NUTRITIONAL REGULATION OF INSULIN AND GLUCAGON SECRETION IN SHEEP

by

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INTRODUCTION

Ruminants have a four-compartment stomach consisting of the rumen, the reticulum, omasum and abomasum or true stomach. The rumen is the largest of the four compartments and functions as a fermentation vat for 70 - 80 percent of ingested digestible dry matter. This arrangement and highly specialized nature of the ruminant stomach has given rise to a digestive mechanism and energy metabolism different from those found in other mammals.

In a reciprocally beneficial symbiotic relationship, the billions of microbes which live in the rumen ferment plant materials consumed by the host animal to form chiefly carbon dioxide, methane and volatile acids. The gases are excreted and the acids are absorbed and oxidized for energy. As a result of rumen fermentation only a very small amount of dietary glucose is available to the animal, hence ruminants have low blood glucose which remains low even after feeding. Thus, as a consequence of the unique digestive system, glucose and other nutritional factors which regulate insulin secretion in monogastrics seem to have a diminished role in ruminants.

Since insulin is needed in ruminants as in nonruminants for the regulation of energy storage and growth, other factors must have assumed the role of regulating insulin secretion in ruminants. Knowledge of these factors is at present limited but necessary for a better understanding of the transformation of feed into meat and milk in ruminants.

Insulin is secreted by the beta cells of the pancreatic islets of Langerhan. In association with the beta cells are alpha cells which secrete glucagon. Until recently the function of glucagon has been obscure; however, it is now attracting the interest of many scientists because of the possibility that it may have a local function which is to stimulate insulin secretion. It seems appropriate, therefore, to study the secretion of insulin and glucagon together.

The inability to assay plasma levels of these hormones had in the past prevented the unequivocal assessment of secretion studies. Better interpretations are now possible because of improved techniques. Particularly important in this regard are the various immunoassay techniques. It was the purpose of this study to develop a radioimmunoassay for glucagon and to investigate the effects of some nutritional factors on the secretion of insulin and glucagon in sheep.

LITERATURE REVIEW

Control of Insulin and Glucagon Secretion

Nutritional status

By measuring changes in the number of alpha and beta cells (Hellman and Hellerstrom, 1959) or changes in liver glycogen and plasma free fatty acids (FFA)(Fuller and Diller, 1970) indirect evidence has been presented to show that during the fed state insulin secretion increases and falls during starvation in the rat; the converse being true for glucagon. More direct measurements of insulin and glucagon secretion in man and dog by radioimmunoassay methods have confirmed that insulin levels are high during the fed state and fall with starvation (Nestel, Carrol and Havenstein, 1970; Unger et al., 1963; Vance, Buchanan and Williams, 1968; Anguilar-Parada, Eisentraut and Unger, 1969). Glucagon results have been controversial. Unger et al. (1963) and Anguilar-Parada, Eisentraut and Unger (1969) have reported a rise in glucagon during starvation in man. This was not confirmed by Vance, Buchanan, and Williams (1968) in either human subjects or dogs. On the contrary, they found glucagon to rise and fall together with insulin.

Prolonged starvation apparently does not result in the establishment of a steady basal level of insulin. Freinkel, Mager and Vinnick (1968) have observed a diurnal variation in plasma insulin levels during starvation. Insulin was found to be significantly higher in the mornings than in the evenings. They concluded there is a rhythmic cyclicity for insulin secretion which is intrinsically determined and can be sustained

without exogenous stimulation to insulinogenesis.

Carbohydrates

The elevation of blood glucose concentration above the fasting range (60 - 100 mg/100 ml) by oral or intravenous glucose administration to man or laboratory animals results in increased insulin secretion (Randle et al., 1968; Karam et al., 1966; Pallotta and Kennedy, 1968). Ruminants have been shown to respond in a similar manner to intravenous injections of glucose (Boda, 1964; Horino et al., 1968). The stimulatory effect of glucose on insulin secretion has also been demonstrated in vitro. The rate of insulin secretion was found to vary with glucose concentration above 35 - 50 mg/100 ml (Grodsky et al., 1963; Coore and Randle, 1964).

The effect of glucose on glucagon secretion appears to be dependent on the route of administration. Buchanan et al. (1967) and Unger et al. (1968) have shown that glucose introduced intrajejunal or intraduodenal in dogs stimulates release of glucagon which is of intestinal rather than pancreatic origin. On the other hand, glucose infusion depresses and hypoglycemia stimulates pancreatic glucagon secretion (Foa et al., 1949; 1952; Unger et al., 1962; 1968; Ohneda et al., 1968). The in vitro studies of Vance et al. (1968) have confirmed that glucagon secretion decreases as the concentration of glucose increases.

The effects of other monosaccharides on the secretion of insulin and glucagon have also been investigated. Most of these studies have dealt more with insulin than glucagon. Mannose, fructose and glucosamine have been demonstrated to stimulate insulin secretion (Randle et al., 1968).

Fructose was effective only at very high concentrations (above 500 mg/100 ml) while glucosamine was only slightly effective. Glucosamine may also inhibit glucose-induced insulin secretion (Randle et al., 1968; Karam et al., 1966; Martin and Bambers, 1965). It has also been reported that galactose, 2-deoxyglucose, N-acetylglucosamine, d-ribose, d-arabinose, mannoheptulose and phloridzin are either without effect on or inhibit insulin secretion (Randle et al., 1968; Karam et al., 1966; Vance et al., 1968). The literature is, however, filled with conflicting observations on the effects of these sugars on insulin secretion. Pozza et al. (1958) found galactose stimulatory and fructose ineffective in dogs. Boda (1964) reported that both fructose and galactose stimulated insulin secretion in sheep. However, Grodsky et al. (1963) observed that galactose could not stimulate insulin secretion from isolated rat pancreas. These discrepancies are probably due to differences in dosages, species or assay techniques.

The effects of 2-deoxyglucose, glucosamine and fructose have been introduced as evidence to discount the direct involvement of the glucose molecule in the stimulation of insulin secretion (Frohman, 1969). Glucosamine and 2-deoxyglucose inhibit glucose-induced insulin secretion by inhibiting glucose metabolism. Glucosamine is thought to competitively inhibit glucose phosphorylation (Martin and Bambers, 1965). Frohman (1969) has stated that fructose stimulation of insulin secretion may simply result from its conversion to glucose since insulin secretion is not stimulated by fructose in patients with "essential fructose intolerance", where a deficiency of 1-phosphofructoaldolase exists.

This enzyme converts fructose-1-phosphate to dihydroxyacetone phosphate and glyceraldehyde. However, fructose has been reported to stimulate insulin secretion in the absence of increased blood glucose in patients with glucose-6-phosphatase deficiency (Hug and Schubert, 1967). Consequently, it has been suggested that the trioses or subsequent intermediates in the glycolytic pathway may actually be responsible for stimulating insulin secretion. Using sheep pancreas Montague and Taylor (1968) have shown that citric acid stimulates insulin secretion. However, Randle et al. (1968) failed to show any effect of pyruvate and citrate on insulin secretion in perfused rabbit pancreas.

Although 2-deoxyglucose and mannoheptulose inhibit insulin secretion they have been observed to have no effect on glucagon secretion (Vance et al., 1968). Phloridzin has been found to induce glucagon secretion in dogs. This is probably due to its effect on hypoglycemia (Randle et al., 1968).

Fatty acids and ketones

The effect of volatile fatty acids on insulin secretion has been investigated in ruminants for whom these acids form a major source of energy. Manns and Boda (1967) observed that propionate and butyrate stimulate insulin secretion and hyperglycemia in sheep. Acetate was without effect. By infusing propionate and butyrate into the pancreatic artery, Manns, Boda and Willes (1967) subsequently presented evidence to suggest that butyrate and perhaps propionate may exert their effects directly on the pancreas. In vitro studies of Montague and Taylor (1968) have also demonstrated that citrate, butyrate and octanoate may have a

direct effect on the sheep pancreas. However, they did observe that insulin release was positively correlated with the production of glucose-6-phosphate in the medium. They explained their results as the effect of short chain fatty acids on the intracellular accumulation of citrate which may then affect phosphofructokinase, resulting in an inhibition of glycolysis and accumulation of glucose-6-phosphate. These results do not support the idea that the release of insulin brought about by sugars is mediated by glycolysis; for, in the presence of fatty acids, glycolysis is inhibited, but rather suggest that glucose has to be metabolized by some other pathway in order to stimulate insulin secretion. Presumably the pentose phosphate pathway is involved.

Horino et al. (1968) have confirmed that propionate and butyrate stimulate insulin secretion in sheep as well as cattle while acetate is without effect. They also observed that propionate and butyrate have no effect in nonruminants. Apparently during the course of evolution species acquired insulin secretory response mechanism sensitive to the major nutrients or substrates utilized by that organism.

Other short chain fatty acids -- valerate, isovalerate, hexanoate, heptanoate and octanoate -- have been found to stimulate insulin secretion in sheep (Horino et al., 1968). Increased insulin secretion has also been observed in humans in response to intravenous injections of medium chain triglycerides (composed mainly of C8 and C10 fatty acids) or long chain triglycerides (corn oil) (Pi-Sunyer et al., 1969). In studies involving the measurement of both insulin and glucagon (Seyffert and Madison, 1967; Madison et al., 1968) triglyceride (cottonseed oil emulsion) infusion was

found to stimulate insulin and inhibit glucagon secretion in dogs. The mechanism of action of the long chain fatty acids is thought to be either a direct stimulatory feedback on the beta cells and an inhibitory feedback on the alpha cells or mediated through the action of ketone bodies. The ketone body hypothesis is based on the observations of Madison et al. (1964) in dogs and Sauls (1967) in man that ketone bodies stimulate insulin secretion. However, Horino et al. (1968) and Manns, Boda and Willes (1967) were unable to show any effect of beta hydroxybutyrate on insulin secretion in sheep. Evidence in support of the direct action theory has recently been presented in a study by Crespin et al. (1969). In this study, sodium oleate was infused directly into the superior pancreaticoduodenal artery of anesthetized dogs. They observed a 5 to 20 fold increase in pancreatic venous insulin and a 3 to 6 fold increase in peripheral plasma insulin concentration within ten minutes. Linoleate was found to have the same effect.

Amino acids

By measuring plasma insulin changes in normal subjects following a protein meal or during the infusion of amino acids Floyd and his colleagues have demonstrated that insulin secretion occurs in response to amino acid load (Floyd et al, 1966a; 1966b; Fajans et al., 1967). Small changes in blood glucose concentrations could not account for the increase in insulin secretion. These same workers also showed that individual amino acids differ as to their ability to stimulate insulin secretion (Floyd et al., 1966b; Fajans et al., 1967). Of the amino acids studied the most

potent were arginine and lysine and the least potent was histidine. A mixture of the ten essential amino acids resulted in higher plasma insulin levels than arginine, the most potent amino acid. Other workers have confirmed some of these observations in man (Pallotta and Kennedy, 1968; Fineberg et al., 1970). Hertelendy, Machlin and Kipnis (1969) have also demonstrated that arginine infusion stimulates insulin secretion in sheep.

More recent work by Floyd and his colleagues in which several amino acid pairs were infused into human subjects indicates synergism between some amino acids. Synergism was observed between arginine and leucine, and arginine and phenylalanine; the synergism between arginine and leucine being greater than that between arginine and phenylalanine. However, there was no synergism between arginine and lysine or arginine and histidine or leucine and histidine (Floyd et al., 1970a). Amino acids and glucose have also been shown to have a synergistic effect on insulin secretion (Pallotta and Kennedy, 1968; Floyd et al., 1970b).

The mechanism of action of amino acids on insulin secretion is not yet well understood. It appears that different amino acids may have different mechanisms, for example, chlorpropamide which accentuates the insulin releasing action of leucine and diazoxide which suppresses leucine-induced insulin secretion, were found to have no effect on the insulinogenic activity of arginine or a mixture of eight amino acids (Fajans et al., 1967). Frohman (1969) has suggested that the differences may be due to differences in metabolic pathway.

Infused or ingested amino acids and proteins have also been found to stimulate glucagon secretion (Pek et al., 1968; 1969). However, some differences in response were found. Leucine was found to have no effect on glucagon secretion (Pek et al., 1969) and arginine was found to be more potent in stimulating glucagon secretion than a mixture of the ten essential amino acids (Pek et al., 1968).

Cations

Using isolated perfused rat pancreas Grodsky and Bennett (1966) have shown that there is a specific requirement for calcium for the insulin secretion induced by glucose. Manganese was not essential nor could it substitute for calcium. They also observed that potassium directly stimulated insulin secretion in the absence of glucose when its concentration was increased from 4 to 8 mEq per litter. Other workers have confirmed these observations (Milner and Hales, 1968; Hales and Milner, 1968). Hales and Milner (1968) made the additional observation that extracellular sodium is a prerequisite for secretion and suggested that depolarization and/or influx of sodium across beta cell membrane leads to insulin secretion.

Little or no information is available with respect to glucagon secretion. Since the primary effects of the cations are on the membrane, the same principles may also apply to glucagon.

Intestinal factors

McIntyre et al. (1964; 1965) reported that the hyperglycemia induced by oral or intrajejunal glucose in man was more effective in stimulating

insulin secretion than a comparable hyperglycemia induced by intravenous glucose. They attributed their findings to an intestinal factor(s) which affected insulin secretion. Dupre and Beck (1966) supported this hypothesis by showing that extracts of porcine intestinal mucosa infused simultaneously with glucose greatly elevated insulin-like activity than when glucose alone was administered intravenously. Evidence has accumulated to implicate glucagon, secretin, gastrin, and pancreozymin. In 1948, Sutherland and deDuve demonstrated the existence of a "glucagon-like" hyperglycemic factor in the intestine. More recent studies have established that the intestinal tract does indeed contain substances which are both immunologically and biochemically related to glucagon (Valverde et al., 1968; 1969). Orci et al. (1968) have presented structural evidence for glucagon producing cells in the intestinal mucosa of the rat. It has also been shown that there is release of glucagon after oral glucose but not after intravenous administration, and that the rise in glucagon is not of pancreatic origin (Unger et al., 1968; Buchanan et al., 1967).

Since the demonstration by several workers that glucagon stimulates insulin secretion both in vivo (Samols, Marri and Marks, 1966; Ketterer, Eisentraut, and Unger, 1967; Sussman and Vaughan, 1967) and in vitro (Grodsky et al., 1967) it has been suggested that the role of intestinal glucagon is to stimulate insulin secretion and that pancreatic glucagon may also have a local function.

By using purified extracts, Unger et al. (1967), Dupre et al. (1969), and Kahil, McIlhaney and Jordan (1970) have demonstrated that secretin, gastrin and pancreozymin stimulate the secretion of both insulin and glucagon. Because small changes in insulin secretion in normal subjects may remain undetected as the results of hepatic clearance and the dilution of hepatic vein plasma, Kaess, Schlierf and von Mikulicz-Radecki (1970) have shown by by-passing the liver that the secretion of insulin produced by the liberation of endogenous secretin occurs at physiological doses of HCl and may be proportional to the rate of influx of H^+ into the duodenum.

