose release from the liver ranged from 34 to 41 mmol/hr which is indicative of normal hepatic gluconeogenesis in fed sheep (26). Hindquarter net flux always showed a net uptake of glucose from the blood and ranged from 2.7 to 10.5 mmol/hr. Only during lactation did hindquarter uptake of glucose increase significantly by four-fold compared to controls.

Free Fatty Acids

Arterial plasma concentrations of FFA increased significantly from 173 μM in controls by 80, 122 and 29 % during mid- and late-gestation and lactation, respectively, and decreased significantly by 58 % during the weaned state (Table 3). Portal-drained viscera net flux, indicative of omental lipolysis, in the controls was 3.4 umol/min which was not different than zero, but increased significantly by eight- and nine-fold during mid- and late-gestation, respectively. Portal-drained viscera net flux during lactation was four-fold higher than control, however statistical significance was only P>.1. Furthermore, PDV net flux went from a net release of FFA in controls to a net uptake during the weaned state, however statistical significance was only at the P>.1

TABLE 2. Glucose arterial concentrations and net fluxes in open, gestating, lactating and weaning ewes.[1,2]

Physiological state	Exp	Obs	Artery	Portal-drained viscera	Liver	Hindquarters
			mg/dl	----------m mol/h----------		
Open	9	44	55.2 ± 1.4	9.0 ± 1.4	38.4 ± 5.4	-2.7 ± 2.3
Mid Gestation	7	40	45.0 ± 1.6**	5.0 ± 2.7	35.3 ± 4.5	-7.5 ± 3.5
Late Gestation	8	48	46.5 ± 1.0**	6.3 ± 4.6	41.2 ± 5.2	-6.2 ± 3.4
Lactation	9	54	46.4 ± 0.5**	3.3 ± 1.9	40.3 ± 2.9	-10.5 ± 1.7*
Weaned	4	24	52.9 ± 0.8	-4.3 ± 3.2*	33.6 ± 2.7	-9.6 ± 2.5

[1]Means ± SEM.

[2]Statistically different from open ewes; *P<.05, **P<.01.

TABLE 3. Free fatty acid arterial concentrations and net fluxes in open, gestating, lactating and weaning ewes.[1,2]

Physiological state	Exp	Obs	Artery	Portal-drained viscera	Liver	Hepatic extraction ratio	Hindquarters
			μM	--------μmol/min----------			μmol/min
Open	5	32	173 ± 11	3.4 ± 5.8	-35.6 ± 4.9	0.11 ± 0.01	7.2 ± 2.6
Mid Gestation	7	40	313 ± 24**	27.9 ± 6.3*	-93.0 ± 10.8**	0.16 ± 0.02**	43.9 ± 10.0**
Late Gestation	8	48	384 ± 23**	30.5 ± 10.6**	-101.6 ± 15.2**	0.11 ± 0.01	29.3 ± 8.4*
Lactation	9	54	223 ± 11*	14.4 ± 3.4	-53.0 ± 5.3	0.11 ± 0.01	19.8 ± 3.2
Weaned	4	24	101 ± 8*	-8.2 ± 5.2	-13.1 ± 3.6	0.07 ± 0.02	3.7 ± 2.7

[1]Means ± SEM.

[2]Statistically different from open ewes; *P<.05; **P<.01.

level. Liver net flux was always indicative of hepatic removal of FFA from the blood. Liver net flux in controls was 35.6 µmol/min and, during mid- and late-gestation, uptake of FFA increased by 2.6- and 2.9-fold, respectively. Liver net flux remained elevated during lactation but returned to control values in the weaned state. Hepatic extraction of FFA was 11 % in control sheep. During mid-gestation this increased significantly to 16 % . Hindquarter net flux always showed a net release of FFA into the circulation, indicative of rump lipolysis. Control values for hindquarter net flux was 7.2 µmol/min and this increased by six- and four-fold during mid- and late-gestation, respectively. Free fatty acid release by the hindquarter remained elevated during lactation, however statistical significance was only at the P>.1 level. During the weaned state, hindquarter net flux of FFA returned to values similar to the control.

Acetoacetate

Concentrations of acetoacetate in arterial blood was 29.2 µM in controls and changed little during mid- and late-gestation but increased by 61 and 27 % during lactation and weaned states, respectively (Table 4). Portal-drained viscera net flux in controls showed a net production of acetoacetate at a rate of 43.5 µmol/min, indicative of alimentary ketogenesis. Production of acetoacetate tended to decrease (P>.1) during mid-gestation and decreased significantly by 23 % during late-gestation. During lactation and weaned states, PDV production of acetoacetate increased significantly by 40 and 44 % , respectively.

The liver net flux always indicated a net removal of acetoacetate

TABLE 4. Acetoacetate arterial concentrations and net fluxes in open, gestating, lactating and weaning ewes.[1,2]

Physiological state	Exp	Obs	Artery	Portal-drained viscera	Liver	Hindquarters	Hindquarters extraction ratio
			μM	---------- μmol/min----------			
Open	9	44	29.2 ± 1.5	43.5 ± 4.7	-42.2 ± 4.1	-5.7 ± 1.2	-0.13 ± 0.02
Mid Gestation	7	40	30.9 ± 1.5	35.0 ± 2.4	-42.6 ± 3.6	-4.9 ± 1.3	-0.10 ± 0.03
Late Gestation	8	48	31.3 ± 1.8	33.4 ± 2.7†	-28.7 ± 3.8*	-4.2 ± 2.0	-0.04 ± 0.03**
Lactation	9	54	47.1 ± 2.4**	61.0 ± 4.9**	-69.0 ± 5.2**	-9.4 ± 1.6†	-0.10 ± 0.02
Weaned	4	24	37.1 ± 3.9*	62.6 ± 2.8**	-68.9 ± 5.0**	-4.9 ± 1.5	-0.09 ± 0.04

[1]Means ± SEM.

[2]Statistically different from open ewes; †P<.1, *P<.05, **P<.01.

from the circulation. Liver net flux of control animals was -42.2 μmol/min and a similar rate was observed during mid-gestation. However, during late-gestation, liver removal of acetoacetate from blood decreased by 32 % . Liver uptake of acetoacetate increased by 64 and 63 % during lactation and weaned states, respectively.

Like the liver, the hindquarters were always removing acetoacetate from the blood. Control animal hindquarters were taking up acetoacetate at a rate of 5.7 μmol/min and rates were similar during mid- and late-gestation as well as weaned states. However, there was a significant increase in hindquarter uptake of acetoacetate by 65 % during lactation. The hindquarter extraction ratio ranged from 9 to 13 % in controls and during mid-gestation, lactation and weaned, however a significant decrease in hindquarter extraction occurred during late-gestation when extraction was reduced by 70 % .

3-hydroxybutyrate

Arterial blood concentration of 3-hydroxybutyrate was 563 μM in control animals and increased by 32, 48, 71 and 99 % during mid- and late-gestation, lactation and weaned states, respectively (Table 5). Portal-drained viscera net flux always showed a net release of 3-hydroxybutyrate, again an indication of alimentary ketogenesis. Portal-drained viscera release of 3-hydroxybutyrate in controls was 270 umol/min and fluxes were similar during mid- and late-gestation. However, significant increases in PDV release of 3-hydroxybutyrate of 27 and 53 % were observed during lactation and weaned states, respectively. Liver net flux always showed hepatic production of 3-hydroxybutyrate,

TABLE 5. 3-hydroxybutyrate arterial concentrations and net fluxes in open, gestating, lactating and weaning ewes.[1,2]

Physiological state	Exp	Obs	Artery	Portal-drained visera	Liver	Hindquarters	Hindquarters extraction ratio
			μM	----------μmol/min----------			
Open	9	44	563 ± 32	270 ± 37	266 ± 38	-110 ± 22	0.13 ± 0.02
Mid Gestation	7	40	739 ± 48**	254 ± 33	362 ± 37	-140 ± 16	0.13 ± 0.02
Late Gestation	8	48	836 ± 46**	262 ± 23	557 ± 92**	-91 ± 13	0.06 ± 0.01**
Lactation	9	54	963 ± 23**	343 ± 24*	336 ± 20	-268 ± 19**	0.15 ± 0.01
Weaned	4	24	1118 ± 42**	412 ± 25**	278 ± 30	-197 ± 22**	0.14 ± 0.02

[1]Mean ± SEM.

[2]Statistically different from open ewes; *P<.05, **P<.01.

indicative of hepatic ketogenesis. The liver was producing 3-hydroxybutyrate at a rate of 266 µmol/min in controls and tended (P>.1) to increase during mid-gestation to a new hepatic production rate of 362 µmol/min, an increase of 36 %. During late-gestation, liver net flux increased even further by 109 % to 557 µmol/min. Again, during lactation, hepatic production tended (P>.1) to be elevated with a net flux 26 % higher than that of controls. During weaned states, liver production of 3-hydroxybutyrate returned to rates similar to that of controls.

The hindquarters were always removing 3-hydroxybutyrate from the circulation. Hindquarter net flux was similar between controls and mid- and late-gestating animals, ranging from -91 to -140 µmol/min. Hindquarter uptake of 3-hydroxybutyrate increased by 44 and 79 % during lactation and weaned states, respectively. Hindquarter extraction ratio was similar between controls and mid-gestating, lactating and weaning animal ranging from 13 to 15 %. Similar to observations for acetoacetate, there was a 54 % decrease in hindquarter extraction to 6 % during late-gestation.

Insulin

Arterial plasma concentration of insulin was 71.9 µU/ml in control animals and decreased during mid- and late-gestation, lactation and weaned states by 38, 55, 73 and 55 %, respectively (Table 6). Portal-drained viscera net release, an indication of pancreatic insulin production, was 29.2 mU/min in control sheep. Pancreatic production decreased by 48, 46, 77 and 25 % during mid- and late-gestation,

TABLE 6. Insulin arterial concentrations and net fluxes in open, gestating, lactating and weaning ewes.[1,2]

Physiological state	Exp	Obs	Artery	Portal-drained viscera	Liver	Hepatic extraction ratio	Hindquarters
			µU/ml	----------mU/min----------			mU/min
Open	8	38	71.9 ± 6.8	29.2 ± 3.1	-12.3 ± 2.3	0.07 ± 0.01	-1.9 ± 1.5
Mid Gestation	6	34	44.8 ± 4.2**	15.2 ± 1.8**	-9.2 ± 1.4	0.10 ± 0.02	-0.1 ± 0.5
Late Gestation	7	42	32.3 ± 3.6**	15.7 ± 2.0**	-6.2 ± 1.3**	0.10 ± 0.02	-1.6 ± 0.8
Lactation	9	54	19.3 ± 1.2**	6.8 ± 0.8**	-4.9 ± 0.8**	0.11 ± 0.02	-0.1 ± 0.5
Weaned	4	24	32.7 ± 4.4**	21.9 ± 5.1*	-9.2 ± 2.6	0.11 ± 0.03	-0.4 ± 1.3

[1]Means ± SEM.

[2]Statistically different from open ewes, *P<.05, **P<.01.

lactation and weaning sheep, respectively. The hepatic net flux always showed the liver to be removing insulin from the circulation. The liver of control animals was removing insulin from the blood at a rate of 12.3 mU/min. There was a tendency ($P>.1$) for hepatic removal of insulin to decline during mid-gestation and weaned states. During late-gestation and lactation, a significant decrease in hepatic uptake of insulin by 50 and 60 % was observed, respectively. However, hepatic extraction ratio remained unchanged between controls and the various aforementioned physiological states and ranged from 7 to 11 %. The hindquarters were always removing insulin from the blood but at rates so small that the net flux values were not significantly different from zero.

