ENDOCRINE RESPONSES TO AGE IN GROWING LAMBS AND CALVES.

by

STEPHEN KWAME ARTHUR

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ABSTRACT

Growth hormone (GH), insulin, and glucose levels were measured in blood taken from Outaouais Arcott (a breed developed hybridizing the Finnish Landrace, Suffolk and Shrophire breeds) lambs at 67 and 157 days of age during 120 min intravenous infusions of saline (0.9% NaCl, Sal), low insulin (0.2 mU/kg/min, LI), high insulin (6.0 mU/kg/min, HI) and adrenaline (0.3 ug/kg/min, Adr) following a 12-hour fast. Glycerol was measured for only Sal and Adr infusions. Specific insulin binding (SIB) to mononuclear leukocytes (MNL) was also determined in the lambs at three-week intervals from 12 to 27 weeks of age, and monthly in Holstein calves from birth to 20 weeks of age.

Pretreatment insulin and glucose levels (measured at 20 min intervals for 1 hr prior to treatment) increased while GH decreased with age in lambs ($P<0.05$). Adrenaline increased glycerol levels at both ages, but caused hyperglycemia only at 157 days of age. An elevation in GH level with HI was seen in younger lambs. Hypoglycemia induced by HI was delayed at 157 days of age and LI had no hypoglycemic effect at either age. With age, SIB increased ($P<0.05$) in lambs but decreased in calves ($P<0.05$).

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Chapter I

INTRODUCTION

According to Turner (1978), hormonal function is modified by the stage of maturity in growing mammals. Age-related variations in plasma/serum insulin levels and in insulin activity have been observed in both monogastrics (Kappy and Plotnick 1980; Vandergrift et al. 1985) and ruminants (Grizard et al. 1987; Kappy 1983). The effect of age probably reflects changes in metabolic needs of the animal (Bergen 1974) and/or increases in population, size, and distribution of various cell types, especially adipocytes (Hood 1983). The need for growth and protein accretion prevails in the initial stages of development of every animal, while the conservation of nutrients takes precedence in adulthood (Turner 1978). Consequently, the extent of protein and energy metabolism, both of which are under the influence of insulin, changes with age in mammals (Bergen 1974). The changes are likely to be mediated through circulating hormone levels and/or sensitivity of insulin target organs.

The observation that blood cells mirror the major target tissues for insulin receptor characteristics (Beck-Nielsen 1980; Kappy and Plotnick 1980) led to several blood cell

insulin receptor studies partly because of the easy availability of the cells (Ector et al 1983; Kappy 1983; Khan et al. 1986; Kennedy et al. 1987; Kennedy et al. 1988b). Insulin binding to monouclear leukocytes (MNL) in ruminants is important because erythrocytes of adult cattle and sheep bind no insulin (Kappy 1983; Kappy et al. 1981). There have been no direct studies on the effect of age on MNL insulin binding in ruminants.

The present study was therefore aimed at understanding how age modulates the effects of hormones, particularly insulin and adrenaline, by examining sensitivity to insulin and adrenaline in growing lambs as well as insulin binding to MNL in lambs and calves as they mature.

Chapter II

LITERATURE REVIEW

2.1 DETERMINANTS OF INSULIN ACTION

For insulin to elicit its biologic effects, an appropriate minimum amount of hormone must circulate within the body at any given time. In addition, target organs should recognise and be able to respond to insulin. Insulin binds to the target organ or specific cell surface receptors to form hormone-receptor complexes (Kahn 1979). Subsequent to this is the creation one or more signals across the cell surface. The signal(s) could be depicted in several ways. These include production of a mediator, changes in membrane structure, alterations in the ionic potential of the membrane or even release of specific protein kinases or enzymes (Seals and Czech 1982). Irrespective of the nature of the signal or the form taken, the signal influences a number of secondary steps which direct the expression of the biologic effects of insulin. Disturbance of any of the above steps could thus affect insulin action (Kolterman et al. 1982).

In summary, insulin action primarily depends on the presence of the hormone in the blood, the binding of the hormone to the receptor, and post-receptor events in

particular cell types. The number of hormone-receptor complexes formed as a result of the initial binding is also important and depends on receptor characteristics, namely the number and the affinity of the receptor for the hormone (Etherton 1982). The events occurring beyond the insulin-receptor interaction equally play a significant role in the perpetuation of insulin action (Kahn 1979). The relationship between age and these factors are discussed below. Note, however, that these factors are interdependent, and therefore they will be considered together, where appropriate.

2.2 CIRCULATING INSULIN LEVELS

Insulin is produced by the beta cells (ß cells) of the pancreas (see Orci et al. 1988). The extent of production, release, and, hence, plasma concentrations is controlled by the sensitivity of the ß cells to plasma metabolites like glucose, amino acids or volatile fatty acids (Sasaki et al. 1982), hormones (including growth hormone (GH) (Waghorn et al. 1987), somatostatin (Hellman and Lernmark 1969) and epinephrine (Oda et al. 1988)), as well as certain neuronal signals (Weekes 1986). Another factor that regulates peripheral insulin levels is the degree of metabolic clearance (Reaven et al. 1982). Age or any agent or phenomenon that affects any of the insulin regulatory factors could alter plasma insulin levels.

Machlin et al. (1968) have observed that growing pigs show a proportionate rise in their plasma insulin level with increases in age. The reverse relationship was found for GH and age. Similar results have been found for fasted steers (Trenkle and Topel 1978), growing lambs (Godden and Weekes 1981), and bulls (Martin et al. 1979). Higher insulin level and lower GH level were associated with advanced age, increased body weight and enhanced carcass adiposity. There was also a negative relationship between insulin level and the amount of muscle in the carcass (Trenkle and Topel 1978). In contrast to peripheral insulin level, Yki-Jarvinen and Koivisto (1983) have reported that in vivo sensitivity of glucose metabolism increases with quantity of muscle and decreases with rising adiposity.

In growing mammals, the obese individuals tend to have higher insulin level than their corresponding leaner counterparts (McNiven 1984; Vandergrift et al. 1985; McCann et al. 1986). A similar trend occurs between adult or older mammals compared to the young (Grizard et al. 1987) presumably as result of the tendency to deposit more fat with increasing maturity (Turner 1978). Elevated insulin levels in the blood have been highly correlated with adiposity (Baile et al. 1983), although Kappy (1983) observed that plasma insulin concentrations of an ewe and its fetus were similar. Early maturing cattle breeds, in spite of their lower mature body weights, tend to have

higher plasma insulin levels (Trenkle 1981). Marked rises in insulin as a result of feeding have been observed in older lambs (Godden and Weekes 1981). Increased sensitivity of the pancreatic ß cell with age has been offered as a possible explanation for these observations (Godden and Weekes 1981). To the contrary, however, Gregory et al. (1980) reported that although postprandial insulin levels were enhanced by age in both Hereford and Friesian steers, the leaner breed exhibited a larger increase. Certain homeorrhetic needs may also modulate the insulin secretory and/or islet cell responses to various stimuli (Vernon et al. 1985). Among other things, ageing, excessive activity, and cold exposure have all resulted in a decline in insulin secretory responses (Molina et al. 1985; Bines and Hart 1982; Sasaki et al. 1982).

2.3 INSULIN BINDING TO VARIOUS CELL TYPES

Insulin receptors from a variety of tissues share similarities like the presence of two subunits, namely the alpha (α) and beta (ß) subunits (Kahn 1979). Nevertheless, marked variations exist in morphology and activity among insulin receptors, depending on the cell type or the species considered (Gammeltoft 1984). In addition, in the normal ageing process of mammals, the size, number, and distribution of various cell types alter to meet changing anatomical and functional needs of the growing individual (Bergen 1974). Hence, binding characteristics of the target

cells for insulin are likely to undergo some changes as the animal matures.

Adipose, muscle, and liver cells are considered the three major target tissues for insulin (Glieman et al. 1980). The presence of insulin receptors on different types of adipose, muscle, and liver cells, in both normal and diseased mammals, has been established (Forgue and Freychet 1975; Olefsky et al. 1976). Insulin binding to blood cells (erythrocytes and monocytes) also takes the same form as the major target tissues for insulin (Gambhir et al. 1977; Beck-Nielsen 1980; Kappy and Plotnick 1980). Considering the accessibility of blood cells, this observation is important to the study of insulin binding characteristics.

2.3.1 Adipose Cells

Insulin binding to fat cells decreases with ageing in humans (Balazi et al. 1978; Bolinder et al. 1983b). The decline in insulin action with age is explained by both receptor and post-receptor abnormalities. Similar results have been reported for rats (Olefsky 1976b) where decreased insulin secretion was also attributed to irreversible changes in the islet ß cell with age (Reaven et al. 1983). Changes in rat sensitivity to insulin (in vivo) with age was also associated with diet (Reaven et al. 1983). Diminished insulin binding was reported for rats fed high glucose or fat diets (Olefsky and Seakow 1978) while increased

adipocyte insulin binding in fasted rats has been observed (Olefsky 1976b). The increase in binding was attributed to alterations in receptor affinity and not to changes in receptor numbers. Bolinder et al. (1983a) observed higher insulin binding to subcutaneous adipose tissues which are bigger than omental adipose tissue in humans, irrespective of whether binding was considered per fat cell number or per fat cell surface area. The difference in binding between the two adipose cell types was explained by changes in receptor affinity and not in receptor number. These observations support the earlier report of Glieman et al. (1980) indicating increased binding to large fat cells. McNiven (1984) has indicated that the observed insulin resistance of large fat cells is more dependent on their inability to use glucose in metabolism (a post-receptor effect) than on a defect in insulin binding or sugar transport.

Results from a number of studies indicate that adipocytes from swine show little or no response to insulin binding especially in older animals (Etherton and Chung 1981). Adipocytes from mature pigs (100kg) were reportedly unable to increase oxidation of glucose and lipogenesis in the presence of insulin (O'Hea and Leveille 1970). Insulin stimulation of glucose oxidation and lipid production in market-weight pigs (90-100kg) was approximately 25% the level of stimulation observed in adipocytes from young rats

(150g) (Etherton and Chung 1981). Adipocytes from 12kg pigs however, metabolised glucose in response to insulin two times faster than those from market weight pigs. Insulin binding to pig adipocytes was considered nominal compared to young rats (Yang and Baldwin 1973; Etherton and Chung 1981), but Walton et al. (1984) showed that insulin can stimulate pig adipocytes in vitro even in short term-cultures. The authors explained that a lack of response would from contamination of reagents used for incubations. This suggestion reinforces the assertion by Etherton et al. (1984) that bovine serum albumin (BSA) from particular sources reduced the insulin-binding ability of swine adipocytes. The same BSA type promoted lipid production in a proportionate manner, even without addition of exogenous insulin to the medium. The purity of BSA used in assays must be examined to avoid contamination with insulin or insulin-like growth factors (IGF) (Etherton et al. 1984).

Earlier reports indicated that ruminant (sheep/cattle) adipocytes bound very little insulin irrespective of the length of culture, especially in older cattle (Vernon 1980; Prior and Smith 1982). This finding was partly confirmed in a study in which adipose cells from 10 month old bulls were neither responsive to physiological nor pharmacological doses of insulin in a 3-hour culture (Vernon et al. 1985b). The bovine fat cells however, responded and bound insulin in a long term culture of about 3 days. Similar results were

obtained with sheep adipocyte (Vernon et al. 1981). Vernon et al. (1985b) noted that the degree of insulin binding to bovine adipocytes was higher than that of female rats under similar conditions. Furthermore, they suggested that the lack of binding in ruminant adipocytes previously reported might be a peculiar characteristic of older animals since insulin binding to rat adipocytes decreased with age. In addition, Vernon et al. (1981) noted that the type of collagenase in their incubation media was different from that of other researchers. Apparently, the collagenase used by the others had high tryptic activity leading to insulin receptor loss and consequently to a lack of insulin binding in the fat cells (Vernon et al. 1981). One conclusion drawn from these findings is that the ruminant adipocytes bind insulin, and have a transient period of metabolic insensitivity upon removal from the animal, which accounts for the lack of response in short-term incubations done immediately after their removal from the body. (Vernon et al. 1981).

On the other hand, Etherton and Evock (1986) have shown that bovine adipose cells synthesize lipids in response to insulin when cultured for as short as 2 hours provided a pure BSA is used in the buffer. They observed that insulin is required for adipocytes to exhibit their lipogenic ability in culture. Due to these conflicting results, further studies are required to clarify the issue before any

conclusions about age-dependent insulin binding to adipose cells can be made.

2.3.2 Liver Cells

Messerole and Etherton (1984) reported a reduction in insulin binding to liver cells of lean (Yorkshire) swine with age, unlike their obese (Ossabaw) counterparts. At physiological insulin concentrations up to 2.5ng, liver cells of Yorkshire pigs showed a progressive decline in insulin binding from 85 days to 173 days of age. Insulin binding fluctuated in the obese, but, for lean and obese pigs of similar age and/or live weight, insulin binding to liver cells was persistently higher in the lean breed than the obese up to 140 days of age. At 173 days of age there was no difference in binding between the breeds. In addition, there were no differences in binding at higher-than-physiological insulin levels (>5ng/ml). A reduction in binding affinity with age was observed only for obese swine. Liver microsomes of pigs did not experience a drop in binding as a result of constant exposure to insulin (down regulation) (Messerole and Etherton 1984). Both chicken and rat liver cells have been shown to exhibit insulin binding (Krupp and Lane 1981).

Gill and Hart (1980) showed that ruminant liver cells can bind insulin. They also reported that ovine hepatocytes bound more insulin at Day 50 of lactation than at Day 20,

due to a rise in receptor numbers with time of lactation (Gill and Hart 1980). There was no difference in binding between unmated and lactating ewes at Day 20 of lactation.

2.3.3 Muscle Cells

Insulin binding to various types of muscle have been documented using heart (Forgue and Freychet 1975) and skeletal muscle (Olefsky et al. 1976; Hom and Goodner 1984). Muscle cells also exhibit varied sensitivities to insulin. The skeletal muscle is the most sensitive to insulin-promoted glucose uptake and deposition (DeFronzo et al. 1981). In vivo glucose uptake in the rat is more sensitive to insulin than is protein synthesis in the skeletal muscle (Garlick et al. 1983). The perfused rat heart, however, is more responsive to protein synthesis than glucose uptake effects of insulin (Flaim et al. 1983).

According to Jeanrenaud (1978), at the onset of obesity, muscles are more susceptible to insulin resistance than other insulin target tissues. Also, heart and skeletal muscle tissue respond to insulin-stimulated glucose uptake more than adipose tissue (Hom et al. 1984). Lean mice had more insulin receptors in their soleus muscle and were more sensitive to insulin than were genetcally obese mice. The insensitivity of the muscle of obese mice was correctible by fasting (Forgue and Freychet 1975). In another study of rat soleus muscle tissue, Grundleger et al. (1980) found that

there was a reduction in insulin binding, insulin receptor number and insulin affinity in both lean and obese mice with advancing age. The magnitude of the decline in insulin binding was the same in both genotypes up to the sixth week. After this period, the rate of decline in the obese was greater than that of the lean (Grundleger et al. 1980). These results are in direct contrast to the results found by Messerole and Etherton (1984) who examined liver cells in lean and obese breeds of pigs. Glucose utilization by the rat muscle tissue also declined with age, but glucose transport was unaffected. The results of this experiment suggest that insulin action in a target cell is sometimes independent of the binding characteristics, as was the case for glucose transport. Crettaz and Jeaneraud (1980) have noted that both receptor and non-receptor defects characterise insulin resistance in muscle cells, and notable among the latter defects are glucose transport and phosphorylation.

2.3.4 Blood cells

The observation that blood cells mirror the major target tissues for insulin receptor characteristics (Beck-Nielsen 1980; Kappy and Plotnick 1980) has given a high impetus to blood cell insulin receptor studies due to the easier accessibility of these cells. As a result, there has been a plethora of insulin binding studies using red blood cells and monocytes (Ector et al. 1983; Kappy and Plotnick 1980;

Kappy 1983; Kappy et al. 1981; Khan et al. 1986; Neufield et al. 1978). Cells from adult animals were almost invariably used in these studies; so there is still a paucity of information on the changes in insulin binding of blood cells as an animal matures, especially in the ruminant.

According to Ector et al. (1983), insulin binding to erythrocytes in gilts is reduced from birth to market-weight (about 100kg). The highest insulin binding to erythrocytes was recorded at birth (4-6 days) followed by weaning (34-44 days) and the growing phases (80-100 days). There was no significant difference in binding between the growth and finishing phases. This was true in both the fed and fasted states. Binding was inversely related to growth rate during weaning to growth phases, and to lean percentage of the carcass at finishing (Ector et al. 1983). In humans, studies with neonates, children, and adults have shown that insulin binding to red blood cells decreases with age and stage of growth (Kappy and Plotnick 1979; Hendricks et al. 1980). Similar observations were made with monocytes (Thorsson and Hintz 1977) and adipose cells (Balazi et al. 1978). Variation in erythrocyte insulin binding over age probably results from the higher number and affinity of insulin receptors in younger mammals (Neufield et al. 1978; Thorsson and Hintz 1977). Similar results have been reported for blood cells in general (Kelly et al. 1974; Kappy et al. 1981). In another study, Khan et al. (1986) found that

erythrocytes of patients with Fredereich's ataxia (a neurological abberration which leaves the patient more prone to diabetes mellitus) and their controls exhibited similar decreases in insulin binding with age.

Binding of insulin to erythrocytes decreases with age in cattle (Kappy 1983) and sheep (Kappy et al. 1981). A progressive decrease in number of insulin receptors (to zero) on the cell surface due to the appearance of erythrocytes of the adult type has been offered as a possible cause of this phenomenon. Kappy (1983) has suggested that changes in osmotic fragility of erythrocytes which affects insulin binding, occur with age. According to the author, a new set of erythrocytes, having greater osmotic fragility replaces the existing ones as ruminants age. The more fragile cells bind less insulin leading to the observed decrease in binding with age. This replacement of fetal erythrocytes occurs within 2 months of birth (Kappy 1983). Another proposal is that the loss of insulin binding to the erythrocytes may reflect the metabolic and/or physiological transformation of the animals from a monogastric to a ruminant stage (Kappy et al. 1981). This event could then signify varying insulin requirements with age, that is, from the period when glucose and amino acids are major substrates for metabolism to one in which volatile fatty acids constitute the main raw materials for energy and heat production (Kappy et al. 1981).

Mononuclear leukocytes (MNL) comprise approximately 15-20% monocytes, 80-85% lymphocytes, 1% granulocytes and some erythrocytes and platelets. However, monocytes account for 80% of the total insulin binding in MNL cell suspensions (Beck-Nielsen et al. 1977). Neufield et al. (1978) studied the insulin binding behaviour of monocytes from infants of pregnant diabetic mothers, normal infants and non-diabetic adults. Monocyte insulin binding in the two groups of infants was higher than that in adults. A difference in both receptor numbers and binding affinity accounted for the variation in binding rate (Neufield et al. 1978). Curiously, the infants of hyperinsulinemic diabetic mothers exhibited a high insulin binding affinity and a high number of insulin receptors even under conditions of high circulating insulin levels. This contrasts with the general finding that hyperinsulinemia causes a reduction in insulin receptor numbers through down-regulation (Olefsky 1976).

Increased insulin binding to monocytes during pregnancy has also been shown in young healthy women. Pregnant women had markedly higher monocyte insulin binding values than non-pregnant women due to high numbers of receptor (Puavilai et al. 1982). Reduced insulin sensitivity in gravid women is therefore probably caused by post-receptor events (Puavilai et al. 1982). In this regard, Beck-Nielsen and Pedersen (1978) found a highly positive association between monocyte insulin binding and sensitivity to insulin in young

healthy individuals. They also indicated that monocyte insulin binding is markedly affected by the composition and quantity of a subject's diet. The dietary effects are manifested via changes in binding affinity and not receptor numbers. Reduced monocyte insulin binding in Fredereich's ataxia has also been attributed to a decline in receptor affinity (Khan et al. 1986). Moderate exercise resulted in a rise in insulin binding to monocytes and erythrocytes (Pedersen et al. 1980; Michel et al. 1984), while exercising to the point of fatigue led to reduction in insulin binding (Michel et al. 1984).

Insulin binding to ruminant mononuclear leukocytes (MNL) is of particular interest since it has been shown that the erythrocytes of adult ruminants bind no insulin (Kappy 1983, Kappy et al. 1981). Unfortunately, not much has been reported for monocyte insulin binding in ruminants. Recently, however, Kennedy et al. (1987) reported insulin binding to mononuclear leukocyte (MNL) in Holstein cows at different stages of lactation. Specific insulin binding to MNL was markedly reduced at the 12th wk of lactation as compared to 4th and/or 6th week. By proportion, the highest binding was recorded in the 4th wk of lactation. Differences in binding were attributed to changes in receptor number and not affinity (Kennedy et al. 1987). Insulin binding to MNL receptors in clonidine-treated male lambs has also been reported (Kennedy et al. 1988b). Specific insulin binding

was higher in Suffolk than Outaouais Arcott (a hybrid of Finnish Landrace, Suffolk and Shropshire) lambs. This finding suggests an effect of breed on insulin binding to MNL. In another study, Kennedy et al. (1988a) observed a higher insulin binding to MNL receptors in heifers than in steers. Furthermore, the authors indicated that insulin receptor parameters could provide a reliable estimate of some carcass characteristics in heifers. Considering the accessibility of MNL cells and the relative lack of information on insulin binding to ruminants, the above observation deserves furtherattention. In addition, the suggestion may find practical application in the production of lean meat, which is of considerable importance to the animal industry at present.

2.4 WHOLE ANIMAL AND TISSUE RESPONSE TO INSULIN

Insulin affects both energy and protein metabolism (Turner 1978; Bergen 1974). The importance of insulin in all aspects of growth and development is also evidenced by insulin deficiency symptoms (Williams 1981). The effect of insulin on glucose regulation, which has been the subject of many studies, will be discussed with particular emphasis on the role of receptors. Changes in insulin response due to age are also of special interest. Other non-glucoregulatory functions of insulin, especially the effects on protein accretion, though beyond the scope of this discussion, are acknowledged.

2.4.1 Changes in insulin action

The prevailing insulin levels do not always correspond to the biologic effects expected of the hormone. Insulin action may be altered for many reasons. The hormone's action could be blocked, mimicked, attenuated, or masked by insulin antibodies or insulin antagonists (Kahn et al. 1977). Moreover, the insulin produced could be defective and hence unable to act efficiently (Newsholme 1982). However, the most common and frequent occurrence is the change in the response of the end organs due to changes in insulin receptor characteristics or post-receptor changes (Kolterman et al. 1980; Newsholme 1982). For this reason, the behaviour of a tissue/organ in the presence of insulin is one of the major indices of the effectiveness of insulin action. By definition, sensitivity to insulin refers to the ability of insulin to reduce blood glucose levels or to promote glucose uptake from the blood into target tissues (Vranic et al. 1980).