Other hormones

The inhibitory effect of epinephrine on insulin secretion has been demonstrated in man (Porte and Williams, 1966; Karam et al., 1966), monkey (Kris et al., 1966), dog (Altszuler et al., 1967; Kosaka et al., 1964), rat (Wright and Malaisse, 1968), pigs (Hertelendy et al., 1966), and sheep (Hertelendy, Machlin and Kipnis, 1969). It is believed that epinephrine exerts its effect by stimulating alpha adrenergic receptors of the beta cells (Porte, 1969). Epinephrine is probably responsible for the inhibitory effect of 2-deoxyglucose (Karam et al., 1966; Laszlo et al., 1961; Wegienka, Grasso and Forsham, 1966). There is paucity of information about the effect of epinephrine on glucagon secretion. When given via the portal vein, epinephrine was found to be a poor stimulator of hepatic glycogenolysis as compared with systemic administration. This has been interpreted to mean that epinephrine stimulates glucagon secretion

(Edzinli and Sokal, 1966).

It has been reported that corticotropin (ACTH) stimulates insulin secretion (Genuth and Lebovitz, 1965; Sussman and Vaughan, 1967). Cortisone was also found to elevate plasma insulin in hamsters (Campbell, Rastogi, and Hausler, 1966) and man (Klink and Estrich, 1964) and sheep (Bassett and Wallace, 1967). The mechanism of action of ACTH and cortisone appears to be different. Studies by Lebovitz and Pooler (1967a; 1967b) suggest that ACTH like glucagon exerts its effect by activating the adenyl cyclase system of the beta cell. Cortisone on the other hand stimulates insulin secretion by virtue of its ability to induce hyperglycemia. There are reports that ACTH and cortisone may stimulate the activity of pancreatic alpha cells (Cavallero and Mosca, 1953; Foa and Galansino, 1962). The significance of these observations is dubious because of lack of specificity of the methods used for the determination of glucagon.

Malaisse, Malaisse-Lagae and McCraw (1967) studied the effect of thyroxine on insulin secretion in rats. Thyroidectomy reduced insulin secretion. This was restored to normal by the administration of thyroxine (15 µg/kg). Treatment of normal rats with high doses of thyroxine (200 µg/kg) resulted in decreased secretion of insulin. However, in vitro studies showed no effect of thyroxine on insulin secretion. Thyroxine is thought to exert its effect on insulin secretion by enhancing glucose absorption.

It has been observed that insulin response to arginine is diminished in ateliotic dwarfs (Merimee, Burgess and Rabinowitz, 1967) but enhanced in

acromegalics (Fineberg et al., 1970). The latter authors concluded that the capacity of the pancreatic cells to release insulin is increased with prolonged exposure to excessive quantities of growth hormone (GH). These observations are in agreement with in vitro studies (Martin and Gagliardino, 1967) using pancreas from hypophysectomized and normal rats. However, GH probably does not have a direct effect on insulin secretion since in vitro incubation with the hormone does not result in enhanced insulin release (Bouman and Bosboom, 1965). Early reports that GH stimulated glucagon secretion (Abrams et al., 1953; Clarke et al., 1956) were later contradicted by the work of Sirek, Sirek and Best (1957). These workers found that the hyperglycemic factor released after treating dogs with growth hormone persisted after pancreatectomy. It was suggested that the hyperglycemic factor might be other substances like serotonin or catecholamines. It appears that although the anterior pituitary may have an indirect influence on the activity of the alpha cells and the secretion of glucagon, any evidence for a direct relationship between GH and glucagon secretion is, at best, circumstantial (Foa, 1964).

Estrogens (or gonadal steroids) appear to stimulate insulin secretion. Spellacy, Carlson and Birk (1967) observed higher insulin levels during glucose load in a group of women on oral contraceptive tablets (Enovid). Similar observations have been made in sheep and cattle fed diethyl stilbestrol (DES) (Trenkle, 1969). The mechanism of action is not known but it is thought to be mediated via its effect on growth hormone.

Oxytocin, vasopressin and angiotension II have been reported to affect insulin secretion (Frohman, 1969). The significance of these observations is unknown since it is difficult to distinguish physiologic from pharmacologic effects.

Neural regulation

The discovery of nerve cells in rat embryonic pancreatic bud (Pictet et al., 1969) and observations from numerous studies involving the use of epinephrine (reviewed above under "other hormones") have suggested that the nervous system may play a role in pancreatic cytodifferentiation and secretion. Parasympathomimetic or cholinergic agents stimulate insulin secretion (Kaneto et al., 1968) while the inhibitory effect of epinephrine is abolished by alpha-adrenergic blocking agents. Beta cell adrenergic receptors have been divided into two classes -- alpha and beta receptors. Alpha blockade stimulates and beta blockade inhibits insulin secretion. Conversely, stimulation of the alpha receptors inhibits and stimulation of the beta receptors stimulates insulin secretion (Porte, 1967; Gale et al., 1970). The inhibition of insulin secretion by epinephrine is mediated through the activation of alpha adrenergic receptors. It has been reported by Turtle and Kipnis (1967) that the stimulation of beta adrenergic receptors may involve the stimulation of adenyl cyclase.

Frohman et al. (1967) and Kaneto et al. (1967) have demonstrated that the parasympathetic system is involved in insulin secretion. Stimulation of the vagus was found to cause immediate release of insulin. However, the findings of Nelson et al. (1967) are at variance with the above

observation. Nelson and his coworkers found no increased insulin secretion due to vagal stimulation in dogs; instead they got a transient fall. They justified their results by arguing that since insulin hypoglycemia stimulates vagus function, it seemed inappropriate for vagal activity to augment insulin release further and thus perpetuate the hypoglycemic state.

By radioautography of tritiated norepinephrine Esterhuizen et al. (1968) have shown that some of the nerves between the beta cells in the cat contain norepinephrine granules. Cholinergic nerves surrounding the islet cells have also been identified (Daniel and Henderson, 1967). Stimulation of these nerves could result in very high local concentrations of norepinephrine or acetyl choline which would inhibit or stimulate insulin secretion as the case may be.

The central nervous system pathways controlling insulin secretion have not been completely identified but the ventral hypothalamus appears to be involved. Rats with ventromedial hypothalamic lesions have been shown to have enlarged islets or elevated insulin levels (Hales and Kennedy, 1964; Frohman et al., 1969; Han et al., 1970). It appears that normally the ventromedial region of the hypothalamus exerts inhibitory influences on the islets, since lesions result in enlargement of the islets and elevation of plasma insulin.

The effect of the nervous system on the secretion of glucagon has not received much attention. The rise in blood glucose observed after vagal stimulation strongly suggests that the vagus may influence glucagon secretion in the same manner as insulin secretion. This rise in glucose

is not blocked by phentolamine, an alpha adrenergic blocking agent (Frohman, Ezdinli and Javid, 1967).

The nervous system appears to be important but not absolutely necessary for the secretion of pancreatic hormones. Animals with transplanted denervated pancreas are able to maintain normal blood glucose for weeks (Ota et al., 1968).

Stress and exercise

In a study in which men were subjected to physical stress and exercise Schalch (1967) found no changes in plasma insulin levels despite elevated plasma glucose and FFA. Using rats, Wright and Malaisse (1968) have demonstrated that the effect of exercise on insulin secretion is mediated via epinephrine. Exercise or epinephrine injection reduced insulin secretion; and after blockade of the rats sympathetic system, exercise induced a fall instead of a rise in blood sugar concentration, an abnormally small increase in FFA and no increase in body temperature. They concluded that endogenous epinephrine released during stress or exercise is sufficient to suppress insulin secretion even under conditions or hyperglycemia.

STUDIES ON THE RADIOIMMUNOASSAY OF GLUCAGON

Principles

The principles of the radioimmunoassay of protein and peptide hormones have been reviewed by Berson et al. (1964). Essentially it is based on the competition between labeled and unlabeled hormone for antibody. The binding of antibody to I^{125}-labeled hormone is reduced or inhibited in the presence of unlabeled hormone; the degree of inhibition being progressively greater as the amount of unlabeled hormone is increased. If known concentrations of hormone are used a standard curve can be obtained by plotting percentage of labeled hormone bound to antibody against concentration of unlabeled hormone. The concentration of endogenous hormones in plasma is determined by comparison with the curve relating percentage labeled hormone bound to concentration of hormone in known standard solutions.

There are several variations of the radioimmunoassay of peptide hormones depending on the specific hormone or techniques for separating free hormone from hormone-antibody complex. There are, of course, other little details like reagents, temperature and duration of incubation which vary from one laboratory to the other. The studies to develop a radioimmunoassay for glucagon were patterned after a modification (Trenkle, 1968) of Herbert's coated charcoal method for insulin (Herbert et al., 1965). In the modified procedure, antibody to bovine insulin produced in guinea pigs is incubated in sodium barbital buffer pH 8.6 for five days at $4^{o}C$ with solutions of standard bovine insulin or the plasma samples being assayed before addition of I^{125}-insulin. The tubes are incubated for

additional two days at 4°C before addition of 1.5 ml of a suspension of coated charcoal (7.5g Norit A, 0.75g dextran-80 and 3g bovine serum albumin per 100 ml of the pH 7.4 acetate-barbital buffer defined by Herbert et al. (1965)). After an adsorption time of 1 - 2 hours the tubes are centrifuged at 4°C for twelve minutes at the speed of 2000 g. The supernatant containing insulin-antibody complex and degraded insulin is removed by aspiration and the radioactivity adsorbed to charcoal (or the labeled insulin no bound to antibody) counted in an automatic gamma counter.

Investigations

Materials and method

Antibody production Three rabbits weighing 2.4 to 2.8 kg and four guinea pigs weighing 420 to 470g were immunized at monthly intervals for the first five months by subcutaneous injections of glucagon preparation as described by Unger et al. (1961). The rabbits received 1 mg glucagon per animal and the guinea pigs 0.5 mg per animal at each immunization. Because no detectable antibody was found in these animals during the first five months, the doses and schedule of injection were changed. The guinea pigs were placed on 1 mg per animal per injection and the immunization interval reduced to two weeks. The rabbits were continued on 1 mg per animal but at the new schedule. After five more injections, the animals with low antibody titer were abandoned. Those with reasonably high titer were immunized again for the last time and bled twice, ten and thirteen days later. Plasma from these animals was diluted 1:10 with barbital buffer

pH 8.6 containing 2.5 mg bovine serum albumin per ml and frozen.

Glucagon (Calbiochem, Lot No. 72669) for injection was prepared by suspending the hormone in an emulsion of saline and complete Freund's adjuvant (1/1 V/V) to a final concentration of 1 mg per ml. Antibody production from each animal was studied by bleeding the animals ten days after each injection beginning from the third injection. The plasma or its dilution was incubated with a known amount of I^{125}-glucagon for two days at $4^{o}C$. At the end of this period free I^{125}-glucagon was separated from antibody-bound I^{125}-glucagon by adsorption to the coated charcoal described previously. Reduction in free I^{125}-glucagon was regarded as an indication of antibody formation.

The feasibility of constructing a standard curve was examined by the addition of different quantities of unlabeled glucagon to the reaction mixtures and noting the corresponding decrease in the binding of I^{125}-glucagon.

Since commercial glucagon may contain insulin as an impurity the presence of antibody to insulin was also tested by incubating the antibody preparation with I^{125}-insulin. When antibody to insulin was detected in the preparation, the cross reaction between I^{125}-insulin and antibody to glucagon, and I^{125}-glucagon and antibody to insulin was studied. Two approaches were followed: (1) known amounts of I^{125}-glucagon and I^{125}-insulin were added separately and in combination to assay tubes containing different dilutions of the antibody preparation. The tubes were incubated for three days and the total activity bound to antibody was determined.

(2) Progressively increasing concentrations of unlabeled insulin were added to tubes containing constant amounts of the antibody preparation and I^{125}-glucagon to study the ability of unlabeled insulin to inhibit the binding of I^{125}-glucagon.

Glucagon or insulin was iodinated with I^{125} by the chloramine-T method of Greenwood and Hunter (1963). The labeled hormones were purified on cellulose column as described by Berson and Yalow (1961).

Assay conditions Optimum conditions for assaying glucagon using the modified coated charcoal method described for insulin was investigated.

The rate of the binding reaction between antibody and I^{125} glucagon was studied by setting up several tubes containing constant amounts of antibody and I^{125}-glucagon and incubating at $4^{o}C$ over varying lengths of time. The degree of binding at these periods was determined. The amount of antibody used was such that only about 50 percent of the radioactivity added could be maximally bound. The pH of both the barbital (or incubation) and the acetate-barbital (or charcoal) buffers was varied and the resultant effect on binding measured.

The time required for the complete adsorption of all free glucagon to coated charcoal could be a critical factor in the assay. This was studied by adding coated charcoal suspension to tubes of four sets of standard curve assays. The four sets were limited to 5, 10, 15, or 20 minutes adsorption time respectively.

Glucagon is readily degraded by proteinases in plasma (Hazzard et al., 1968; Unger et al., 1961). The effect of adding proteinase inhibitors to

standard curve and recovery assays containing plasma was studied by using trasylol and soybean trypsin inhibitor (SBTI). The ability of the assay to give repeatable plasma concentrations was also studied. Three lambs were bled and plasma from each animal divided into three portions and stored frozen. The portions were assayed on different days. The effect of plasma volume assayed on glucagon concentration (expressed in ng/ml) was also investigated.

Results and discussion

Antibody production After the fifth immunization, no detectable antibody titer was found in either the rabbits or the guinea pigs. However, when the dose of glucagon was increased and the interval between injections shortened to two weeks, detectable titers were observed; but it was not until after the tenth immunization that a reasonably high titer was found in one of the guinea pigs. Table 1 shows different dilutions of plasmas from guinea pig no. 31 and their binding capacity. For assays, 0.1 ml of 1:200 dilution of the plasma was used. The 1:200 dilution was chosen because it was the highest dilution that could bind about 50 percent of I^{125}-glucagon activity. The antibody production in this study was poor. This may have been due to four factors -- method of preparation of antigen, method of administration, dose and time between immunizations. Studies by Assan et al. (1965) and Worobec et al. (1967) indicate that satisfactory antibody production is possible only when (1) glucagon is stabilized by conjugation to serum albumin or adsorbed on to

Table 1. Antibody dilutions and percentage of I^{125}-glucagon and I^{125}-insulin activities bound

Dilutions	% I^{125}-glucagon activity bound	% I^{125}-insulin activity bound
1:10	61.9	87.4
1:100	56.2	84.8
1:200	50.2	83.1
1:500	31.3	78.8
1:1000	13.7	68.9

polyvinylpyrrolidone or beeswax (2) higher doses of glucagon are used (3) animals are immunized every two days or every week, and (4) glucagon is administered intramuscularly.