Glucagon

Arterial plasma concentration of glucagon was 195 pg/ml in control animals and remained the same during mid-gestating, lactating and weaning animals (Table 7). During late-gestation a significant increase occured when the arterial concentration of glucagon increased by 25 % over the control value. Portal-drained viscera net flux, which refers to pancreatic as well as alimentary glucagon production, showed similar production rates between controls and the various different physiological states except during late-gestation and weaned states when significant increases were observed. The liver net flux always presented a negative rate indicating constant removal of glucagon from the blood. Livers of control animals were removing glucagon from the circulation at a rate of 1 to 2 µg/hr. Liver removal of glucagon increased significantly by 52 and 67 % during lactation and weaned

TABLE 7. Glucagon arterial concentrations and net fluxes in open, gestating, lactating, and weaning ewes.[1,2]

Physiological state	Exp	Obs	Artery	Portal-drained viscera	Liver	Hindquarters	Insulin glucagon ratio
			pg/ml	----------µg/h----------			
Open	9	44	195 ± 11	4.2 ± 0.5	-1.2 ± 0.4	-0.9 ± 0.2	9.4 ± 0.8
Mid Gestation	7	40	203 ± 13	5.1 ± 0.7	-1.9 ± 0.4	-0.4 ± 0.2	6.5 ± 0.8**
Late Gestation	8	48	260 ± 14**	5.3 ± 0.5†	-1.4 ± 0.4	-0.6 ± 0.3	2.7 ± 0.2**
Lactation	9	54	215 ± 6	4.5 ± 0.4	-2.5 ± 0.3*	-1.0 ± 0.1	2.1 ± 0.1**
Weaned	4	24	214 ± 5	5.6 ± 0.5†	-3.6 ± 0.7**	-0.7 ± 0.3	3.5 ± 0.4**

[1]Means ± SEM.

[2]Statistically different from open ewes, †P<.1, *P<.05, **P<.01.

states, respectively. The hindquarters were always removing glucagon from the blood at rates ranging from 0.4 to 1.0 μg/hr. Finally, insulin to glucagon ratio in control animals was 9.4. The ratio decreased significantly by 31, 71, 78 and 63 % during mid- and late-gestation, lactation and weaned states, respectively.

CHAPTER V

DISCUSSION

Glucose Metabolism

Arterial concentrations and net fluxes of glucose indicate normal metabolism occurring in open ewes. Similar concentrations and rates of hepatic gluconeogenesis were evident in results found by other laboratories (20,70,81). Arterial concentrations of glucose decreased in mid- and late-gestation and lactation without changes in rates of hepatic gluconeogenesis. Other researchers found similar decreases in arterial concentrations of glucose in pregnant (26,158) and lactating (79) compared to nonpregnant, nonlactating animals. However simultaneous measurements of hepatic gluconeogenesis were not determined in these reports.

Since hepatic *de novo* production of glucose remained relatively constant between the various physiological states and since renal gluconeogenesis remains the same between fed, open and pregnant sheep (83,84), the decreases in arterial concentrations of glucose observed must have been due to increased glucose utilization by some tissues. However, no significant hindquarter uptake of glucose was observed in either mid- or late-gestation. Although neither uterine or umbilical fluxes were measured directly, uptake of glucose by these tissues in both mid- and late-gestation may have increased. During mid-gestation, the decreased arterial concentration of glucose is perhaps due to

increased utilization by the uterus since in the first two months of gestation, the major part of the ewes weight increase is due to uterine growth (168). However, during late-gestation, when fetal growth accounts for the major portion of the maternal weight gain (168), the decreased arterial concentration of glucose is perhaps due to increased utilization by the utero-placental-fetal tissues. The ovine placenta has an increased rate of glucose utilization compared to the fetus. The utero-placental tissue takes up one-third of the maternally produced glucose of which only 33 % is directed to the fetal tissue (67).

The decrease in arterial glucose concentration during lactation may be due partially to an increase in mammary gland utilization of glucose. Other studies have reported that the mammary gland increases its oxidation (3) and utilization (39) of glucose during lactation. Although mammary venoarterial differences were not measured during this study, the increase in glucose uptake by the hindquarters observed during lactation is perhaps indicative of an increased mammary gland utilization of glucose. The above speculation is contingent upon whether or not the hindquarter catheter tips were located in such a way as to be a measure of mammary blood flow. In addition, the utilization of glucose by peripheral tissues of sheep decreases during lactation (167) thus increasing availability of glucose for milk synthesis by the mammary gland.

During weaning, arterial glucose concentration rose to near control values probably since demands for glucose by the utero-placental-fetal and mammary tissues decreased.

Although the insulin to glucagon ratio decreased during both

gestation and lactation, a condition which favors increased hepatic gluconeogenesis, glucose production by the liver remained the same in pregnant and lactating ewes. These results contradict previous reports which indicated an increased liver production of glucose during gestation (21,26,39,154) and lactation (39) perhaps due to an increase in the activity of liver gluconeogenic enzymes (107,152).

FFA Metabolism

Arterial concentrations and net fluxes of FFA similar to those reported here in open ewes have been demonstrated (70,81,97,143). This indicates normal rates of omental and hindquarter lipolysis and hepatic utilization were occurring in open animals. However, during mid- and late-gestation, arterial FFA concentrations were elevated due to increased omental and hindquarter lipolysis and despite a concentration dependent increased hepatic extraction. Other researchers have noted similar increases in arterial concentrations of FFA during mid- (166) and late-gestation (30,39,134,166,168) as well as similar hepatic extraction ratios (70,81).

The increased FFA release from the PDV and hindquarter can be explained by the decreased insulin to glucagon ratio evident during both mid- and late-gestation which favors a condition of fat mobilization vs re-esterification.

Although there is no apparent placental transfer of FFA to the sheep fetus (76) or uterine FFA oxidation (128), FFA have been reported to be the principle energy substrates of muscle during rest (78) as well as exercise (29). If this is true for the gestating ewe, FFA could

spare glucose for preferential use by the developing utero-placental-fetal tissues.

Also, the increases in FFA taken up by the liver could be utilized for the production of ketone bodies, again due to the lower insulin to glucagon ratio during gestation which enhances glucagon's ketogenic action upon the liver due to alleviation of malonyl-CoA inhibition of carnitine acyltransferase I (112,113). The increased arterial concentration of FFA, evident during gestation, resulted in an increase in concentration dependent hepatic uptake of FFA and subsequent elevated hepatic ketogenesis.

Arterial concentration of FFA remained elevated during lactation compared to controls, but was somewhat lower than in gestating animals. Similar observations have been reported by Chaiyabuti et al. (39). The arterial plasma level of FFA remained elevated due to four- and 2.9-fold increases in omental and hindquarter lipolysis, respectively, and despite a 47 % increase in hepatic uptake compared to controls. Although these increases were not statistically significant on an individual tissue basis, it seems apparent that there was some degree of physiological significance when looking at the whole body. Furthermore, fat mobilization during lactation appears to be due to a complete disappearance of re-esterification (115) in light of the low insulin to glucagon ratio. Since the mammary gland extracts neglible amounts of FFA, glucose availability for milk secreting tissue could by enhanced by extra mammary tissue utilization of FFA thus sparing glucose for milk synthesis (18).

After weaning, the ewe's arterial concentration of FFA fell 42 %

below control values. Although no statistical significant net fluxes were observed during the weaning state, the decreased FFA concentration can perhaps be attributed to a 49 % decrease in hindquarter lipolysis and more importantly, a PDV net uptake rather than a net release of FFA. Since PDV represents a measure of omental metabolism and since the omental fat pads are the most active tissue with respect to lipogenesis (75), it seems reasonable to suggest that during the weaning state, the body is replenishing lost fat reserves mobilized during lactation as well as gestation.

Ketone Body Metabolism

Control animals had arterial concentrations and net fluxes of acetoacetate and 3-hydroxybutyrate similar to open, fed ewes as previously reported (70,81,137,143). Arterial concentrations of acetoacetate remained the same throughout gestation, however PDV production and subsequent hepatic uptake decreased significantly during late-gestation. The decreased PDV production of acetoacetate might be due to underfeeding, however feed requirements were adjusted to meet the increased energy demands of gestation. The decrease in hepatic extraction is concentration dependent. In other words, the liver takes up nearly all the acetoacetate produced by the PDV which agrees with work reported by Heitmann et al. (70). Concomitant with increases or decreases in PDV acetoacetate production are subsequent increases or decreases in hepatic uptake. Since the activity of 3-oxoacid CoA transferase is non-existent in the ruminant liver (175), the apparent hepatic utilization of acetoacetate seems paradoxical. Perhaps what is

occurring is a conversion of acetoacetate to 3-hydroxybutyrate due to the presence of 3-hydroxybutyrate dehydrogenase in the liver (88) and dependent upon the redox potential (108).

Hindquarter extraction ratio is in agreement with previously published values (70,78) except during late-gestation when a significant 69 % decrease was observed. The decrease in acetoacetate extraction suggests a shunting of the ketone body away from the hindquarter and towards the utero-placental tissue. Although little oxidation of acetoacetate occurs in the pregnant uterus (127), the placental activity of 3-hydroxybutyrate dehydrogenase increases during late-gestation (49) and therefore acetoacetate could convert to 3-hydroxybutyrate for oxidation by the placenta (49,147) as well as the uterus (127).

During lactation, arterial concentration of acetoacetate increased by 61 % perhaps due in part to increased PDV production. One factor which could have elevated PDV production of acetoacetate is 3-hydroxybutyrate. Infusion of 3-hydroxybutyrate into normal sheep resulted in an increase in PDV release of acetoacetate (68). Also, the mammary gland could possibly have contributed to the increased arterial acetoacetate since mammary release of acetoacetate has been demonstrated during ketosis (91). During lactation in this study, both arterial concentration and PDV production of 3-hydroxybutyrate were elevated.

The liver removed all of the acetoacetate produced by the PDV thus favoring the theory that the mammary gland contributes to increased arterial acetoacetate.

The increased hindquarters utilization of acetoacetate during lactation could also be an indication of increased mammary utilization,

again, dependent upon hindquarter catheter location being a measure of mammary blood flow. The mammary gland has been shown to take up acetoacetate, however it utilizes 3-hydroxybutyrate to a much greater extent (91).

During weaning, arterial concentration of acetoacetate remained elevated, although 21 % less than during lactation, despite increased PDV release and subsequent complete hepatic removal. Again, arterial concentration and PDV production of 3-hydroxybutyrate were elevated which could explain the elevated PDV release of acetoacetate. However, the reason for an increase in arterial acetoacetate remains unknown. 3-hydroxybutyrate arterial concentration and net fluxes in control animals were also similar to values published by others (70) and are indicative of normal ketone body metabolism occurring in open sheep. Portal-drained viscera production of 3-hydroxybutyrate remained unchanged during pregnancy however, arterial concentrations rose significantly. These changes are due to increased hepatic release of 3-hydroxybutyrate during mid- and late-gestation of 36 and 109 % , respectively. Increased hepatic uptake of FFA combined with hepatic uptake and possible conversion of acetoacetate to 3-hydroxybutyrate during mid- and late-gestation appear sufficient to explain the increased arterial 3-hydroxybutyrate resulting from elevated hepatic ketogenesis. The lowered insulin to glucagon ratio during both phases of gestation favors glucagon's action promoting hepatic but not alimentary (32) ketogenesis.

Also, like the acetoacetate results, hindquarter extraction of 3-hydroxybutyrate decreased during late-gestation by 54 % suggesting a redirection of the ketone for use by the utero-placental tissue. Both

uterus and placenta oxidize 3-hydroxybutyrate in significant quantities (49,127,147).

The increased arterial concentration of 3-hydroxybutyrate during lactation appears to be primarily due to increased alimentary ketogenesis since hepatic uptake of FFA and subsequent ketone production by the liver decreased during this time. Other researchers (39) have reported increases in 3-hydroxybutyrate during lactation.

The 1.4-fold increase in hindquarter uptake of 3-hydroxybutyrate during lactation not only indicates increased rump skeletal muscle utilization but also suggests an increased mammary removal from the circulation for milk fat synthesis, again, depending upon catheter tip location. The cow mammary gland synthesizes milk fatty acids from 3-hydroxybutyrate (85,104,106,124,151) which is taken up by the gland at a rate eight-fold greater than acetoacetate (91).

The further increase in 3-hydroxybutyrate arterial concentration evident during weaning appears to be due to a more substantial increase in alimentary ketogenesis, again, perhaps due to overfeeding. Since the energy demands of gestation and lactation are no longer taxing the body during weaning, perhaps glucose is becoming the preferred energy substrate over FFA and ketone bodies. Although the hindquarters are taking up a significant amount of 3-hydroxybutyrate, the increased uptake is merely concentration dependent. If the weaned phase of this study had been carried on for a longer period of time, perhaps when mammary involution became complete, a more gradual return to control values might have become evident.