2.4.2 Assessment of insulin action

Criteria to measure insulin sensitivity extend beyond the apparently limited definition given above (Puavilai et al. 1982). Insulin sensitivity has been determined by the degree of reduction in glucose level in response to endogenous insulin, to exogenous insulin administered with or without glucose (Grizard et al. 1987). The degree of glucose removal following an exogenous supply of glucose (glucose

tolerance) can also be used to measure insulin action (Waghorn et al. 1987). In addition, the ratio of glucose to insulin (G/I ratio) in both the fed and fasted states has provided an index for insulin sensitivity (DeFronzo et al. 1979). An attempt has been made to study insulin action by perfusing the forearm of humans with insulin and measuring glucose uptake (Rabinowitz and Zieler 1962). This method of assessment has not been widely accepted because it has produced variable results. Also, Greenfield et al. (1981) have pointed out that it requires a lot of skill to perform the perfusion study. Another measure of insulin action insulin suppression test (IST) in which epinephrine, propranolol, insulin, and glucose are infused at constant rates to curtail internal insulin release. The plateau level of glucose at a specific serum insulin level is an indicator of insulin resistance (Greenfield et al. 1981). From the IST is derived the pancreatic clamp, whereby somatostatin replaces epinephrine and propranolol in the inhibition of the pancreas. Endogenous pancreatic hormones are then replaced by infusion of basal amounts of insulin and glucagon (Harano et al. 1977; Nagulesparan et al. 1979). The advantage in this technique is that a particular hormonal or substrate change can be studied without interference from insulin and glucagon feedback (Cherrington and Steiner 1982). Modification of these methods gave rise to the euglycemic clamp technique which determines tissue sensitivity to insulin and the sensitivity of the ß cell to

glucose (DeFronzo et al. 1979). Insulin, together with a known quantity of glucose is infused to attain a constant level of glucose (euglycemic plateau). The amount of glucose infused to maintain the euglycemia under steady state conditions is a measure of the amount of glucose uptake by the tissues in response to insulin (DeFronzo et al. 1979). The euglycemic clamp is based on two assumptions. The first is that, due to negative feedback, endogenous glucose production is markedly inhibited with a rise in insulin. Secondly, the balance between glucose absorption and expenditure is zero, since steady state conditions exist (DeFronzo et al. 1979). Each of the criterion is limited by its inability to account for other counter-regulatory effects stimulated by the excessive amounts of catecholamines or by the rise in insulin or glucose. The euglycemic clamp technique reduces the opposing influences to a minimum and has, thus, become the most popular. The technique has also been adapted to measure glucose utilization and production (Rizza et al. 1981) as well as the amount of glucose oxidized or stored (DeFronzo et al. 1981; Felber et al. 1981).

The common denominator of all these different criteria is that, among other things, they are used to ascertain the level of resistance to insulin action in a given subject or tissue, especially in the diseased state (Greenfield et al. 1981). Results from insulin dose-response curves provide a

standard criterion for interpretation of the action of insulin (Kolterman et al. 1982). A uniform assessment of the causes of any alterations in the action of the hormone is also made possible by dose-response curves. The shape of the curve could thus be predicted, and reasons assigned for any deviations (Kolterman et al. 1982; DeFronzo et al. 1979). Explanation of how the curves are utilized will be considered under the appropriate section.

2.4.3 Insulin effects on hepatic glucose production

It is pertinent to discuss briefly the action of insulin on hepatic glucose production since the liver plays a prominent role in glucose metabolism (Cherrington and Steiner 1982). One of the basic assumptions of the glucose clamp technique is the inhibition of endogenous glucose production by insulin (DeFronzo et al. 1979). Examination of the precise effect of insulin and/or glucose on hepatic glucose output is therefore central to the validity of the various clamp techniques used to study insulin action.

According to Cherrington and Steiner (1982), pharmacological doses of insulin have long been shown to inhibit glucose production in the liver (see Bearn et al 1952 and Madison et al. 1960 for review). Subsequent studies have indicated that physiological levels and minor increments in insulin are equally potent in suppressing glucose production by the liver (DeFronzo et al. 1978; Long

et al. 1971). Severe hyperinsulinemia has also been shown to completely block hepatic glucose production in both obese and non-obese humans (Kolterman et al. 1980). There was a marked drop in hepatic glucose output following a moderate infusion of glucose, which caused mild hyperinsulinemia and hyperglycemia without altering glucagon levels. Glucose production was unaffected when diabetic men, unresponsive to insulin, were subjected to the same level of glucose infusion (Long et al. 1971). Euglycemic studies by Chiasson et al. (1976, 1979) showed that an increase in insulin level of about 100 U/ml suppressed liver glucose output in overnight fasted dogs and humans. This finding implies a reduction in glycogenolysis since Cherrington (1981) has noted that in humans almost all the glucose produced overnight is derived from glycogen. Due to a decline in glucagon level that accompanied hyperinsulinemia, Chiasson et al. (1979) hesitated in drawing conclusions about the effects of insulin on glucose production by the liver. Their observations were, however, confirmed by Steiner et al. (1981) subjected conscious dogs to the pancreatic clamp technique. Glucose production was inhibited as a result of increases in insulin level even without a decline in glucagon level. Furthermore, DeFronzo et al. (1979) and Rizza et al. (1981) have reported that overnight fasted normal men are sensitive to slight increases in insulin level. A 90-100 U/ml rise in portal insulin would completely block net glucose output by the liver or reduce it to below

10% of the basal amount (DeFronzo et al. 1978). In addition, approximately 29 U/ml of insulin was enough to cause 50% suppression of endogenous glucose production while about twice this amount was needed for half-maximum inhibition of glucose utilization in humans (Rizza et al. 1981). Thus, hepatic glucose output is more sensitive to a rise in insulin than glucose utilization in humans and dogs. Indeed, basal insulin levels are believed to exert similar inhibitory effects on glucose production in dogs (Cherrington et al. 1978; Steiner et al. 1981) and humans (Felig and Wahren 1971).

Ruminants also respond to the suppressive effects of insulin on endogenous glucose production. However, they are considered less responsive (Brockman 1978). Compared to humans, sheep are virtually insensitive and unresponsive to insulin effects on liver glucose production (Weekes et al. 1983). Whereas a 50-60 U/ml level of insulin was enough to totally block glucose production by the liver in humans, insulin concentration of about 300 U/ml only decreased endogenous glucose to 50% of the basal level in sheep, and total inhibition could not be attained (Weekes et al. 1983). Ironically, there was no significant difference between the amount of insulin required for half-maximum glucose utilization by the liver in sheep and humans, meaning that the two species have similar sensitivities in this regard. In effect, unlike humans, hepatic glucose production is less

sensitive to insulin than is glucose utilization in sheep (Weekes et al. 1983). The responsiveness of the liver to glucose uptake was considerably lower in ruminants as compared to humans (Weekes 1983). Above all, the dose-response curve for insulin effects on hepatic glucose uptake in obese patients was reportedly shifted to the right (Kolterman et al. 1980). This shift to the right represented a decrease in sensitivity, and changes in sensitivity to insulin are usually due to changes in the number of insulin receptors (Kolterman et al. 1982). Thus, obese idividuals are insensitive to the inhibitory effects of insulin on glucose production, and the abnormality present is a reduction in number of insulin receptors.

2.5 RECEPTOR AND POST-RECEPTOR EFFECTS

The importance of the number and affinity of insulin receptors and the target organ in the perpetuation of insulin action has already been noted (Kahn 1979). The peripheral insulin level, though equally important, is not critical in most cases.

Olefsky (1975) showed that in rat adipocytes, only a fraction of the insulin receptors present need to be filled to elicit maximum effects of the hormone. In other words, there are always some 'spare' receptors which need not be occupied with insulin even when maximum insulin action is achieved. This observation has led to what has been

generally accepted as the 'spare receptor theory'. The presence of 'spare' receptors has since been confirmed both in vitro and in vivo. The inhibitory effects of insulin on hepatic glucose was achieved with only 11% receptor occupancy (Kolterman et al. 1980). Half-maximum and total response were achieved with 11% and 50% receptor occupancies, respectively, in both erythrocytes and adipose cells (Kolterman et al. 1980; Rizza et al. 1981). However, the fraction of receptor sites filled for maximum insulin effects depends on the target tissue (cell type) and the specific effect of insulin being examined (Kolterman et al. 1980).

The concept of spare receptors provided the basis upon which dose response curves are interpreted (Kolterman et al. 1982). In this regard, a distinction is made between insulin sensitivity and insulin responsiveness (Kahn 1978; Kahn 1980). In a system with spare receptors, provided that adequate amounts of hormone are available, minor differences in number or affinity of receptors lead to a leftward or rightward movement of the dose-response curve, even though the maximum response to insulin remains unaltered (Kolterman et al. 1982). The shifts in the curve signal changes in insulin sensitivity. The rightward shift represents a decrease in receptor number and/or affinity and a decline in sensitivity. A higher-than-normal quantity of hormone is, therefore, required to achieve half the potential capacity

of insulin action. A shift to the left means an increase in sensitivity, whereby lower-than-normal concentrations are needed for the submaximal action of insulin (Kahn 1980). In practice, decline in sensitivity is a more frequent occurrence especially in diseased states like obesity or diabetes (Williams 1981). Nevertheless, increases in sensitivity do occur. Both physical exercise and growth hormone deficiency to increase insulin sensitivity (Koivisto et al. 1980; Parkes and Bassett 1985). For example, well-trained athletes exhibit excellent glucose metabolic activities, in spite of their lower-than-normal insulin levels. This finding strongly supports the association of high sensitivity to insulin with exercise (Bonen et al. 1985).

There are situations where the concentration of insulin required for 50% of the maximum insulin action is quite normal (normal sensitivity) but the maximum insulin response is lowered. Such a situation signifies a decrease in insulin responsiveness (Kahn 1980). Reduced insulin responsiveness is mainly but not exclusively a result of a change in post-receptor steps (Weekes et al. 1983). Under certain circumstances both changes in sensitvity and changes in responsiveness occur simultaneously (Kolterman et al. 1980). Characterization of the insulin dose-response curve, has provided a popular foundation for the study of the glucoregulatory actions of insulin. Careful assessment of

the whole body or in vivo dose-response curves is advised, because they only portray the total response; individual tissues and/or routes of metabolism may respond differently when various actions of insulin are tested separately (Brady et al. 1981). For instance, Howard et al. (1984), showed that the anti-lipolytic action of insulin is hardly affected in obese humans although glucose modulation by insulin was severely impaired in the same subjects. Bolinder et al. (1983b) observed that human adipocytes were more sensitive to insulin suppression of lipid breakdown than oxidation of glucose. Differences were also found between subcutaneous and omental adipose tissues in their response to insulin (Bolinder et al. 1983b). Receptor loss of such magnitude that would drastically reduce or leave no spare receptors should theoretically be expected to lower maximum hormone response (responsiveness), while the possibility that some post-receptor changes could shift the dose-response curve horizontally cannot be precluded (Weekes et al. 1983). However, a total loss of spare receptors would eliminate the plateau in most dose-response curves (Kolterman et al. 1980). Such a consideration should provide a useful guide to the interpretation of dose-response curves.

Having explored the issue of spare insulin receptors, and the context within which dose-response curves are frequently used to interprete changes in sensitivity and/or responsiveness, it is worthwhile to consider some

circumstances under which the above scheme has been applied, with particular reference to the effects of insulin on glucose regulation in mammals.

2.5.1 Insulin action in monogastrics

A strong relationship exists between glucose tolerance and insulin sensitivity as measured by intravenous glucose and insulin tolerance tests in dogs and pigs (Heard and Henry 1969) and in normal men (Beck-Nielsen and Pedersen 1978). Also, both insulin binding and insulin sensitivity were markedly reduced following a high level of sucrose and/or fat intake (Beck-Nielsen and Pedersen 1978). Studies with obese diabetics confirmed the importance of the receptor and/or the sensitivity of the end organ in the occurrence of insulin resistance (Beck-Nielsen and Pedersen 1979). Similar results have been reported for erythrocytes of hyperinsulinemic obese children along with a rise in serum insulin (Asayama et al. 1982). This was found to be true both during fasting and after glucose challenge. In vivo insulin sensitivity (as determined by the Insulin Suppression Test, described previously), was also closely associated with specific insulin binding and insulin receptor concentration (Asayama et al. 1982). Contending that their results confirmed earlier reports of DeFronzo et al. (1979) and Kolterman et al. (1980), the authors suggested that resistance to insulin in obesity was solely due to decline in insulin receptors, irrespective of the

criteria used in testing for resistance, be it insulin suppression or the euglycemic clamp (Asayama et al. 1982). This suggestion is not in total agreement with the observation that post-receptor effects also contribute to the impairment of target tissues in obese monogastrics and ageing humans (Bolinder et al. 1983b). In another study, Kolterman et al. (1980) characterised the insulin resistance in humans using a modification of the euglycemic clamp technique, which afforded a comprehensive review of responses to insulin in both obese and non-obese individuals. They noted that although receptor defects prevail in all cases of insulin resistance in the obese, post-receptor events could also exacerbate the level of resistance especially in the presence of high ambient insulin levels. Glucose removal was comparatively slower in the obese than their controls at the insulin infusion rate 40 U/ml/min. The dose-response curve shifted to the right in all obese subjects. This reduced sensitivity signified a defect at the receptor level (Kolterman et al. 1982). The majority of the obese patients also showed a concomitant lowering of maximum glucose uptake an indication of a drop in insulin responsiveness (Kahn 1980). A number of the obese however, showed normal responses to insulin. The deviation in these subjects, therefore, only resided at the receptor level and not beyond (Kolterman et al. 1980). In view of the above, insulin response in the obese is best described as heterogenous, since it could either be marked by

decreases in sensitivity alone, or in combination with reduced responsiveness (Kolterman et al. 1980). Further scrutiny showed that the subjects with moderate insulin resistance (moderate hyperinsulineamia) exhibited only the receptor abnormality, while under conditions of severe hyperinsulineamia or pronounced insulin resistance, both receptor and post-receptor disturbances manifested (Kolterman et al. 1980). A resistance to insulin's stimulation of glucose use in muscle and adipose tissue was also observed (Kolterman et al. 1980). In this regard, Yki-Jarvinien and Koivisto (1983) observed that insulin-stimulated glucose metabolism in vivo is enhanced by high muscle content of the body, but declines with increase in fat content, implying that reduced responsiveness was strictly due to a reduction in responsive tissue (muscle) as fat content increased. Cherrington and Steiner (1982) have noted that insulin and its receptors modulate glucose production and utilization in both hepatic and extrahepatic tissues. This observation is perhaps an apt summary of what has been discussed above, for non-ruminants.

2.5.2 Insulin action in ruminants

Comparatively little is known about the extent to which receptor and post-receptor effects alter insulin action in ruminants, particularly farm animals (Weekes 1986). Basic metabolic differences exist between ruminants and non-ruminants in glucose availability and use. Ruminants rely

mainly on endogenous glucose for their metabolic needs (Brockman 1982). Due to this fact, there is predominant glucose manufacture in the liver (Brockman 1982). The need for efficient removal of exogenous glucose may, therefore, not be as critical in ruminants as one would expect for non-ruminants. In fact, Weekes et al. (1983) has stated that the ruminant liver cannot use much glucose since it possesses no glucokinase. Nevertheless, ruminants experience the same gluco-regulatory effects of insulin as occurs in monogastrics, including the ability to block hepatic glucose output (Brockman 1983) and the ability to promote glucose absorption by the target tissues especially muscle (Prior et al. 1984). The scant information available suggests that ruminants show a reduced response to the glucoregulatory effects of insulin compared to non-ruminants. Weekes et al. (1983) showed that adult sheep were less-sensitive and less-responsive to the inhibitory effect of insulin on glucose production by the liver than their human counterparts. This implies that both receptor and non-receptor parameters may be involved in the observed differences in response to insulin. Janes et al. (1985) have observed that insulin accounts for a significantly small fraction of the basal glucose uptake in sheep. In addition, they noted that peripheral glucose removal is neither as responsive nor sensitive in sheep as in man. However, according to Weekes et al. (1983), although adult sheep were less responsive than man in terms of peripheral glucose uptake, the

senstivities to insulin were of comparable magnitude in both species. Half-maximum glucose removal from the periphery of humans and sheep occurred at insulin concentrations of 58 U/ml and 52 U/ml, respectively (Weekes et al. 1983). Morever, responses in coldstressed sheep were comparable to those of normal humans in the same study. Therefore, the the target organs in sheep have the capability to respond effectively to insulin, given the appropriate circumstances (Weekes et al. 1983). Added to this, Janes et al. (1985) have indicated that there is a decline in response to insulin-stimulated glucose utilization in sheep, due to the inability of the cells to pick up the available glucose (rather than glucose deficiency), thus emphasizing in part the role of the receptor. Another observation is that obese heifers, like obese non-ruminants, were less sensitive to insulin than their lean counterparts (McCann et al. 1986). Whereas insulin resistance in obese monogastrics may result from both receptor and non-receptor impairment of the target organ, only receptor defects have been associated with insulin resistance in ruminants to date (McCann and Reimers 1985). The role of the receptor in insulin responses in ruminants cannot, therefore, be overemphasized.

2.5.3 Age and insulin action

The metabolic needs of an animal changes from birth to adulthood (Bergen 1974). Along with changes in metabolic needs are modifications in the levels and function of

metabolic hormones like insulin and GH (Turner 1978; Machlin et al. 1968). Changes in peripheral insulin levels with age have been documented in humans and other animals. However, except under certain specific circumstances, such as complete lack of insulin, there is usually enough insulin to fill the available receptor sites of any tissue (Heard and Henry. 1969). This observation agrees with the spare receptor theory outlined above. It also suggests that changes in serum/plasma insulin levels, though useful in certain diagnoses, cannot entirely explain the effects of age on insulin action. The marked variation in serum/plasma insulin levels with age, might, therefore result from a variety of factors of which receptor characteristics are but one of them.

Studies on the direct effects of age on receptor parameters are lacking. Nevertheless, changes in insulin binding, sensitivity and responsiveness (all of which reflect alterations in both receptor and non-receptor effects on the target organ) occur as mammals mature (Vandergrift et al. 1985; Kappy and Plotnick 1980; Kappy 1983; Kappy et al. 1981; Bolinder et al. 1983b). Turner (1978) reported that at a young age, mammals are less able to tolerate glucose but there is a progressive improvement in their ability to handle glucose up to adulthood. Twenty-day old rat fetuses were unresponsive to insulin even though their peripheral insulin levels were higher than those of

their mothers (Clark et al. 1968). However, studies with male Sprague-Dawlwey rats between 1.5 to 12 months of age showed that both glucose-stimulated insulin secretion and insulin-stimulated glucose uptake decline with age (Reaven et al. 1983). Erythroid cells show a decline in number of insulin receptors with age in humans (Asayama et al. 1982), in sheep (Kappy et al. 1981) and, in cattle (Kappy 1983). Heard and Henry (1969) noted that dependence on insulin develops with age, and once achieved, glucose uptake is modulated by sensitivity to insulin. With advancing maturity and adulthood, as energy deposition takes precedence over protein accretion, sensitivities to insulin are perhaps altered once more (Turner 1978). This might explain the observation of Cacciari et al. (1978) that, for a given insulin concentration, younger people utilize glucose more efficiently than adults. Increased fat deposition may also account for changes in insulin sensitivity with age (Bergen 1974). In this regard, obese animals have always been shown to be less sensitive to insulin than lean animals of comparable age in both monogastrics (Kolterman et al. 1980; Wangsness et al. 1981) and ruminants (McCann et al. 1986). However, Weekes (1986) noted that in spite of an enhanced fat or protein deposition with increasing maturity, tissue responsiveness to insulin glucose uptake and metabolism were hardly changed. Sensitivity to insulin declined moderately due to a slight drop in number and affinity of insulin receptors. Sheep

metabolic removal of insulin tended to decline markedly with age (Reaven et al. 1982). Reduced metabolic excretion of insulin may partly explain the higher postprandial insulin levels in older sheep (Godden and Weekes 1981; Sasaki et al. 1984).

Even if the direct effects of age on insulin utilization cannot be measured, the fact that older animals tend to show an increase in peripheral insulin levels, just like the obese, is an indication that they require higher concentrations of insulin for their optimum body function and, therefore, are less sensitive to insulin (Grizard et al. 1987). Changes in binding affinity due to age in various target tissues for insulin have been discussed previously. Mention should be made that there are situations where binding characteristics alone cannot be used to interprete insulin sensitivity and action. One such instance is pregnancy. Pregnant humans exhibit a reduced sensitivity to insulin but binding is enhanced and remains so throughout the pregnancy (Felig and Soman 1979). Presumably there are post-receptor changes in the pregnant human.

2.6 EFFECTS OF ADRENALINE ON CIRCULATING HORMONE LEVELS

The sympathetic nervous system, which releases catecholamines, plays a prominent role in the modulation of metabolism and circulation in mammals (Young et al. 1980; Bassett 1970). Even though there is consensus in the overall

metabolic effects of catecholamines (and their derivatives) in mammals, some inter-species variations occur in their effectiveness (Basett 1970; Oda et al. 1988). Variable responses to adrenaline have been reported for peripheral glucose and plasma free fatty acids (FFA) in ruminants (Graham and Philips 1981).

According to Radloff and Schultz (1966) ruminants are less sensitive to adrenaline and noradrenaline than non-ruminants (Radloff and Schultz 1966). Moreover, very moderate rises in FFA were reported for cattle and sheep following injection of a high dose of adrenaline (Khachadurian et al. 1967; Sidhu and Emery 1972). But, according to Himms-Hagen (1967) and Bassett (1970), responses to catecholamines depend, among other things, on the method by which the hormone is administered. Adult Merino wethers, when infused with adrenaline over a prolonged period, had higher responses (as measured by the rise in peripheral glucose, lactate and FFA) than when given the same dose of adrenaline once by intravenous injection (Bassett 1970). Either route of adrenaline administration was, however, effective in blocking increases in plasma insulin levels. These results are consistent with the works of Hertelendy et al. (1969) and Oda et al. (1988). They also reaffirm the view that sheep behave like humans and and infrahuman species in their response to adrenaline, both qualitatively (Himms-Hagen 1967) and quantitatively (Porte

1967). Therefore, ruminants may not be very different from non-ruminants in their sensitivity to catecholamines as has been suggested elsewhere (Bassett 1970). Indeed, Gooden et al. (1986) observed a 4-fold increase in both plasma FFA and glycerol in response to noradrenaline in Karakul ewes in vivo. Jaster and Wegner (1981) have also reported stimulation of glycerol release by adrenaline in dairy cow adipocytes.