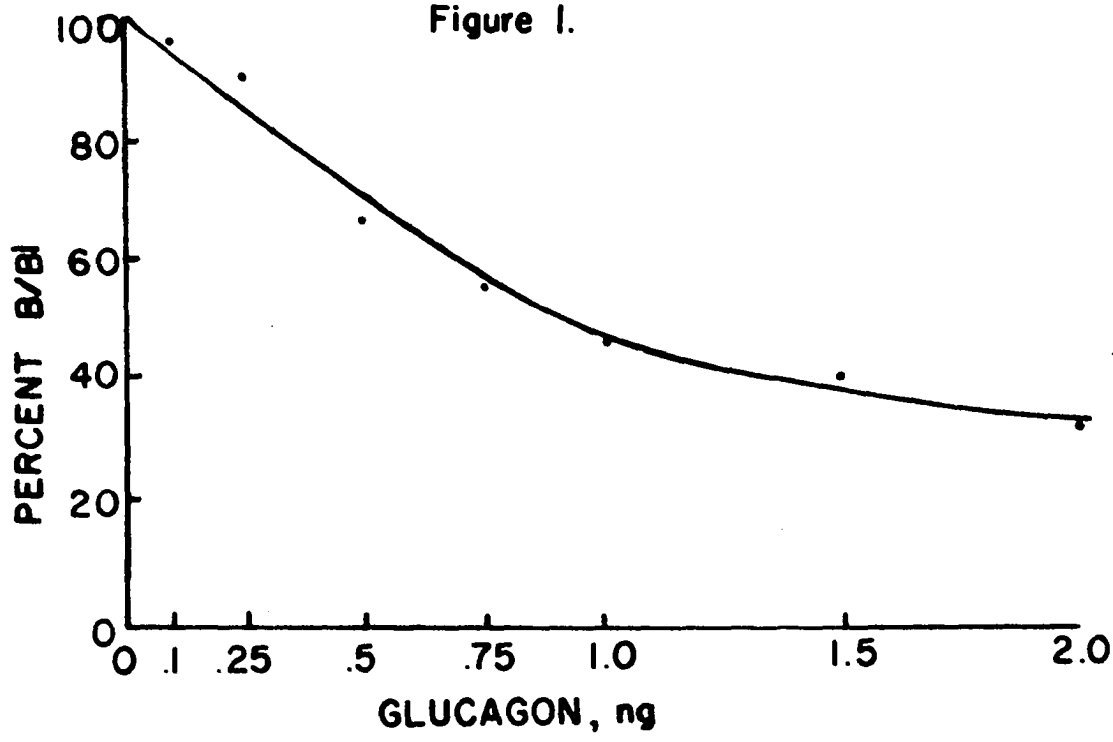
Figure 1 shows a standard curve which demonstrates the specificity of the binding between antibody and I^{125}-glucagon. As the quantity of unlabeled glucagon added to the reaction mixture increased, the binding of I^{125}-glucagon decreased. Beyond 1.5 ng the curve flattens out. So for assaying plasma samples, standards of 0.1 to 1.5 ng were used.

As suspected, the antibody preparation contained antibody to insulin (Table 1). In fact the insulin antibody titer was higher than that of glucagon. The main reason for this is that insulin is a more antigenic molecule. Table 2 and Figure 2, however, show that neither the presence of insulin antibody nor the addition of I^{125}-insulin interferes with the specificity of the binding between antibody to glucagon and I^{125}-glucagon. If I^{125}-glucagon and I^{125}-insulin were bound to the same antibody one would expect the total activity bound in column C to be less than the sum

Figure 1. Glucagon standard curve (B^I equals the radioactivity bound to antibody when no unlabeled hormone was added and B is the activity bound to antibody in the standard or sample tubes)

Figure 2. The ability unlabeled insulin and glucagon to compete with I^{125}-glucagon for glucagon antibody

Figure 1.



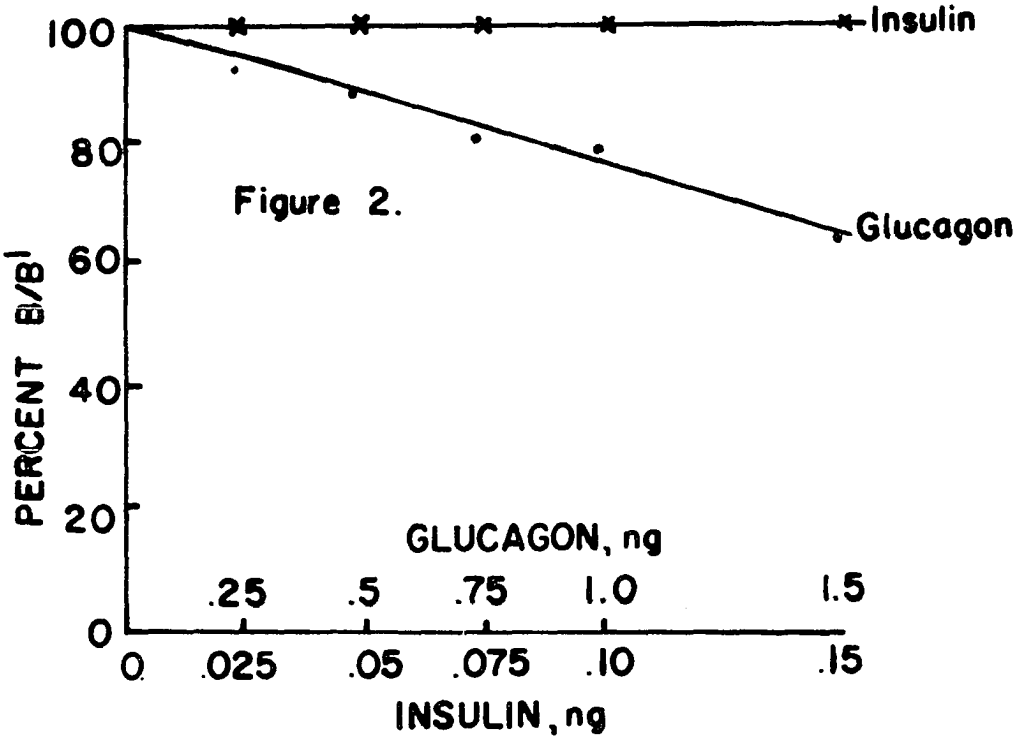


Figure 2.

of columns A and B (i.e., column D). But the results show that the total activity bound in column C equals the sum of A and B. This additivity in activity shows that the hormones are independently bound to different antibodies. The noninterference of insulin or its antibody in the glucagon binding reaction is further illustrated in Figure 2, which shows that unlabeled insulin does not inhibit the binding of I^{125}-glucagon to antibody to glucagon.

Table 2. Specificity of I^{125}-glucagon for antibody to glucagon

Treatments		Total activity bound (cpm) A I^{125}-glucagon	B I^{125}-insulin	C I^{125}-glucagon +I^{125}-insulin	D Sum of counts in A and B
Antibody dilutions	1:10	649.0	1584.1	2212.7	2233.1
	1:100	606.9	1537.3	2163.2	2144.2
	1:200	609.4	1507.4	2117.9	2116.8
	1:500	516.6	1428.5	1922.1	1945.1
	1:1000	355.9	1250.0	1547.3	1605.9

Assay conditions Figure 3 shows that the binding reaction between I^{125}-glucagon and antibody to glucagon is fast and about complete within six hours of incubation. About 83 percent of the maximum binding had taken place during this period. Thereafter, binding increased at a progressively slower rate. Since the completion of the reaction is not necessary for establishing a standard curve, it would seem that a twelve hour incubation time could be adequate. However, for routine assays, an

Figure 3. Rate of the binding reaction between I^{125}-glucagon and glucagon antibody (percent I^{125}-glucagon bound to antibody vs. time)

Figure 4. The effect of charcoal adsorption time on the standard curve

Figure 3.

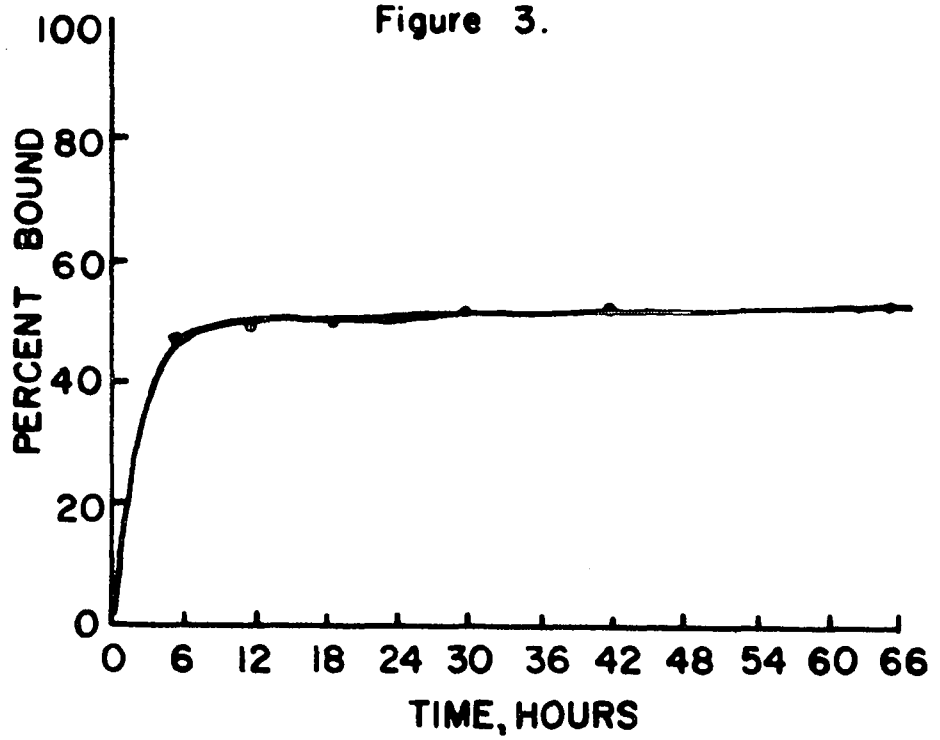
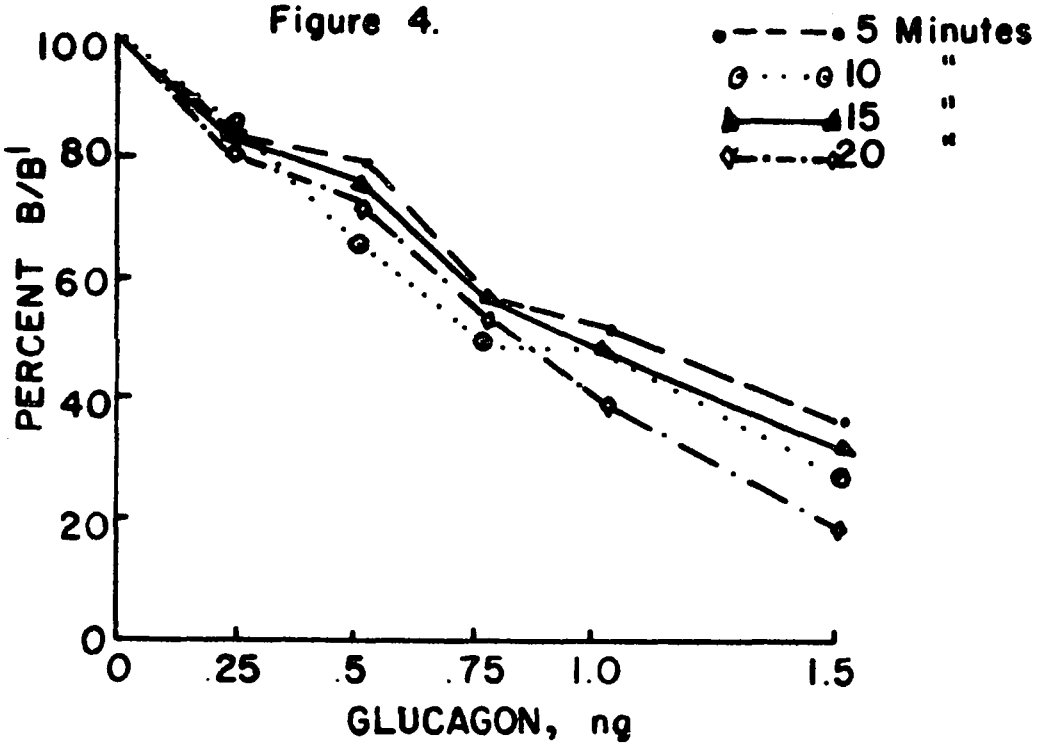


Figure 4.



incubation time of 48 hours was allowed. This practice was justified by the observation that such lengthy incubations did not result in any appreciable degradation of I^{125}-glucagon.

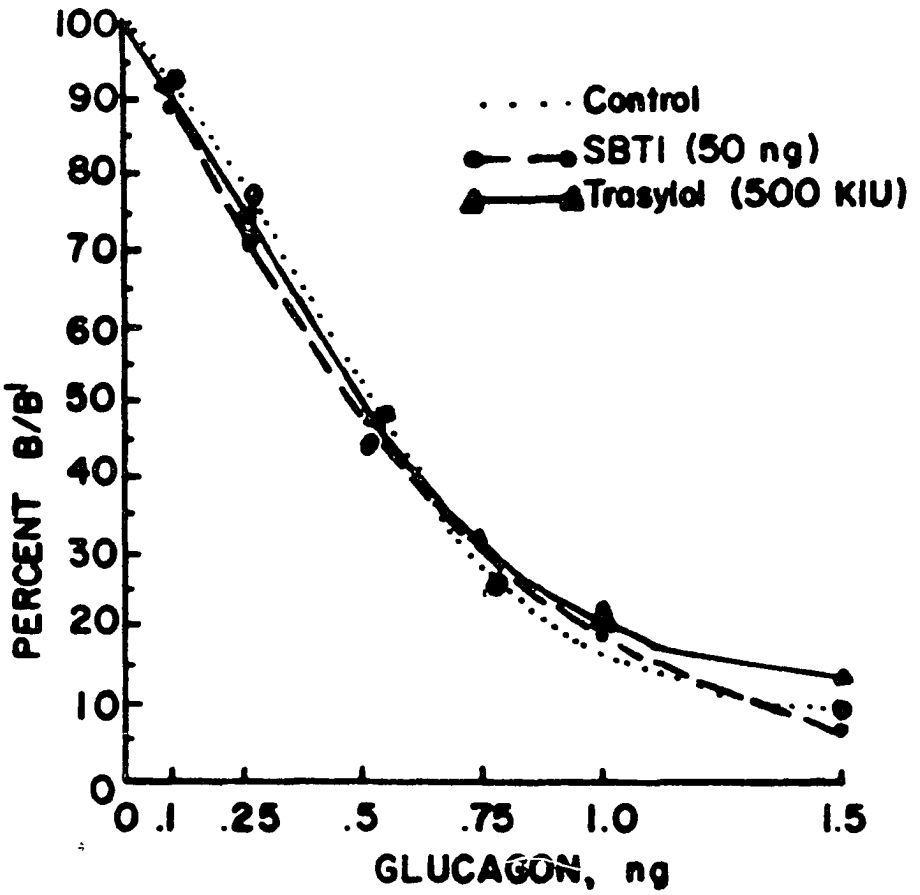
Varying the pH of either the incubation or charcoal buffer between 8 - 8.6 and 7.4 - 8.6 respectively had no appreciable effect on the binding or adsorption reactions (Table 3). The combination of incubation buffer pH 8.6 and charcoal buffer pH 7.4 which produced the best binding was adopted.