Insulin and Glucagon Metabolism

Open ewes apparently exhibited normal concentrations and fluxes of insulin and glucagon since values reported in this study are in agreement with earlier findings (70). Arterial insulin concentrations decreased during mid-gestation with a further decline during late-gestation. The decreased arterial concentrations of insulin during mid- and late-gestation were due to 48 and 46 % declines in pancreatic production, respectively. Decreases in arterial concentrations of insulin during gestation have also been reported elsewhere (30,166,168).

Hepatic extraction values remained unchanged throughout the study and agree closely with values published by Brockman and Bergman (34). Although the liver removed significantly less insulin from the circulation during late-gestation and lactation compared to control values, the hepatic removal of insulin throughout all phases of the study are within ranges reported by Brockman and Bergman (34). Also, it might be possible that the decreased hepatic uptake of insulin evident during late-gestation is related to insulin resistance noted during this period (100,101) which is characterized by a decreased sensitivity of the liver and peripheral tissues to insulin. Perhaps the decrease in sensitivity to insulin of the hepatic and peripheral tissue is due to the presence of the conceptus. The increased insulin resistance would enhance hepatic ketogenesis and peripheral lipolysis which are both evident during late-gestation. Insulin has a direct anti-ketogenic effect upon the liver of rats (96) and sheep (33) which would be eliminated by a decrease in hepatic insulin sensitivity. The increased lipolysis by the hindquarter and omental fat pads during gestation may

have been due to a fall in serum progesterone resulting in a decrease in adipocyte insulin receptors concomitant with a lowered serum insulin (56). Furthermore, the lipolytic effects of glucagon upon adipose tissue are more evident in the absence of insulin.

Umbilical and placental V-A differences were not measured directly in this study. However, since insulin promotes umbilical glucose extraction (47) without affecting placental transfer to the fetus (66), it appears that the decreased arterial insulin evident during gestation was actually a favorable condition promoting fetal development by reducing extra-utero-placental-fetal tissue glucose utilization.

The further decline in arterial concentration of insulin during lactation was undoubtedly due to a significant reduction in pancreatic production. Similar changes in arterial concentration, pancreatic output, and hepatic uptake have been reported in lactating animals (39,79,105,162,168). In lactating rats, decreased pancreatic insulin production was observed and explained in part by a decrease in Ca+2 content of the islet cells (74).

Insulin appears to have no direct effect upon mammary ketone or glucose metabolism (66,94). Therefore, the lowered insulin observed during lactation is actually advantageous to the mammary gland, enabling it to more favorably compete with insulin-dependent tissues for available nutrients. The lowered insulin during lactation also promotes FFA mobilization from adipose tissue stores and thus is ultimately responsible for the increased FFA levels prevalent during this period.

During weaning, arterial insulin remained lower than in controls, however the concentration began to rise due to an increase in PDV

production. Hepatic uptake returned to normal showing an uptake of approximately 50 % of the insulin released by the pancreas. Insulin concentrations were shown to return to normal values 95-135 days following lactation (168). If the weaning phase of this study had been carried out for a longer period of time, perhaps insulin levels would have returned to control values.

Arterial concentrations of glucagon for the most part remained relatively stable throughout this study, except for a significant increase noted during late-gestation. This increase could be attributed to an increased PDV release of glucagon. Therefore, during late-gestation, the rise in glucagon concomitant with lowered insulin concentration is probably responsible for the increased lipolysis and subsequent hepatic ketogenesis.

Arterial concentration of glucagon returned to normal values during lactation due to a two-fold increase in liver uptake combined with a slight decrease in PDV release. In cows, glucagon concentration remained the same throughout lactation (71) while in rats, glucagon was shown to have no effect upon mammary lipogenesis (136). Thus glucagon appears to have a relatively passive role during lactation.

Portal-drained viscera production of glucagon increased during weaning, however hepatic uptake increased thus maintaining normal arterial concentrations.

Hepatic extraction of glucagon throughout the study was approximately 7 % which is the same as that reported by Brockman et al. (35).

Conclusion

In conclusion, due to decreased pancreatic production of insulin during gestation and lactation, the insulin to glucagon ratio was lowered to a level which allowed glucagon to exert its lipolytic and ketogenic effects which resulted in increased FFA and ketone body concentrations in the blood. Also, since glucagon concentrations and net fluxes for the most part showed little change throughout this study, the influence of glucagon upon changes in lipolysis and ketogenesis depend primarily upon insulin concentrations as evidenced by the insulin to glucagon ratio. It was during times of low insulin and low insulin to glucagon ratio that major changes in concentration and fluxes of glucose, FFA and ketone bodies occurred. Furthermore, since glucose concentrations were lowered during gestation and lactation, the elevated FFA and ketone body concentrations in the circulation may serve to spare glucose for increased fetal and mammary tissue utilization.

Therefore, it appears that glucagon plays a relatively passive role during gestation and lactation while insulin activates the major changes in metabolism primarily due to its absence.

LIST OF REFERENCES

REFERENCES

1 Aiello, R. J., T. M. Kenna, and J. H. Herbein. 1984. Hepatic gluconeogeneic and ketogenic interrelationships in the lactating cow. J. Dairy Sci. 67:1707.

2 Andrews, A. H. 1982. Effects of glucose and propylene glycol on pregnancy toxaemia in ewes. Vet. Rec. 110:84.

3 Annison, E. F. and J. L. Linzell. 1964. The oxidation and utilization of glucose and acetate by the mammary gland of the goat in relation to their overall metabolism and to milk formation. J. Physiol. 175:372.

4 Annison, E. F., J. L. Linzell, and C. E. West. 1968. Mammary and whole animal metabolism of glucose and fatty acids in fasting lactating goats. J. Physiol. 197:445.

5 Annison, E. F., K. J. Hill, and D. Lewis. 1957. Studies on the portal blood of sheep. Biochem. J. 66:592.

6 Ash, R. and G. D. Baird. 1973. Activation of volatile fatty acids in bovine liver and rumen epithelium. Biochem. J. 136:311.

7 Baird, G. D. 1982. Primary ketosis in the high-producing dairy cow: Clinical and subclinical disorders, treatment, prevention, and outlook. J. Dairy Sci. 65:1.

8 Baird, G. D. 1977. Aspects of ruminant intermediary metabolism in relation to ketosis. Biochem. Rev. 5:819.

9 Baird, G. D. and R. J. Heitzman. 1971. Mode of action of a glucocorticoid on bovine intermediary metabolism. Biochim. Biophys. Acta. 252:184.

10 Baird, G. D., J. G. Van Der Walt, and E. N. Bergman. 1983. Whole-body metabolism of glucose and lactate in productive sheep and cows. Br. J. Nutr. 50:249.

11 Baird, G. D., H. W. Symonds, and R. Ash. 1974. Determination of portal and hepatic metabolite production rates in the adult dairy cow. Proc. Nutr. Soc. 33:70A.

12 Baird, G. D., R. J. Heitzman, and K. G. Hibbitt. 1972. Effects of starvation on intermediary metabolism in the lactating cow. Biochem. J. 128:1311.

13 Baird, G. D., K. G. Hibbitt, and J. Lee. 1970. Enzymes involved in acetoacetate formation in various bovine tissues. Biochem. J. 117:703.

14 Baird, G. D., R. J. Heitzman, K. G. Hibbitt, and G. D. Hunter. 1974. Bovine ketosis: A review with recommendations for control and treatment. Part II. Br. Vet. J. 130:318.

15 Baird, G. D., K. G. Hibbitt, G. D. Hunter, P. Lund, M. Stubbs, and H. A. Krebs. 1968. Biochemical aspects of bovine ketosis. Biochem. J. 107:683.

16 Balmain, J. H. and T. J. Folley. 1951. Further observations on the in vitro stimulation by insulin of fat synthesis by lactating mammary gland slices. Biochem J. 49:663.

17 Bauman, D. E. and W. B. Currie. 1980. Partitioning of nutrients during pregnancy and lactation: A review of mechanisms involving homeostasis and homeorhesis. J. Dairy Sci. 63:1514.

18 Bell, A. W. 1980. Lipid metabolism in liver and selected tissues and in the whole body of ruminant animals. Prog. Lipid Res. 18:117.

19 Bergman, E. N. 1971. Hyperketonemia-ketogenesis and ketone body metabolism. J. Dairy Sci. 54:936.

20 Bergman, E. N. 1968. Glycerol turnover in the nonpregnant and ketotic pregnant sheep. Am. J. Physiol. 215:865.

21 Bergman, E. N. 1964. Glucose turnover rates in pregnant and nonpregnant sheep. Nature. 202:1333.

22 Bergman, E. N. 1963. Quantitative aspects of glucose metabolism in pregnant and nonpregnant sheep. Am. J. Physiol. 204:147.

23 Bergman, E. N. and D. E. Hogue. 1967. Glucose turnover and oxidation rates in lactating sheep. Am. J. Physiol. 213:1378.

24 Bergman, E. N. and K. Kon. 1964. Acetoacetate turnover and oxidation rates in ovine pregnancy ketosis. Am. J. Physiol. 206:449.

25 Bergman, E. N. and K. Kon. 1964. Factors affecting acetoacetate production rates by normal and ketotic pregnant sheep. Am. J. Physiol. 206:453.

26 Bergman, E. N., M. L. Katz, and C. F. Kaufman. 1970. Quantitative aspects of hepatic and portal glucose metabolism and turnover in sheep. Am. J. Physiol. 219:785.

27 Bergman, E. N., W. E. Roe, and K. Kon. 1966. Quantitative aspects of propionate metabolism and gluconeogenesis in sheep. Am. J. Physiol. 211:793.

28 Bickerstaffe, R. and E. F. Annison. 1974. The metabolism of glucose, acetate, lipids and amino acids in lactating dairy cows. J. Agric. Sci., Camb. 82:71.

29 Bird, A. R., K. E. Chandler, and A. W. Bell. 1981. Effects of exercise and plane of nutrition on nutrient utilization by the hind limb of the sheep. Aust. J. Biol Sci. 34:541.

30 Blom, A. K., K. Hove, and J. J. Nedkvitne. 1976. Plasma insulin and growth hormone concentrations in pregnant sheep II: Post-absorptive levels in mid- and late pregnancy. Acta Endocrinol. 82:553.

31 Brockman, R. P. 1979. Roles for insulin and glucagon in the development of ruminant ketosis --- A review. Can. Vet. J. 20:121.

32 Brockman, R. P. 1976. Effects of glucagon and insulin on lipolysis and ketogenesis in sheep. Can. J. Comp. Med. 40:166.

33 Brockman, R. P. and B. Laarveld. 1985. Effects of insulin on net hepatic metabolism of acetate and 3-hydroxybutyrate in sheep (Ovis Aries). Comp. Biochem Physiol. 81A:255.

34 Brockman, R. P. and E. N. Bergman. 1975. Quantitative aspects of insulin secretion and its hepatic and renal removal in sheep. Am. J. Physiol. 229:1338.

35 Brockman, R. P., J. G. Manns, and E. N. Bergman. 1976. Quantitative aspects of secretion and hepatic removal of glucagon in sheep. Can. J. Physiol. Pharmacol. 54:666.

36 Brockman, R. P., E. N. Bergman, P. K. Joo, and J. G. Manns. 1975. Effects of glucagon and insulin on net hepatic metabolism of glucose precursors in sheep. Am. J. Physiol. 229:1344.

37 Burnol, A., A. Leturque, P. Ferre, and J. Girard. 1983. Glucose metabolism during lactation in the rat: Quantitative and regulatory aspects. Am. J. Physiol. 245:E351.

38 Bush, R. S. and L. P. Milligan. 1971. Enzymes of ketogenesis in bovine rumen epithelium. Can. J. Anim. Sci. 51:129.

39 Chaiyabutr, N., A. Faulkner, and M. Peaker. 1982. Glucose metabolism *in vivo* in fed and 48 h starved goats during pregnancy and lactation. Br. J. Nutr. 47:87.

40 Chaiyabutr, N., A. Faulkner, and M. Peaker. 1980. The utilization of glucose for the synthesis of milk components in the fed and starved lactating goat in vivo. Biochem. J. 186:301.

41 Chandler, K. D., B. J. Leury, A. R. Bird, and A. W. Bell. 1985. Effects of undernutrition and exercise during late pregnancy on uterine, fetal and uteroplacental metabolism in the ewe. Br. J. Nutr. 53:625.