Contrary to many reports (Bassett 1970; Hertelendy et al 1969; Sasaki and Takahashi 1980; Oda et al. 1988), Grisdale-Helland et al. (1986) reported that epinephrine infusion acutely raised insulin levels in growing lambs. This observation is perhaps an age-related phenomenon of sympathetic activity (Young et al. 1980). Whereas Bassett (1970) reported that adrenaline was more potent than noradrenaline in elevating plasma glucose and lactate levels in matured sheep, Alexander et al (1968) found that the two catecholamines were equally effective in this regard in young lambs, perhaps due to the pharmacological doses used. Hertelendy et al. (1969) have observed that adrenaline stimulates insulin release (via ß-adrenergic activity) when the dominant insulin-suppressive α-adrenergic blockade is effectively removed. Unlike modulation of glucagon, there is a consensus on the stimulatory effects of ß-adrenergic and inhibitory effects of α-adrenergic activity on insulin in all mammals (Oda et al. 1988). It is, thus, possible that

the α-adrenergic activity of adrenaline was somehow impaired in the study of Grisdale-Helland et al. (1986). There is evidence for the sympatho-adrenal modulation of insulin secretion in response to glucose administration in cold-exposed sheep (Sasaki and Takahashi 1980). Enhanced glucose response to adrenaline infusion has been observed in both cold-exposed (Graham and Phillips 1981) and exercising sheep (Brockman 1982). These observations corroborate reports that enhanced sympathetic nervous activity, acting via the α-adrenergic mechanism, suppresses insulin release in sheep under the stress of cold environments (Sasaki et al. 1982; Sasaki and Takahashi 1980) or exercise (Brockman and Halvorson 1981). The net result is a pronounced glucose release and uptake (Sasaki et al. 1982; Brockman and Halvorson 1981; Graham and Phillips 1981). Similar results have been reported for the Pietrain pig in which there was also a reduced sensitivity to insulin (Gregory et al. 1977). Consistent with these reports is Brockman's (1982) observation that extirpation of the nerve supply to the adrenals in exercising sheep increased insulin levels, thereby causing a reduced rise in glucose. The increase in glucagon level was, however, not affected. These results support the belief that endocrine activity of the adrenal medulla modulates hepatic and muscle glycogen breakdown via control of insulin secretion in exercising sheep (Brockman 1982).

In the light of the above, note that Basset (1970) has suggested that the output of FFA and secretion of insulin represent more sensitive measures of adrenaline action in sheep than the peripheral glucose and lactate concentrations. He observed a persistent rise in FFA and a sustained inhibition of insulin secretion, even at adrenaline infusion rates which caused minimal rise in glucose or lactate levels (Bassett 1970).

Chapter III

MATERIALS AND METHODS

3.1 ANIMALS AND DIET

Outaouais Arcott (a hybrid of about 50% Finnish Landrace, 25% Suffolk and 25% Shropshire, developed by the Animal Research Centre, Agriculture Canada, Ottawa) ram lambs born within three days of each other were used. Prior to weaning at 6 weeks of age each lamb was treated with 1.5mg selenium, 69 I.U. vitamin E (Distocel BTI Products Inc., Montreal, Quebec) and 5ml Covexin-8 (Burroughs Wellcome Inc., Kirkland, Quebec).

After weaning, 10 lambs were put on a Westfalia Separator Automatic Feeder System (Westfalia Systemat, Centrico Inc, Elk Grove, IL) for efficient monitoring of feed intake. Modifications made to the feeder system for use with sheep have been previously reported by Boila et al. (1990).

The animals were fed a pelleted ration consisting of 49% barley, 40% alfalfa meal, 10% canola meal, and 1% vitamin-mineral premix containing vitamin A and D at 5000 IU/kg. The diet was calculated to contain 72.6% total digestible nutrients (TDN), 18.3% protein, 0.91% Ca and 0.46% P on a dry matter (DM) basis.

3.2 MANAGEMENT

The animals were housed in a 6 m by 4 m pen. Lambs had free access to water and cobalt-iodized salt. Weighing was done once weekly (see Appendix 1 for mean lamb weights for each week). On the basis of uniformity of weight, eight lambs were selected at 65 days old from the original ten for the trial.

At 67 (age 1) and 157 (age 2) days of age (i.e., lean and fat growth phases respectively), the lambs were subjected to a hormone sensitivity test (HST) (see below). In addition, all lambs were blood sampled for mononuclear leukocyte (MNL) and specific insulin binding (SIB) determination on six occasions (see below).

3.3 HORMONE SENSITIVITY TEST (HST)

A day before each test period the lambs were sheared around the neck and indwelling clear vinyl catheters (Dural Plastics, Australia) were inserted; one into the right and the other into the left jugular vein for infusion and sampling, respectively. The infusion and sampling catheters were inserted to depths of 25 cm and 15 cm, respectively. Catheters were taped and covered with "vet wrap" for protection. place. The catheters were left in-situ for each HST period lasting 9 days. The lambs were divided into two groups of four each for the convenience of testing one group in the morning and the other in the afternoon of the same

day. One day before the HST the lambs were placed in individual 2.4 by 1.2 m metabolism crates where they remained for the duration of the 9 day HST. During this 9 day period, the lambs had free access water, but feed was denied for 12 hours before and 3 hours of treatment (see below).

On a test day, each lamb was infused with saline (0.9% NaCl) for 1 hr (pretreatment period) followed by a 2hr challenge with one of 4 treatments, saline (Sal) (0.9% NaCl), low insulin (LI) dose (0.2 mU/kg/min ovine insulin, Sigma, St Louis, Mo.), high insulin (HI) dose (6.0 mU/kg/min) and adrenaline (Adr) (Sigma) (0.3 ug/kg/min). Infusions were given every second day, allowing for a one day rest period between treatments. Infusates were freshly prepared in sterile saline on the day of infusion with ascorbic acid (0.1g/L) added as an antioxidant. The adrenaline solution was ice-chilled at all times.

A multichannel peristaltic pump (Technicon Autoanalyzer Pump) was used to deliver the infusates at the rate of 0.6 ml/min. The infusion times were arranged to simultaneously infuse one group (4 animals) in the morning and the second in the afternoon of the same day. Two blood samples (3ml and 2ml) were taken from each lamb every 20, 10 and 20 min for the 1st, 2nd and 3rd hours of infusion, respectively. Serum for GH, insulin, and glycerol assays was harvested from the 3ml sample and stored at -20C after allowing the blood to

clot at room temperature. The 2ml sample was withdrawn into heparinized (0.5U) syringes, gently inverted, and transferred to ice-chilled 10x75 mm test tubes. The blood was centrifuged at 4C and the plasma for glucose determination harvested and stored at -20C. After the last day of the HST, the catheters were removed. Each lamb was given 150 000 IU penicillin G benzathine and 150 000 IU penicillin G procaine (Derapen, Ayerst Laboratories, Montreal, Que) to prevent infection and was then returned to the pen for the resumption of normal activity.

3.3.1 Insulin and GH Assays

A double antibody radioimmunoassay (RIA) procedure (Kennedy et al. 1987) with ovine insulin (Sigma, St Louis, Mo.) standards was used to measure serum insulin. Guinea pig anti-bovine insulin (Miles Laboratories, Elkhart, Ind) and Goat anti-guinea pig serum (Antibodies Inc., Davis, Calif) were used as 1st and 2nd antibodies respectively. Sample were analysed in 2 assays, each containing 9 replicates of pooled serum to determine within assay variations. Intra and inter-assay coefficients of variation were 8.5% and 9% respectively. The lowest insulin level detectable was 0.6 ng/ml. Growth hormone (GH) was assayed by a double antibody RIA (Kennedy et al. 1988b). Rabbit anti-ovine growth hormone serum (NIAMDD, Bethesda, Md) and sheep anti-rabbit serum (Antibodies Inc., Davis, Calif.) were used as 1st and 2nd antibodies respectively. Samples were analysed

in 2 assays each containing pooled serum replicates to determine variations. Intra and interassay coefficients of variation were 11% and 12.6% respectively. The lowest GH level detectable was 1.0ng/ml. Serum samples for both insulin and GH were assayed in duplicate.

3.3.2 Glucose Determination

Plasma glucose concentration was assayed in duplicate using the Technicon Autoanalyzer II (Neocuproine method No. AA-2) previously mentioned by Kennedy et al. (1988b).

3.3.3 Glycerol Determination

Serum glycerol was assayed using a UV-method (Glycerol Kit, Boehringer Mannheim, Dorval, Quebec, Cat. No. 148 270). Solutions were read against water at a wavelength of 340nm. Recovery of standards for variation was 98% .

3.4 SPECIFIC INSULIN BINDING (SIB) DETERMINATION

For the SIB assays, the lambs were divided into 2 groups of 4 each. Blood samples (180 ml) were taken from four lambs at 83 days and four at 85 days of age. Sampling was repeated at 21-day intervals on 5 consecutive occasions. The 6th and final stage of SIB determination was included to help explain the unusually high binding observed at Stage 5.

Blood was taken by venipunture into a 250 ml sterilized plastic bottle containing 10 ml of chilled diK-EDTA solution

(4.5% pH 7.4) as anticoagulant. The bottle was gently inverted about 3 times. White blood cells (WBC) were harvested within an hour of blood withdrawal. The remaining blood cells and some plasma were re-infused into the respective donors the same day. Details of the blood cell isolation and return process are provided in Appendix 2.

MNL isolation and cell insulin binding assays were done on the day of blood collection. The assay method was as reported by Kennedy et al. (1987). Whole blood was, however, centrifuged with a force of about 700g (2000 rpm). This followed preliminary tests which showed that maximum cell yield occurred at this force. MNL cells were counted in triplicate Unopette Test 5856 (Becton-Dickinson, Becton, Dickinson and Co., Rutherford, N.J.) microcollection method for dilution and an American Optical Hemotocytometer for counting. Samples were diluted in Tris-HCL buffer to contain $60x10^6$ cells, and incubated at 15C for 100min in the presence of [^{125}I]-insulin (New England Nuclear Products, N. Billerica, MA). The tracer insulin was included at 40 pg/tube (13000 - 20000 cpm/tube) depending on the age of the [^{125}I]-insulin.

3.5 DAIRY CALF TRIAL

Ten Holstein heifer calves from the Glenlea Research Station, University of Manitoba were made available for MNL specific insulin binding determination. The calves were weaned at 5 weeks of age. Calves were fed the following diets; calf starter at 2 weeks, calf grower at 6 weeks, and a dairy ration supplemented with good quality hay at 3-4 months. Diet compositions are given at given at Appendix 3. Mean birthweight for the calves was 42 kg. A 250 ml blood sample was taken from each calf within 2 days of birth and every month thereafter for 5 consecutive months (monthly calf weights are given in Appendix 4). The blood sampling and processing procedures were exactly as reported for the ram lambs. However, the number of cells per binding tube was $30x10^6$ due the low white blood cell counts in the very young calves. There was no return of blood cells to the donor calves after sampling. Tracer insulin concentrations ranged from 10000 to 20000 cpm/tube (40 pg/tube) depending on the age of the [^{125}I]-insulin. One calf was eliminated from the trial due to illness.

3.6 STATISTICAL ANALYSIS

Analysis of variance using a General Linear Model (GLM) (SAS Institute Inc., 1986) was used to examine the responses to infusion results. The day of infusion (Day) and the period (Per) within the day (i.e., morning or afternoon) on which the different groups of animals (ID) were respectively

infused were included in the statistical model. Unless otherwise stated, means were compared using the predicted means test (SAS Institute Inc., 1986).

Of the variables studied, response for each animal within each treatment and age was determined by subtracting the average values for the first infusion hour (pretreatment period) from the 2nd or 3rd hours of infusion respectively. From examination of Treatment and Age means of a variable over the entire infusion period, the hour of maximum response was determined for subsequent analysis. From this the mean response in hour 3 was used for glucose and insulin, and hour 2 response was used for GH and glycerol respectively. Only the adrenaline and saline (control) treatments were compared for glycerol.

Specific insulin binding results were analysed using the General Linear Model (GLM, SAS Institute Inc., 1986). Only specific insulin binding at physiological insulin incubation concentrations (5 ng/ml or less) were analyzed. Age means were compared using the Neuman-Keuls test (Steel and Torrie, 1980). Unless otherwise stated, number of observations in each analysis was 8 for sheep and 10 for calves.

Chapter IV

RESULTS

4.1 PRETREATMENT HORMONE LEVELS

Pretreatment glucose and insulin levels were higher ($P<0.01$) and GH was lower ($P<0.01$) than levels during treatment in older lambs (Table 1).

4.2 GLUCOSE LEVELS

In order to assess time of maximum response, means for all the sampling times were calculated and are shown in Figs. 1 and 2 respectively. Results for adrenaline infusion was not available for one lamb (n=7). From these figures, it can be seen that neither Sal nor LI had a marked effect on plasma glucose level. For HI, asymptotic glucose levels occurred within 60 to 70 minutes of treatment in the younger lambs (Fig. 1). In the older lambs, however, asymptoticity for glucose level was yet to be achieved after 120 minutes of HI treatment (Fig. 2). A slight rise in glucose levels due to Adr occurred in age 1 within 60 minutes of infusion and remained constant thereafter (Fig. 1). Adrenaline markedly raised glucose level in age 2 lambs; whereas, glucose levels increased for about 70 minutes after which there was a slight decline in glucose level (Fig. 2).

TABLE 1. Pre-treatment* least square means ± SE for plasma glucose, serum insulin and serum Growth Hormone (GH) concentrations in lambs.

	Glucose (mg/dl)	Insulin (ng/ml)	GH (ng/ml)
Age (Days)			
67	66.0 ± 2.0	1.6 ± 0.2	10.8 ± 1.2
157	79.4 ± 2.0	3.3 ± 0.2	3.3 ± 1.9
Levels of significance (P=)			
Age	0.0001	0.0001	0.0001
Trt	0.9150	0.7828	0.4629
Age x Trt	0.8181	0.7830	0.8065

* Pre-treatment samples were collected during Hour 1, prior to the implementation of the treatments.

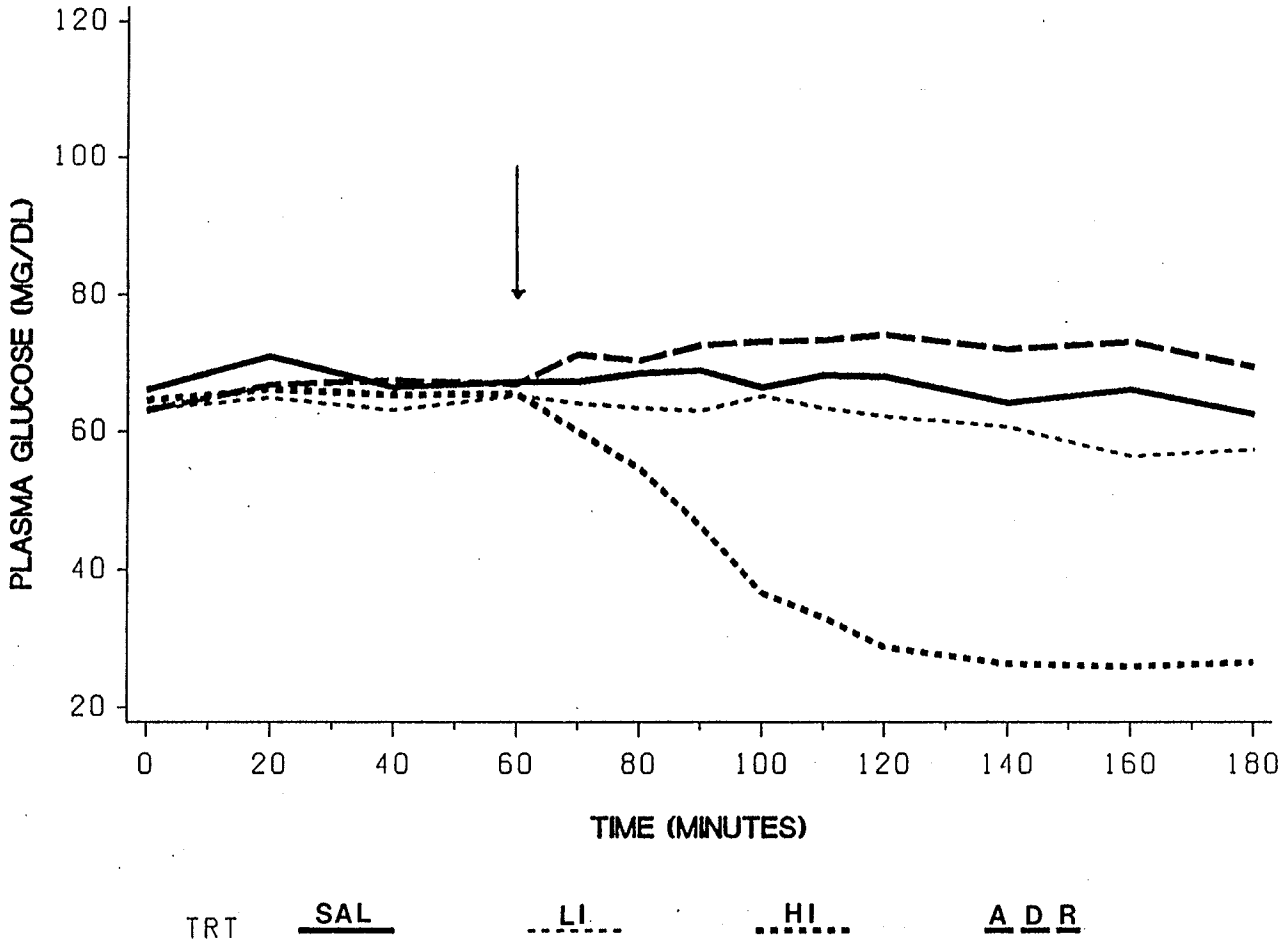

Figure 1. Mean glucose profile during infusion of Saline, Low Insulin, High Insulin and Adrenaline in ram lambs at 67 days of age. Arrow indicates start of treatment.

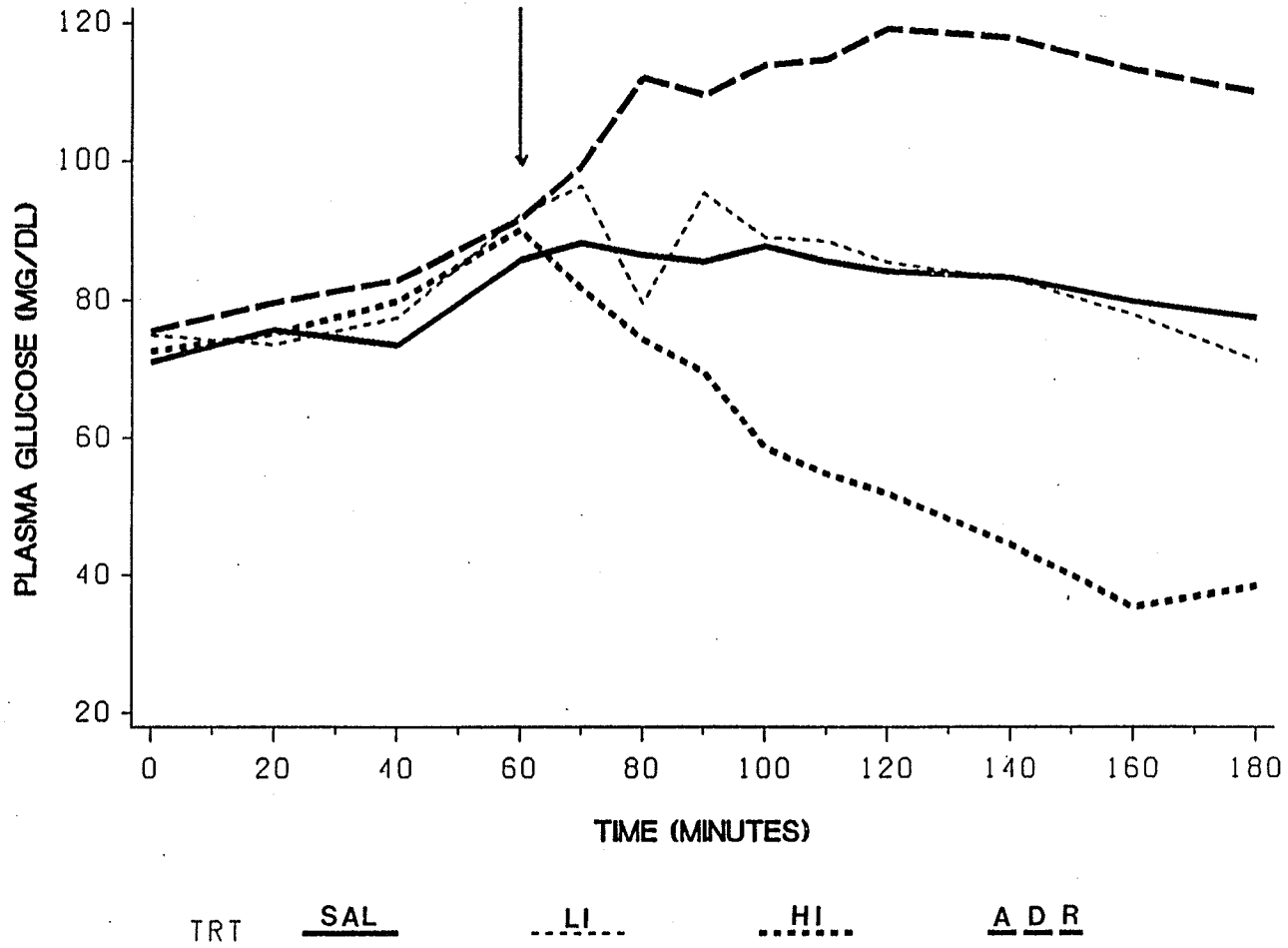

Figure 2. Mean glucose profile during infusion of Saline, Low Insulin, High Insulin and Adrenaline in ram lambs at 157 days of age. Arrow indicates start of treatment.

Mean plasma glucose levels for the pretreatment and treatment samples at both ages are shown in Figs. 3 and 4 respectively.

Day(Age), Per and Animal(Per) did not affect glucose response. Thus, responses found were similar a.m. and p.m., and did not change during the 9 day HST at either age. The Age x Treatment interaction was significant ($P<0.03$) (Table 2). At both ages, HI significantly ($P<0.05$) increased the glucose response (hypoglycemia) compared to Sal. Adrenaline (Adr) resulted in a significant ($P<0.05$) increase in glucose (hyperglycemia) in relation to the control treatment only at age 2 and the glucose response to Adr was greater at age 2 than at age 1. Glucose response to LI was never different from the response to Sal.