Table 3. Effect of pH on radioactivity bound to antibody

Charcoal buffer pH	Total activity bound (cpm) Incubation buffer pH 8.0	8.6
7.4	787	857
8.0	815	838
8.6	818	821

The adsorption of free glucagon to coated charcoal appeared to be instantaneous. There was practically no difference between five minutes and twenty minutes adsorption time. However, allowing the adsorption to take place for fifteen minutes or more resulted in a smooth standard curve (Figure 4). In other words, the points lie on or very close to the curve. Adsorption time of twenty minutes or more was used in routine assays.

Figure 5. The effect of proteinase inhibitors on the standard curve


100
90
80
70
60
50
40
30
20
10
0
PERCENT B/B¹
0 .1 .25 .5 .75 1.0 1.5
GLUCAGON, ng
Control
SBTI (50 ng)
Trasylol (500 KIU)

Trasylol and SBTI had no significant ($p>0.05$) effect on the standard curve (Figure 5). Apparently there was no appreciable degradation of I^{125}-glucagon when incubated in buffer in the absence of plasma. However, when unlabeled glucagon was added to plasma, recovery was very poor, thus indicating some degradation. Recovery was improved by the addition of 1000 ng SBTI (Table 4). Observations by Hazzard et al. (1968) suggest that endogenous glucagon is not susceptible to similar degradation except, perhaps, when samples have not been stored frozen or have been subjected to

Table 4. The effect of SBTI on the recovery of unlabeled glucagon added to plasma

Exogenous glucagon (ng) added to 0.2 ml plasma	Percent recovery	
	Control	1000 ng SBTI
0.10	60	70
0.25	64	72
0.50	51	62
0.75	46	52

frequent freezing and thawing. In assaying plasma samples, the major problem is usually the degradation of labeled hormone since endogenous glucagon does not seem to undergo marked degradation. This problem, however, does not seem to be real when plasma samples assayed do not exceed 0.2 ml. In any case, unlike the double antibody method, the charcoal method offers a way of estimating I^{125}-glucagon degradation or "serum damage". This is done by adding control tubes containing plasma

and all other components of the reaction mixture, except glucagon antibody. It appears, therefore, that with the charcoal method, reasonably accurate data could be obtained without adding trasylol or any other proteinase inhibitor to plasma samples. Perhaps glucagon assays can best be improved by using high dilutions of high titer antibody and labeled glucagon of high specific activity.

Figure 5 shows a comparison of the glucagon concentrations of the same plasma samples assayed on three different dates. The difference between concentrations of the same sample estimated on different dates was small. However, the relative differences between samples were maintained. These observations indicate that under similar conditions, the coated charcoal radioimmunoassay method can be expected to give consistent and reproducible results.

Table 5. Variability of glucagon measurements

	Glucagon concentration ng/ml			
Sample No.	Day 1	Day 2	Day 3	Mean
85	0.550	0.500	0.500	0.517 ± 0.016
99	1.000	1.075	0.925	1.000 ± 0.043
299	0.700	0.650	0.650	0.666 ± 0.017
579	0.450	0.450	0.475	0.458 ± 0.014

Glucagon concentrations (ng/ml) obtained by assaying different volumes of the same plasma sample are shown in Table 6. The smallest volume, 0.1 ml, gave the highest concentration of 0.80 ng/ml which was about one and three-fifths times the concentration of glucagon as assayed from 0.2 ml and 0.4 ml of plasma. The 0.2 ml and 0.4 ml volumes gave reasonably similar results. The concentration of glucagon in 0.1 ml of plasma was found to be less than 0.1 ng which was the lowest standard used in the assay. Thus the concentration assigned to the 0.1 ml volume was determined from extrapolation and probably explains the exaggerated value. The use of 0.2 ml sample volume in routine assays was adopted.

Table 6. The effect of plasma volume assayed on glucagon concentration (ng/ml)

Volume of plasma	Glucagon concentration, ng/ml
0.1 ml	0.800
0.2 ml	0.550
0.4 ml	0.475

Assay Procedure

As a result of the preceding investigations a procedure for the routine assay of sheep plasma glucagon by the modified coated charcoal immunoassay was established. This is summarized in the flow diagram shown in Figure 6.

Figure 6. A flow diagram for the modified coated charcoal glucagon immunoassay

0.20 ml glucagon standard or plasma
+ 0.25 ml barbital buffer (containing bovine serum albumin, 2.5 mg/ml)
+ 0.1 ml antibody preparation 1:200 dilution (sufficient to bind approximately 50 percent of the labeled glucagon in the absence of competing unlabeled hormone)
Mix

$4^{o}C$ ↓ 48 hrs.

+ 0.05 ml I^{125}-glucagon (with approximate activity of 2000 cpm)
Mix

$4^{o}C$ ↓ 24 hrs.

+ 0.2 ml normal plasma added to tubes containing no plasma; and 0.2 ml barbital buffer added to plasma tubes.
+ 1.0 ml coated charcoal suspension (7.5 g Norit A, 0.75 g dextran-80 and 3 g bovine serum albumin per 100 ml of the pH 7.4 acetate-barbital buffer)
Mix

$4^{o}C$ ↓ 1 hr.
(adsorption of free glucagon)

Centrifuge
$4^{o}C$, 2000g, 12 mins.

Supernatant (discard) | Charcoal (count)

STUDIES ON THE EFFECTS OF SOME NUTRITIONAL FACTORS ON THE SECRETION OF INSULIN AND GLUCAGON IN SHEEP

Experimental Procedure

Experiment 1. The effect of increasing levels of glucose, propionate and butyrate on insulin secretion

A 45.5 kg lamb with a catheter in the jugular vein was used for this study. The treatments and experimental design are shown in Table 7.

Table 7. Experimental design for Experiment 1

Periods	Period I	Period II	Period III
Treatments	Glucose	Propionate	Butyrate
	0.3 mM/kg BW	0.5 mM/kg BW	0.25 mM/kg BW
	0.6 mM/kg BW	1.0 mM/kg BW	0.5 mM/kg BW
	1.0 mM/kg BW	2.0 mM/kg BW	1.0 mM/kg BW

During each period, the order in which the different levels were administered was randomly determined. After an overnight fast, the lamb was given an intravenous injection of the predetermined treatment and heparinized blood samples obtained at -30, 0, 10, 20, and 30 minutes after injection. The metabolites were made up in 40 ml volumes and injected over a five minute period. Glucose was dissolved in saline while propionate and butyrate were diluted in water and neutralized to pH 7.4.

During each period, an interval of one day was allowed between treatments. A two day interval was allowed between periods.

During collection, the blood samples were chilled in ice or placed in a refrigerator at 4°C and later centrifuged at 4°C and 11000 g for twenty minutes and the plasma frozen until analyzed for insulin and glucose. All blood samples in other experiments were handled and processed in this way. Insulin was analyzed by the modified coated charcoal immunoassay method described previously. Glucose was analyzed by the Technicon Auto-Analyzer method.

The lamb was fed 0.91 kg per day of a 50 percent grain diet. The diet was composed of 35 percent cracked shelled corn; 10 percent ground corn cobs; 40 percent ground alfalfa hay; 6 percent soybean meal; 8 percent molasses, and 1 percent trace mineralized salt. Except where otherwise stated, all lambs used in other experiments were maintained on this diet.

Experiment 2. The effect of different diets on rumen volatile fatty acids and plasma insulin levels

The purpose of this experiment was to relate changes in rumen volatile fatty acids resulting from different diets to changes in plasma insulin levels. Six lambs (three pairs) with an average weight of 30 kg were used in a 3 X 3 Latin square design to study the effects of three diets on rumen volatile fatty acid patterns and plasma insulin levels. The lambs were kept in metabolism crates and fed 0.727 kg per day of 80 percent grain diet or 0.91 kg per day of 50 percent grain diet or 1.14 kg per day of hay.

The rations were approximately isonitrogenous and isocaloric. The animals were maintained on one diet for three weeks at the end of which they were fed a different diet. The first two weeks on each diet served as an adjustment period. During the third week, jugular blood and rumen fluid samples were obtained on two alternate days, four hours after feeding.

Rumen samples taken for volatile fatty acid analysis were obtained by means of a stomach tube (Raun and Burroughs, 1962) and prepared as described by Cottyn and Boucque (1968). The apparatus used was a dual column Aerograph, 1520 gas chromatograph with hydrogen flame ionization detectors and columns packed with twenty percent neopentyl-glycylsuccinate, two percent H_3PO_4 on 60-80 mesh firebrick. The carrier gas was nitrogen. The temperatures were 195°C, 160°C and 205°C at the injectors, column oven, and detectors, respectively.

The blood samples were prepared and analyzed for glucose and insulin as previously described.

The hay fed in this experiment was in the form of long, baled alfalfa hay. The 50 percent grain diet was of the same composition as that described in Experiment 1. The composition of the 80 percent grain diet was 20 percent ground alfalfa hay; 6 percent soybean meal; 65 percent rolled corn; 8 percent molasses; and 1 percent trace mineralized salt.

Experiment 3. The effect of feeding on rumen volatile fatty acid concentrations and plasma insulin and glucagon levels in sheep

In Experiment 2, varying the level of grain in the diet was found to

have little or no effect on either proportions of rumen volatile fatty acids or plasma insulin levels. This was probably due to restricted feed intake and the fact that the animals on the hay diet tended to select the leaves and to reject the stems. The purpose of this experiment was to further study the possible role of rumen produced volatile fatty acids on insulin secretion by measuring volatile fatty acid production and plasma insulin levels after feeding.

Four lambs with an average weight of 36 kg and previously trained to consume all their daily ration of 0.91 kg at one time were used in this study. The animals were fed and rumen fluid samples obtained at -2, -1, 0, 1.5, 3, 5, 7, 12, and 24 hours post prandial. Two days later, jugular catheters were placed in each animal and blood samples obtained at the same time intervals after feeding. The rumen fluid and blood samples were prepared for analysis as previously described. The blood samples were analyzed for insulin, glucagon, free fatty acids and glucose. Plasma free fatty acid levels were determined by the method of Ko and Royer (1967). The other parameters were analyzed as previously described.

Experiment 4. The effect of feeding on insulin secretion in milk-fed calves

The main purpose of this experiment was to study the effect of feeding and starvation on insulin secretion in milk-fed, non-ruminating ruminants. Three-month-old calves with an average weight of 82 kg and maintained on milk from birth were used in this study. After an overnight fast the animals were each fed 450 g of Doughboy Calf Milk Replacer (Formula 308). This "formula" contained 20 percent crude protein and 20 percent fat.

Blood samples were obtained by means of catheters placed in the jugular veins at 0, 0.5, 1, 1.5, 2, 2.5, 3, 6, 12 and 24 hours after feeding. After 24 hours fasting, the animals were fed again and the last samples obtained one hour later. The blood samples were analyzed for insulin, glucose and FFA as previously described.

Experiment 5. The effect of intravenous infusion of casein hydrolysate on insulin and glucagon secretion

The primary purpose of this experiment was to study the effect of amino acids contained in casein hydrolysate on insulin secretion. A preliminary experiment was run in which the effect of a 5 percent solution (w/v) was studied. The casein hydrolysate (General Biochemicals, Chagrin Falls, Ohio, Lot No. 83027) was dissolved in 1N NaOH and neutralized to pH 7.4 with HCl; distilled water was added to make up the volume. A 26.3 kg lamb had catheters placed in both right and left jugular veins. One catheter was used for infusion and the other for withdrawing blood. The solution was infused for one hour after an overnight fast at the rate of 3 mls per minute by means of a peristaltic infusion pump (Harvard Apparatus Co., Dover, Mass., Model 600-120). Blood samples were obtained at -30, 0, 15, 30, 45, 60, 75, 90, 105 and 120 minutes after the beginning of the infusion. The experiment was repeated five days later.

Plasma insulin and glucose were measured as described previously. Plasma alpha amino nitrogen was measured by the method of Spackman, Stein and Moore (1958) modified for determination with the Technicon-Auto-Analyzer.

Hydrindantin was substituted for stannous chloride as the reducing agent in the ninhydrin reagent.

After examining the data obtained from this preliminary study it was concluded that the amino acid concentration in the 5 percent casein hydrolysate solution was not high enough to stimulate insulin secretion. Another experiment involving four lambs with an average weight of 40 kg was conducted. A 10 percent casein hydrolysate solution was used in this experiment which was carried out essentially in the same manner as the preliminary study. Blood samples were obtained at -30, -15, 0, 15, 30, 45, 60, 90 and 120 minutes after the beginning of the infusion of the test solution. The main difference was that saline was infused for 45 minutes during the control period. This means that the first three samples taken at -30, -15 and 0 minutes were obtained during saline infusion. Plasma glucagon was measured in addition to the other factors measured in the preliminary experiment.

Experiment 6. The effect of oleic acid on insulin and glucagon secretion

Two lambs with an average weight of 35 kg were fasted overnight and injected intravenously with 20 ml emulsion of 1.5 ml oleic acid in 30 ml of plasma; the pH was adjusted to 7.4. The plasma used had previously been obtained from the respective animals. Blood samples were obtained by means of jugular catheter at -30, -15, 0, 15, 30, 45, 60, 90 and 120 minutes after injection. The samples were analyzed for free fatty acids, glucose, insulin and glucagon.

Experiment 7. The effect of butyrate on glucagon secretion

Four lambs with an average weight of about 39 kg had catheters placed in their jugular veins. After an overnight fast they were injected intravenously with a 20 ml solution of butyric acid (1 mM per kilogram body weight) neutralized to pH 7.4 with sodium hydroxide. Heparinized blood samples were obtained at -30, -15, 0, 15, 30, 45, 60, 90, and 120 minutes after injection. The samples were analyzed for glucose, insulin and glucagon as previously described. The experiment was repeated using two lambs. This time blood samples were obtained at shorter intervals: -10, -5, 0, 5, 10, 15, 20, 25 and 30 minutes after injection.

Experiment 8. The effect of glucagon on plasma metabolites and insulin secretion

Glucagon (1 mg suspended in 10 ml of saline) was intravenously injected into two lambs weighing 33 and 43 kg, respectively. The lambs were fasted overnight prior to the inception of the experiment and blood samples were obtained by means of jugular catheters at -10, -5, 0, 5, 10, 15, 20, 25, 30 and 40 minutes after injection. The samples were analyzed for glucagon, insulin, glucose, urea nitrogen, alpha amino nitrogen, and free fatty acids. Urea nitrogen was measured by the Technicon Auto-Analyzer method. Other parameters were analyzed as previously described. Glucagon fractional turnover rate and half life were also estimated.