42 Char, V. C. and R. K. Creasy. 1976. Acetate as a metabolic substrate in the fetal lamb. Am. J. Physiol. 230:357.

43 Christie, W. W. and R. C. Noble. 1982. Fatty acid biosynthesis in sheep placenta and maternal and fetal adipose tissue. Biol. Neonate. 42:79.

44 Comline, R. S. and M. Silver. 1976. Some aspects of foetal and utero-placental metabolism in cows with indwelling umbilical and uterine vascular catheters. J. Physiol. 260:571.

45 Costa, N. D., G. H. McIntosh, and A. M. Snoswell. 1976. Production of endogenous acetate by the liver in lactating ewes. Aust. J. Biol. Sci. 29:33.

46 Crabtree, B., D. J. Taylor, J. E. Coombs, R. A. Smith, S. P. Templer, and G. H. Smith. 1981. The activities and intracellular distributions of enzymes of carbohydrate, lipid and ketone-body metabolism in lactating mammary glands from ruminants and non-ruminants. Biochem. J. 196:747.

47 Crandell, S. S., P. A. Palma, and F. H. Morriss. 1982. Effect of maternal serum insulin on umbilical extraction of glucose and lactate in fed and fasted sheep. Am. J. Obstet. Gynecol. 142:219.

48 deBoer, G., A. Trenkle, and J. W. Young. 1985. Glucagon, insulin, growth hormone, and some blood metabolites during energy restriction ketonemia of lactating cows. J. Dairy Sci. 68:326.

49 Edwards, E. M., J. M. Rattenbury, G. C. E. Varnam, U. K. Dhand, M. K. Jeacock, and D. A. L. Shepherd. 1977. Enzyme activities in the sheep placenta during the last three months of pregnancy. Biochimica et Biophysica Acta. 497:133.

50 Elphick, M. C. and D. Hull. 1977. The transfer of free fatty acids across the rabbit placenta. J. Physiol. 264:751.

51 Emmanuel, B. 1980. Oxidation of butyrate to ketone bodies and CO2 in the rumen epithelium, liver, kidney, heart and lung of camel (Camelus Dromedarius), sheep (Ovis Aries) and goat (Capra Hircus). Comp. Biochem. Physiol. 65B:699.

52 Emmanuel, B. and L. P. Milligan. 1983. Butyrate:acetoacetyl-CoA transferase activity in bovine rumen epithelium. Can. J. Anim. Sci. 63:355.

53 Felts, J. M. and P. A. Mayes. 1965. Lack of uptake and oxidation of chylomicron triglyceride to CO2 and ketone bodies by the perfused rat liver. Nature. 206:195.

54 Fleet, I. R. and T. B. Mepham. 1985. Mammary uptake of amino acids and glucose throughout lactation in Friesland sheep. J. Dairy Res. 52:229.

55 Flint, D. J. 1980. Changes in the number of insulin receptors of isolated rat hepatocytes during pregnancy and lactation. Biochimica et Biophysica Acta. 628:322.

56 Flint, D. J., R. A. Clegg, and R. G. Vernon. 1980. Regulation of adipocyte insulin receptor number and metabolism during late-pregnancy. Mol. Cell. Endocrinol. 20:101.

57 Forsberg, N. E., R. L. Baldwin, and N. E. Smith. 1985. Roles of glucose and its interactions with acetate in maintenance and biosynthesis in bovine mammary tissue. J. Dairy Sci. 68:2544.

58 Fox, F. H. 1971. Clinical diagnosis and treatment of ketosis. J. Dairy Sci. 54:974.

59 Girard, J., P. H. Duee, P. Ferre, J. P. Pegorier, F. Escriva, and J. F. Decaux. 1985. Fatty acid oxidation and ketogenesis during development. Reprod. Nutr. Develop. 25:303.

60 Hallford, D. M. and D. W. Sanson. 1983. Serum profiles determined during ovine pregnancy toxemia. Agri-Practice 4:27.

61 Hanson, R. W. and F. J. Ballard. 1967. The relative significance of acetate and glucose as precursors for lipid synthesis in liver and adipose tissue from ruminants. Biochem. J. 105:529.

62 Hart, I. C. 1983. Endocrine control on nutrient partition in lactating ruminants. Proc. Nutr. Soc. 42:181.

63 Hart, I. C., J. A. Bines, S. V. Morant, and J. L. Ridley. 1978. Endocrine control of energy metabolism in the cow: Comparison of the levels of hormones (prolactin, growth hormone, insulin and thyroxine) and metabolites in the plasma of high- and low-yielding cattle at various stages of lactation. J. Endocrinol. 77:333.

64 Hawkins, R. A. and D. H. Williamson. 1972. Measurements of substrate uptake by mammary gland of the rat. Biochem. J. 129:1171.

65 Hawkins, R. A., E. H. Williamson, and H. A. Krebs. 1971. Ketone body utilization by adult and suckling rat brain in vivo. Biochem. J. 122:13.

66 Hay, W. W. Jr., J. W. Sparks, M. Gilbert, F. C. Battaglia, and G. Meschia. 1984. Effect of insulin on glucose uptake by the maternal hindlimb and uterus, and by the fetus in conscious pregnant sheep. J. Endocrinol. 100:119.

67 Hay, W. W. Jr., J. W. Sparks, R. B. Wilkening, F. C. Battaglia, and G. Meschia. 1983. Partition of maternal glucose production between conceptus and maternal tissues in sheep. Am. J. Physiol. 245:E347.

68 Heitmann, R. N. and J. M. Fernandez. 1986. Autoregulation of alimentary and hepatic ketogenesis in sheep. J. Dairy Sci. 69:1270.

69 Heitmann, R. N. and E. N. Bergman. 1981. Glutamate interconversions and glucogenicity in the sheep. Am. J. Physiol. 241:E465.

70 Heitmann, R. N., S. C. Sensenig, C. K. Reynolds, J. M. Fernandez, and D. J. Dawes. 1986. Changes in energy metabolite and regulatory hormone concentrations and net fluxes across splanchnic and peripheral tissue in fed and progressively fasted ewes. J. Nutr. 116:2516.

71 Herbein, J. H., R. J. Aiello, L. I. Eckler, R. E. Pearson, and R. M. Akers. 1985. Glucagon, insulin, growth hormone, and glucose concentrations in blood plasma of lactating dairy cows. J. Dairy Sci. 68:320.

72 Hird, F. J. R. and R. H. Symons. 1961. The mode of formation of ketone bodies from butyrate by tissue from the rumen and omasum of the sheep. Biochimica et Biophysica Acta. 46:457.

73 Hove, K. and A. K. Blom. 1976. Plasma insulin and growth hormone concentrations in pregnant sheep I: Diurnal variations in mid- and late-pregnancy. Acta Endocrinol. 82:544.

74 Hubinont, C. J., S. P. Dufrane, P. Garcia-Morales, I. Valverde, A. Sener, and W. J. Malaisse. 1986. Influence of lactation upon pancreatic islet function. Endocrinol. 118:687.

75 Ingle, D. L., D. E. Bauman, and U. S. Garrigus. 1972. Lipogenesis in the ruminant: In vivo site of fatty acid synthesis in sheep. J. Nutr. 102:617.

76 James, E., G. Meschia, and F. C. Battaglia. 1971. A-V differences of free fatty acids and glycerol in the ovine umbilical circulation. Proc. Soc. Exp. Biol. Med. 138:823.

77 James, E. J., J. R. Raye, E. L. Gresham, E. L. Makowski, G. Meschia, and F. C. Battaglia. 1972. Fetal oxygen consumption, carbon dioxide production, and glucose uptake in a chronic sheep preparation. Pedriatrics. 50:361.

78 Jarrett, I. G., O. H. Filsell, and F. J. Ballard. 1976. Utilization of oxidizable substrates by the sheep hind limb: Effects of starvation and exercise. Metabolism. 25:523.

79 Jones, R. G., V. Ilic, and D. H. Williamson. 1984. Physiological significance of altered insulin metabolism in the conscious rat during lactation. Biochem. J. 220:455.

80 Katz, M. L. and E. N. Bergman. 1969. Simultaneous measurements of hepatic and portal venous blood flow in the sheep and dog. Am. J. Physiol. 216:946.

81 Katz, M. L. and E. N. Bergman. 1969. Hepatic and portal metabolism of glucose, free fatty acids, and ketone bodies in the sheep. Am. J. Physiol. 216:953.

82 Katz, M. L. and E. N. Bergman. 1969. A method for simultaneous cannulation of the major splanchnic blood vessels of the sheep. Am. J. Vet. Res. 30:655.

83 Kaufman, C. F. and E. N. Bergman. 1974. Renal ketone body metabolism and gluconeogenesis in normal and hypoglycemic sheep. Am. J. Physiol. 226:827.

84 Kaufman, C. F. and E. N. Bergman. 1971. Renal glucose, free fatty acid, and ketone body metabolism in the unanesthetized sheep. Am. J. Physiol. 221:967.

85 King, K. R., J. M. Gooden, and E. F. Annison. 1985. Acetate metabolism in the mammary gland of the lactating ewe. Aust. J. Biol. Sci. 38:23.

86 Kleiber, M., A. L. Black, M. A. Brown, C. F. Baxter, J. R. Luick, and F. H. Stadtman. 1955. Glucose as a precursor of milk constituents in the intact dairy cow. Biochimica et Biophysica Acta. 17:252.

87 Knowles, S. E., I. G. Jarrett, O. H. Filsell, and F. H. Ballard. 1974. Production and utilization of acetate in mammals. Biochem. J. 142:401.

88 Koundakjian, P. P. and A. M. Snoswell. 1970. Ketone body and fatty acid metabolism in sheep tissues. Biochem. J. 119:49.

89 Kronfeld, D. S. 1972. Ketosis in pregnant sheep and lactating cows. A Review. Aust. Vet. J. 48:680.

90 Kronfeld, D. S. 1957. A comparison of normal concentrations of reducing sugar, volatile fatty acids, and ketone bodies in the blood of lambs, pregnant ewes, and non-pregnant adult ewes. Aust. J. Agr. Res. 8:202.

91 Kronfeld, D. S., F. Raggi, and C. F. Ramberg, Jr. 1968. Mammary blood flow and ketone body metabolism in normal, fasted, and ketotic cows. Am. J. Physiol. 215:218.

92 Kronfeld, D. S., G. P. Mayer, J. McD. Robertson, and F. Raggi. 1963. Depression of milk secretion during insulin administration. J. Dairy Sci. 46:559.

93 Kulhanek, J. F., G. Meschia, E. L. Makowski, and F. C. Battaglia. 1974. Changes in DNA content and urea permeability of the sheep placenta. Am. J. Physiol. 226:1257.

94 Laarveld, B., R. K. Chaplin, and R. P. Brockman. 1985. Effects of insulin on the metabolism of acetate, 3-hydroxybutyrate and triglycerides by the bovine mammary gland. Comp. Biochem. Physiol. 82B:265.

95 Laarveld, B., D. A. Christensen, and R. P. Brockman. 1981. The effect of insulin on net metabolism of glucose and amino acids by the bovine mammary gland. Endocrinol. 108:2217.

96 Laker, M. E. and P. A. Mayes. 1984. Investigations into the direct effects of insulin on hepatic ketogenesis, lipoprotein secretion and pyruvate dehydrogenase activity. Biochimica et Biophysica Acta. 795:427.

97 Leat, W. M. F. 1974. Variation in plasma glucose and free fatty acid concentrations in sheep associated with season, pregnancy and lactation. J. Agric. Sci., Camb. 82:181.

98 Leat, W. M. F. 1966. Utilization of free fatty acids by starved and pregnant sheep. Biochem. J. 101:317.

99 Leighton, B., A. R. Nicholas, and C. I. Pogson. 1983. The pathway of ketogenesis in rumen epithelium of the sheep. Biochem. J. 216:769.

100 Leturque, A., A. F. Burnol, P. Ferre, and J. Girard. 1984. Pregnancy-induced insulin resistance in the rat: Assessment by glucose clamp technique. Am. J. Physiol. 246:E25.

101 Leturque, A., P. Ferre, A. Burnol, J. Kande, P. Maulard, and J. Girard. 1986. Glucose utilization rates and insulin sensitivity in vivo in tissues of virgin and pregnant rats. Diabetes. 35:172.