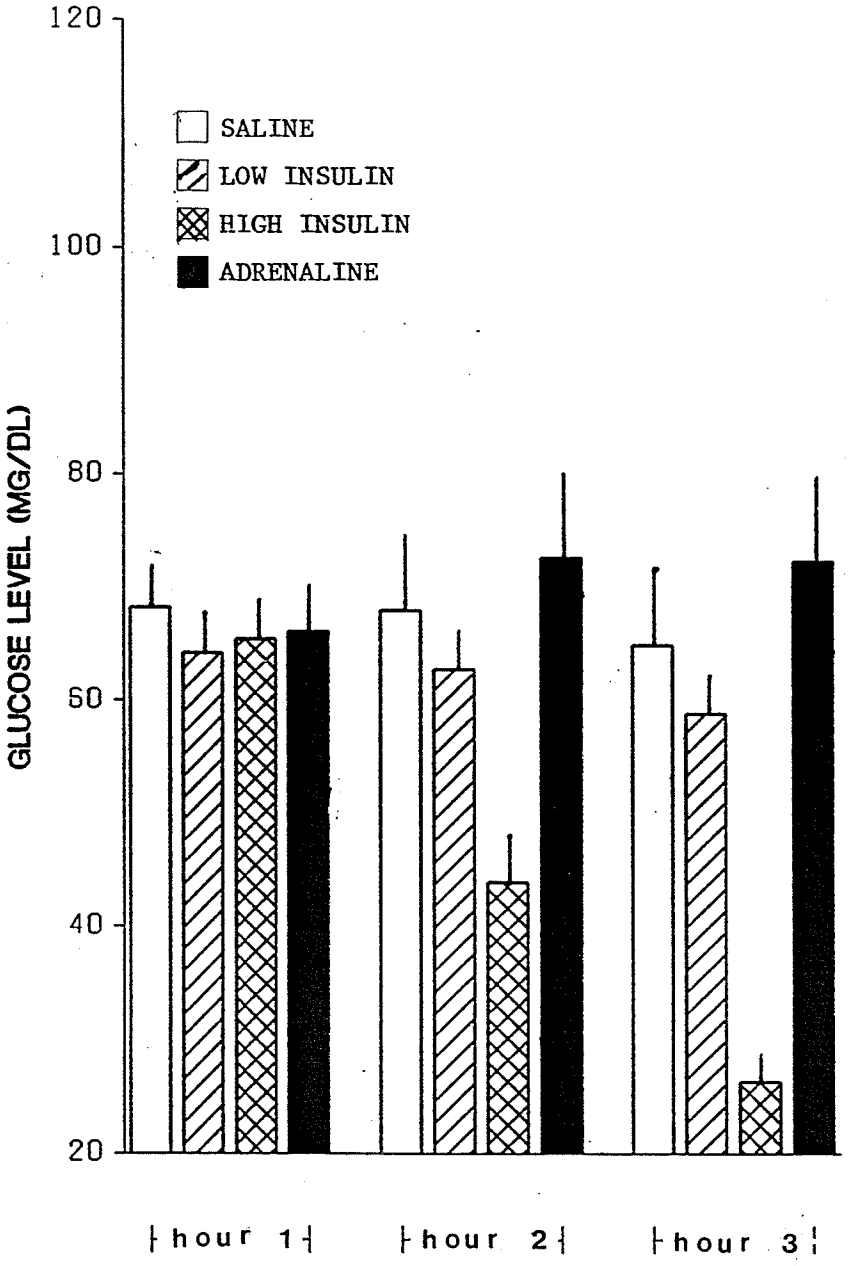

Figure 3. Hourly mean + SE of glucose levels during Saline, Low Insulin, High Insulin and Adrenaline infusions in lambs at 67 days of age.

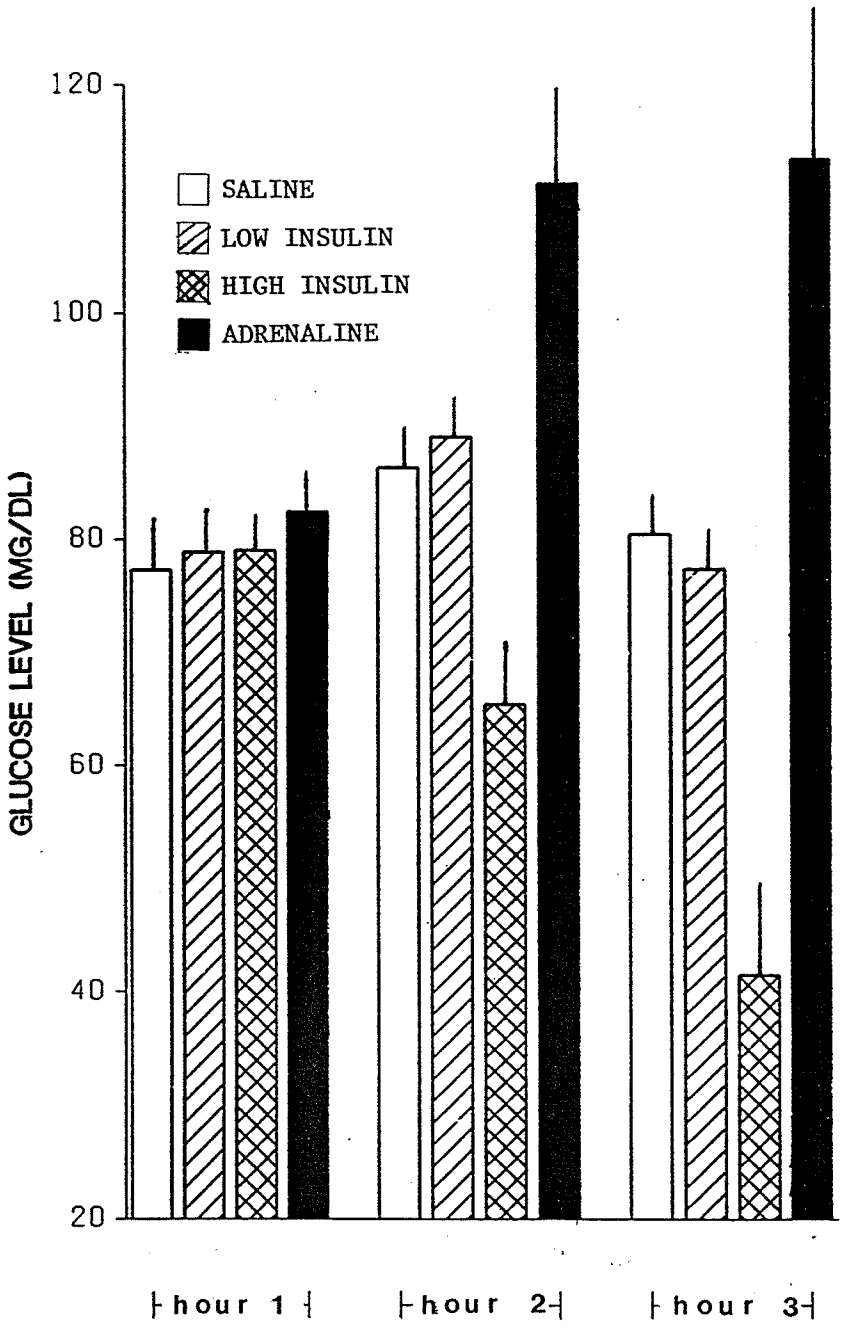

Figure 4. Hourly mean + SE of glucose levels during Saline, Low Insulin, High Insulin and Adrenaline infusions in lambs at 157 days of age.

TABLE 2. Least square means ± SE plasma glucose, serum insulin and serum growth hormone (GH) response+ to infusion.*

	Glucose (mg/dl)		Insulin (ng/ml)		GH (ng/ml)	
Age						
1	-11.2 ± 2.1		4.0 ± 0.7		4.9 ± 2.3	
2	-1.2 ± 2.0		3.7 ± 0.7		1.8 ± 2.1	
Treatment						
Sal	-0.1 ± 2.8		-0.1 ± 1.1		-0.9 ± 3.0	
LI	-4.6 ± 2.9		0.0 ± 1.1		0.6 ± 3.0	
HI	-38.4 ± 2.8		14.6 ± 1.0a		16.2 ± 3.3	
Adr	18.3 ± 2.9		0.8 ± 1.0		-2.7 ± 3.2	
Age x Treatment	**Age 1**	**Age 2**	**Age 1**	**Age 2**	**Age 1**	**Age 2**
Sal	-3.3	3.1	-0.2	0.1	-2.9	1.2
LI	-7.7	-1.5	-0.2	0.1	0.2	1.1
HI	-39.1a	-37.5a	15.7	13.6	27.4ab	5.0b
Adr	5.5b	31.0ab	0.5	1.0	-5.2	-0.3
SE‡	3.9		1.5		4.3	
Level of Significance (P=)						
Age	0.0011		0.7991		0.3285	
Trt	0.0001		0.0001		0.0006	
Per	0.6583		0.1100		0.9081	
ID(Per)	0.3230		0.5818		0.7089	
Day(Age)	0.2410		0.3782		0.0299	
Age x Trt	0.0259		0.7637		0.0146	

+ Hour 3 response for glucose and insulin; Hour 2 for GH.
* Main effect means were compared instead of Age X Treatment interaction means only if the latter is not significant.
‡ SE were as given except for Age 1 LI where SE for glucose, insulin and GH were 4.3, 1.6 and 4.6 respectively; SE for GH, Age 1 HI was 5.1 and SE for glucose, Age 1 Adr was 4.3.
a Mean differed from saline control ($P<0.05$).
b Within a treatment Age 2 differed from Age 1 ($P<0.05$).

4.3 INSULIN

Table 2 shows that the treatment effect on serum insulin response was significant ($P<0.05$) but there was no significant Age x Treatment interaction. Insulin level was increased by HI, but not changed by Sal, LI, nor Adr. There were no effects of Age, Per, Animal(Per) and Day(Age) on serum insulin.

Although the magnitude of the insulin response to HI was similar at both ages, the increase was gradual for about 50 minutes in age 1, but there was a sharp insulin rise within 10 minutes of infusion in age 2 (Figs. 5 and 6). Mean serum insulin levels for each hour of infusion at both ages are shown in Figures 7 and 8.

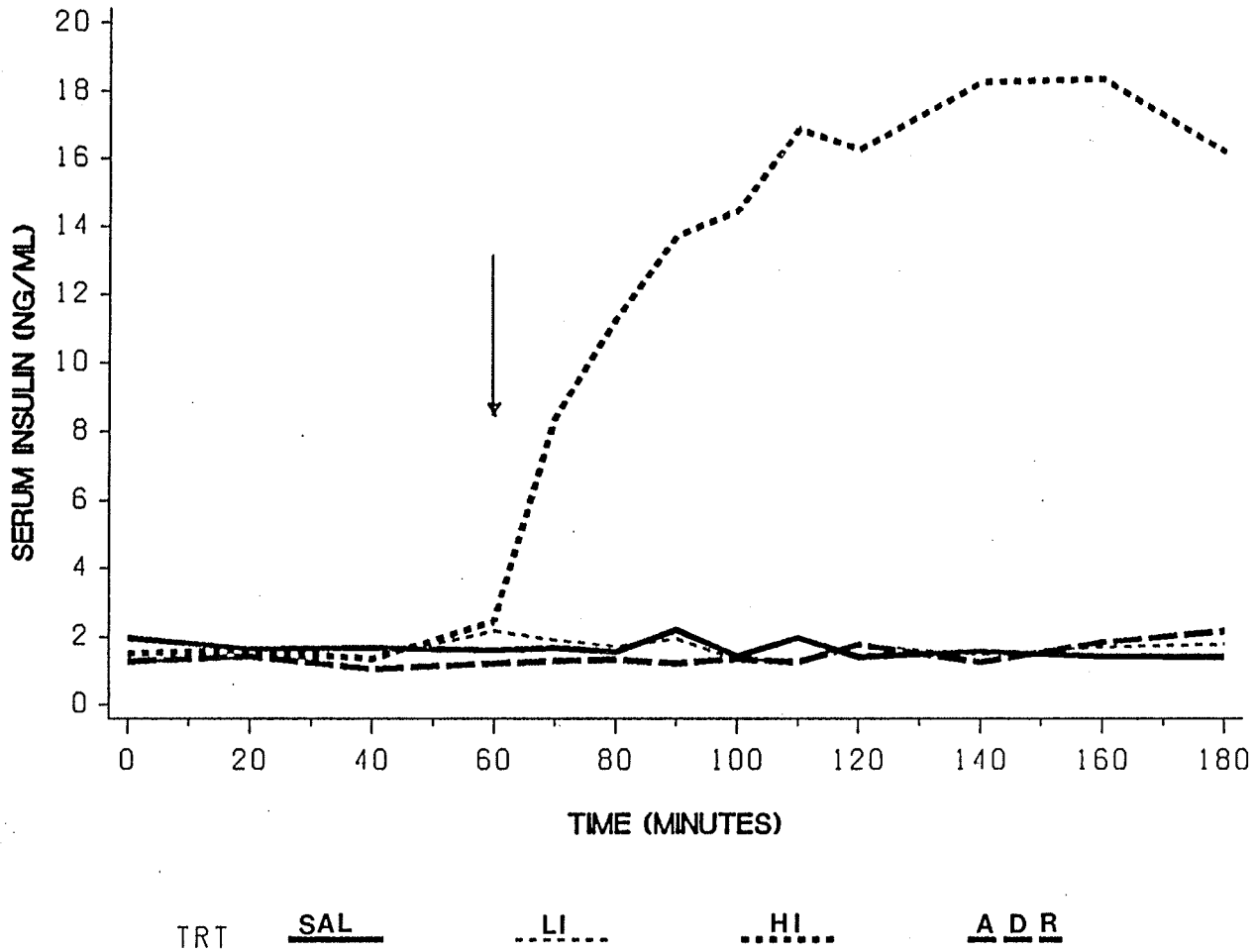

Figure 5. Mean insulin profile during infusion of Saline, Low Insulin, High Insulin and Adrenaline in ram lambs at 67 days of age. Arrow indicates start of treamtment.

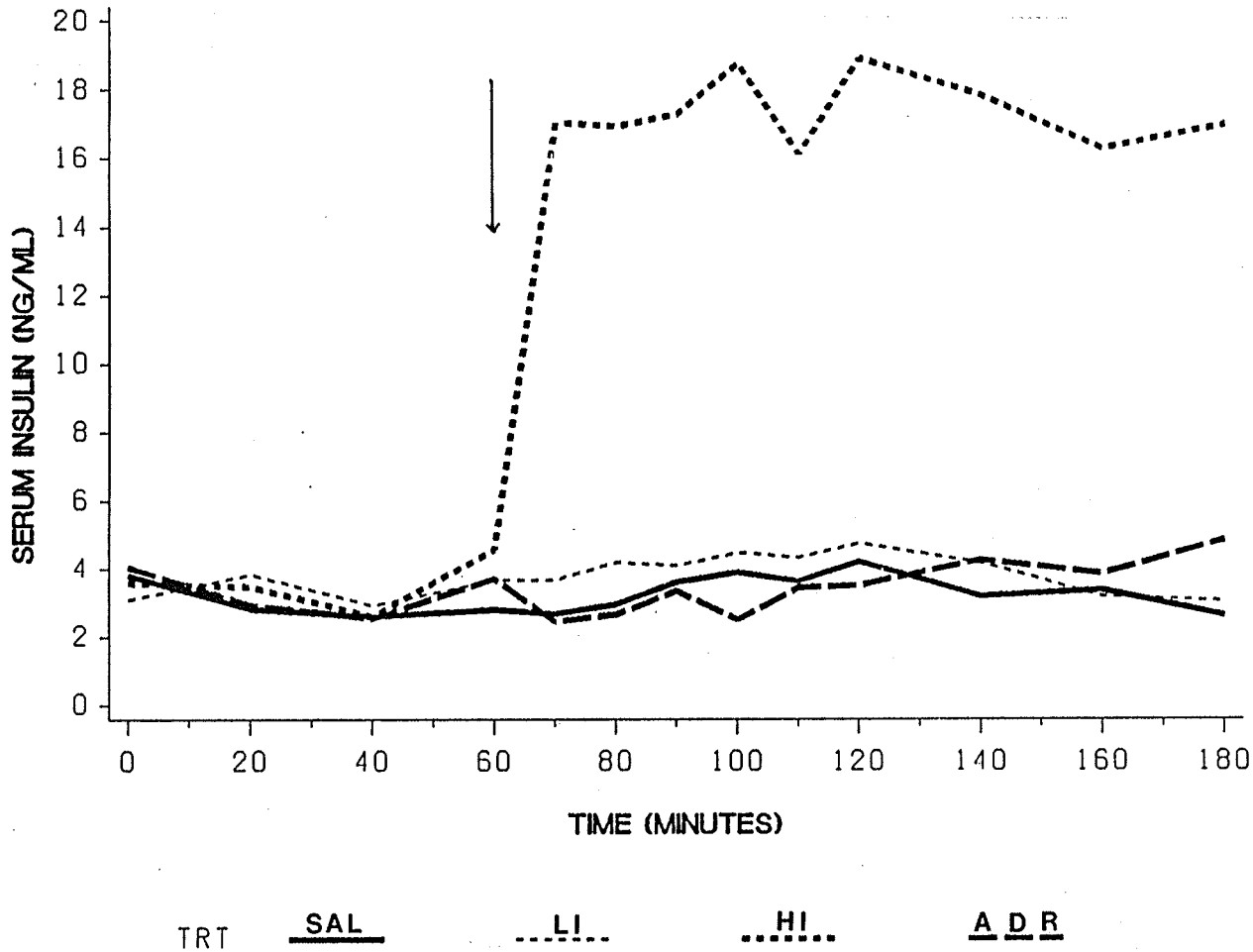

Figure 6. Mean insulin profile during infusion of Saline, Low Insulin, High Insulin and Adrenaline in ram lambs at 157 days of age. Arrow indicates start of treamtment.

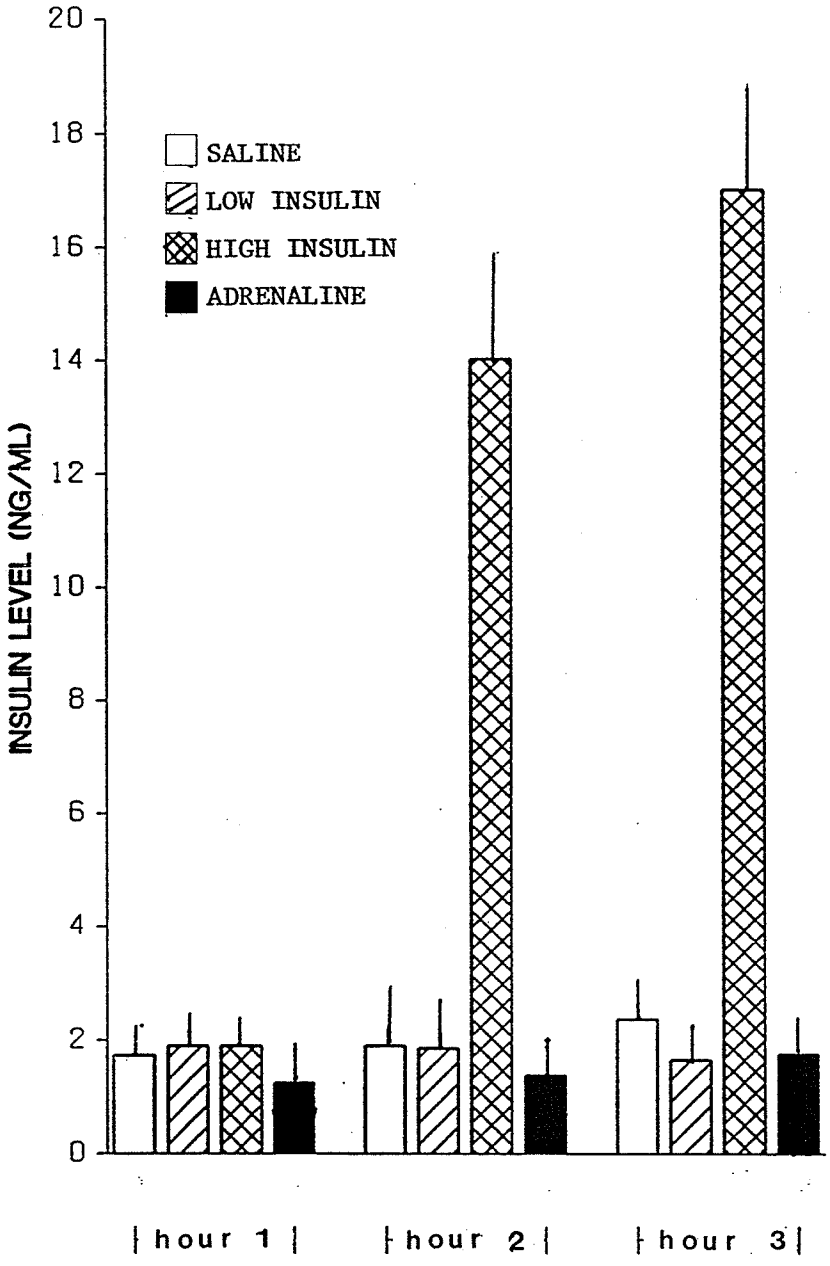

Figure 7. Hourly mean + SE of insulin levels during Saline, Low Insulin, High Insulin and Adrenaline infusions in lambs at 67 days of age.