Results and Discussion

Experiment 1. The effect of increasing levels of glucose, propionate and butyrate on insulin secretion

The results are shown in Table 8. Plasma insulin levels increased as the concentrations of glucose, propionate and butyrate were increased ($p < 0.05$). On a molar basis, butyrate was more insulinogenic than either glucose or propionate. Plasma glucose changes were also proportional to the concentrations of propionate and butyrate injected ($p < 0.05$). Glucose injection resulted in higher plasma glucose levels which did not vary to any great extent with the concentration of glucose injected. Glucose level *per se* does not appear to be the main factor regulating insulin secretion induced by propionate and butyrate. Glucose injection resulted in higher plasma glucose concentrations and less insulin secretion in contrast to lower glucose concentrations and more insulin secretion from propionate and butyrate. This suggests that propionate and butyrate may also have a direct effect on the pancreas. The reason for the high potency of butyrate in provoking insulin secretion is unknown.

The mechanism of hyperglycemia from propionate and butyrate appears to be different. Propionate is converted to glucose through a pathway involving methyl malonyl CoA and subsequent conversion to succinate. The metabolism of butyrate on the other hand does not yield glucose (Armstrong, 1965). Phillips, Black and Moller (1965) observed that butyrate infusion activated liver phosphorylase and depleted liver glycogen. This suggests that the hyperglycemic effect of butyrate may

Table 8. The effect of different concentrations of glucose, propionate and butyrate on plasma insulin and glucose levels

GLUCOSE	0.3 mM/kg BW[a]	0.6 mM/kg BW	1.0 mM/kg BW
Plasma Glucose mg/100 ml[b]	120.3	142.7	143.4
Plasma insulin ng/ml[b]	1.65	1.96	5.07
PROPIONATE	**0.5 mM/kg BW**	**1.0 mM/kg BW**	**2.0 mM/kg BW**
Plasma glucose mg/100 ml[b]	82.9	105.4	131.2
Plasma insulin ng/ml[b]	4.44	10.77	17.28
BUTYRATE	**0.25 mM/kg BW**	**0.5 mM/kg BW**	**1.0 mM/kg BW**
Plasma glucose mg/100 ml[b]	82.2	112.1	149.1
Plasma insulin ng/ml[b]	7.60	10.31	32.05

[a]BW is the abbreviation for body weight.

[b]Values are the means for samples taken at 10, 20 and 30 minutes after injection of test solution.

be brought about by glycogenolysis.

Experiment 2. The effect of different diets on rumen volatile fatty acids and plasma insulin levels

Increasing the level of grain in the diet had no significant effect on either the plasma insulin or plasma glucose levels (Table 9). The effect of the diets on acetate production was highly significant ($p < 0.01$).

The difference was due to the hay diet which resulted in a relatively high acetate concentration. The acetate values for the 50 percent and 80 percent grain diets were similar but lower than for the hay diet. Propionate and butyrate production were not significantly affected by dietary treatment. Comparison of the means for the different treatments indicated that the mean propionate production from the hay diet was greater ($p < 0.05$) than the mean propionate production from the 50 percent grain diet but not different from the 80 percent grain diet. Butyrate concentration tended to be higher in the grain diets.

On the whole, the hay diet resulted in more volatile fatty acid production than the grain diets. Similar observations were made by Luther (1964). The failure to show a direct relationship between level of grain and propionate production may have been due to two factors. Firstly, the hay diet was in the form of long hay and the lambs tended to select the leaves and tender parts, leaving the stems. Secondly, the diets were fed in restricted amounts. The lack of effect of the dietary treatments on propionate and butyrate was reflected on the insulin levels.

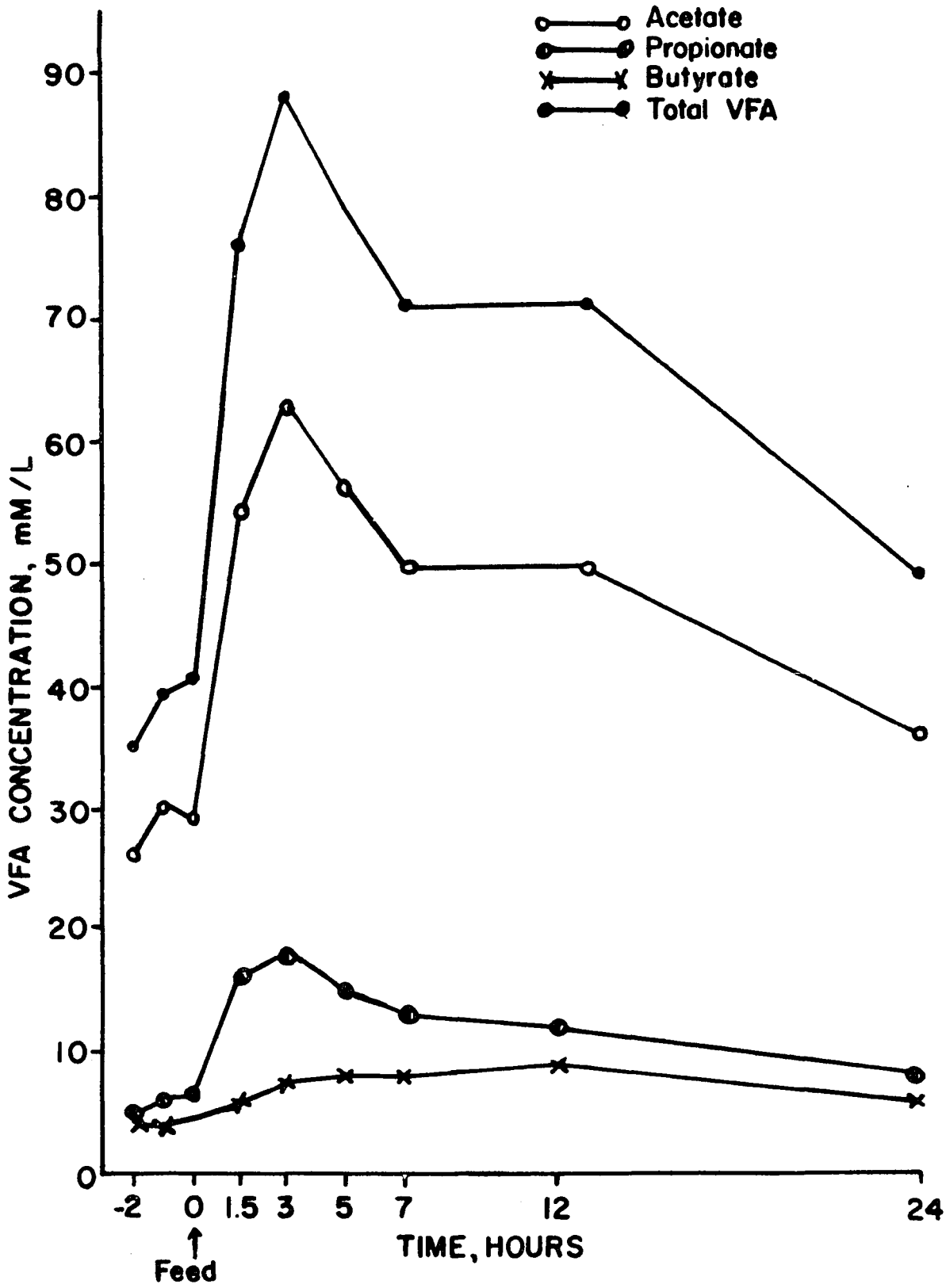
Table 9. The effect of different diets on rumen volatile fatty acids and plasma insulin levels

	Hay	50% grain	80% grain
No. of lambs	6	6	6
Plasma insulin ng/ml	0.53	0.49	0.38
Plasma glucose mg/100 ml	70.6	72.27	70.7
Rumen acetate mM/L	64.72	42.05	43.4
Rumen propionate mM/L	17.15	10.28	14.73
Rumen butyrate mM/L	4.9	5.7	7.7

Experiment 3. The effect of feeding on rumen volatile fatty acid concentrations and plasma insulin and glucagon levels in sheep

The mean rumen volatile fatty acid concentrations are shown in Figure 7. Following feeding, there was a rise in the concentration of acetate, propionate and butyrate ($p < 0.01$). Acetate concentrations reached the highest level three hours after feeding and then declined to a plateau between seven and twelve hours after feeding. By twenty-four hours post-prandial the levels had declined to about pre-feeding concentrations. The changes in propionate concentration paralleled those of acetate. The peak level also occurred three hours after feeding; however, there was no conspicuous plateauing between seven and twelve hours post-prandial. The rise in butyrate concentration was more gradual and the highest level was reached twelve hours after feeding. The butyrate

Figure 7. Effect of time after feeding on rumen volatile fatty acid (VFA) concentrations in adult sheep


Acetate
Propionate
Butyrate
Total VFA
VFA CONCENTRATION, mM/L
0
10
20
30
40
50
60
70
80
90
-2
0
1.5
3
5
7
12
24
TIME, HOURS
Feed

pattern is perhaps a reflection of rapid absorption and utilization by the rumen wall.

The effects of feeding on plasma hormones and metabolites are shown in Figure 8. There was little change in the mean glucose levels following feeding. The mean plasma free fatty acid levels declined after feeding to reach their lowest concentrations between three and five hours and then to rise to a significantly high level ($p < 0.05$) at twenty-four hours after feeding. The mean insulin levels increased following feeding and then dropped as fasting progressed. The increase achieved significance ($p < 0.05$) only at 1.5 hours after feeding. Plasma glucagon levels showed no significant changes.

Since volatile fatty acid absorption varies directly with concentration it perhaps can be assumed that blood acetate, propionate and butyrate levels paralleled rumen concentrations. Schambye (1951) observed that portal blood concentrations of volatile fatty acids varied according to rumen concentration. The elevated insulin levels can therefore be attributed to the volatile fatty acids and other nutrients.

Experiment 4. The effect of feeding on insulin secretion in milk-fed calves

Figure 9 shows the effect of feeding on plasma glucose, insulin and free fatty acids in milk-fed calves. Feeding markedly elevated plasma glucose and insulin levels ($p < 0.01$). By thirty minutes after feeding plasma glucose had risen from 54 mg/100 ml to 164 mg/100 ml. This rise continued until two hours after feeding. Thereafter, glucose levels

Figure 8. Effect of time after feeding on plasma glucose, free fatty acids (FFA), insulin and glucagon concentrations in adult sheep

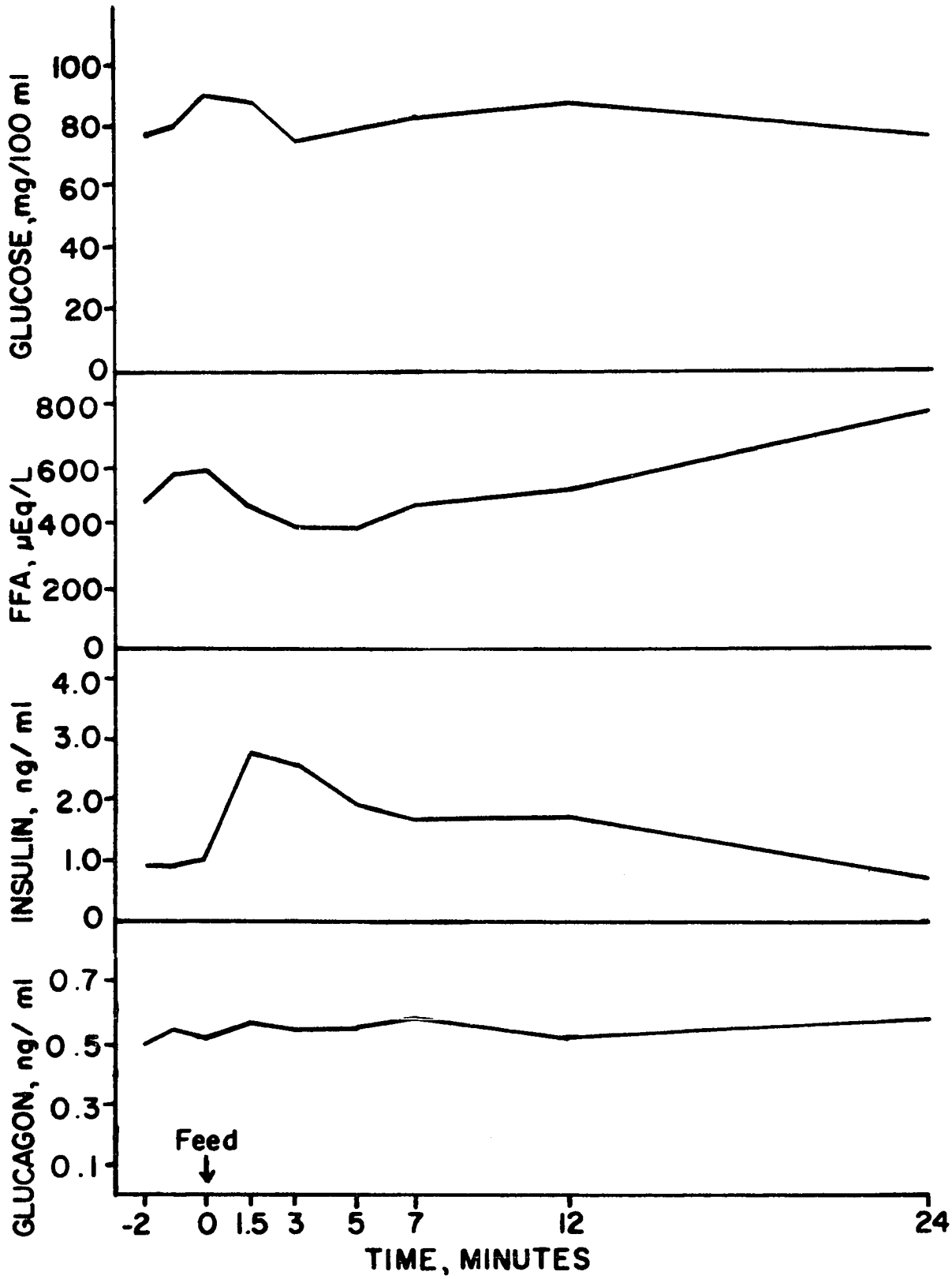
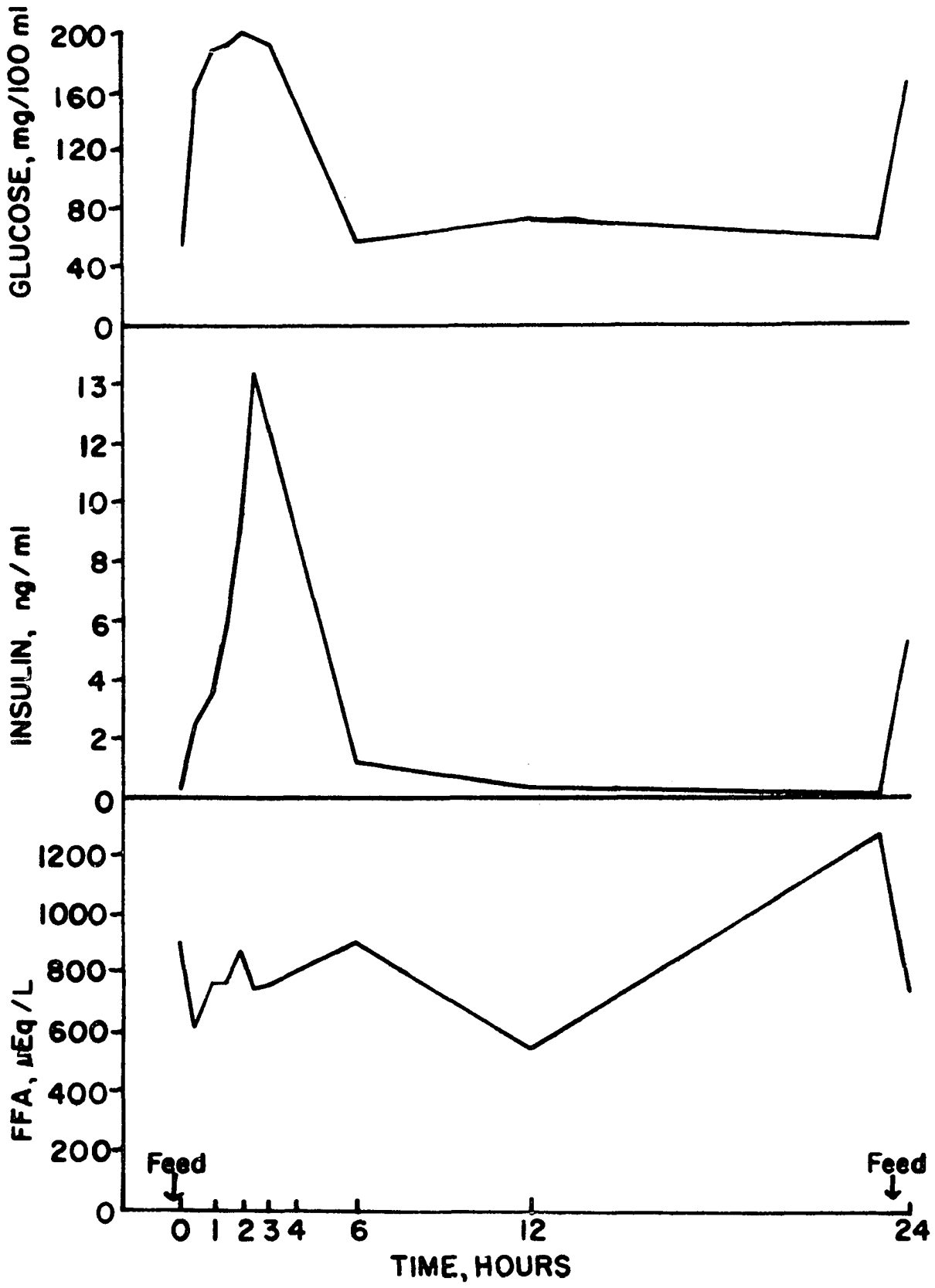