102 Lindsay, D. B. 1959. The significance of carbohydrate in ruminant metabolism. Vet. Res. Annotations. 5:103.

103 Lindsay, D. B. and B. P. Setchell. 1976. The oxidation of glucose, ketone bodies and acetate by the brain of normal and ketonaemic sheep. J. Physiol. 259:801.

104 Linzell, J. L., E. F. Annison, S. Fazakerley, and R. A. Leng. 1967. The incorporation of acetate, stearate and D(-)-3-hydroxybutyrate into milk fat by the isolated perfused mammary gland of the goat. Biochem. J. 104:34.

105 Lomax, M. A., G. D. Baird, C. B. Mallinson, and H. W. Symonds. 1979. Differences between lactating and nonlactating dairy cows in concentration and secretion rate of insulin. Biochem. J. 180:281.

106 Luick, J. R., and M. Kleiber. 1967. Synthesis of milk fatty acids from D, L $3C^{14}$-betahydroxybutrate. Fed. Proc. 26:632 (abst 2105).

107 Mackie, W. S. and R. M. Campbell. 1972. Effects of pregnancy and lactation on the activities of some gluconeogenic and urea-cycle enzymes in sheep liver. J. Agric. Sci., Camb. 79:423.

108 Martin, D. W. Jr., P. A. Mayes, V. W. Rodwell, and D. K. Granner. 1985. Harper's Review of Biochemistry, 20th. ed., Lange Medical Publications, Los Altos, Ca.

109 Martin, R. J., T. G. Ramsay, and R. B. S. Harris. 1984. Central role of insulin in growth and development. Dom. Anim. Endocrinol. 1:89.

110 Mayes, P. A. and J. M. Felts. 1967. Regulation of fat metabolism in the liver. Nature. 215:716.

111 Maynard, L. A., J. K. Loosli, H. F. Hintz, and R. G. Warner, ed. 1979. The expanding field of nutrition. Page 1 in Animal Nutrition. 7th ed. McGraw-Hill Co., New York, N. Y.

112 McGarry, J. D. and D. W. Foster. 1979. In support of the roles of malonyl-CoA and carnitine acyltransferase I in the regulation of hepatic fatty acid oxidation and ketogenesis. J. Biol. Chem. 254:8163.

113 McGarry, J. D., G. F. Leatherman, and D. W. Foster. 1978. Carnitine palmitoyltransferase I. J. Biol. Chem. 253:4128.

114 Mellenberger, R. W., D. E. Bauman, and D. R. Nelson. 1973. Metabolic adaptations during lactogenesis. Biochem. J. 136:741.

115 Metz, S. H. M. and S. G. van den Bergh. 1977. Regulation of fat mobilization in adipose tissue of dairy cows in the period around parturition. Neth. J. Agric. Sci. 25:198.

116 Mills, S. E., D. C. Beitz, and J. W. Young. 1986. Evidence for impaired metabolism in liver during induced lactation ketosis of dairy cows. J. Dairy Sci. 69:362.

117 Morris, B. 1963. The metabolism of free fatty acids and chylomicron triglycerides by the isolated perfused liver of the rat. J. Physiol. 168:564.

118 Morriss, F. H. Jr., R. D. H. Boyd, E. L. Makowski, G. Meschia, and F. C. Battaglia. 1974. Umbilical V-A differences of acetoacetate and 3-hydroxybutyrate in fed and starved ewes. Proc. Soc. Exp. Biol. Med. 145:879.

119 Morriss, F. H. Jr., C. R. Rosenfeld, R. Resnik, G. Meschia, E. L. Makowski, and F. C. Battaglia. 1974. Growth of uterine oxygen and glucose uptakes during pregnancy in sheep. Gynecol. Invest. 5:230.

120 Noble, R. C., W. Steele, and J. H. Moore. 1971. The plasma lipids of the ewe during pregnancy and lactation. Res. Vet. Sci. 12:47.

121 Noble, R. C., J. H. Shand, A. W. Bell, G. E. Thompson, and J. H. Moore. 1978. The transfer of free palmitic and linoleic acids across the ovine placenta. Lipids. 13:610.

122 Oddy, V. H., J. M. Gooden, G. M. Hough, E. Teleni, and E. F. Annison. 1985. Partitioning of nutrients in Merino ewes. II. Glucose utilization by skeletal muscle, the pregnant uterus and the lactating mammary gland in relation to whole body glucose utilization. Aust. J. Biol. Sci. 38:95.

123 Owen, D. E., A. P. Morgan, H. G. Kemp, J. M. Sullivan, M. G. Herrera, and G. F. Cahill. 1967. Brain metabolism during fasting. J. Clin. Invest. 46:1589.

124 Palmquist, D. L., C. L. Davis, R. E. Brown, and D. S. Sachan. 1967. Availability and metabolism of various substrates in ruminants. V. Entry rate into the body and incorporation into milk fat of D(-)3-hydroxybutyrate. J. Dairy Sci. 52:633.

125 Paxson, C. L. Jr., F. H. Morriss, Jr., and E. W. Adcock III. 1978. Effect of uterine artery insulin infusions on umbilical glucose uptake in sheep. Pediat. Res. 12:864.

126 Pethick, D. W. and D. B. Lindsay. 1982. Acetate metabolism in lactating sheep. Br. J. Nutr. 48:319.

127 Pethick, D. W. and D. B. Lindsay. 1982. Metabolism of ketone bodies in pregnant sheep. Br. J. Nutr. 48:549.

128 Pethick, D. W., D. B. Lindsay, P. J. Barker, and A. J. Northrop. 1983. The metabolism of circulating nonesterified fatty acids by the whole animal, hind-limb muscle and uterus of pregnant ewes. Br. J. Nutr. 49:129.

129 Pethick, D. W., D. B. Lindsay, P. J. Barker, and A. J. Northrop. 1981. Acetate supply and utilization by the tissues of sheep *in vivo*. Br. J. Nutr. 46:97.

130 Philipps, A. F., P. W. Porte, and J. R. Raye. 1985. Relationship between resting glucose consumption and insulin secretion in the ovine fetus. Biol. Neonate. 48:85.

131 Portman, O. W., R. E. Behrman, and P. Soltys. 1969. Transfer of free fatty acids across the primate placenta. Am. J. Physiol. 216:143.

132 Posner, B. I. 1974. Insulin receptors in human and animal placental tissue. Diabetes. 23:209.

133 Prior, R. L. and R. K. Christenson. 1978. Insulin and glucose effects on glucose metabolism in pregnant and nonpregnant ewes. J. Anim. Sci. 46:201.

134 Reid, R. L. and N. T. Hinks. 1962. Studies on the carbohydrate metabolism of sheep. XVIII. The metabolism of glucose, free fatty acids, ketones, and amino acids in late pregnancy and lactation. Aust. J. Agr. Res. 13:1112.

135 Robinson, A$_{14}$ M. and D. H. Williamson. 1978. Utilization of D-3-hydroxy [3-^{14}C]butyrate for lipogenesis *in vivo* in lactating rat mammary gland. Biochem. J. 176:635.

136 Robson, N. A., R. A. Clegg, and V. A. Zammit. 1984. Regulation of peripheral lipogenesis by glucagon. Biochem. J. 217:743.

137 Roe, W. E., E. N. Bergman, and K. Kon. 1966. Absorption of ketone bodies and other metabolites via the portal blood of sheep. Am. J. Vet. Res. 27:729.

138 Ruderman, N. B. and M. N. Goodman. 1973. Regulation of ketone body metabolism in skeletal muscle. Am. J. Physiol. 224:1391.

139 Ruderman, N. B., C. R. S. Houghton, and R. Hems. 1971. Evaluation of the isolated perfused hindquarter for the study of muscle metabolism. Biochem. J. 124:639.

140 Schwalm, J. W. and L. H. Schultz. 1976. Relationship of insulin concentration to blood metabolites in the dairy cow. J. Dairy Sci. 59:555.

141 Seccombe, D. W., P. G. R. Harding, and F. Possmayer. 1977. Fetal utilization of maternally derived ketone bodies for lipogenesis in the rat. Biochimica et Biophysica Acta. 488:402.

142 Seeds, A. E., L. S. Leung, S. J. Stys, K. E. Clark, and P. T. Russell. 1980. Comparison of human and sheep chorion laeve permeability to glucose, 3-hydroxybutyrate, and glycerol. Am. J. Obstet. Gynecol. 138:604.

143 Sensenig, S. C., D. J. Dawes, and R. N. Heitmann. 1985. Energy metabolite concentrations and net fluxes across splanchnic and peripheral tissues in pregnant ewes. J. Anim. Sci. 61 (Suppl.), 454 (abst).

144 Seto, K., T. Tsuda, and M. Umezu. 1955. The mechanism of the ketone bodies production with rumen epithelium. I. The ketone bodies production from volatile fatty acids with rumen epithelium and the influence of substances concerned with the citric acid cycle on their production. Tohoku J. Agric. Res. 4:91.

145 Seifert, C. D., M. Graf, G. Janson, A. Kuhn, and H. D. Soling. 1974. Formation of free acetate by isolated perfused livers from normal, starved and diabetic rats. Biochem. Biophys. Res. Comm. 57:901.

146 Shambaugh, G. E. III. 1985. Ketone body metabolism in the mother and fetus. Fed. Proc. 44:2347.

147 Shambaugh, G. E. III, S. C. Mrozak, and N. Freinkel. 1977. Fetal fuels. I. Utilization of ketones by isolated tissues at various stages of maturation and maternal nutrition during late gestation. Metabolism. 26:623.

148 Shand, J. H. and R. C. Noble. 1979. Delta-9 and delta-6 desaturase activities of the ovine placenta and their role in the supply of fatty acids to the fetus. Biol. Neonate. 36:298.

149 Shaw, J. C. and C. B. Knodt. 1941. The utilization of 3-hydroxybutyric acid by the lactating mammary gland. J. Biol. Chem. 138:287.

150 Simmons, M. A., M. D. Jones, Jr., F. C. Battaglia, and G. Meschia. 1978. Insulin effect on fetal glucose utilization. Pediat. Res. 12:90.

151 Smith, G. H. and S. McCarthy. 1969. Synthesis of milk fat from 3-hydroxybutyrate and acetate in mammary tissue in the cow. Biochimica et Biophysica Acta. 176:664.

152 Smith, R. W. and A. Walsh. 1982. Effects of pregnancy and lactation on the activities in sheep liver of some enzymes of glucose metabolism. J. Agric. Sci., Camb. 98:563.

153 Somogyi, M. 1945. Determination of blood sugar. J. Biol. Chem. 160:69.

154 Steel, J. W. and R. A. Leng. 1973. Effects of plane of nutrition and pregnancy on gluconeogenesis in sheep. 1. The kinetics of glucose metabolism. Br. J. Nutr. 30:451.

155 Steel, J. W. and R. A. Leng. 1973. Effects of plane of nutrition and pregnancy on gluconeogenesis in sheep. 2. Synthesis of glucose from ruminal propionate. Br. J. Nutr. 30:475.

156 Storry, J. E. and J. A. F. Rook. 1965. Effect in the cow of intraruminal infusions of volatile fatty acids and of lactic acid on the secretion of the component fatty acids of the milk fat and on the composition of blood. Biochem. J. 96:210.

157 Unger, R. H. 1971. Glucagon and the insulin:glucagon ratio in diabetes and other catabolic illnesses. Diabetes. 20:834.

158 van der Walt, J. G., J. Procos, and F. J. Labuschagne. 1980. Glucose turnover, tolerance and insulin response in wethers, ewes and pregnant ewes in the fed and fasted state. Onderstepoort J. Vet. Res. 47:173.

159 van Duyne, C. M., H. R. Parker, R. J. Havel, and L. W. Holm. 1960. Free fatty acid metabolism in fetal and newborn sheep. Am. J. Physiol. 199:987.

160 Varnam, G. C. E., M. K. Jeacock, and D. A. L. Shepherd. 1978. Hepatic ketone-body metabolism in developing sheep and pregnant ewes. Br. J. Nutr. 40:359.

161 Varnam, G. C. E., M. K. Jeacock, and D. A. L. Shepherd. 1978. Activities of ketone body utilising enzymes in tissues of fed and fasted sheep. Res. Vet. Sci. 24:124.