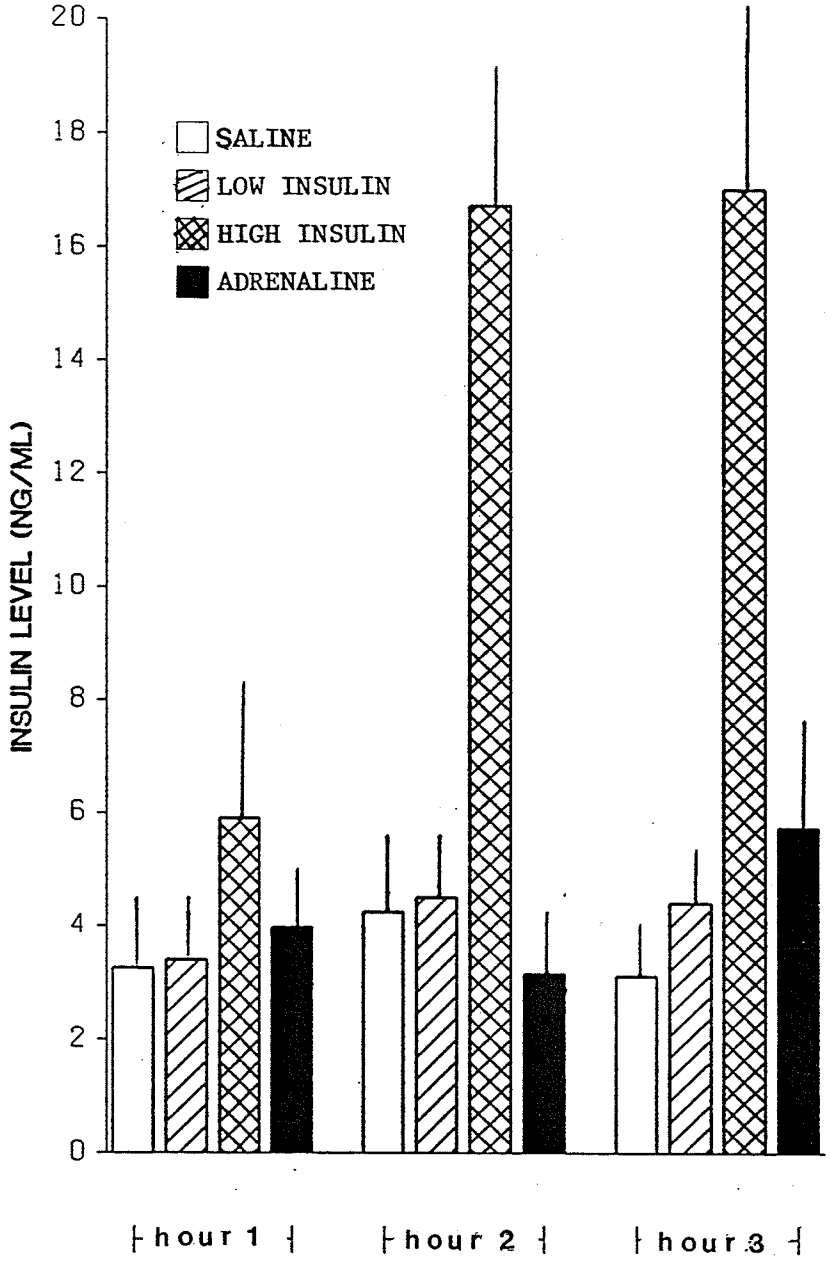

Figure 8. Hourly mean + SE of insulin levels during Saline, Low Insulin, High Insulin and Adrenaline infusions in lambs at 157 days of age.

4.4 GROWTH HORMONE (GH)

GH concentrations during infusion are shown in Figures 9 and 10. In general, GH concentration appeared higher and much more variable in age 1 than in age 2 (Figs. 9 and 10). The maximum GH response to HI in age 1 occurred at 20 minutes of infusion, after which there was a drop to pre-treatment levels within 80 minutes. In age 2 a slight rise in GH occurred at 20 minutes of insulin infusion and GH returned to baseline by 60 minutes of HI infusion. Sal, LI, and Adr had no effect on GH (Figs 9 and 10). Hourly means (Figs. 11 and 12) demonstrated that response was maximum in hour 2. Analysis of hour 2 respone (Table 2) showed a significant Age x Trt effect ($P<0.05$) in that HI caused a significant increase in GH only at age 1.

4.5 GLYCEROL

The plasma glycerol levels of Adr infused lambs was 28% greater ($P<0.05$) than that of salineinfused lambs (Table 3). There was no significant ($P>0.05$) effect of age or age x treatment on plasma glycerol level.

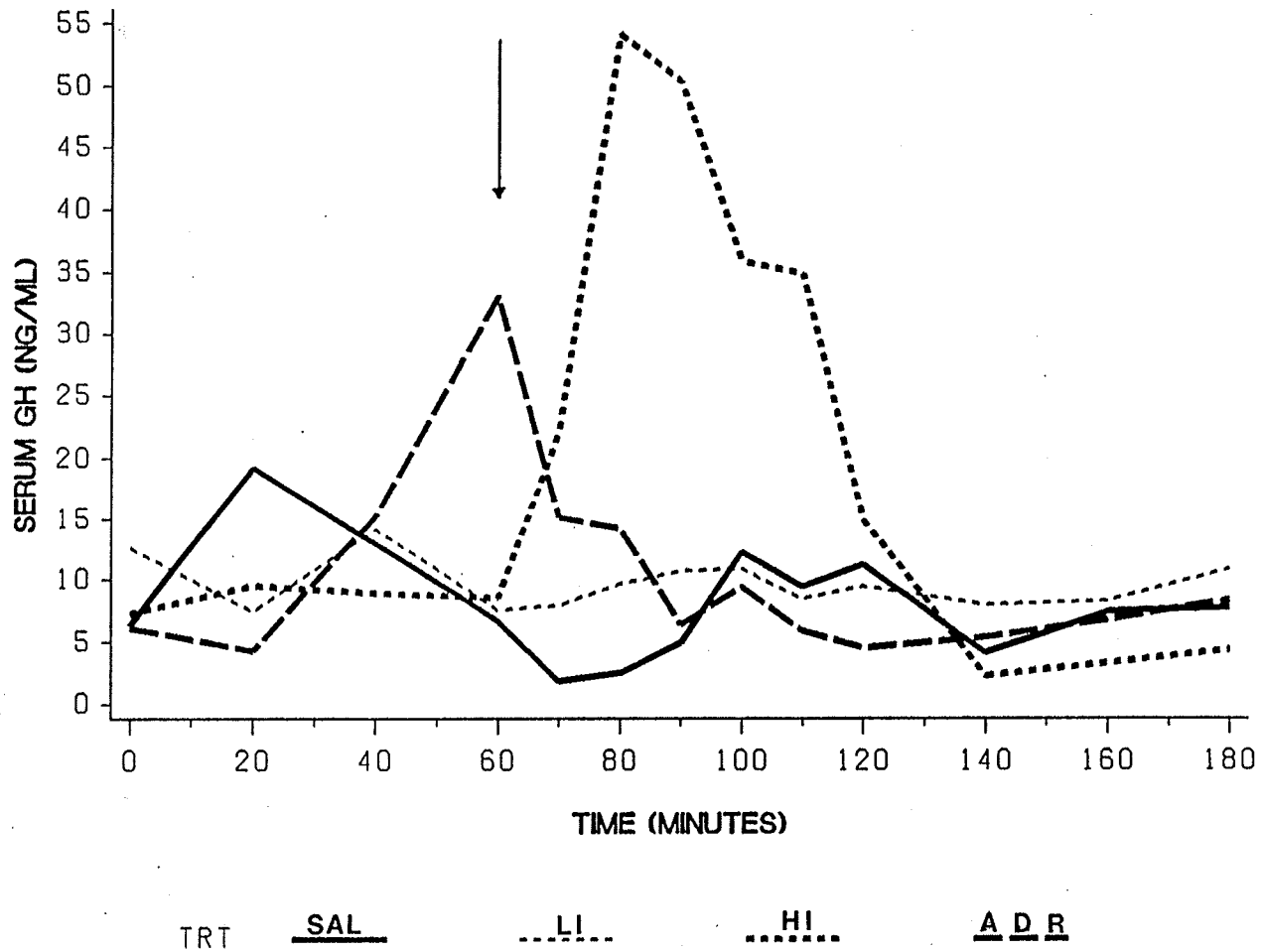

Figure 9. Mean GH profile during infusion of Saline, Low Insulin, High Insulin and Adrenaline in ram lambs at 67 days of age. Arrow indicates start of treatment.

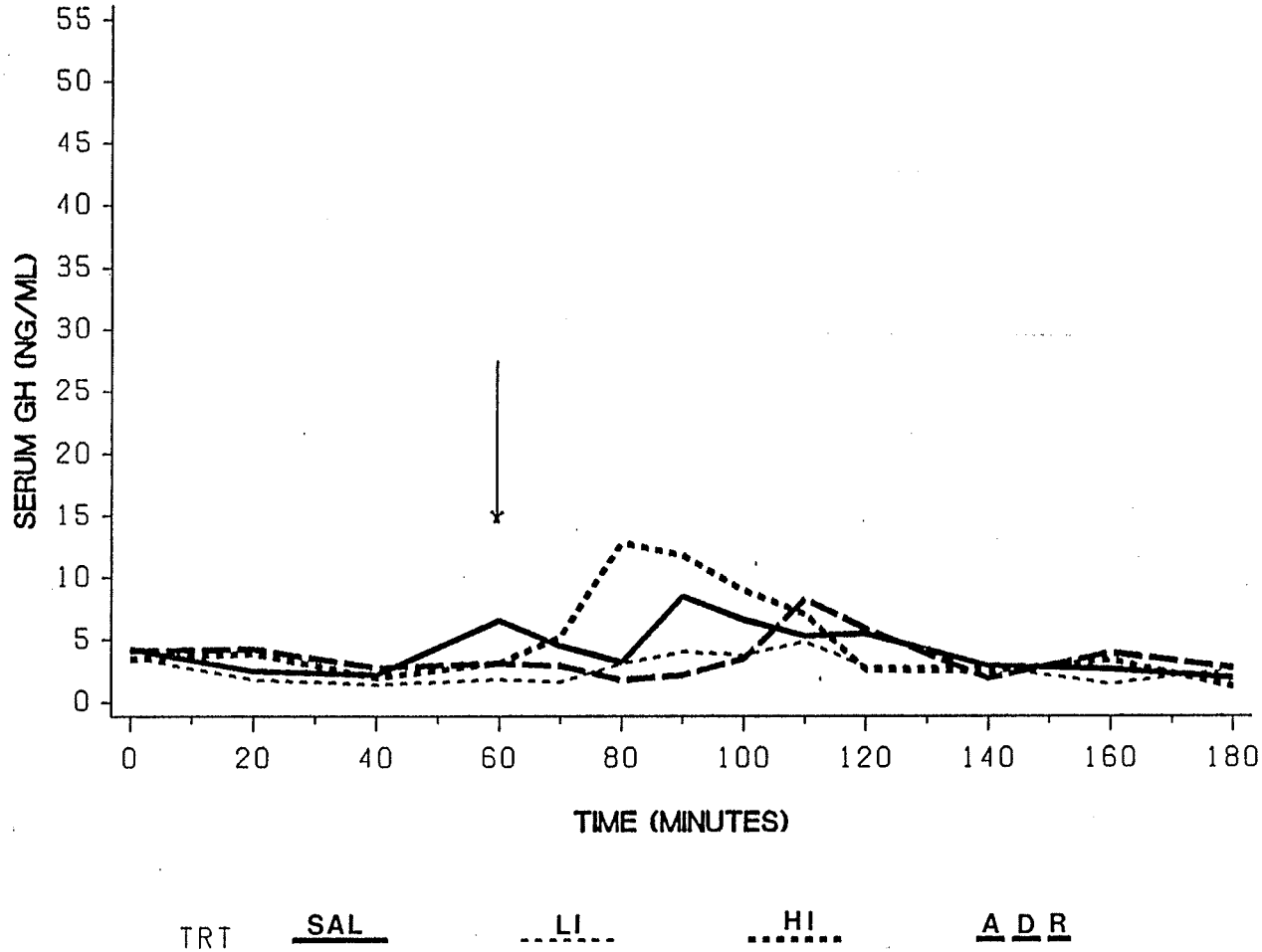

Figure 10. Mean GH profile during infusion of Saline, Low Insulin, High Insulin and Adrenaline in ram lambs at 157 days of age. Arrow indicates start of treatment.

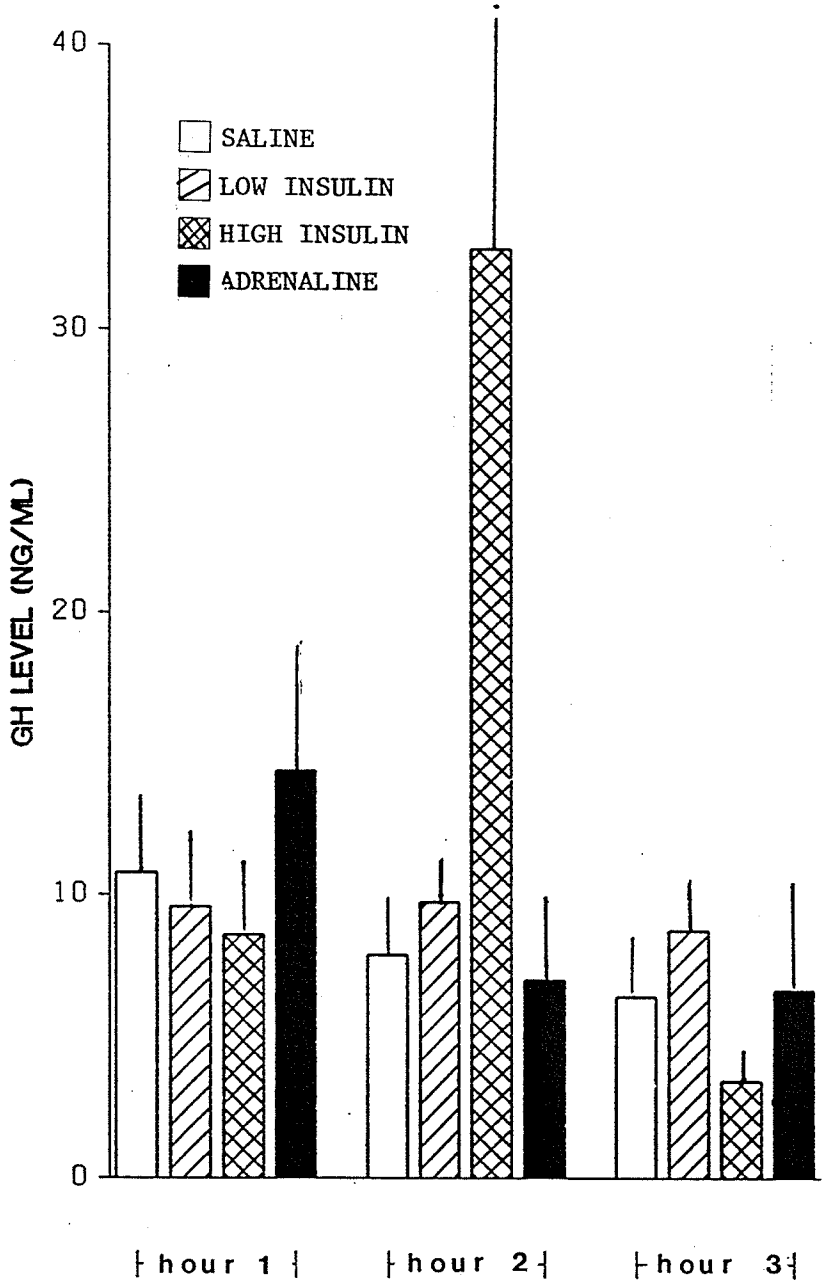

Figure 11. Hourly mean + SE of GH levels during Saline, Low Insulin, High Insulin and Adrenaline in lambs at 67 days of age.

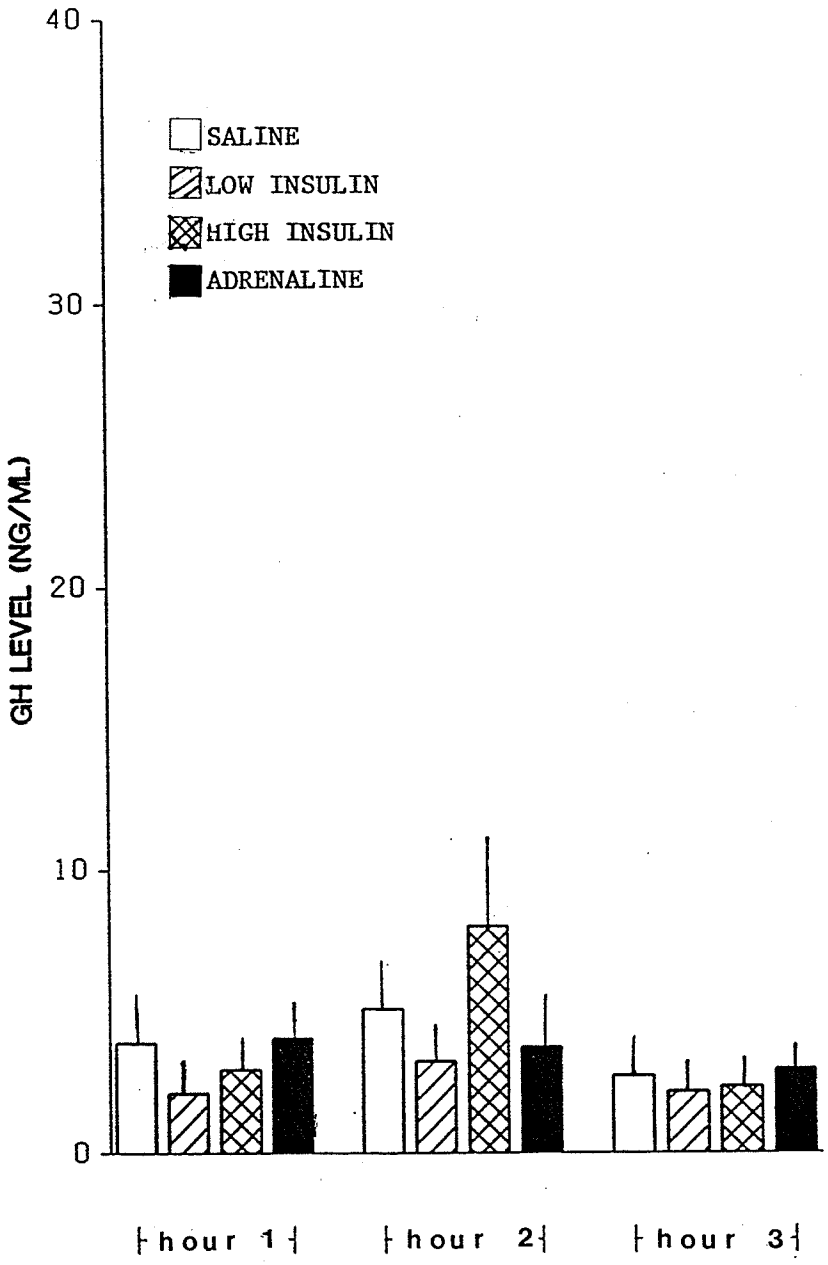

Figure 12. Hourly mean + SE of GH levels during Saline, Low Insulin, High Insulin and Adrenaline in lambs at 157 days of age.

TABLE 3. Least square means ± SE for plasma glycerol following adrenaline infusion*.

	Glycerol (mM)
Treatment	
Sal	0.18
Adr	0.23
SE	0.02
Levels of significance (P=)	
Age	0.8410
Trt	0.0338
Age x Trt	0.6570

* Samples collected during Hour 2 were used for analysis.

4.6 MONONUCLEAR LEUKOCYTE (MNL) INSULIN BINDING

4.6.1 Ram Lambs

Figure 13 shows SIB to MNL at different stages of growth. Both age and insulin incubation concentration significantly affected SIB ($P>0.05$) to MNL. SIB decreased with increasing insulin concentration. However, there was no interaction ($P<0.05$) between age and concentration. The main effect means for age are given in Table 4. Although SIB tended to increase with age the effect of age was not always consistent. There was no difference in SIB between 12 and 15 weeks of age, nor between 21 and 27 weeks of age, respectively (Table 4). Specific insulin binding (SIB) at 24 weeks of age was higher than at all other ages ($P<0.05$) (Table 4) but SIB at 18 weeks and older was higher than at 12 or 15 weeks of age.

4.6.2 Dairy Calves

Figure 14 shows SIB to MNL of heifer calves at different stages of growth. Both age and insulin incubation concentration significantly affected SIB ($P<0.05$) to MNL. SIB decreased with increasing insulin concentration ($P>0.05$). However, there was no interaction ($P<0.05$) between age and concentration. The main effect means for age are given in Table 5. Specific insulin binding appeared to follow a downward trend with increasing maturity of calves. However, SIB was not significantly depressed until 12 weeks of age (Table 5). Also, there were no differences in binding

from 4 to 16 weeks, and from 12 to 20 weeks of age. The main difference in SIB was, therefore, a decrease ($P<0.05$) from one week to 12 weeks of age.

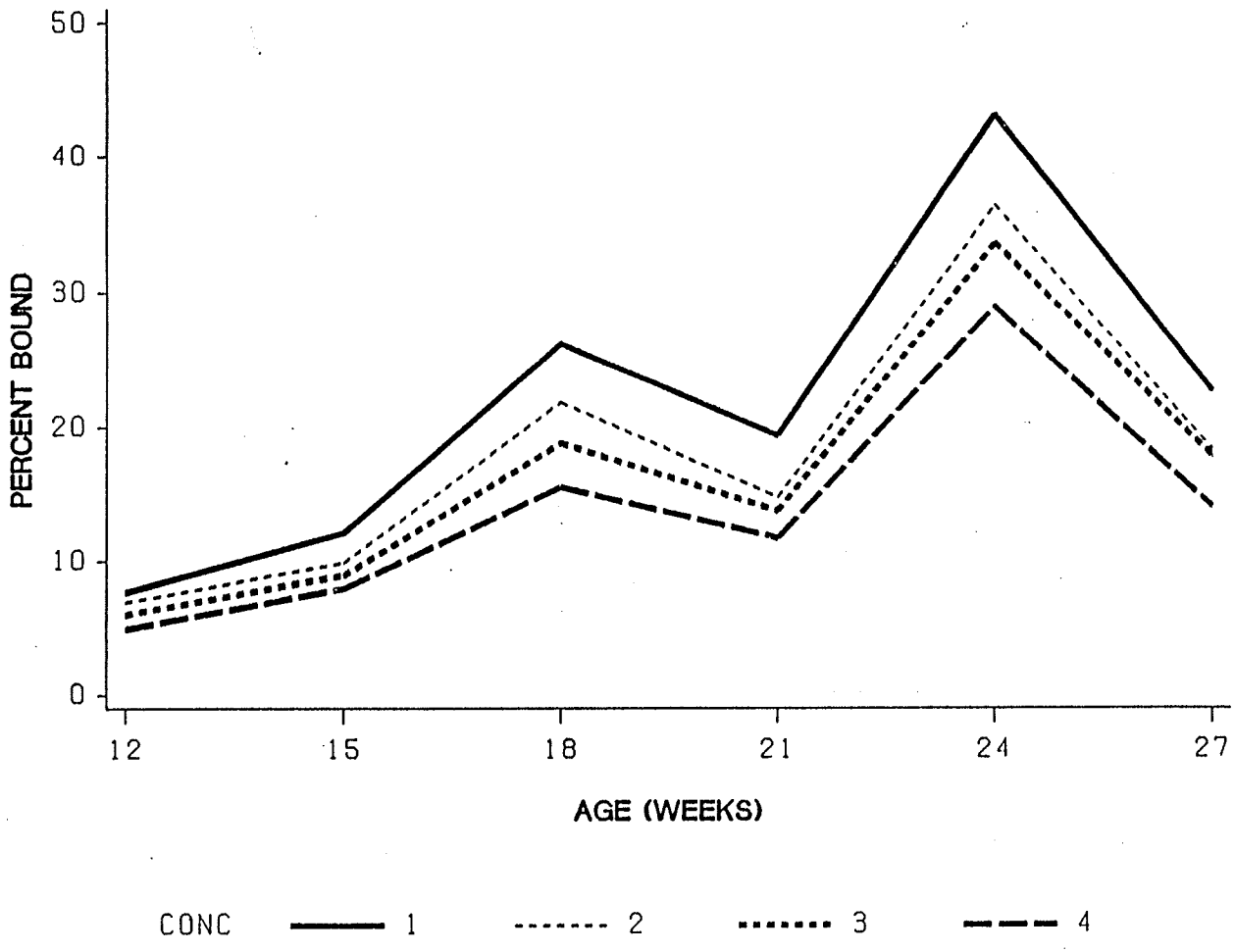

Figure 13. Effect of age of lamb on MNL specific insulin binding (% bound) at insulin concentrations of 1 (0.1 ng/ml), 2 (0.4 ng/ml), 3 (1.0 ng/ml) and 4 (5.0 ng/ml) in ram lambs. Each binding tube had 60x10 cells.