GLUCOSE, mg/100 ml
100
80
60
40
20
0
FFA, μEq/L
800
600
400
200
0
INSULIN, ng/ml
4.0
3.0
2.0
1.0
0
GLUCAGON, ng/ml
0.7
0.5
0.3
0.1
Feed
-2
0
1.5
3
5
7
12
24
TIME, MINUTES

Figure 9. Effect of time after feeding on plasma glucose, insulin and free fatty acids (FFA) concentrations in milk-fed calves


GLUCOSE, mg/100 ml
200
160
120
80
40
0
INSULIN, ng/ml
13
12
10
8
6
4
2
0
FFA, μEq/L
1200
1000
800
600
400
200
0
Feed
Feed
0 1 2 3 4 6 12 24
TIME, HOURS

declined and by six hours after feeding had reached pre-feeding levels. Refeeding after twenty-four hours fast resulted in an immediate increase in plasma glucose. Changes in plasma insulin levels paralleled the changes in glucose concentrations. The immediate effect of feeding on plasma free fatty acid level was to lower it. This, however, was short-lived and the plasma free fatty acid levels remained relatively high until after six hours past feeding when there was a decline. By twenty-four hours after feeding plasma free fatty acids had risen to their highest point. Refeeding resulted in the lowering of the plasma free fatty acids. The reason for the relatively high plasma free fatty acid levels between one and six hours after feeding is not clear. It may have been due to the high fat content (20 percent) of the milk replacer fed to the calves.

Experiment 5. The effect of intravenous infusion of casein hydrolysate on insulin and glucagon secretion

The result of the preliminary experiment in which 5 percent casein hydrolysate was used is shown in Table 10. The infusion of 5 percent casein hydrolysate had no appreciable effect on either plasma glucose or plasma insulin. The elevation in plasma alpha amino nitrogen was statistically significant ($p < 0.01$).

By increasing the concentration of the casein hydrolysate solution to 10 percent, greater elevation in plasma alpha amino nitrogen was achieved (Figure 10). As the solution was infused the alpha amino nitrogen concentrations gradually increased ($p < 0.01$) until the termination of the infusion. The changes in glucose and insulin levels were significant ($p < 0.05$) and paralleled the changes in alpha amino nitrogen. However,

Figure 10. Effect of infusing 10 percent casein hydrolysate solution on plasma alpha amino nitrogen (AAN), glucose, insulin and glucagon concentrations

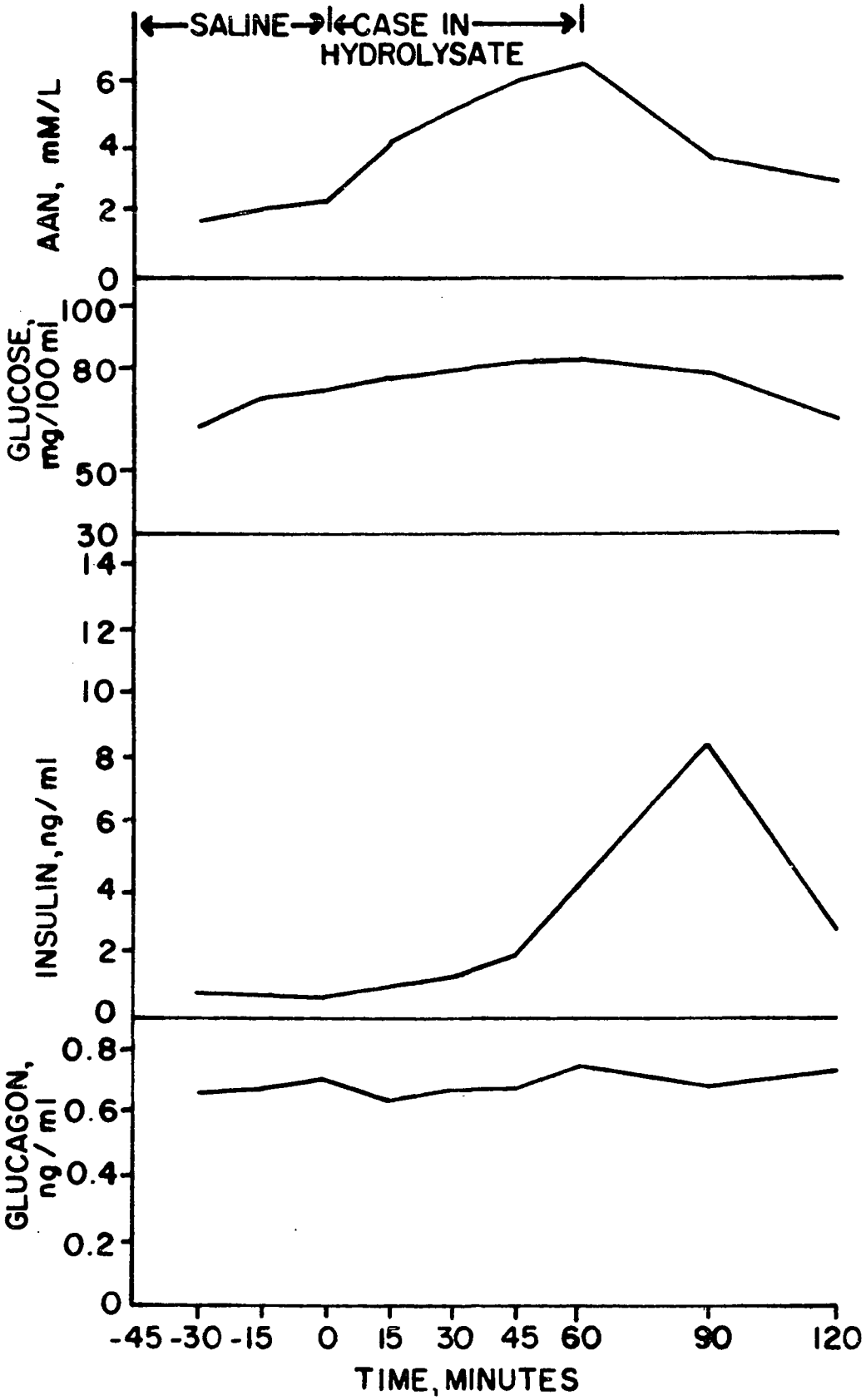

←SALINE→|←CASE IN→|
HYDROLYSATE
AAN, mM/L
6
4
2
0
GLUCOSE, mg/100 ml
100
80
50
30
INSULIN, ng/ml
14
12
10
8
6
4
2
0
GLUCAGON, ng/ml
0.8
0.6
0.4
0.2
0
-45 -30 -15 0 15 30 45 60 90 120
TIME, MINUTES

Table 10. The effect of 5 percent casein hydrolysate on plasma insulin, alpha amino nitrogen and glucose concentrations

Time (min.) after infusion[a]	AAN[b] mM/L	GLUCOSE mg/100 ml	INSULIN mg/ml
-30	3.0	73.2	1.06
0	3.5	84.1	1.94
15	4.0	72.2	2.79
30	4.1	74.8	1.87
45	4.3	70.2	1.31
60	4.6	81.4	2.50
75	3.2	66.7	1.16
90	2.3	62.4	1.11
105	2.7	74.2	1.00
120	2.8	68.2	0.99

[a]Casein hydrolysate was infused between 0 and 60 minutes.

[b]AAN = alpha amino nitrogen.

insulin levels continued to rise until thirty minutes after the infusion had been stopped. This increase in insulin level at a time when the alpha amino nitrogen concentration was falling suggests that the level of alpha amino nitrogen *per se* was not the main factor controlling insulin secretion.

Glucagon secretion was not appreciably affected by the infusion of casein hydrolysate. This is not in agreement with the work of Pek et al. (1968; 1969) who found the infusion or ingestion of amino acids to stimulate glucagon secretion in nonruminants.

Experiment 6. The effect of oleic acid on insulin and glucagon secretion

From Figure 11, it can be seen that the injection of 1 ml of oleic acid elevated plasma free fatty acid levels in the two lambs ($p < 0.01$). Glucose levels were not significantly affected. Insulin concentrations were elevated three-fold ($p < 0.01$) fifteen minutes after injection and thereafter declined. Oleic acid appeared to have an inhibitory effect on glucagon secretion. In the two animals used in this study, glucagon levels tended to go down following injection; however, this trend was not statistically significant. These observations are in agreement with those reported by others in nonruminants (Seyffert and Madison, 1967; Madison et al., 1968; Pi-Sunyer et al., 1969; Crespin et al., 1969).

Experiment 7. The effect of butyrate on glucagon secretion

The results are shown in Figure 12 and 13. Butyrate significantly elevated glucagon levels from 0.93 to 1.31 ng ($p < 0.01$) within the first fifteen minutes. By thirty minutes after injection the glucagon concentrations had fallen to basal levels. Although the elevation of plasma glucagon levels amounted to only about 50 percent, the highest level in all the animals was observed at fifteen minutes after injection. This time period coincided with the peak elevation in plasma insulin ($p < 0.01$) and plasma glucose ($p < 0.01$) levels. The insulin and glucose data confirmed previous work by Manns and Boda (1967) and Horino et al., (1968).

Since blood samples were obtained at fifteen minute intervals it was not possible to establish the exact time relationships between the peak

Figure 11. Effect of injecting plasma emulsion of oleic acid on plasma free fatty acid (FFA), glucose, insulin and glucagon concentrations

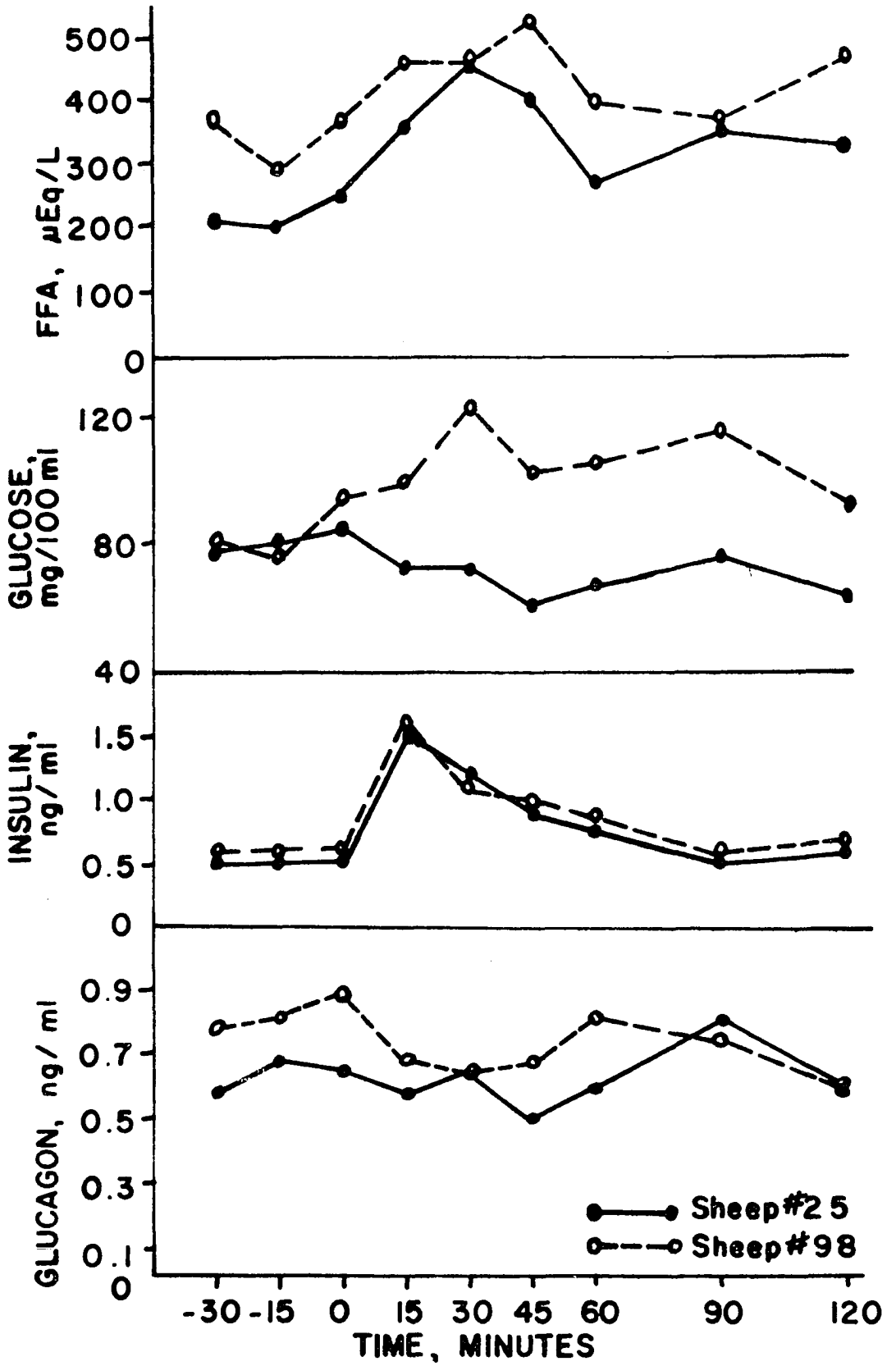

FFA, μEq/L
500
400
300
200
100
0
GLUCOSE, mg/100ml
120
80
40
INSULIN, ng/ml
1.5
1.0
0.5
0
GLUCAGON, ng/ml
0.9
0.7
0.5
0.3
0.1
0
Sheep#25
Sheep#98
-30 -15 0 15 30 45 60 90 120
TIME, MINUTES