162 Vasilatos, R. and P. J. Wangsness. 1981. Diurnal variations in plasma insulin and growth hormone associated with two stages of lactation in high producing dairy cows. Endocrinol. 108:300.

163 Vernon, R. G. and E. Finley. 1985. Regulation of lipolysis during pregnancy and lactation in sheep. Biochem. J. 230:651.

164 Vernon, R. G. and D. J. Flint. 1984. Adipose tissue: Metabolic adaptation during lactation. Symp. Zool. Soc. Lond. 51:119.

165 Vernon, R. G. and D. J. Flint. 1983. Control of fatty acid synthesis in lactation. Proc. Nutr. Soc. 42:315.

166 Vernon, R. G., R. A. Clegg, and D. J. Flint. 1985. Adaptations of adipose tissue metabolism and number of insulin receptors in pregnant sheep. Comp. Biochem. Physiol. 81B:909.

167 Vernon, R. G., E. Taylor, and A. Faulkner. 1984. Regulation of glucose metabolism in tissues of the sheep hind-limb: Response to lactation. Can. J. Anim. Sci. 64(Suppl):302.

168 Vernon, R. G., R. A. Clegg, and D. J. Flint. 1981. Metabolism of sheep adipose tissue during pregnancy and lactation. Biochem. J. 200:307.

169 Wallace, L. R. 1945. The growth of lambs before and after birth in relation to the level of nutrition. II. J. Agric. Sci., Camb. 38:243.

170 Wastney, M. E., J. E. Wolff, and R. Bickerstaffe. 1983. Glucose turnover and hepatocyte glucose production of starved and toxaemic pregnant sheep. Aust. J. Biol. Sci. 36:271.

171 Watson, H. R. and D. B. Lindsay. 1972. 3-Hydroxybutyrate dehydrogenase in tissues from normal and ketonaemic sheep. Biochem. J. 128:53.

172 Weidermann, M. J. and H. A. Krebs. 1969. The fuel of respiration of rat kidney cortex. Biochem. J. 112:149.

173 West, C. E. and E. F. Annison. 1964. Metabolism of palmitate in sheep. Biochem. J. 92:573.

174 Williamson, D. H., J. Mellanby, and H. A. Krebs. 1962. Enzymic determination of D(-)-3-hydroxybutyric acid and acetoacetic acid in blood. Biochem. J. 82:90.

175 Williamson, D. H., M. W. Bates, M. A. Page, and H. A. Krebs. 1971. Activities of enzymes involved in acetoacetate utilization in adult mammalian tissues. Biochem. J. 121:41.

176 Wilson, S., J. C. MacRae, and P. J. Buttery. 1983. Glucose production and utilization in non-pregnant, pregnant and lactating ewes. Br. J. Nutr. 50:303.

177 Yalow, R. S. 1976. Methods in Radioimmunoassay of Peptide Hormones, North-Holland, Amsterdam.

178 Yelverton, J. T., E. A. Henderson, and R. W. Dougherty. 1969. A chronically implanted arterial catheter for unanesthetized animals. Cornell Vet. 59:466.

APPENDICES

APPENDIX 1

Glucose Analysis

Sigma Chemical Co. #510 enzymatic colorimetric *in vitro* determination for whole blood, plasma or serum is based upon the following coupled enzymatic reactions:

$$\text{1) glucose} + 2\,H_2O + O_2 \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + 2\,H_2O_2$$

$$\text{2) } H_2O_2 + \text{o-Dianisidine (colorless)} \xrightarrow{\text{peroxidase}} \text{oxidized o-Dianisidine (brown color)}$$

The intensity of the brown color is measured at 450 nm and is proportional to the original glucose concentration.

Reagents

A) PGO enzyme, stock # 510-6 (preweighed capsules).

--contains glucose oxidase, peroxidase, and buffer salts.
--one capsule is diluted with H_2O to 100 ml, and stored in an amber bottle at 0-5 degrees C.

B) o-Dianisidine Dihydrochloride, stock # 510-50.

--preweighed vial is diluted with H_2O to 20 ml and stored at 0-5 degrees C.
--1.6 ml of this is added to 100 ml of PGO enzyme solution.

C) Glucose Standard Solution, stock # 535-100.

--consists of a solution of beta-D-glucose, 100 mg/dl in benzoic acid, 0.1 % and stored at 0-5 degrees C.

D) Barium Hydroxide Solution, stock # 14-3, 0.3N.

E) Zinc Sulfate Solution, stock # 14-4, $ZnSO_4 * 7H_2O$, 5%.

Specimen Collection and Preparation

Blood should be collected in a container with suitable anticoagulant. Immediately after sample is collected, pipette 0.2 ml blood in test tube containing 1.8 ml of distilled H2O, then vortex. To each tube add 1 ml barium hydroxide, vortex, then add 1 ml zinc sulfate and vortex again. Blank and standard are prepared in a similar manner except 0.2 ml of distilled H2O and 0.2 ml of glucose standard are used, respectively, in place of the blood. Tubes are then centrifuged at 3g for 15 min at 0-5 degrees C.

Analysis

Prepare unknown, blank, and standard tubes in duplicate as follows:

To each tube add 0.25 ml of supernatant from the respective centrifuged unknown, blank, and standard tubes followed by 2.5 ml of PGO/color solution and vortex. Incubate all tubes at 37 degrees C for 30 min in a waterbath or at room temperature for 45 min.

Avoid direct exposure to sunlight or bright daylight. Following incubation, remove all tubes from the waterbath, if necessary, and read at 450 nm. Readings should be completed within 30 min.

Calculation

$$\text{Blood Glucose (mg/dl)} = \frac{\text{absorbance unknown}}{\text{absorbance standard}} \times 100$$

APPENDIX 2

Free Fatty Acid Analysis

NEFA - C kit from WAKO and is based upon the following reactions:

A) FFA are activated to CoA ester by acyl CoA synthetase (ACS)

$$R\text{-}COOH + ATP + CoA \xrightarrow{ACS} acyl\text{-}CoA + AMP + PP$$

B) Acyl-CoA is oxidized by acyl CoA oxidase (ACOD) to produce hydrogen peroxide

$$acyl\text{-}CoA + O2 \xrightarrow{ACOD} 2,3\ trans\text{-}enoyl\text{-}CoA + H2O2$$

C) Hydrogen peroxide is acted on by peroxidase (POD) in the presence of 3-methyl-N-ethyl-N-beta-hydroxyethyl-aniline (MEHA) and 4-aminoantipyrine (AAP) to form a product with purple color which is measured colorimetrically at 550 nm.

$$2\ H2O2 + MEHA + AAP \longrightarrow purple\ quinone\ product + 4\ H2O$$

Reagents

A) Color Reagent "A". each vial contains:

- acyl-CoA synthetase
- ascorbate oxidase
- CoA
- ATP
- 4 aminoantipyrine

B) Diluent for color reagent "A". bottle contains:

- phosphate buffer, pH 6.9 (0.05 M)
- magnesium chloride
- surfactant
- stabilizers

C) Color Reagent "B". each vial contains:

acyl-CoA oxidase
peroxidase
MEHA

D) Diluent for color reagent "B". bottle contains:

phenoxyethanol
surfactant

E) NEFA standard. each vial contains aqueous solution with:

oleic acid (1.0 mM)
surfactant
stabilizers

Procedure

A) Preparation of standards:

1) Dilute the standard provided (1000 µeq/l) with distilled H2O to provide 125, 250, and 500 µeq/l standards. Validation tests show distilled H2O is an acceptable diluent.

B) Preparation of reagents:

1) Mix color reagent "A" by adding 10 ml of the specific "A" diluent to each vial. Gently invert the vial until contents are completely dissolved and then combine with 13.3 ml of distilled H2O.

2) Mix color reagent "B" by adding 20 ml of the specific "B" diluent per vial. Gently invert until contents are dissolved then combine with 33.3 ml distilled H2O.

3) Solutions should be stored between 2-8 degrees C and are stable for 5 days. Do not freeze solutions or expose them to direct sunlight.

C) Assay

1) Assay each sample and standard in duplicate.

2) Add .4 ml distilled H2O to each tube.

3) Add .1 ml of serum, plasma, or standard to each tube, followed by .35 ml of the combined reagent "A" plus H2O mix. Vortex, and refrigerate for up to 60 min if necessary.

4) Incubate tubes in a waterbath at 37 degrees C for exactly 20 min.

5) Following incubation add 0.8 ml of the combined reagent "B" plus H2O mix to each tube, vortex, and incubate a second time at 37 degrees C for exactly 20 min.

6) Remove tubes from waterbath, allow to equilibrate at room temperature for 5 min, then record the OD at 550 nm using a blank prepared with reagents as zero.

(volume in ml)

tube	standard	plasma	reagent"A"+H2O	reagent"B"+H2O
blank	--	--	0.35	0.80
std in µeq/l				
125	.025	--	"	"
250	"	--	"	"
500	"	--	"	"
1000	"	--	"	"
unknown	--	0.10	0.35	0.80

Comments

1) Do not mix reagents and supplies from test kits bearing different lot numbers.

2) The assay is sensitive to heparin and plasma samples using heparin as an anticoagulant are unsuitable.

3) Samples which are hemolyzed may yield inaccurate results.

4) Ascorbic acid interferes so ascorbate oxidase is automatically included in reagent "A" mixture in the WAKO kit.

APPENDIX 3

Acetoacetate Analysis

Treatment of Blood

1) Mix equal volumes of whole blood and 1 M $HClO_4$ immediately after sampling.

2) Centrifuge at 3g for approximately 15 min.

3) Neutralize excess $HClO_4$ with KOH so that the final pH is between 6 and 8.

4) Centrifuge off the potassium perchlorate formed at aproximately 3g for 15 min then pour off the supernatant.

5) Samples must be analyzed the same day as sampling.

Reagents

1) Phosphate buffer (pH 6.8, 0.1 M)

   1.36 g KH_2PO_4 ---> 100 ml

   1.74 g K_2HPO_4 ---> 100 ml

   Mix equal volumes of each solution, check pH and add one or the other to balance pH.

2) NADH (approx. 1mM)
   (Boehringer Mannheim, grade II, 98 % pure, disodium salt, cat # 128023)

   .005 g ---> 6 ml H_2O

   Make up on day of use.

3) 3-hydroxybutyrate dehydrogenase
   (Boehringer Mannheim, grade II, 5mg/ml, cat # 127841)

4) Acetoacetate standards
   (Sigma Chemical Co., lithium salt, 90-95 % pure, cat # A-8509, stock solution 1.5 mM)

   0.01620 g ---> 100 ml H_2O (Make up on day of use.)

Dilute to the following concentrations for the working standards: 0.15; 0.09; 0.075; 0.045; and 0.0225 mM.

Use 2 zero concentration standards as blanks.

The standards must be taken through the entire procedure as small amounts of perchlorate will affect enzyme activity. Pipette into conical centrifuge tubes:

1 ml standard and 1 ml 1 M HClO4.

Neutralize to pH 6-8.

Centrifuge at 3g for 15 min.

Standard dilutions:

A) 0.15 ---> 2 ml of 1.5 mM + 18 ml H2O

B) 0.09 ---> 12 ml of A + 8 ml H2O

C) 0.075 --> 4 ml of A + 4 ml H2O

D) 0.045 --> 10 ml of B + 10 ml H2O

E) 0.0225 -> 10 ml of D + 10 ml H2O

Assay Procedure

Pipette into 12 x 75 mm culture tubes:

buffer - 0.5 ml

sample/standard - 1.0 ml for fed sheep, (0.5 ml + 0.5 ml H2O for fasted sheep)

NADH - 0.05 ml

read E1 at 340 nm

add 0.005 ml 3-hydroxybutyrate dehydrogenase and incubate at room temperature for approximately 20 min.

read E2 at 340 nm

Important:

NADH will "go off" slowly at room temperature but at about the same rate in all samples. The zero concentration acetoacetate standards can be used as a control for this. Pipette the NADH into all of the cuvettes at known time intervals, i.e. 15-60 s. Then read E1 in all cuvettes at the same timed intervals. Add enzyme at same time intervals. Incubate. Read E2 at same timed intervals.

Calculations

Calculate E1 - E2 for all standards, samples and blank:

change in E of standard - change in E of blank

change in E of sample - change in E of blank

Plot a standard curve of change in E of standard vs concentration.

Read the concentration of the samples from the curve.

Multiply by appropriate dilution factors.