TABLE 4. Least square means ± SE for insulin bound to lamb MNL at different stages of growth.

Age (weeks)	Insulin Bound (%)
12	6.2 ± 1.9a
15	9.7 ± 1.8a
18	20.6 ± 1.8b
21	15.0 ± 1.8c
24	35.5 ± 1.8d
27	17.8 ± 1.9bc

a-d Means with different letters differed significantly ($P<0.05$).

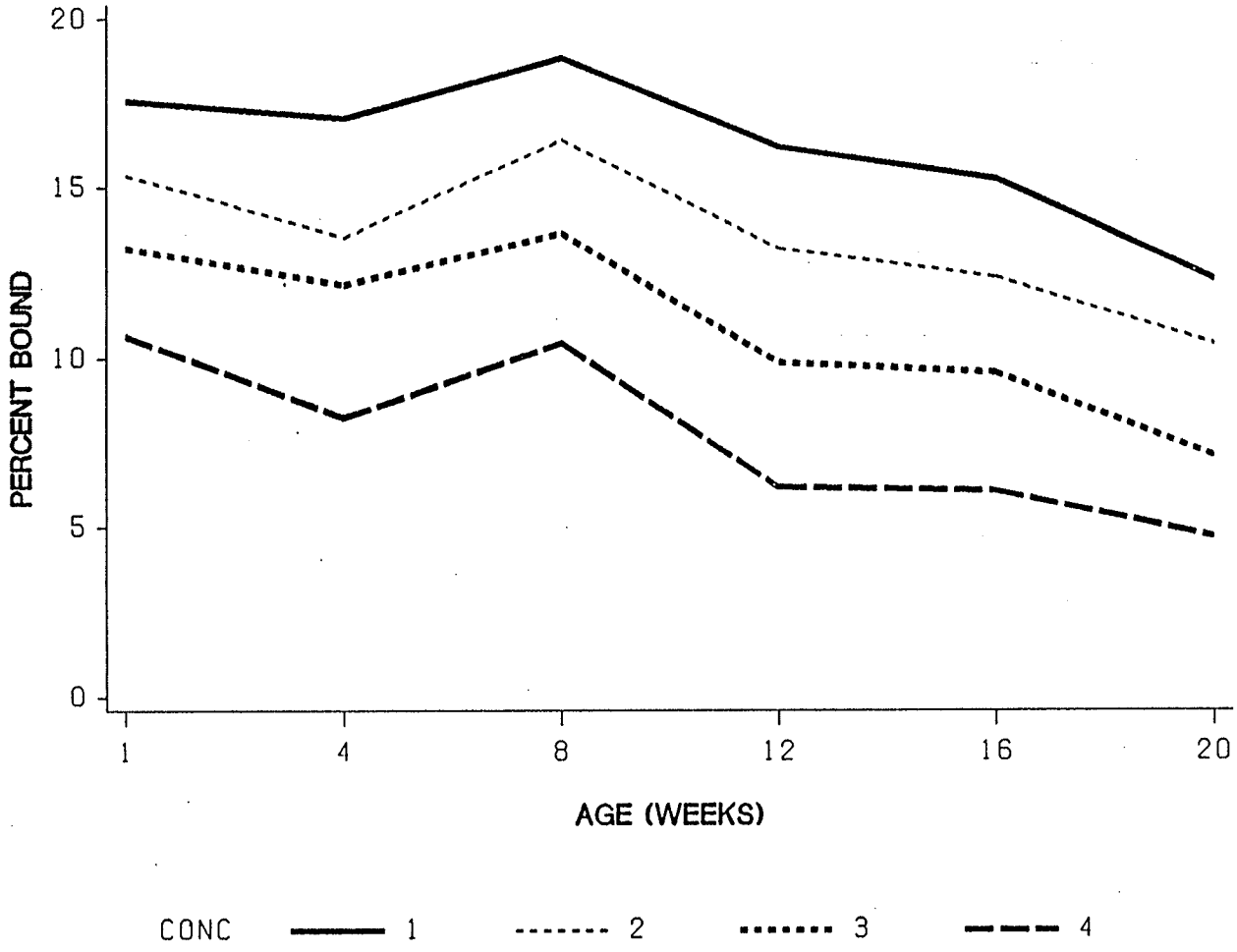

Figure 14. Effect of age of calf on MNL specific insulin binding (% bound) at insulin concentrations of 1 (0.1 ng/ml), 2 (0.4 ng/ml), 3 (1.0ng/ml) and 4 (5.0 ng/ml) in dairy calves. Each binding tube had 30x10 cells.

TABLE 5. Least square means ± SE for insulin bound to calf MNL at different stages of growth.

Age (weeks)	Insulin Bound (%)
1	14.2 ± 1.9a
4	12.8 ± 1.0ab
8	13.9 ± 1.0ab
12	11.4 ± 0.9bc
16	11.3 ± 0.9bc
20	9.2 ± 0.9c

a-d Means with different letters differed significantly ($P<0.05$).

Chapter V

DISCUSSION

The metabolic needs of an animal changes from birth to adulthood (Bergen 1974). Along with changes in metabolic needs are modifications in the levels and function of metabolic hormones like insulin and growth hormone (Turner 1978; Machlin et al. 1968) and plasma metabolites. Machlin et al. (1968) observed that there is a proportionate rise in plasma/serum insulin level with increasing age in growing pigs. The reverse was found to be true for growth hormone (GH) levels. Similar results have been found for fasted steers (Trenkle and Topel 1978), both large- and small-framed steers (Verde and Trenkle, 1987), growing lambs (Godden and Weekes 1981; Johnsson et al. 1985; Johns and Bergen 1976) and bulls (Martin et al. 1979).

In the current study, pretreatment concentrations of serum insulin and serum GH followed the same trend as reported previously with an increase in serum insulin and a decline in serum GH between 67 and 157 days of age. These changes would result in an increase in the ratio of insulin to GH with age, as was found by Kennedy et al. (1988b) for Outaouais Arcott intact male lambs (same breed and gender used in the present trial). Surprisingly, this could not be

said of control Suffolk lambs in the same study (Kennedy et al. 1988b). The present study can only associate higher insulin levels and lower GH (or a rising I:GH ratio) with advancing age. However, it has been reported that together with age, changes in insulin and GH levels could be attributed to increasing body weight and enhanced carcass adiposity. A negative correlation between insulin levels and the amount of muscle in the carcass has also been noted (Trenkle and Topel 1978).

The preceding observations about changes in insulin and GH levels with age support the assertion of Turner (1978) that growth and protein accretion prevails in the initial stages of development of every animal while conservation of nutrients takes precedence in adulthood. This suggests that the higher GH levels and lower insulin levels in age 1 (of the current study) may have acted to stimulate muscle and bone growth at the expense of fat accumulation. By age 2, the ratio would favour fat deposition over protein accretion. Searle et al. (1972) have noted that the extent of insulin stimulation of muscle and fat storage varies with increasing maturity.

Pretreatment glucose as well as serum insulin concentrations increased by age 2 in this study. These findings agree with the results of Kennnedy et al. (1988b). High insulin levels do not always correspond to low glucose concentration (Long et al. 1971). The doubling of serum

insulin with age in the present study was associated with a 20% increase in plasma glucose and suggests insulin insensitvity in the older lambs. High GH levels cause insulin insensitivity (Davidson 1987), but in the present study, GH decreased with age.

Insulin hypoglycemia is a result of both insulin stimulation of glucose removal from circulation and insulin inhibition of gluconeogenesis in the liver. Declines in insulin-stimulated glucose uptake with age have been reported in Sprague-Dawley rats in vivo (Reaven et al. 1983) and in muscle of growing lambs (in vitro) (Scharrer et al. 1977). Weekes et al. (1983) observed that adult sheep were less-sensitive and less-responsive than humans (Rizza et al. 1981) to the inhibitory effect of insulin on glucose production in the liver. Although the magnitude of hypoglycemia induced by supraphysiological insulin infusion, HI, was the same in both ages in the present study, hypoglycemic glucose concentrations levelled (indicating maximum response) within 60 minutes in age 1, but took longer (about 120 minutes) to occur in age 2. The delayed hypoglycemic response in older lambs reflects decreased sensitivity to insulin, increased rate of insulin clearance and/or enhanced insulin counter-regulatory activity with age. Heard and Henry (1969) observed that dependance on insulin develops with age in normal pigs and dogs, and once achieved, glucose uptake is modulated by

sensitivity to insulin. Cacciari et al. (1978) also noted that in humans the young are more sensitive to insulin-induced glucose disposal than adults. With advancing maturity energy deposition takes precedence over protein accretion, and perhaps sensitivity to insulin is reduced (Turner 1978). Whole animal response needs to be differentiated from tissue responses since there is a heterogeniety of tissue responses to insulin (Brady et al. 1981). An increase in body fat may change insulin sensitivity with age (Bergen 1974). Weekes (1986) noted that tissue responsiveness and glucose uptake and metabolism were only slightly changed with increasing maturity. In this regard, the observation by Yki-Jarvinen and Koivisto (1983) that insulin-stimulated glucose metabolism in vivo is enhanced by high muscle content of the body, but declines with increase in fat content is relevant. Hence, reduced sensitivity may be due to a reduction in responsive tissue (muscle) as fat content increased with age. This contention, together with the previous discussions which point to the fact that muscle growth predominates adiposity at a younger age, suggests that body composition changes may underlie the age effect of hypoglycemic response to HI.

As mentioned above the slow decline in glucose in age 2 may be due to the influence of active insulin counter-regulatory effects of age 2; particularly effects of adrenaline. Adrenaline is thought to be a major insulin

counter-regulatory hormone during insulin-induced hypoglycemia (Oda et al. 1988). A curious observation in the present study was that adrenaline caused hyperglycemia only in age 2. The present results indicate that the lambs became more sensitive to the glucoregulatory effects of adrenaline as they matured. Adrenaline causes hyperglycemia either by directly stimulating glycogenolysis and gluconeogenesis (Ganong 1981) or indirectly by causing glucagon release (Oda et al. 1988). Graham and Phillips (1981) have also shown variation in lamb hyperglycemic response to the present dose of adrenaline; plasma glucose response to adrenaline was greater in cold- than warm-exposed sheep and was more depressed by fasting in the former. The present results suggest that adrenaline may not play an insulin counter-regulatory role in younger ruminants. Should this be the case, then the relatively quick achievement of HI induced hypoglycemia in the young rams may have resulted from the abscence of adrenaline counter-regulatory effects.

The lack of glucose response to LI in the present trial, even at age 2, is noteworthy, considering the fact that Weekes et al. (1983) used a similar dose to obtain half-maximum hypoglycemic response in adult sheep. However, the clamp technique used by Weekes et al. (1983) avoids insulin counter-regulatory effects because glucose is clamped and therefore hypoglycemia does not occur. Perhaps a low

hypoglycemic effect of LI in the present study was masked by insulin counter-regulatory mechanisms. The sensitivity of young lambs to low insulin doses has not been previously studied.

Adrenaline increased plasma glycerol levels in the current study, but the effect was not age-dependent. Thus, a lipolytic response to adrenaline appeared to be fully functional at an early age in lambs. That the hyperglycemic but not the lipolytic response to adrenaline increased with age suggests that the age effect is something other than a decrease in rate of adrenaline clearance from the blood. Increases in glycerol release in response to both adrenaline and noradrenaline have been reported in dairy cows (Jaster and Wegner 1981) and Karakul ewes (Gooden et al. 1986) respectively. Bassett (1978) has suggested that secretion of insulin represents a more sensitive measure of adrenaline action in sheep than peripheral glucose concentrations. This theory is not supported by the present study where adrenaline had a marked hyperglycemic but not hyperinsulinemic effect in lambs at age 2.

The large GH response to insulin observed at age 1 may be related to the generally elevated activity of the somatotrophs in young animals. Previous studies suggest that GH promotes low insulin sensitivity/responsiveness. In the present study, however, young lambs showed a relatively rapid hypoglycemic response to insulin even in the presence

of a very large GH response. Thus, there was no evidence that the GH response was glucose-sparring (Hart et al. 1985).

Another observation from the current study is that, during HI infusion, serum insulin peaked sooner in the older lambs but the hypoglycemic response took longer to reach maximum. This further suggests that the older lambs were less sensitive to insulin or had a more functional adrenaline counter-regulatory mechanism. A reduced sensitivity to insulin in older lambs may be an age-effect related to circulating insulin levels. The rise in peripheral insulin in ageing mammals is a direct consequence of fat deposition or enhanced level of adiposity (Bergen 1974; McNiven 1984). In other words, excess fat deposition is associated with insulin resistance. The older lambs in the present study were not necessarily obese, but their increased body fat content might have muted their response to insulin, due to a lowered sensitivity of the fat cells. Smith et al. (1979) have suggested that large amounts of fat from lipolytically active fat cells possibly reach the liver, impair hepatic insulin uptake, and culminate in the observed hyperinsulinemia associated with ageing and obesity. This is not supported by the present results where similar insulin levels were found in lambs at 2 ages receiving HI.

The observation that red and white blood cells mirror the major target tissues for insulin receptor characteristics (Beck-Nielsen 1980; Kappy and Plotnick 1980) led to several blood cell insulin receptor studies (Ector et al 1983; Kappy 1983; Khan et al. 1986; Kennedy et al. 1987 and Kennedy et al. 1988b). Insulin binding to ruminant mononuclear leukocytes (MNL) is of particular interest since it has been shown that erythroctes of adult cattle and sheep bind no insulin (Kappy 1983; Kappy et al. 1981). Mononuclear leukocyte insulin binding at tracer insulin concentration (maximum binding, at $24x10^6$ cells per binding tube) was 7% and 16% for 21 week old Outaouis Arcott and Suffolk lambs respectively (Kennedy et al. 1988b). This binding for Outaouais Arcott was similar to that in the present study, in which maximum binding was 20% for 21-week-old ram lambs (at $60x10^6$ cells per binding tube). Belluk (1988) reported 16.4% maximum binding ($60x10^6$ cells per binding tube) for Suffolk lambs at 22 weeks of age. An unusually high SIB in the present study for lambs at 24 weeks of age prompted an extension of the study to 27 weeks to confirm the rise in SIB with age. Specific insulin binding (SIB) at 27 was lower than SIB at 24, similar to SIB at 18 but higher than SIB at 12 and/or 15 weeks of age. These results indicate that the rise in SIB followed a linear trend with age up to 24 weeks of age. The reason for the decline in SIB at 27 (or the rise at 24) weeks is unknown and there are no other studies of the effect of age on MNL insulin binding in ruminants for

comparison. In retrospect, extension of the study to 30 weeks might have helped to explain whether SIB at 24 weeks was atypical or whether SIB in sheep followed a rhythmic pattern at the stages of growth studied. In general, there was an increase in SIB with age after the 15th week. But in human studies, monocyte insulin binding was found to decrease with age (Thorsson and Hintz, 1977; Neufield et al. 1978). The human studies compared infants and adults. Perhaps the wide age gap was a major contributing factor to the observed decrease in insulin binding with age. The maximum age difference in the present study (15 weeks) may not be long enough for major changes in the physiology of the lambs. Indeed, Ector et al. (1983) noted that although insulin binding to erythrocytes in gilts decreased from birth (4-6 days), to weaning (34-44 days), and then in the growing phase (80-100 days), there was no difference between the growth and finishing phases.

Serum insulin level at 23 weeks (age 2) was greater than at 10 weeks (age 1), while the reverse was true for GH in the ram lambs. Thus, increased SIB was associated with elevated insulin levels. These results conflict with the general finding that circulating insulin levels down-regulate insulin receptor numbers (Olefsky 1976). The higher insulin level observed at 23 weeks of age in this study in itself may not constitute a hyperinsulineamia of enough magnitude to down-regulate insulin receptors

significantly. Another published exception is the results of Neufield et al. (1978) who found that infants of hyperinsulinemic diabetic mothers exhibited a high insulin binding affinity and a high number of insulin receptors even under conditions of high circulating insulin levels. Increase in SIB, however, did not alter maximum response to insulin in the lambs studied but delayed the response, suggesting a decline in insulin sensitivity. Kennedy et al. (1988b) have pointed out that the factors influencing insulin receptors in ruminants, particularly the complex interrelationships between circulating hormones like insulin and GH, need further study.

According to Kennedy et al. (1987), maximum insulin binding in dairy cows dropped from 17.9% to 8.4% by the 12th week of lactation. In beef steers and heifers, binding of 7.6% and 6.1%, respectively, were reported (Kennedy et al. 1988a). The number of cells per binding tube in these reports was $60x10^6$. The results of the dairy calf trial in the present study support the report of Kennedy et al. (1987) of MNL insulin binding to dairy cows and to older ruminants in general (Kennedy et al. 1988a; Kennedy et al. 1988b). For the dairy calves, SIB at tracer insulin concentrations was 17% in the first week of birth, and had dropped to 14% on the 20th week. From the above, it appears that insulin binding declined with age in cattle. This decline in insulin binding with age agrees with the results

of the human monocyte studies reported above (Thorsson and Hintz, 1977; Neufield et al. 1978), as well as the erythrocyte study in gilts (Ector et al. 1983). There were no differences in SIB between 12 to 20 weeks in the dairy calf study, just as Ector et al. (1983) reported no difference in erythrocyte insulin binding between growing (80-100 days) and finishing phases in gilts. However, SIB increased from 12 to 21 weeks in the ram lambs. This apparent difference in binding behaviour between the rams and the calves at a similar chronological age may not be unusual. Given the differences in size and rate of development between sheep and cattle, perhaps chronological age alone cannot form a basis for comparing the ram lambs and dairy calves. A decline in SIB is most common in the trials (present calf trial included) where SIB is studied from birth. Studies of such nature are lacking in lambs. The calves in the present trial would have been physiologically younger than the lambs. However, no major shifts in binding occurred at least a month after weaning. Probably, the shift in binding was related to factors other than weaning. Besides, the growth-promoting effects of insulin (eg. stimulation of DNA) and the gluco-regulatory effects are not only influenced by age, but by other factors including body mass (fat or muscle content) as well as circulating hormones. Above all, both breed (Kennedy et al. 1988b) and gender (Kennedy et al. 1988a) have been shown to affect SIB to MNL in ruminants.

Chapter VI

SUMMARY AND CONCLUSIONS

Serum insulin and plasma glucose increased while serum GH decreased with age in lambs. Compared to the young, older sheep had delayed insulin-induced hypoglycemia and had a low GH response to insulin. The present results suggest that the glucose counter-regulatory effects of adrenaline may increase dramatically with age in lambs and that this underlies a slow hypoglycemic response to high insulin doses. The hyperglycemic but not lipolytic response to adrenaline increased with age in the lambs.

MNL insulin binding of young ruminants is age-dependent. Specific insulin binding to dairy calf MNL declined at 12 weeks of age while it increased after 18 weeks of age in lambs. Firm conclusions cannot be made about the lambs since the binding at 24 weeks appeared unusually high. The duration of the study did not permit further verification of this rise with age. Any interpretation of the binding data has to consider the fact that, beside breed and gender, the effects of age may be mediated through variables like body composition and levels of circulating hormones. Studies involving age should be interpreted in a broader perspective to include both chronological and physiological age since the latter is equally if not more important.

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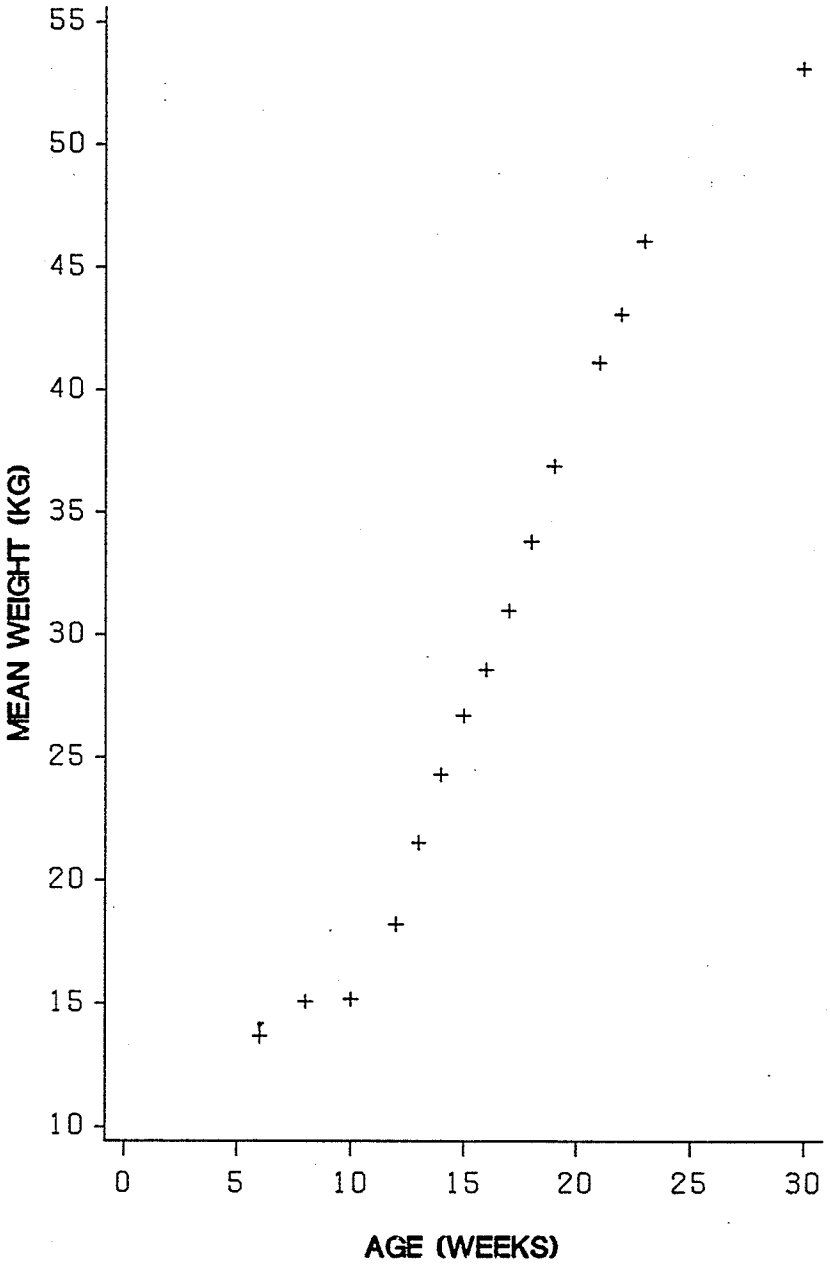
APPENDICES

Appendix 1
Profile of Mean Weekly Lamb Weights



A.2 APPENDIX 2

A.2.1 Separation of WBC and return of blood cells

The RBC portion of the blood together with about two-thirds of the plasma contained therein was returned to their respective donors blood-sampled for MNL determination. This was to reduce to a minimum any physiological stress due to blood loss.