Figure 12. Effect of intravenous injection of butyrate (1 mM/kg) on plasma glucose, insulin and glucagon concentrations

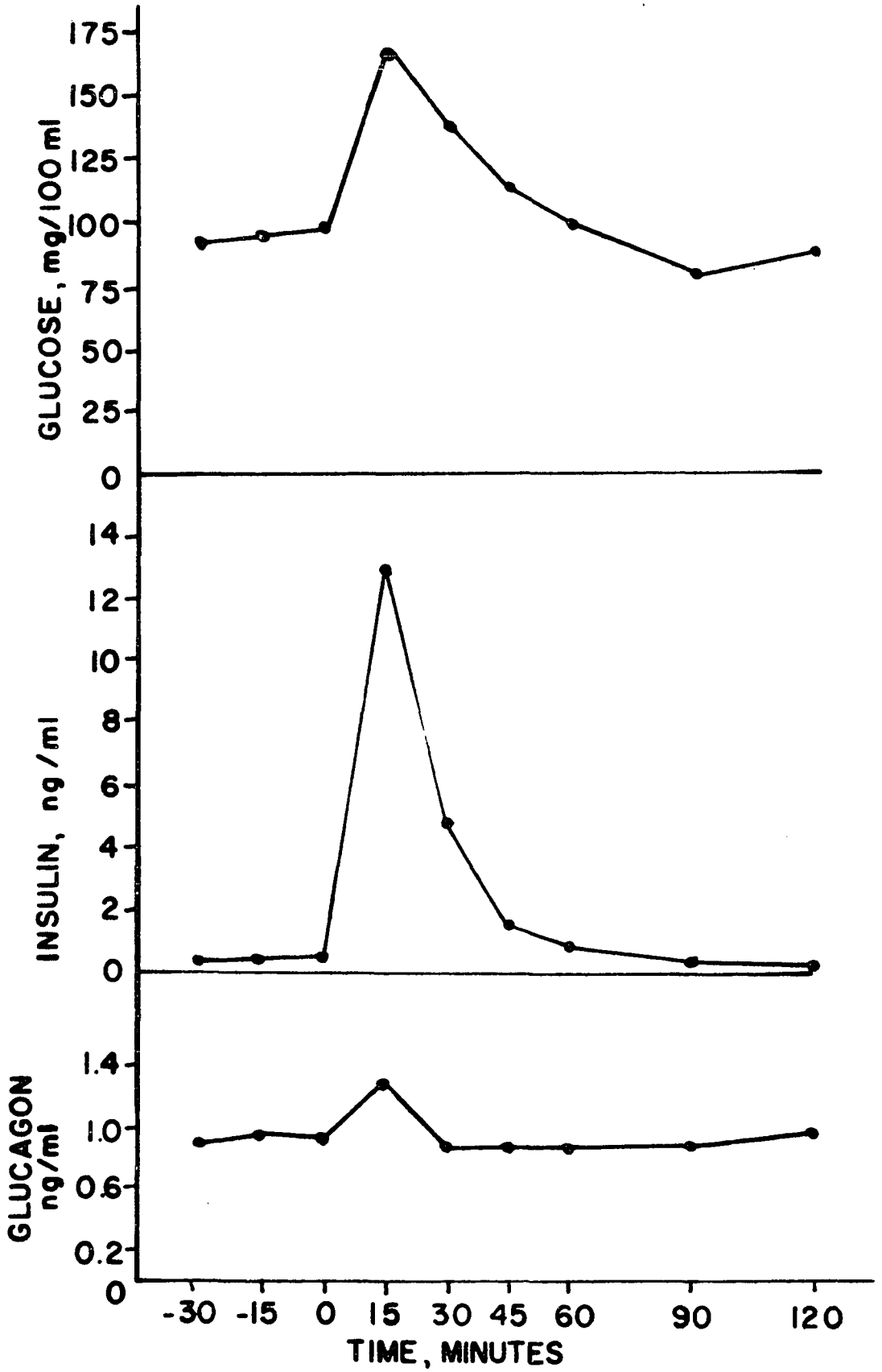
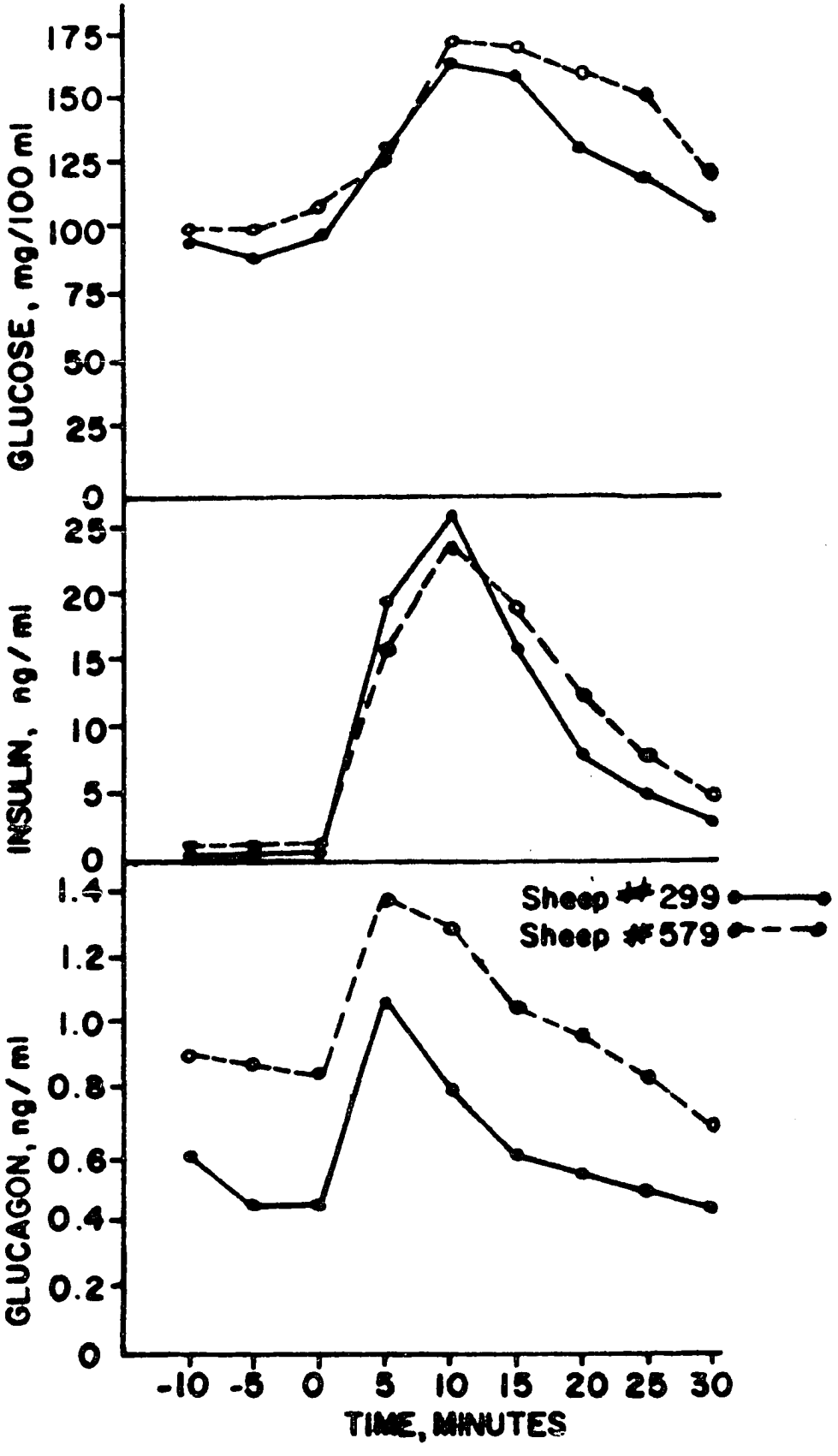

GLUCOSE, mg/100 ml
175
150
125
100
75
50
25
0
INSULIN, ng/ml
14
12
10
8
6
4
2
0
GLUCAGON ng/ml
1.4
1.0
0.6
0.2
0
-30 -15 0 15 30 45 60 90 120
TIME, MINUTES

Figure 13. Time relationships between peaks in glucose, insulin and glucagon concentrations induced by butyrate

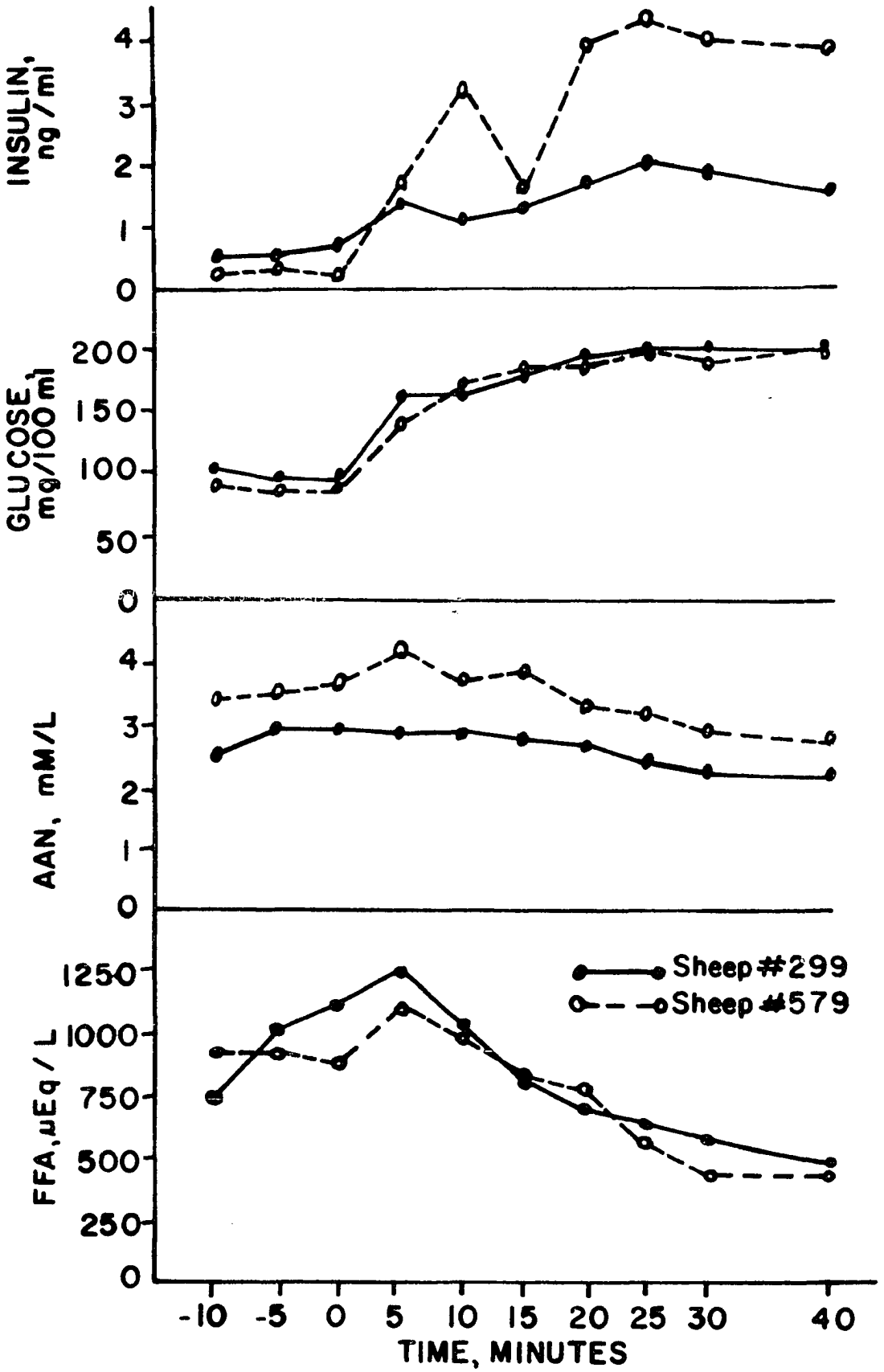

GLUCOSE, mg/100 ml
175
150
125
100
75
50
25
0
INSULIN, ng/ml
25
20
15
10
5
0
GLUCAGON, ng/ml
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Sheep #299
Sheep #579
-10 -5 0 5 10 15 20 25 30
TIME, MINUTES

concentrations of glucagon, insulin and glucose. The experiment was repeated using two lambs and blood samples obtained at five minute intervals. The individual results from two animals -- sheep #299 and #579 -- are shown in Figure 13. Again the effectiveness of butyrate in stimulating glucagon secretion was observed. Glucagon, insulin and glucose levels were significantly elevated ($p < 0.01$). Rather noteworthy is the observation that peaks in glucagon secretion occurred before peaks in insulin or glucose levels: suggesting that glucagon might mediate either or both insulin secretion and hyperglycemia. Because of the shorter time interval between samples, higher insulin and glucagon concentrations were recorded at peak secretion. Sheep #579 with the higher glucagon levels also had the higher plasma glucose levels. This may have resulted in the slower decline in insulin levels from the peak concentration.

Experiment 8. The effect of glucagon on plasma metabolites and insulin secretion

Changes of plasma insulin and plasma metabolites following the injection of glucagon are shown in Figure 14. Plasma insulin levels were elevated 4 - 20 fold ($p < 0.05$) after glucagon injection. The two lambs showed two insulin peaks. The first and smaller peaks occurred between five and ten minutes after injection. Then the insulin levels dropped to rise to higher points, ten to fifteen minutes later. Thereafter the insulin levels were relatively high until the termination of the experiment

Figure 14. Effect of intravenous injection of glucagon on plasma insulin, glucose, alpha amino nitrogen (AAN), and free fatty acids (FFA) concentrations


INSULIN, ng/ml
4
3
2
1
0
GLUCOSE, mg/100ml
200
150
100
50
0
AAN, mM/L
4
3
2
1
0
FFA, μEq/L
1250
1000
750
500
250
0
Sheep #299
Sheep #579
-10
-5
0
5
10
15
20
25
30
40
TIME, MINUTES

at forty minutes after injection. This multiphasic nature of insulin secretion has been observed by other workers and is thought to represent the immediate effect of a stimulus on the release of stored insulin followed by its effect on synthesis and secretion or different storage compartments (Curry et al., 1968; Floyd et al., 1970[a]).