APPENDIX 4

3-Hydroxybutyrate Analysis

Treatment of Blood

1) Mix equal volumes of whole blood and 1 M $HClO_4$ immediately after sampling.

2) Centrifuge at 3g for approximately 15 min.

3) Neutralize excess $HClO_4$ with KOH so that the final pH is between 6 and 8.

4) Centrifuge off the potassium perchlorate formed at approximately 3g for 15 min then pour off the supernatant.

5) Samples may be stored at -20 degrees C for at least one week.

Reagents

1) Tris-HCl buffer (pH 8.5, 0.1 M)

   2.42 g tris ----> ~50 ml H_2O

   pH ----------> 8.5 with 1 M HCl

   final volume ---> 200 ml

2) Hydrazine-tris buffer (burnt bacon buffer)
   (make up on day of use)

   2.5 ml hydrazine hydrate

   0.05 g EDTA

   12.5 ml 1 M HCl

   volume ---> 50 ml with tris-HCl buffer

   check pH ~8.5

3) NAD+ (~14 mM)
(Boehringer Mannheim, grade II, free acid, 98 % pure, cat # 127990)

0.03 g ---> 3 ml

4) 3-hydroxybutyrate dehydrogenase
(Boehringer Mannheim, 5 mg/ml, cat # 127841)

5) 3-hydroxybutyrate standards (DL-3-hydroxybutyric acid)
(Sigma Chemical Co., cat # H-6501, sodium salt, 98 % pure, stock solution 2 mM)

0.0656 g ---> 250 ml H2O

Working standards:

2.0; 1.6; 1.2; 0.8; and 0.4 mM.

the standards must go through the whole procedure and are treated as described in the acetoacetate assay.

Standards:

2.0 = 100 % ---> 20 ml 2.0 mM + 0 ml H2O

1.6 = 80 % ---> 16 ml 2.0 mM + 4 ml H2O

1.2 = 60 % ---> 12 ml 2.0 mM + 8 ml H2O

0.8 = 40 % ---> 8 ml 2.0 mM + 12 ml H2O

Assay Procedure

Pipette into culture tubes:

sample or standard	0.25 ml - fed	0.1 ml -fasted
H2O	0.75 ml - fed	0.9 ml - fasted
buffer	0.5 ml	
NAD+	0.05 ml	

Read E1 at 340 nm

add 0.005 ml 3-hydroxybutyrate dehydrogenase and incubate at room temperature for about 45 min.

Read E2 at 340 nm

Important:

NAD+ and hydrazine form a complex which absorbs at 340 nm. Therefore, a slow constant increase in absorbance occurs. This is similar to the acetoacetate assay but in reverse. So pipette the NAD+ and enzyme and take the absorbance readings at timed intervals as described for the acetoacetate assay.

Calculation

Same as for acetoacetate, except change in E is calculated from E2 minus E1.

APPENDIX 5

Insulin Analysis

Reagents

1) $NaH_2PO_4 * H_2O$
   Fisher Scientific Co.

2) NaCl
   Fisher Scientific Co.

3) Disodium ethylene diamine tetraacetate (EDTA)
   Fisher Scientific Co.

4) Merthiolate (thimerosal)
   Sigma Chemical Co.

5) 5 M NaOH
   100 g ---> 500 ml H_2O

6) Bovine serum albumin (BSA) RIA grade
   Sigma Chemical Co.

7) Guinea pig serum (GPS)
   Research Products International (RPI)

8) Bovine pancreatic crystalline insulin
   Sigma Chemical Co.

9) Guinea pig anti-bovine insulin (1st antibody)
   Miles Laboratories, Inc.

10) 125I-insulin
    Amersham Corporation

11) Goat anti-guinea pig IgG (2nd antibody)
    RPI

12) Borosilicate culture tubes 12 x 75 mm
    Fisher Scientific Co.

Reagent Preparation (for 3 assays of 123 tubes each)

1) Phosphate buffered saline (PBS-EDTA)

Dissolve in 800ml double distilled (DD) H2O:

1.38 g $NaH_2PO_4 * H_2O$

8.77 g NaCl

9.30 g EDTA * 2 H_2O disodium salt

Adjust pH to 7.6 with 5 M NaOH

Add 100 mg thimerosal and bring to 1000 ml in a volumetric flask and store at 2-4 degrees C.

2) 1 % BSA-PBS

Add 1 % BSA to cold PBS in a beaker and allow to dissolve (this takes approx. 2-3 hr.) while kept at 2-4 degrees C. After BSA is dissolved, readjust pH to 7.6 using 5 M NaOH. Bring to desired amount in volumetric flask and store at 2-4 degrees C.

Note: a total of 350 ml is needed. 250 ml can be made on day 1, this is enough to set up assays and dilute labeled insulin. On day 3, mix 100 ml to dilute 2nd antibody.

3) 0.25 % GPS-1 % BSA-PBS-EDTA

Add 250 µl GPS to 1 % BSA-PBS and bring to 100 ml in a volumetric flask.

4) 1st antibody

Reconstitute lyophilized guinea pig anti-bovine insulin according to manufacturers instructions.

Use 1 ml cold DD H_2O and then add 9 ml 1 % BSA-PBS-EDTA to give a 1/10 dilution. This solution is then frozen in 0.15 ml aliquots. (use polystyrene tubes).

A 1/8000 dilution is used for the assay. To obtain this dilution, 0.05 ml of the 1/10 dilution is diluted to 40 ml using 1 % BSA-0.25 % GPS-PBS-EDTA.

5) Insulin standards

78.43 mg of bovine insulin (Sigma # I5500) was diluted in 500 ml of 1 % BSA-PBS-EDTA (at room temperature with 1-2 hr stirring) to yield a 4 U/ml solution. Our insulin lot contained 25.5 U/mg therefore, 78.43 mg = 2000 U.

1 ml of this 4 U/ml solution was then diluted to 100 ml in 1 % BSA-PBS-EDTA to give a 40 mU/ml solution. The 40 mU/ml solution was frozen in 0.5 ml aliquots using liquid nitrogen and stored at -20 degrees C for future use.

To prepare standard solution, 0.2 ml of the 40 mU/ml solution was diluted to 4 ml using 1 % BSA-PBS-EDTA to give a 2 mU/ml solution. The following dilutions of the 2 mU/ml solution are then used to give solutions utilized for a standard curve:

Standard	2 mU/ml solution	BSA-PBS-EDTA
200 µU/ml	1.0 ml	9.0 ml
120	0.6	9.4
80	0.4	9.6
40	0.2	9.8
20	0.1	9.9
12	0.06	9.94
8	0.04	9.96
4	0.02	9.98
1	0.005	9.995

200 µl of these solutions is then used in each standard tube.

6) Labelled insulin

Labelled insulin is diluted with 1 % BSA-PBS-EDTA to give a solution containing enough 125I-insulin so that approximately 10,000 cpm are in a 100 µl sample.

7) 2nd antibody

RPI antibody is thawed and diluted 1/80 using 1 % BSA-PBS. Currently, RPI antibody is frozen in 1 ml aliquots which can be diluted to 80 ml. This would provide enough 2nd antibody for 3 assays plus some left over which could be quick frozen and thawed prior to reuse.

8) Guinea pig serum (GPS)

Guinea pig serum should be frozen in 400 µl aliquots.

Procedures

Day 1 - add plasma/standards, diluent and 1st antibody

Day 2 - add 125I-insulin

Day 3 - add 2nd antibody

Day 5 - centrifuge, pour off and count precipitate.

Day 1

Label culture tubes. Usually, 3 assays are run containing 123 tubes each. Currently, the centrifuge can hold 120 tubes (the 3 total tubes are not centrifuged) and a standard curve is prepared for each centrifuge load.

Mix 1 % BSA with PBS-EDTA before use (usually the night before) and have its pH at 7.6.

Thaw an aliquot each of 1st antibody, insulin standard solution and guinea pig serum. Prepare insulin standard solutions, GPS-BSA-PBS-EDTA (100 ml is sufficient for 3 assays) and 1st antibody as previously described.

Dispense BSA-PBS-EDTA and GPS-BSA-PBS into tubes as needed, standard solutions and plasma samples. Add 1st antibody to all but total and non-specific binding tubes. Vortex tubes and incubate 24 hr at 2-4 degrees C.

Day 2

Prepare labelled insulin and add 100 µl to all tubes, vortex, and incubate 24 hr at 2-4 degrees C.

Day 3

Prepare 2nd antibody and add 200 µl to all but total tubes, vortex and incubate 48 hr at 2-4 degrees C.

Day 5

Centrifuge at 3g for 30-35 min (do not centrifuge or decant total tubes).
Pour off supernatant into waste bottle (quickly and gently) and place upside down on Kimwipes in a foil lined box for at least 15 min.

Wipe out the top half of each tube with a Kimwipe on a wooden applicator stick and count for one minute.

Assay Protocol Using 400 µl Samples

(volumes in µl)

tube #	plasma or std	PBS $\bar{a}$ GPS	PBS $\bar{c}$ GPS	1st Ab	125I	2nd Ab
1-3 total	--	--	--	--	100	--
4-6 N.S.	--	500	100	--	"	200
7-9 B 0	--	400	"	100	"	"
10-12	std 1 200	200	"	"	"	"
13-15	4 "	"	"	"	"	"
16-18	8 "	"	"	"	"	"
19-21	12 "	"	"	"	"	"
22-24	20 "	"	"	"	"	"
25-27	40 "	"	"	"	"	"
28-30	80 "	"	"	"	"	"
31-33	120 "	"	"	"	"	"
34-36	200 "	"	"	"	"	"
37-39	pool calf 400	--	"	"	"	"
40-42	pool cow "	--	"	"	"	"
43-44	un-known "	--	"	"	"	"
122-123	" 400	--	100	100	100	200

If plasma samples average greater than 20 µU/ml then the following protocol using 200 µl samples could be used with good confidence in results:

Assay Protocol Using 200 µl Samples

(volumes in µl)

tube #		plasma or std	PBS $\bar{c}$ GPS	1st Ab	125I	2nd Ab
1-3 total		--	--	--	100	--
4-6 N.S.		--	400	--	"	200
7-9 B 0		--	300	100	"	"
	std					
10-12	1	200	100	"	"	"
13-15	5	200	"	"	"	"
16-18	10	200	"	"	"	"
19-21	20	200	"	"	"	"
22-24	40	200	"	"	"	"
25-27	80	200	"	"	"	"
28-30	120	200	"	"	"	"
31-33	200	200	"	"	"	"
34-36	pool calf	"	"	"	"	"
37-39	pool cow	"	"	"	"	"
40-41	un-known	"	"	"	"	"
122-123		"	200	100	100	100	200

Points to Remember:

Keep all reagents and tubes on ice and cold as much as possible.

Calculate volumes of reagents needed before hand and mix accordingly to avoid wastes.

Keep frozen reagents in a non-frost free freezer.

BSA-PBS-EDTA should not be kept more than 4 weeks at 2-4 degrees C.

GPS-BSA-PBS-EDTA should not be saved and used again after more than 2 or 3 days.

Use "scrupulously" cleaned glass ware.

"Precision" pipetting is essential.

Pipette solutions directly to the bottom of culture tubes as much as possible.

Centrifuge using at least 1.5g in a refrigerated centrifuge (2-4 degrees C).

APPENDIX 6

Glucagon Analysis

Reagents

1) Glycine
   (Fisher Scientific Co. # G-46)

2) Disodium Ethylenediamine Tetraacetate (Na2EDTA)
   (Fisher Scientific Co. # 5-311)

3) Merthiolate (thimerosal)
   (Sigma Chemical Co. # T5152)

4) 3 M NaOH

5) 10 M NaOH

6) Borosilicate Culture Tubes 12 x 75
   (Fisher Scientific Co. # 14 962 10B)

7) Bovine Serum Albumin (BSA) RIA grade
   (Sigma Chemical Co. A 7888)

8) Benzamidine Hydrochloride
   (Sigma Chemical Co. # B6506)

9) Normal Rabbit Serum (NRS)
   (Miles Laboratories, Inc. # 64-291; 50 ml lyophilized)

10) Crystalline Beef-Pork Glucagon
    (Eli Lilly Co. 500 mg)

11) 30 K Rabbit Anti-Glucagon Antibody (1st Ab) 25 µl
    (Roger H. Unger, Dept. of Internal Med., University of Texas, Health Science Center at Dallas, 5323 Harry Hines Blvd., Dallas, Tx. 75235)

12) 125I-Glucagon
    (Cambridge Medical Diagnostics, Co. # 120,
    10 µCi, stable for 10 weeks)

13) Goat Anti-Rabbit IgG (2nd Ab)
    (Research Products International,
    RPT # 500090, 10 ml)

14) 0.02 N HCl

15) Polyethylene Glycol
(Sigma Chemical Co., P2139)

Reagent Preparation

1) 0.2 M glycine-merthiolate

dissolve in ~ 900 ml double distilled (DD) H2O:

15 g glycine

0.1 g thimerosal

adjust pH to 8.8 with 3 M NaOH

final volume ---> 1000 ml with DD H2O

store at 2-4 degrees C.