All materials and equipment used for blood sampling, processing and re-infusion were sterile. Glass containers and pipettes were siliconized to prevent the WBC sticking to their sides.

Whole blood was sampled by venipuncture into a 250ml plastic bottle containing 10ml of sterile anticoagulant (4.5% diK-EDTA, pH 7.4). Aliquots of 45ml each were poured into pre-labelled 50ml plastic tubes. Care was taken not to touch the tip of the sterilized tubes. The 250ml plastic bottles were covered at all times except when blood was being transferred into or out of them. Where necessary, the 45 ml aliquots were balanced with sterile saline (0.9% NaCL) prior to centrifugation at 4C, 2000rpm in a centrifuge previously wiped with 95% ethyl alcohol.

Approximately two thirds of the plasma was transferred into the original 250ml bottle using a macrotranspipettor with sterile tips. Siliconized pipettes were used to aspirate the WBC (seen as a 'buffy coat' - a whitish opaque

middle layer) together with traces of plasma and RBC, prior to returninig the RBC to the 250 ml bottle. The 50 ml bottles were rinsed with sterile saline and the wash also placed in the 250 ml bottles. The WBC was covered and stored at 4C for processing the same day.

A.2.1.1 Re-infusion

To facilitate cell re-infusion, obturator catheters (Becton and Dixon Co., St. Louis. Mo. 16G4; cat. no. 6759) were inserted into the jugular vein of each lamb and properly taped with "vet wrap" tape.

The covered 250ml bottles and their contents were allowed to stand at room temperature for 30-60min. The bottles were then put in a water bath at 39 ± 3C. After allowing for equilibrium in temperature, each bottle was gently inverted a few times and cells were returned to the lamb by withdrawing portions of the blood into a sterile 35cc syringe and injecting the cells into the donor-lambs via the catheter. The catheter was periodically flushed with sterile saline when necessary.

Appendix 3
Table 1. Composition of Calf Diets

Ingredients	Diet Calf Starter (2 weeks) %	Calf Grower (6 weeks) %	Dairy Ration (3-4months) %
Barley	50.0	53.4	40.1
Oats	16.0	-	49.6
Ground Alfalfa Hay	15.0	16.0	-
Canola Meal	9.7	7.0	-
Molasses	3.0	2.0	-
Tallow	3.0	2.0	-
Rock Phosphate	1.5	-	-
Urea	0.8	0.8	0.5
Cobalt Iodized Salt	0.5	-	0.4
Dairy Herd Premix*	0.5	-	-
Beet Pulp	-	16.0	-
Fishmeal (4% Protein)	-	0.6	-
Bio Phosphate	-	1.0	1.0
Dairy Mineral Mix*	-	0.8	0.6
Dairy Vitamin Mix*	-	0.4	0.3
Soybean Meal (48%)	-	-	5.0
Limestone	-	-	1.2
Maggox (54% Mg)	-	-	0.4

*

Dairy Mineral Mix		%
Copper Sulphate	(25% Cu)	0.4
Selenium	(200 mg/kg)	11.0
Zinc Oxide	(72% Zn)	0.6
Magnesium Oxide	(54% Mg)	10.0
Manganese Oxide	(60% Mn)	0.6
Potassium Chloride	(50.54% K)	23.5
Cobalt Iodized Salt	(22.8% Na, 35.6% Cl)	53.9

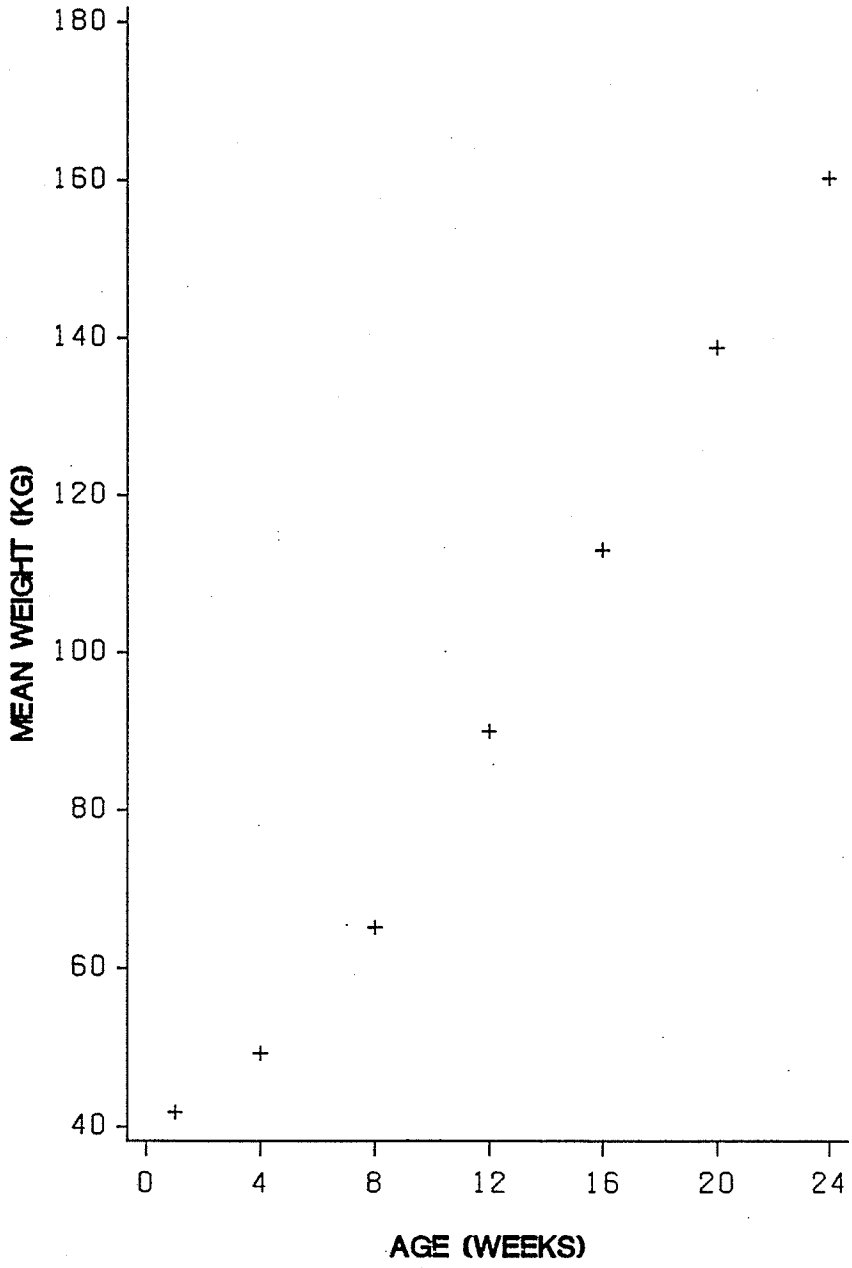
Dairy Herd Premix (Composition in 2.5 Kg)

Vitamin A	2 200 000 IU
Vitamin D	1 100 000 IU
Vitamin E	5 500 IU
Zinc Oxide	27.500 g
Manganese Oxide	27.500 g
Potassium Iodide	0.625 g
Cobalt Chloride	0.202 g
Magnesium Oxide	999.000 g

Dairy Vitamin Mix (Composition in 20 Kg)

Vitamin A (500 000 IU/g)	70 g
Vitamin D (500 000 IU/g)	20 g
Vitamin E (20 000 IU/g)	3 g
Wheat Middlings	19907 g

Appendix 4
Profile of Mean Monthly Calf Weights



APPENDIX 5

GLUCOSE RAW DATA FOR HORMONE SENSITIVITY TEST

ID	TRT	AGE	GLUCOSE (10xmg/dl) 0	20	40	60	70	80	90	100	120	140	160	180 min
1	1	0610	0613 .	0601	0564	0550	0551	0570	0572	0600	0607	0579	0574	
1	2	0758	0760	0780	0765	0767	0764	0785	0790	0761	0746	0790	0756	.
2	1	0806	0937 .	0874	0883	0990	0947	0936	1000	0917	0864	0883	0800	
2	2	0718	0725	0739	0735	0770	0880	0867	0881	0920	0852	0900	0872	0908
3	1	0677	0728	0730	0753	0725	0748	0735	0728	0702	0736	0717	0740	0733
3	2	0708	0710	0740	0970	0940	0878	0855	0930	0865	0840	0870	0855	0845
4	1	0858	0920	0887	0858	0896	0839	0930	0814	0882	0925	0839	0858	0776
4	2	0736	0798	0810	0800	0836	0866	0898	0769	0887	0899	0875	0820	.
5	1	0766	0771	0752	0697	0770	0813	0792	0777	0751	0746	.	0785	0762
5	2	0575	0585	0590	0600	0590	0610	0595	0700	0570	0520	0620	0635	0600
6	1	0480	0533	0480	0530	0473	0480	0470	0475	0480	0460	0458	0438	0408
6	2	0779	1016 .	1179	1211	1153	1211	1158	1095	1053	0971	0876	0779	
7	1	0580	0675	0625	0595	0570	0570	0580	0560	0575	0565	0565	0565	0520
7	2	0770	0804	0844	1120	1214	1207	0945	1084	1022	1112	0939	0858	0796
8	1	0510	0505	0515	0475	0505	0493	0510	0460	0500	0495	0445	0445	0433
8	2	0635	0657	0639	0699	0733	0566	0695	0707	0729	0712	0697	0712	0711
1	2	0509	0528	0459	0497	0501	0511	0461	.	.	.	.	.	.
1	2	0703	0723	0744	0790	0836	0771	0873	0904	0841	0831	0804	0714	0726
2	1	0650	0660	0650	0670	0670	0650	0600	0590	0595	0565	0605	0575	.
2	2	0834	0860	0894	0922	0950	0925	0940	0930	0925	0887	0912	0900	0861
3	1	0695	0710	0773	0810	0855	0815	0835	0813	0773	0773	0733	0683	0688
3	2	0728	0738	0738	0776	0764	0757	0771	0770	0746	0718	0740	0763	0714
4	1	0707	0705	0693	0705	0623	0600	0623	0642	0623	0579	0523	0538	0609
4	2	0739	0784 .	1012	0994	0936	1222	0922	0866	0843	0822	0791	0757	
5	1	0652	0677	0638	0666	0629	0617	0632	0604	0645	0613	0555	0523	0499
5	2	0609	0625	0650 .	0970	0940	0865	0826	0755	0740	0690	0665	0560	
6	1	0694	0713	0710	0700	0670	0670	0665	0705	0675	0694	0710	.	0590
6	2	0791	0532	0715	0635	0795	0358	0715	0560	0758	0744	0739	0664	0438
7	1	0480	0497	0507	0502	0507	0535	0543	0546	0495	0503	0497	0468	0468
7	2	0855	0877	0895	1542	1585	0840	1425	1361	1342	1207	1076	0920	0816
8	1	0673	0706	0621	0673	0678	0678	0687	0668	0638	0631	0631	0607	0598
8	2	0736	0746	0787	0790	0814	0836	0818	0851	0845	0867	0866	0810	0817
1	3	0701	0656	0623	0597	0572	0570	0470	0331	0341	0290	0241	0235	0246
1	3	0707	0718	0763 .	0670	0725	0637	0553	0578	0514	0429	0398	.	
2	3	0660	0680	0690	0705	0530	0480	0395	0315	0275	0250	0190	0255	0290
2	3	0827	0844	0878	0889	0743	0700	0545	0475	0431	0393	0332	0306	0259
3	3	0772	0830	0843	0815	0788	0715	0610	0493	0420	0345	0335	0275	0285
3	3	0703	0725	0780	0790	0670	0760	0826	0755	0777	0866	0823	.	0805
4	3	0670	0655	0677	0723	0680	0657	0610	0503	0413	.	0310	0225	0205
4	3	0717	0751	0764	0917	0837	0717	0620	0501	0444	0415	0392	0372	0367
5	3	0569	0597	0567	0578	0516	0452	0392	0306	0372	0389	0310	0296	0293
5	3	0650	0700 .	1160	1095	1010	0895	0795	0685	0630	0525	0420	0370	
6	3	0638	0650	0660	0638	0605	0490	0385	0325	0270	0248	0248	0264	0292
6	3	0779	0800	0810	0831	0820	0708	0432	0354	0324	0284	0263	0253	0253
7	3	0577	0600	0590	0595	0575	0535	0430	0345	0275	0250	0270	0285	0295
7	3	0682	0718	0792	0760	0713	0582	0510	0445	0417	0421	0320	0327	0264
8	3	0575	0627	0580	0593	0540	0482	0419	0318	0289	0246	0207	0241	0222
8	3	0739	0757	0805	0967	0991 .		1096	0812	0733	0638	0480	0407	0368
1	4	0513	0537	0547	0597	0589	0601	0585	0561	0581	0613	0574	0574	0502
1	4	1009	1123	1144	1111	0870	1094	1087	1161	1223	1262	1259	1249	1170
2	4	0529	0725	0706	0711	0789	0765	0770	0804	0779	0794	0765	0725	0716
2	4	0765	0798	0913	0942	0935	1204	1299	1468	1453	1420	1430	1329	1200
3	4	0785	0825	0872	0773	0817	0795	0885	0818	0835	0827	0822	0852	.
3	4	0630	0730	0785	0885	1085	1200	0880	1075	1285	1300	1345	1355	1445
4	4	1 .	.	.	.	.	.	.	.	.	.	.	.	.
4	4	2 0800	0782	0805	0833	0865	0838	0854	0868	0893	0994	0923	0929	0858
5	4	1 0656	0658	0663	0653	0650	0642	0665	0653	0646	0642	0640	0633	0627
5	4	2 0640	0650	0670	0650	0700	0810	0670	0800	0800	0795	0845	0775	0805
6	4	1 0655	0655	0660	0655	0715	0730	0723	0800	0815	0820	0800	0845	0843
6	4	2 0802	0839	0819	0844	1063	1332	1463	1322	1332	1366	1341	1317	1312
7	4	1 0593	0597	0610	0600	0705	0675	0690	0720	0715	0720	0680	0680	0685
7	4	2 0722	0746	0768	1374	1570	1548	1524	1429	1409	1393	1324	1268	1144
8	4	1 0684	0681	0667	0699	0728	0721	0771	0771	0771	0781	0766	0814	0795
8	4	2 0675	0709	0731	0696	0850	0936	0984	0980	0769	0989	0947	0826	0846

APPENDIX 6.

INSULIN RAW DATA FOR HORMONE SENSITIVITY TEST

ID	TRT	AGE	INSULIN(10xng/ml) 0	20	40	60	70	80	90	100	120	140	160	180 min
01	1	1	0035	0016	0018	0019	0030	0014	.	0009	0015	0010	0021	0022	0015
01	1	2	0019	0026	0021	0017	0018	0017	0016	0018	0026	0029	0016	0018	0017
02	1	1	0034	0039	0026	0026	0036	0044	0029	0025	0029	0025	0027	.	0019
02	1	2	0049	.	0021	0043	0024	0059	0035	0048	0043	0039	0036	.	.
03	1	1	0013	0011	0015	0021	0008	0009	0009	0021	0014	.	0012	0012	0009
03	1	2	0026	0029	0028	0037	0032	0031	0041	0033	.	0050	0030	0043	0040
04	1	1	0015	0018	0029	0016	0013	0013	0028	0018	.	.	0015	0016	0017
04	1	2	0060	0052	0029	0016	0015	0022	0010	0013	0018	.	0023	0038	0025
05	1	1	0026	0014	0013	0012	0015	0008	0049	0011	0012	0013	0018	0015	0013
05	1	2	0059	0020	0011	0017	0011	0012	0014	0013	0012	0008	0026	0031	0018
06	1	1	0006	0006	0008	0006	0006	0007	0006	0006	0044	0007	0006	0007	0007
06	1	2	0028	0022	0049	0030	0028	0031	0056	0082	0054	0068	0038	0040	0043
07	1	1	0016	0015	0014	0014	0014	0017	0017	0014	0013	0019	.	0016	0014
07	1	2	0049	0033	0033	0033	0058	.	0080	0071	0077	0059	0066	0038	0021
08	1	1	0013	0013	0011	0015	0012	0013	0017	0011	0012	0011	0011	0012	0019
08	1	2	0014	0016	0017	0031	0029	0035	0036	0033	0024	0043	0020	0027	0020
01	2	1	0013	0017	0013	0053	0035	0040	0061	.	.	.	.	.	.
01	2	2	0026	0023	0024	0027	0035	0042	0031	0028	0028	0052	0026	0019	0023
02	2	1	0013	0010	0012	0029	0035	0013	0013	0012	0013	0017	0014	0014	0015
02	2	2	0049	0034	0024	0028	0034	0040	0029	0038	0040	0028	0037	0039	.
03	2	1	0013	0017	0020	.	0016	0017	0010	0010	0013	0010	0011	0022	0030
03	2	2	0028	0066	0026	0048	0027	0030	0035	0023	0020	0024	0022	0020	0024
04	2	1	0010	0017	0015	0018	0015	0016	0016	0016	0017	0015	0016	0015	0016
04	2	2	0038	0053	0065	0045	.	0052	0044	0052	0059	0052	0048	0043	.
05	2	1	0019	0012	0015	0013	0013	0010	0017	0016	0016	0032	0022	0023	0028
05	2	2	0011	0017	0016	0027	0035	0028	0024	0073	0023	0051	0034	0030	0020
06	2	1	0006	0009	0008	0008	0008	0009	0006	0012	0009	0010	0013	0010	0014
06	2	2	0024	0060	0021	0027	0021	0031	0023	0029	0058	0021	0021	0018	0041
07	2	1	0015	0015	0013	0014	0015	0014	0014	0011	0012	0013	0014	0018	0008
07	2	2	0029	0016	0029	0055	0076	0080	0100	0092	0096	0120	0105	0052	0049
08	2	1	0013	0015	0014	0018	0016	0019	0019	0016	0014	0026	0014	0017	0014
08	2	2	0042	0037	.	0035	0029	0033	0042	0024	0024	0033	.	0033	0026
01	3	1	0011	0010	0020	0009	0055	0080	0088	0093	0071	0083	0133	.	0122
01	3	2	0035	0025	0030	0059	.	0118	0108	0197	0133	0123	0070	0041	0044
02	3	1	0013	0014	0013	0055	0096	0073	0079	0113	0173	0119	0158	0139	0064
02	3	2	0043	0026	0027	0021	0207	0213	0242	0232	0144	0234	0193	0209	0203
03	3	1	0035	0041	0006	0010	0099	0160	0141	0152	0219	0226	0234	0242	0263
03	3	2	0052	0032	0022	.	0032	0019	0025	0029	0020	0033	0030	0020	0020
04	3	1	0016	0014	0015	.	0084	0104	0206	0212	0240	0214	0209	0138	.
04	3	2	0021	0026	0021	0047	0178	0188	0194	0248	0195	0215	0226	0208	0227
05	3	1	0008	0009	.	0009	0153	0167	0168	0144	0137	0143	0151	0145	0133
05	3	2	0018	0091	0016	.	0203	0201	0185	0187	0224	0177	0162	0166	0219
06	3	1	0008	0005	0005	0029	0086	0111	0130	0158	0135	0154	0144	0173	0191
06	3	2	0019	0020	0019	0012	0250	0294	0320	0257	0254	0336	0364	0346	0259
07	3	1	0014	0012	0014	0013	0046	0096	0151	0156	0200	0202	0228	0231	0150
07	3	2	0018	0023	0032	.	0171	0152	0158	0189	0158	0212	0219	0158	0200
08	3	1	0016	0024	0022	0047	0056	.	.	0130	0176	0162	0203	0216	0211
08	3	2	0080	0034	0042	0090	0152	.	0150	0160	0160	0181	0160	0151	0183
01	4	1	0013	0010	0015	0015	0008	0013	0016	0012	0004	0013	0005	0006	0012
01	4	2	0071	0075	0042	0048	.	0046	0034	0035	0085	0041	0040	.	0046
02	4	1	0014	0013	0013	0013	0019	0013	0014	0020	0016	0013	0018	0016	0013
02	4	2	0066	0043	0053	0041	0043	0017	0033	0035	0055	0083	0115	0119	0115
03	4	1	0012	0011	0008	0009	.	0019	.	0011	0009	0015	0015	0056	0080
03	4	2	0021	0025	0023	.	0025	.	0025	0018	0017	0021	0024	0022	0041
04	4	1	0015	0010	0007	0012	0016	0009	0011	0013	0012	0010	0011	0016	0015
04	4	2	0059	0018	0015	0076	0038	0018	0030	0014	0031	0022	.	0017	0015
05	4	1	0014	0010	0007	0012	0016	0009	0011	0013	0012	0010	0011	0016	0015
05	4	2	0032	0017	0009	.	0014	0011	0016	0013	0013	0016	0016	0012	0014
06	4	1	0009	0009	0007	0011	0007	0007	0006	0010	0009	0009	0008	0009	0009
06	4	2	0022	0024	0029	0022	0018	0034	0054	0031	0022	0019	0025	0030	0037
07	4	1	0014	0039	0013	0013	0016	0015	0016	0015	0022	0058	0018	0013	0014
07	4	2	0031	0022	0014	0020	0020	0041	0042	0035	0040	0042	0037	0038	0099
08	4	1	0012	0013	0015	0013	0009	0022	0012	0015	0017	0014	0015	0015	0016
08	4	2	0022	0011	0019	0015	0014	0019	.	0020	0012	0036	0042	0032	0022

.7 APPENDIX 7.

.8 GH RAW DATA FOR HORMONE SENSITIVITY TEST.