2) 0.05 M EDTA-glycine-merthiolate

dissolve in ~ 900 ml 0.2 M glycine-merthiolate:

18.6 g Na2EDTA

adjust pH to 8.8 with 10 M NaOH

final volume ---> 1000 ml with 0.2 M glycine-merthiolate in a volumetric flask store at 2-4 degrees C.

3) 1 % BSA-glycine-merthiolate

dissolve 1 g BSA in 100 ml 0.2 M glycine-merthoilate, check pH (should not change)

use cold buffer in a beaker with a stirring magnet

4) 0.1 M benzamidine-1 % BSA-0.2 M glycine-merthiolate

prepare 1 % BSA-glycine-merthiolate as in # 3 and add 1.566 g benzamidine hydrochloride

check pH, adjust to 8.8 with ~75 µl 3 M NaOH

5) 0.25 % normal rabbit serum (NRS)-EDTA-glycine-merthiolate

dilute 250 µl NRS to 100 ml in 0.05 M EDTA-glycine-merthiolate

mix immediately before use and store at 2-4 degrees C.

6) rabbit anti-glucagon antibody (1st Ab)

the 30 K is shipped lyophilized with a recommended final culture tube dilution of 1:100,000

to reconstitute Ab with this volume and titer:

remove stopper (after tapping lightly to dislodge any Ab on the stopper) and add 5 ml of cold 1 % BSA-glycine-merthiolate, replace the stopper, mix by gentle inversion, let solution sit (@ 2-4 degrees C) for 1 hr, inverting it every 10-15 min, this gives a 1:200 dilution

transfer this solution to a cold 15 ml centrifuge tube (on ice), wash the original tube with 5 ml 1 % BSA-glycine-merthiolate and add to the original 5 ml to give a 1:400 dilution

this solution should be frozen and stored in 500 µl aliquots, using liquid nitrogen and 12 x 75 mm polypropylene culture tubes.

for the assay, use a 1:50 dilution of the 1:400 solution for the working solution, thaw 1 500 µl aliquot/123 tube assay and dilute to 25 ml using 0.25 % NRS-EDTA-glycine-merthiolate

mix immediately before use and keep at 2-4 degrees C.

7) glucagon standard solution

weigh out 10 mg crystalline glucagon; dissolve in 20 ml 0.02 N HCl to give a solution of 0.5 mg/ml, the optical density of this solution should be 1.055 @ 278 nm (Harris et al. 1979),

if the OD is not 1.055, then correct the dilution to give an equivalent final concentration assuming that:

$$\frac{0.5 \text{ mg/ml}}{1.055 \text{ OD}} = \frac{x \text{ mg/ml}}{\text{actual OD}}$$

dilute 1 ml of the 0.5 mg/ml solution to 100 ml in 1 % BSA-glycine-merthiolate to give a 5 mg/µl solution

dilute 0.5 ml of this 5 mg/µl solution to 500 ml using 1 % BSA-glycine-merthiolate to give a 0.005 ng/µl solution

quick freeze this solution in 2.2 ml aliquots (1.917 ml/standard curve/assay are used)

standard solution is used at this dilution,thaw immediately before use and keep at 2-4 degrees C.

8) labelled glucagon

reconstitute using 5.0 ml benzamidine-BSA-glycine-merthiolate and dilute such that 100 µl contains 8000 cpm

9) goat anti-rabbit IgG (2nd Ab)

thaw the 10 ml received from RPI and quick freeze in 1 ml aliquots

one 1 ml aliquot is diluted to 60 ml using 0.2 M glycine-merthiolate

store at 2-4 degrees C.

10) normal rabbit serum (NRS)

should be frozen in 1 ml aliquots

11) polyethylene glycol (PEG)

make up a 6 % solution

store at 2-4 degrees C.

Procedure

Day 1 -- add buffer, standard, plasma and 1st Ab, incubate 12 hr

Day 1 + 12 hr -- add labelled glucagon, incubate 48 hr

Day 3 -- add 2nd Ab and PEG, incubate 1 hr, centrifuge, pour off, count precipitant

make sure there are adequate quantities of all reagents before beginning

mix buffers needed before beginning, usually 12-24 hr before hand

Day 1

Thaw: 3 500 µl aliquots 1st Ab

3 2.1 ml aliquots standard solution

1 aliquot NRS (at least 250 µl)

3 1 ml aliquots of pooled plasma needed

samples (42/assay)

Prepare 100 ml 0.25 % NRS-EDTA-glycine-merthiolate and use to dilute 1st Ab as previously described (1.5 ml 1st Ab ---> 75 ml NRS-buffer)

Dispense 1 % BSA-glycine-merthiolate, standard solution, plasma and 1st Ab as needed, vortex tubes and incubate 12 hr at 2-4 degrees C.

Day 1 + 12 hr

Prepare labelled glucagon, add 100 µl to all tubes, vortex, incubate 48 hr at 2-4 degrees C.

Day 3

Prepare 2nd Ab and 6 % PEG, mix together the necessary volume needed as follows:

5 parts cold 6 % PEG

2 parts 2nd Ab

Add 1 ml of 2nd Ab/PEG mixture to all but total tubes, incubate at room temperature for 1 hr, centrifuge at 3g for 40 min (do not centrifuge or decant total tubes)

Pour off supernatant into a waste bottle (quickly/gently) and place upside down on Kimwipes in a foil lined box for 15 min

Wipe out the top 1/2 of each tube with a Kimwipe on a wooden applicator stick and count for 1 min

dispose of tubes ASAP

Points to Remember

Keep reagents and assay tubes on ice and cold as much as practical/possible

Keep frozen reagents in a conventional freezer (non-frost free)

Do not use buffer with BSA stored longer than 4 weeks

Use scrupulously cleaned glassware

Assay Protocol

(volumes in µl)

tube #		BSA-gly-mer	* std/ plasma	1st Ab	125I	2nd Ab/ PEG
1-3	total	--	--	--	100	--
4-6	N.S.	600	--	--	"	1000
7-9	B 0	400	--	200	"	"
10-12	5	399	1	"	"	"
13-15	15	397	3	"	"	"
16-18	25	395	5	"	"	"
19-21	50	390	10	"	"	"
22-24	100	380	20	"	"	"
25-27	200	360	40	"	"	"
28-30	400	320	80	"	"	"
31-33	800	240	160	"	"	"
34-36	1600	80	320	"	"	"
37-39	pool ewe	100	300	"	"	"
40-42	unknown	"	"	"	"	"
122-123	unknown	100	300	200	100	1000

*standard = 0.005 ng/ml

Unknown concentrations from this standard curve will be the concentration per 300 µl plasma, so a dilution factor of 3.3333 should be used to give the concentration per ml.

Blood Collection

Blood should be collected in tubes containing benzamidine hydrochloride at a final concentration of 0.03 M and Na2EDTA at a final concentration of 1.2 mg/ml

Prepare tubes by adding 50 µl of the following benzamidine-EDTA-saline solution per 3 ml of blood sampled:

dissolve in 1 ml of 0.9 % saline:

0.2347 g benzamidine hydrochloride

0.06 g Na2EDTA

Para-aminohippuric Acid Analysis

Reagents for Filtrate

1) 20 % trichloracetic acid (TCA)

2) Distilled H2O

Method for Filtrate

1) Pipette 1 ml of blood or standard into 15 ml centrifuge tubes containing 5 ml H2O.

2) Pipette 5 ml of blood or standard mixture into second 15 ml centrifuge tube containing 5 ml 20 % TCA.

3) Let stand 60 min or overnight (can be left at this stage - refrigerated)

4) Filter through Whatman # 4 filter paper into 16 x 150 mm glass culture tubes.

5) Add boiling chip to each culture tube and place a marble on top of each tube.

6) Boil over low heat in water bath for 30 min after fine bubbles appear in each tube.

7) Cool at room temperature - remove marbles when cool.

8) Standards from 1.5 % PAH infusion solution are made along with this using same method as for blood filtrate.

   -- Make up a 1:500 dilution for sheep from 1.5 % infusion solution; 1:100 for dog from 0.25 % infusion solution (considered 10:10 dilution).

   -- From this dilution make up standard dilution: 20 % = 2:10; 40 % = 4:10; 60 % = 6:10; 80 % = 8:10; and 100 % = 10:10 (stock dilution). Be sure to run this through the blood filtrate precedure (not necessary to boil).

   -- Blank mixed with 1/2 distilled H2O and 1/2 TCA (20 %).

Reagents for Analysis

1) 1.2 N HCl (1:10 dilution of 12.4 N HCl close enough).

2) $NaNO_2$ (100 mg ---> 100 ml H_2O) freshly made up on day of analysis.

3) Ammonium sulfate (500 mg ---> 100 ml H_2O) made up within a month.

4) Coupling reagent (N-1 Naphthylethylenediamine dihydrochloride) (100 mg ---> 100 ml H_2O) store in a brown bottle in refrigerator indefinitely.

Analysis

1) Pipette 1 ml of filtrate into test tubes (in duplicate).

   Pipette 1 ml of standards into test tubes (in duplicate).

   Pipette 1 ml of blank into test tubes (in duplicate).

2) Add 0.2 ml of 1.2 N HCl to all tubes, mix well.

   Start time clock and add to all tubes:

   -- 0.1 ml $NaNO_2$, mix well.

   -- 0.1 ml ammonium sulfamate - within 3-5 min, mix well.

   -- 0.1 ml coupling reagent - within 3-5 min, mix well.

   Wait 10 min for color development.

   Read on spectrophotometer at 540 nm.

PAH Stock Solution

1) 44.9 g PAH powder (Eastman Kodak Corp., Rochester, N. Y.) (yields 1.5 % solution of sodium salt.)

   10.5 g NaOH

   500 ml physiological sterile saline (PPS)

   Dissolve each reagent in PPS separately. Then add NaOH solution to PAH and stir. May have to heat gently to fully dissolve. Filter through Whatman # 4 filter paper. Titrate filtrate to pH 7.4 with 1 N HCl or 4 M NaOH. Bring final volume to 500 ml.

2) Store frozen in 75 ml or 150 ml aliquots until needed.

3) For 1.5 % PAH infusion solution, thaw 75 ml or 150 ml aliquots and bring to 500 ml or 1000 ml in a volumetric flask with PPS, respectively.

VITA

Scott Charles Sensenig was born in Reading, Pennsylvania on September 12, 1953. He attended elementary school at Lincoln Park Elementary School and received a secondary school education at Wilson High School where he graduated in 1971. Both schools are located in Berks County, Pennsylvania.

After finishing high school, Scott enlisted in the United States Navy where he served aboard the U.S.S. El Paso (LKA 117) as a radarman\operations specialist 3rd class until being honorably discharged in 1974.

Following his military duty, Scott worked as a machinist for the Polymer Corporation, based in Reading, Pennsylvania, from 1974 until 1979.

In early 1980, he enrolled as a part-time student at Albright College and Reading Area Community College, both in Reading, Pennsylvania, before attending Delaware Valley College of Science and Agriculture in Doylestown, Pennsylvania, as a full-time student in the fall of the same year.

Scott received a Bachelor of Science degree in Dairy Husbandry from Delaware Valley College in 1984.

Upon completion of his undergraduate studies, Scott accepted a research assistantship under the guidance of Dr. Richard N. Heitmann at the University of Tennessee, Knoxville, Tennessee, working in the area of animal nutrition.

Scott received his Master of Science degree on June 12, 1987 and plans to continue his education by working on a PhD program at the University of Tennessee, Knoxville, Tennessee.