.9	ID	TRT		AGE	GH (10xng/ml) 0	20	40	60	70	80	90	100	120	140	160	180 min.
1.	01	1	1	0043	0016	0048	0048	0030	0051	0054	0109	0091	.	0018	0140	0041
2.	01	1	2	0024	0018	0017	0029	.	.	0010	.	0023	.	0027	0013	0017
3.	02	1	1	0231	0200	0073	0082	0017	.	0012	0241	0116	0051	0032	.	0062
4.	02	1	2	0054	0026	0020	0077	0054	0026	0020	0016	0009	0015	0019	0012	0014
5.	03	1	1	0066	.	0181	0048	0021	.	.	0296	0317	0420	.	0069	.
6.	03	1	2	.	.	0015	0069	.	0028	0231	0247	0199	0021	0023	0009	0025
7.	04	1	1	.	0312	0122	0026	0017	0011	.	0012	0020	0064	0016	0019	0008
8.	04	1	2	0080	0013	0010	.	.	.	.	0009	0011	0015	0034	0010	0034
9.	05	1	1	0013	.	0012	0015	0011	0011	0064	0031	0013	.	0013	0039	.
10.	05	1	2	0090	.	0011	0008	.	0009	.	.	.	0060	0060	0078	.
11.	06	1	1	.	0319	0272	0120	0028	0022	0108	0236	0131	0084	0029	.	0236
12.	06	1	2	0012	0014	0019	0249	0074	0075	0166	0057	0031	0097	0021	0049	0011
13.	07	1	1	0018	0010	.	.	.	0049	0042	0027	0028	0018	0049	0057	.
14.	07	1	2	0010	0041	0042	0016	.	0042	0075	0054	0069	0054	0017	0016	0012
15.	08	1	1	0013	0300	0203	0133	0009	0010	0021	0034	0046	0041	0137	0126	0044
16.	08	1	2	0030	0038	0043	0013	0008	0014	0009	0016	0032	0125	0035	0026	0024
17.	01	2	1	0019	0023	0277	0030	0066	0067	0000	0000	0000	0000	0000	0000	0000
18.	01	2	2	.	0010	.	0018	.	.	.	0018	.	.	.	0013	.
19.	02	2	1	0027	0016	.	.	.	0190	0191	0170	0139	0113	0103	0056	0046
20.	02	2	2	0025	0013	0024	0024	0015	0056	0029	0041	0018	0013	0033	0019	.
21.	03	2	1	0020	0009	0243	0113	0275	0188	0138	0104	0162	0124	0222	0087	0272
22.	03	2	2	0015	0013	.	0037	0008	0008	0116	0090	0073	0024	0021	0018	0010
23.	04	2	1	.	0026	.	0097	0038	0022	0018	0042	0044	0139	0018	0012	.
24.	04	2	2	0024	0014	0010	0012	0033	0044	0015	0019	0018	0013	0015	0013	0010
25.	05	2	1	0033	0072	0067	0134	0037	0028	0048	0039	0070	0086	0068	0013	0052
26.	05	2	2	0013	0017	0015	0013	.	.	0021	0025	0032	0021	0015	.	.
27.	06	2	1	0109	0035	0038	0015	0016	0154	0163	0141	0081	0107	0042	0300	0167
28.	06	2	2	0040	0019	0009	0017	0015	0017	0017	0012	0020	0022	0050	0019	0058
29.	07	2	1	0169	.	0105	0054	0065	0021	0035	0020	0025	0039	0026	0181	0124
30.	07	2	2	0030	0036	0009	0009	0014	0047	0077	0075	0042	0066	0017	0011	0020
31.	08	2	1	0505	0340	0124	0088	0061	0105	0267	0359	0158	0154	0163	0018	0106
32.	08	2	2	0108	0022	0018	0015	0012	0015	0009	0024	0138	0045	0048	0010	0035
33.	01	3	1	0020	0204	0226	0164	.	0104	0101	0213	0124	0069	0017	0019	0009
34.	01	3	2	0039	0020	0014	0009	0011	.	0046	0133	0111	.	.	0012	.
35.	02	3	1	0026	0033	0027	0056	0086	0065	0076	0153	.	0023	0012	.	.
36.	02	3	2	0017	0014	0010	0055	0036	0035	0037	0013	0011	0015	0032	0035	0018
37.	03	3	1	0179	0047	0071	0048	0049	0705	0472	0590	0588	0370	0013	0031	.
38.	03	3	2	.	.	.	0010	.	0013	0024	.	.	.	0043	0013	0014
39.	04	3	1	.	.	.	.	0540	0495	0368	0319	0306	0113	0068	0019	.
40.	04	3	2	0013	0023	0021	0011	0021	.	0050	0084	0099	0015	0017	0015	0015
41.	05	3	1	0129	0070	0024	0043	0014	0420	0466	0030	0034	0025	0010	0011	0016
42.	05	3	2	0018	0010	.	.	0016	0030	0118	0081	0043	0020	0013	0012	0002
43.	06	3	1	0027	0196	0096	0073	0053	.	.	0321	0194	0034	0014	0011	0073
44.	06	3	2	0091	.	0038	0084	0251	0484	0495	0093	0012	.	0020	0159	0018
45.	07	3	1	.	.	0092	.	.	.	.	.	.	.	.	0077	.
46.	07	3	2	0014	0092	0023	0034	0011	0081	0068	0029	.	0021	0015	0017	0015
47.	08	3	1	0057	0021	.	0129	0591	1461	1542	0888	0850	0419	0029	0067	0079
48.	08	3	2	0050	0077	0012	0018	0022	.	0107	0196	0151	0060	0036	0011	0008
49.	01	4	1	0102	0012	0024	0023	.	.	.	.	0013	0012	0019	0051	0029
50.	01	4	2	.	.	.	0097	0105	0046	.	0018	0037	.	0008	.	0010
51.	02	4	1	.	.	.	.	.	0030	0015	0011	.	0013	0015	0012	0012
52.	02	4	2	0015	0027	0024	0028	0023	0016	0012	0019	0214	0218	0023	0018	0013
53.	03	4	1	0034	0065	0545	0859	0409	0123	.	0038	0045	0025	0020	0050	0049
54.	03	4	2	0068	0064	0057	0009	0008	.	.	.	.	0080	.	0012	0022
55.	04	4	1	0056	0011	0045	0034	.	.	.	0202	0089	0062	0038	0104	0013
56.	04	4	2	0015	.	0012	0012	0041	0014	0014	0009	0011	0009	.	0009	0011
57.	05	4	1	0012	0017	0024	0021	.	.	0010	.	.	.	0012	0019	0009
58.	05	4	2	0071	0071	0050	0054	0024	.	.	.	.	0009	0015	0144	.
59.	06	4	1	0044	0023	.	0973	.	0276	0150	0205	0137	0149	0242	0236	0282
60.	06	4	2	0052	0036	0014	0014	0012	0009	.	0012	.	.	0014	0009	0010
61.	07	4	1	0037	0042	0039	.	0008	.	0127	.	.	.	.	0008	.
62.	07	4	2	0033	0029	.	0020	0012	0012	0031	0139	0125	0032	0034	0027	0049
63.	08	4	1	0147	0132	0244	0076	0040	.	0022	0016	0015	0014	0036	.	0196
64.	08	4	2	0036	0028	0010	0016	0009	0010	0030	0014	0029	0008	0022	0060	0080

APPENDIX 8.

GLYCEROL RAW DATA FOR HORMONE SENSITIVITY TEST.

ID	AGE	GLYCEROL (100xmM) 20	40	60	80	100	120 min.
1	1	19	16	14	29	28	22
1	2	18	16	15	36	44	18
2	1	14	12	10	19	17	16
2	2	19	19	25	36	29	29
3	1	13	15	18	32	32	32
3	2	22	17	27	21	26	29
4	1	36	21	22	32	22	25
4	2	15	17	18	17	33	32
5	1	.	.	16	34	39	33
5	2	.	.	14	22	24	12
6	1	29	27	26	22	29	22
6	2	15	33	24	13	10	10
7	1	14	19	09	18	18	15
7	2	12	11	16	11	08	06
8	1	16	18	17	19	17	15
8	2	20	14	32	31	26	27

APPENDIX 9

RAW DATA FOR LAMB INSULIN BINDING STUDY

ID	STAGE	CELLS	SA	NSB	TOTAL (cpm) - TUBE 20	19	18	.....	1.			
001	1	42.25	376880	108	5574	149	139	141	129	125	144	165	152
	172	205	166	177	263	167	285	203	271	267	351	300	
002	1	59.00	376880	105	5457	129	142	180	135	170	201	190	189
	227	310	524	375	454	539	595	483	632	523	758	641	
003	1	46.50	376880	112	5367	180	154	452	249	260	183	275	231
	295	258	273	421	287	265	288	381	617	514	505	316	
004	1	60.00	376880	104	5332	191	143	116	188	158	151	212	173
	179	180	242	253	323	269	353	381	398	407	553	414	
005	1	46.38	368303	132	7896	304	484	261	294	654	193	387	283
	321	274	469	670	1012	715	857	1016	799	913	890	1206	
006	1	60.00	368303	107	8341	126	122	167	146	162	154	183	215
	185	237	254	225	282	251	352	342	348	361	295	248	
007	1	60.00	368303	70	2066	076	080	089	110	069	082	091	070
	142	085	113	095	110	127	106	107	129	140	160	136	
008	1	35.25	368303	69	2041	000	000	105	108	000	000	108	124
	000	000	155	138	.	.	156	154	.	.	163	169	
001	2	34.25	295708	84	3793	110	099	101	108	117	121	114	128
	153	136	153	157	196	176	224	210	216	220	345	270	
002	2	35.63	295708	108	3777	118	116	120	130	135	154	223	131
	134	138	146	170	158	154	152	162	172	165	152	174	
003	2	39.38	295708	93	3833	169	143	145	166	147	175	168	197
	218	266	347	327	365	390	403	437	450	447	466	505	
004	2	24.75	295708	114	3201	133	169	176	190	181	186	179	193
	185	191	199	227	232	209	241	276	233	239	296	309	
005	2	58.38	292297	103	3786	131	141	149	200	148	153	185	196
	223	237	327	366	445	496	483	470	497	558	570	659	
006	2	60.00	292297	106	3942	145	144	172	161	194	183	182	203
	212	217	324	304	338	334	374	342	399	402	457	436	
007	2	60.00	292297	113	5038	143	132	150	134	152	185	153	174
	338	230	250	260	352	287	260	282	319	310	349	390	
008	2	38.75	292297	195	4833	237	272	251	177	305	297	316	465
	409	388	672	496	655	641	743	656	858	794	812	957	
001	3	41.25	320600	85	5157	120	123	127	140	142	155	169	222
	190	205	297	306	591	302	452	331	536	562	631	629	
002	3	26.25	320600	48	4883	088	082	091	104	282	956	106	048
	126	118	211	188	241	284	385	376	393	381	491	466	
003	3	51.50	320600	051	5108	046	046	046	045	181	156	360	307
	229	311	936	913	1229	470	1651	1169	1849	1856	2185	2159	
004	3	34.63	320600	048	4763	055	050	045	360	437	446	401	260
	409	437	729	681	711	741	776	684	909	903	1126	1048	
005	3	43.13	313244	92	5350	128	109	117	118	132	164	157	154
	199	317	407	966	692	567	793	745	871	810	1044	1046	
006	3	60.00	313244	111	5297	162	169	611	166	176	218	233	210
	257	283	489	444	568	606	745	656	821	693	776	845	
007	3	60.00	313244	100	4907	152	148	166	164	193	185	225	228
	268	243	409	389	526	507	639	674	743	733	1045	.	
008	3	32.25	313244	100	4910	232	240	255	230	277	292	328	320
	342	359	525	561	618	562	824	815	758	787	802	795	
001	4	39.63	376884	082	6124	114	137	145	142	155	244	167	194
	192	203	298	281	368	360	397	365	510	432	549	526	
002	4	39.25	376884	181	6334	231	245	289	306	441	310	354	397
	485	554	918	907	1150	1245	1494	1351	1465	1045	1615	1584	
003	4	29.50	376884	131	5817	188	172	175	187	207	197	253	243
	469	323	563	613	818	893	953	891	1040	1104	.	1275	
004	4	25.63	376884	090	5663	106	124	173	085	108	097	152	127
	143	140	248	192	254	261	361	265	320	325	183	221	
005	4	52.50	368303	136	6194	158	123	143	139	163	157	215	185
	209	217	360	358	456	384	523	441	579	570	661	634	
006	4	37.50	368303	093	6263	138	142	128	125	151	198	165	176
	191	172	313	300	400	380	478	455	488	415	590	585	
007	4	37.50	368303	117	5981	156	230	181	172	224	194	206	321

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62.   255  341  532  412  578  597  682  630  749  788  998  855
63. 008 4 35.63 368303 100 5694  169  193  158  175  167  315  626  200
64.   246  219  350  332  422  386  470  450  499  475  593  425
65. 001 5 47.00 408651 226 4598  763  746  852  868 1176 1087 1300 1276
66.  1460 1374 2163 1970 2468 2294 2590 2574 2710 2697 3361 3006
67. 002 5 39.00 408651 121 6487  155  089  195  189  252  270  362  383
68.   505  493 1146 1088 1552 1707 1887 1719 2128 1895 2435 2474
69. 003 5 32.75 408651 106 6657  155  140  152  170  198  229  283  273
70.   371  390  817  780 1167 1362 1495 1343 1705 1707 1829 1850
71. 004 5 45.25 408651 164 6718  248  232  274  308  359  405  504  477
72.   555  548 1007  978 1390 1194 1522 1425 1595 1645 1890 1921
73. 005 5 60.00 403960 171 6476  273  285  324  331  493  478  663  720
74.  1142 1102 2328 2318 2640 2843 3213 3306 3272 3432 3808 3860
75. 006 5 57.60 403960 109 6525  208  205  260  225  318  312  386  391
76.   509  473  885  894 1107 1102 1447 1466 1470 1509 1702 1642
77. 007 5 60.00 403960 114 6603  232  252  388  240  271  282  332  372
78.   390  429  502  277  794  837  462  963  815 1080 1278 1287
79. 008 5 60.00 403960 133 6608  189  184  205  232  238  252  256  283
80.   364  287  496  499  614  588  901  673  853  779 1048 1036
81. 001 6 50.38 292297 094 4550  175  172  187  217  220  237  260  262
82.   304  313  474  494  522  526  681  745  879  934  865  833
83. 002 6 55.87 292297 095 4705  371  198  150  207  172  213  219  351
84.   205  293  332  417  411  464  417  609  479  579  558  836
85. 003 6 29.88 292297 095 4595  155  215  578  147  290  165  210  192
86.   286  233  490  468  617  413  805  643  715  706  873  872
87. 004 6 48.88 292297 150 4733  324  290  332  334  385  386  436  421
88.   537  495  731  740  881  732 1054 1014 1027  980 1223 1173
89. 005 6 50.50 292297 129 4832  211  294  237  305  256  320  364  357
90.   378  429  521  556  738  746  792  898  679  853 1001  934
91. 006 6 60.00 292297 095 4777  196  216  264  253  276  301  321  355
92.   540  423  636  630  736  763  847  927  940  954 1128 1713
93. 007 6 60.00 292297 096 5017  129  160  148  254  190  232  197  292
94.   185  340  295  435  289  494  383  536  374  592  468  638
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APPENDIX 10.

RAW DATA FOR DAIRY CALF INSULIN BINDING STUDY.

ID	STAGE	CELLS	SA	NSB	TOTAL (cpm) - TUBE 20	19	18	.....	1.			
1	1	29.50	368303	46	5506	146	133	156	.	191	199	224	252
		340	300	551	513	715	716	867	828	1014	925	1187	969
1	2	30.00	266500	83	4349	101	102	105	102	109	129	124	135
		143	138	222	225	281	358	335	353	381	390	443	447
1	3	30.00	394740	109	6647	142	121	152	145	186	154	189	159
		237	216	395	434	538	545	737	719	770	713	972	1016
1	4	30.00	285635	92	5050	136	083	117	186	130	167	152	151
		237	201	280	304	357	373	502	503	512	505	666	660
1	5	30.00	427946	87	6607	151	168	146	168	172	196	209	208
		262	241	421	418	535	606	720	706	677	681	942	1161
1	6	30.00	427943	113	6360	164	226	165	197	270	210	209	186
		240	318	305	399	446	.	623	641	760	832	964	835
1	7	30.00	309673	146	4949	159	305	286	449	216	254	334	540
		.	327	322	306	315	430	796	525	485	571	707	615
2	1	30.00	339628	112	4975	133	130	147	147	177	170	204	224
		224	233	343	389	454	436	512	557	557	525	683	747
2	2	30.00	368303	183	5902	137	135	112	115	148	136	164	223
		330	170	235	421	321	356	653	223	345	675	692	696
2	4	30.00	263462	107	4465	152	151	120	209	176	176	178	188
		195	231	277	303	336	376	465	438	463	494	552	578
2	5	60.00	399324	134	6515	164	181	230	253	.	266	284	333
		317	380	488	568	571	566	839	781	800	732	998	1044
2	6	30.00	399324	130	6209	166	217	217	248	163	308	155	371
		100	267	264	394	334	314	488	466	565	539	707	650
2	7	30.00	285635	119	4927	134	198	220	319	174	221	369	164
		177	418	214	209	229	473	419	351	378	389	533	487
3	1	30.00	339628	119	5217	135	129	147	154	182	176	214	206
		267	281	495	459	608	637	806	777	854	837	1131	1054
3	2	30.00	368303	79	5607	156	104	110	103	130	132	561	448
		324	564	686	380	490	592	785	580	962	705	837	892
3	4	30.00	263462	103	4170	121	120	143	117	134	137	149	140
		218	176	319	284	383	399	493	549	499	428	649	646
3	5	30.00	399324	124	6474	151	187	167	190	164	189	212	237
		278	296	587	513	661	668	875	953	1054	1067	1298	1358
3	6	30.00	399324	119	6447	180	182	172	156	147	204	177	180
		189	202	316	398	462	408	691	815	984	851	1166	1169
3	7	30.00	285635	94	4764	117	118	102	107	115	121	129	120
		156	138	224	234	461	292	420	406	530	468	592	562
4	1	17.25	285635	70	4678	088	075	102	103	088	087	111	085
		112	115	147	299	386	170	243	215	238	198	283	263
4	2	24.13	427946	108	6728	122	144	125	170	167	147	155	202
		243	257	286	290	409	409	521	426	572	536	538	640
4	3	30.00	464030	127	6947	220	359	268	328	381	370	435	413
		528	543	986	979	1179	1155	1574	1473	1498	1480	1769	1773
4	5	30.00	453423	171	7027	341	368	248	317	318	324	298	329
		391	388	709	659	912	935	1464	1461	1722	1747	2150	2131
4	6	30.00	335737	87	5245	130	131	143	149	188	204	186	198
		199	241	382	399	528	551	864	877	984	977	1174	1140
5	1	30.00	427946	131	6008	158	177	135	158	150	114	233	246
		428	104	613	551	789	779	872	751	1003	972	1231	1126
5	2	30.00	316922	140	5605	175	180	233	202	217	225	297	317
		374	354	635	585	728	768	912	921	787	892	1109	1030
5	3	30.00	464030	104	6889	212	220	242	250	440	486	420	391
		553	607	1046	1078	1388	1462	.	1864	1807	1855	2349	2280
5	4	24.13	453423	138	7243	302	386	277	373	325	365	385	435
		436	333	623	514	584	568	966	953	1143	1095	1524	1406
5	5	20.13	335737	177	5244	137	142	160	177	165	179	163	221
		205	217	340	330	450	460	639	607	716	808	900	826
5	6	30.00	242420	75	3674	.	095	084	091	096	098	097	173
		160	199	178	245	220	206	338	327	395	424	498	1654
6	1	21.50	427946	121	6580	156	159	182	197	205	.	280	272

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620.   364  345  619  650  966  794 1052  987 1080  971 1389 1306
630. 6 2 30.00 316922 103 5398  129  140  104  125  118  142  155  170
640.   182  194  290  357  398  410  527  555  466  522  600  709
650. 6 3 30.00 464030  98 6951  132  148  142  169  174  179  235  209
660.   255  466  447  480  565  637  880  818  767  843 1218 1091
670. 6 4 30.00 453423  96 7279  255  283  274  253  303  335  340  351
680.   391  384  592  556  681  676 1202 1146 1378 1390 1778 1862
690. 6 5 30.00 335737 118 5184  179  119  127  120  158  159  178  161
700.   168  148  305  310  404  394  647  571  713  707  850  893
710. 6 6 30.00 242420  70 4561  130  139  141  114    .  103  116  105
720.   199  109  132  209  156  163  258  260  380  427  366  363
730. 7 1 30.00 394740 124 6296  152  147  162  147  190  172  220  182
740.   256  235  466  435  615  596  748  709  828  725  998  978
750. 7 2 30.00 285635  85 5094  127  123  137  132  166  167  185  205
760.   269  274  504  657  693  646  935  938  965  938 1215 1205
770. 7 3 30.00 427946 103 6900  143  165  163  187  220  222  267  275
780.   334  349  627  600  722  724  943  992  986 1044 1328 1279
790. 7 4 30.00 427943 136 6342  205  205  167  190  209  216  256  254
800.   310  307  638  561  785  858 1439 1328 1732 1783 2037 2179
810. 7 5 30.00 309673 106 4902  133  140  119  158  167  235  207  199
820.   194  280  378  408  472  476  745  745  823  811  925  980
830. 7 6 30.00 188416  79 2953  107  099    .  136  155  120  126  132
840.   131  141  227  210  235  290  423  395  621  423  557  540
850. 8 1 24.75 508962 171 7821  294  269  235  265  292  347  384  385
860.   594  541 1000 1039 1340 1432 2023 1938 1878 1886 2614 2539
870. 8 2 30.00 368303 141 5701  185  204  220  247  327  306  310  343
880.   444  592  909  819  952 1009 1327 1349 1523 1504 1965 2100
890. 8 3 30.00 364039 173 5669  339  243  288  337  275  259  403  426
900.   436  407  861  776 1319 1014 1362 1293 1508 1495 1880 1880
910. 8 4 30.00 260424 105 4159  113  121  128  138  154  160  160  179
920.   167  228  336  320  378  394  647  636  704  744  788  828
930. 8 5 30.00 188426  80 3079  088  100  082  102  098  125  101  118
940.   135  132  195  211  246  250  427    .  419  465  504  505
950. 8 6 30.00 390156 113 6606  236  148  155  166  166  178  184  185
960.   272  238  469  402  458  458  716  777  843  766 1082 1048
970. 9 1 30.00 427946 116 6756  160  192  194  195  244  264  339  355
980.   452  450  822  979 1075 1105 1500 1903 1337 1311 1731 1701
990. 9 2 26.50 427943 114 6456    .  210  217  233  255  297  315  351
1000.  375  417  863  834 1207 1396 1942 1669 2179 2342 2745 2839
1010. 9 3 30.00 309673 104 5044  126  151  151  135  140  180  204  186
1020.  192  237  354  394  462  515  742  763  856  714  689 1014
1030. 9 4 30.00 188416 113 3050  099  109    .    .  110  118  102  148
1040.  135  149    .  252  268  291  354  379  472  482  524  492
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