The effect of glucagon on glucose levels was immediate -- rising from 90 and 98 mg/100 ml to 144 and 163 mg/100 ml within five minutes for sheep #579 and 299 respectively ($p < 0.01$). Glucose levels continued to rise with time to plateau at twenty-five minutes after injection. At this time there was about 100 percent increase in plasma glucose level. After fifteen minutes post-injection, plasma alpha amino nitrogen declined ($p < 0.01$). Plasma urea nitrogen (not shown in Figure 14) was not affected by glucagon administration.

As reviewed by Foa and Galansino (1962) glucagon has been reported to have varied and even opposing effects on nitrogen metabolism. The discrepancies may be due to differences in experimental systems and conditions, and the secondary effects of glucagon on epinephrine (Lawrence, 1967) and insulin (Samols et al., 1966; Karam et al., 1966) secretions. In isolated perfused rat liver, glucagon was shown to be protein catabolic, resulting in increased urea production. This catabolic effect was abolished by insulin (Miller, 1960).

Glucagon appeared to have a dual effect on plasma free fatty acids. At five minutes post injection there was an elevation of plasma free fatty acids. Thereafter, the plasma free fatty acid levels declined until the

end of the experiment. The initial elevation of plasma free fatty acids is consistent with the lipolytic effect of glucagon (Beshwer and Ashmore, 1966). The subsequent decline is perhaps another manifestation of the overriding effect of insulin (Sokal, Aydin and Kraus, 1966).

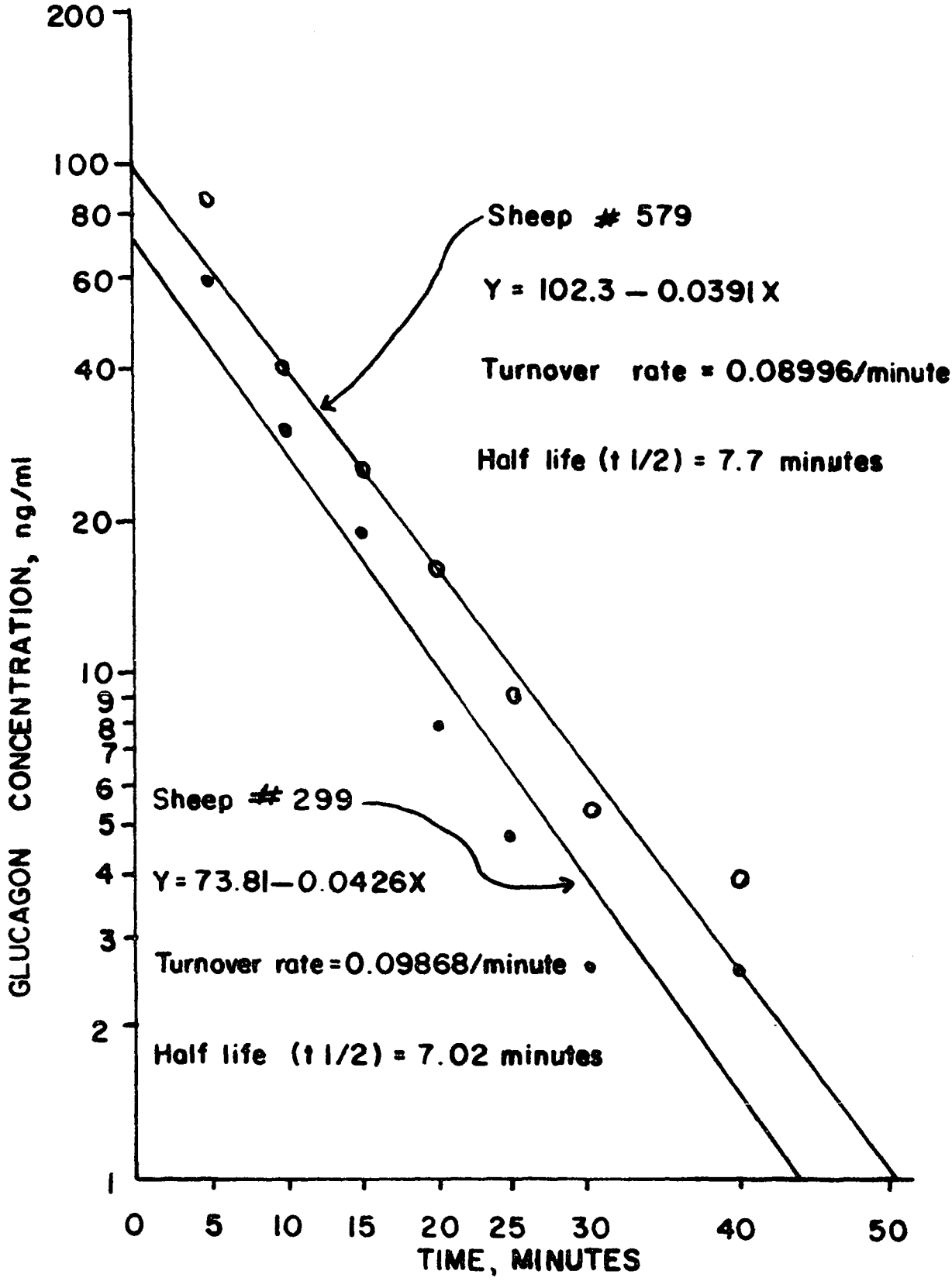
The disappearance of exogenous glucagon was used to determine its fractional turnover rate and half life. Table 11 shows the changes in plasma glucagon levels following the injection of 1 mg exogenous hormone.

Table 11. Disappearance of glucagon in plasma

Time (min.) post-injection	-10	-5	0	5	10	15	20	25	30	40
				Glucagon concentration (ng/ml)						
Sheep #299	.50	.48	.47	59.85	29.75	18.81	7.75	4.66	2.60	2.54
Sheep #579	.58	.60	.62	85.50	40.00	25.00	15.67	9.19	5.25	3.95

These data were used to plot regression lines according to the method described for thyroxine in cattle (Post and Mixner, 1961). This method assumes the disappearance of glucagon to be exponential in nature and to be represented by the regression equation; $Y = ae^{-bx}$, which may be transformed to linear logarithmic form $\ln Y = \ln a - bx$; where Y = plasma glucagon concentration, x = time in minutes, a = Y-axis intercept (value of Y when x = 0), b = regression coefficient or glucagon fractional turnover rate per minute. Figure 15 shows the regression lines plotted on a semi-logarithmic scale. Half life was calculated by dividing the natural

Figure 15. The disappearance of glucagon injected into sheep


200
100
80
60
40
20
10
9
8
7
6
5
4
3
2
1
GLUCAGON CONCENTRATION, ng/ml
Sheep # 579
Y = 102.3 – 0.0391 X
Turnover rate = 0.08996/minute
Half life (t 1/2) = 7.7 minutes
Sheep # 299
Y = 73.81 – 0.0426X
Turnover rate = 0.09868/minute
Half life (t 1/2) = 7.02 minutes
0
5
10
15
20
25
30
40
50
TIME, MINUTES

log of 2 by the turnover rate.

The half life of about seven minutes observed in this experiment is in agreement with values of five to ten minutes reported in man (Samols et al., 1966) and rat (Buchanan et al., 1968).

GENERAL DISCUSSION

Observations by Manns and Boda (1967) and Horino et al. (1968) have indicated that the volatile fatty acids may be more important than glucose in regulating insulin secretion in ruminants. Because of the nutritional importance of the volatile fatty acids the relationship between dose and insulin response was investigated. The effects of other nutritional factors on insulin secretion were also studied. Since insulin is one of the hormones involved in the regulation of growth, it was thought that knowledge of the factors regulating its secretion might prove useful in livestock production.

Increasing the dose of either propionate or butyrate resulted in a corresponding increase in insulin secretion. Diet is known to have a marked effect on the proportion of volatile fatty acids produced in the rumen. By increasing the level of grain in a ration, Donefer et al. (1963) and Luther (1964) have observed a decrease in the molar proportion of acetic acid with a concurrent increase in the molar proportion of butyric acid. Assuming that volatile fatty acid absorption will reflect rumen concentration (Schambye, 1951) one can postulate that increasing the level of grain in a ration will lead to increased insulin secretion. Indeed, some observations in the literature strongly suggest that this may be so. Injection of insulin or feeding high concentrate ration appears to have the effect of depressing milk fat content and milk yield (Gowen and Tobey, 1931; Jorgensen, Schultz and Barr, 1965). In glucose tolerance tests, Hale and King (1958) found that glucose disappeared faster in

grain-fed than hay-fed lambs. Trenkle (1966) observed higher insulin levels in corn-alfalfa fed lambs than in alfalfa hay fed ones. However, the present study was not successful in demonstrating any stimulatory effect of feeding grain on insulin secretion. The results can perhaps be explained on the basis of two factors. Firstly, the hay diet was in the form of long hay and the lambs tended to select the leaves and did not eat the stems. Secondly, diets were fed in restricted amounts. No appreciable differences in volatile fatty acid patterns were observed.

Feeding milk to young calves or hay-concentrate diet to mature lambs resulted in elevated plasma insulin levels. The young calves had never been fed solid food and therefore possessed nonfunctional rumens. The lambs on the other hand, had been on solid food for a long time and had developed functional rumens. It was interesting to note that the nature of insulin response was different in the two types of animals. Insulin release was greater in the milk-fed calves than in the lambs, and while the rise in insulin levels paralleled the rise in plasma glucose concentrations in the calves there was no relationship between plasma glucose levels and plasma insulin levels in the lambs. On the contrary, insulin secretion paralleled rumen volatile fatty acid concentrations in the lambs. This indicates that glucose may be important in regulating insulin secretion in the milk-fed but not in the adult ruminant. The high fat content of the milk replacer may also have contributed to the hyperinsulinemia in the calves.

In nonruminants, nutrients stimulate the secretion of intestinal hormones which have been shown to be insulinogenic (Unger et al., 1968; Buchanan et al., 1967; Unger et al., 1967; Dupre et al., 1969; Kahil et al., 1970). The end result is the amplification of insulin response. This may in part explain the extremely high levels of insulin observed in the milk-fed calves. However, very little is known about the role of these intestinal factors in promoting insulin secretion in the calf or newborn lamb. Knowledge of the relative importance of these hormones in regulating insulin secretion in ruminants is desirable for a better understanding of the effect of feeding. A recent study in adult goats indicates that secretin and cholecystokinin-pancreozymin may not be important in stimulating insulin secretion in ruminants (Baile, Glick and Mayer, 1969).

Increased frequency of feeding has been known to result in faster growth rates in young sheep and cattle (Gordon and Tribe, 1952; Hardison et al., 1957; Horton and Nelson, 1961; Mohrman et al., 1959; Rakes et al., 1957). In some cases a greater total feed intake accompanied the more frequent feedings and this offers a partial explanation for the improved performance. However, the cause of improved performance when total daily feed intake was kept the same is not known. Satter and Baumgardt (1962) fed equal amounts of feed at three different frequencies to some calves and observed that the animals had significantly greater nitrogen retention when fed eight times than when fed four or two times. The most frequent feeding also resulted in high and less fluctuation in volatile fatty acid concentrations. Since insulin is an important anabolic agent (Gaebler,

Liu and Zuchlewski, 1956; Wool et al., 1968) and it has been shown in this study that plasma insulin concentrations rise following feeding, the increased nitrogen retention resulting from frequent feeding could be attributed in part to the effect of insulin.

The importance of animo acids in regulating insulin secretion in ruminants is dubious. An increase in plasma insulin levels was obtained only after a high concentration of casein hydrolysate was infused. It was noted that insulin levels continued to rise after the infusion had been stopped and at a time when alpha amino nitrogen concentration was falling. This suggests that the level of alpha amino nitrogen per se may not be the main factor controlling insulin secretion in ruminants. It is possible that a metabolic product of amino acids, notably urea, may be involved here.

Oleic acid was found to stimulate insulin secretion and to depress glucagon levels. Because of the role of insulin and glucagon on lipolysis Madison et al. (1968) have characterized the effect of long chain fatty acids on insulin and glucagon secretion as feedback stimulation and inhibition which serve to modulate alterations in carbohydrate metabolism. Luyckx and Lefebvre (1970) have presented evidence which indicates that plasma free fatty acids may be important in regulating pancreatic endocrine secretions. Depressing plasma free fatty acid level by nicotinic acid infusion stimulated glucagon secretion while triglyceride infusion had the opposite effect.

The stimulatory effect of butyrate on glucagon secretion indicates that the effect of butyrate on liver phosphorylase and hyperglycemia

(Phillips, Black and Moller, 1965) is mediated by glucagon. Significant in this regard is the observation that the glucagon peak occurred before that of glucose. This effect of butyrate appears to be a direct one since ketone bodies, the metabolic products of butyrate, have been shown to have no effect on hyperglycemia in sheep (Phillips and Black, 1966; Manns, Boda and Willes, 1967; Horino et al., 1968).

The accumulation of ketone bodies results in ketosis, a pathological condition. Menahan, Schultz and Hoekstra (1966) have shown that hypoglycemia aggravates the accumulation of ketone bodies in goats. It is therefore teleologically significant that butyrate although ketogenic is also hyperglycemic through its effect on glucagon. The latter effect may serve to reduce the severity of the ketotic condition. The administration of glucagon to hypoglycemic, hyperketonemic sheep has been reported to increase blood glucose and decrease blood ketone levels (Ho and Reber, 1957; Burtis et al., 1968).

In Experiment 1, it was found that insulin secretion resulting from butyrate was more than could be accounted for on the basis of hyperglycemia. Similar observations have been made by other workers (Manns and Boda, 1967; Horino et al., 1968). Glucagon has been shown to stimulate insulin secretion in nonruminants (Samols et al., 1966; Karam et al., 1966). The present study showed that glucagon also stimulates insulin secretion in the sheep. However, the insulin response following glucagon injection was considerably less than that observed after butyrate injection and a little smaller than would be expected from the hyperglycemia

produced. This reduced response is probably due to the high dose of glucagon used. Plasma glucagon concentrations rose from a mean basal level of 0.54 ng/ml to a mean of 72.68 ng/ml five minutes after the injection of exogenous glucagon in contrast to 1.4 ng/ml after butyrate injection. It is possible that the plasma glucagon concentration following glucagon injection was high enough to stimulate epinephrine secretion (Lawrence, 1967; Lefebvre et al., 1968) which inhibited or modified the effect of glucagon on insulin secretion (Hertelendy et al., 1969). This view is strengthened by the observation that insulin concentrations were higher at the latter part of the experiment when plasma glucagon concentration was declining. Modest elevations in plasma glucagon concentration as observed following butyrate injection may be rather potent in stimulating insulin secretion. It is possible therefore that the hyperinsulinemia observed with butyrate is the result of synergism between glucose and glucagon. This does not preclude a direct effect of butyrate on the beta cells.

Secretion of insulin and glucagon varied depending on the stimulus. Feeding and casein hydrolysate infusion stimulated insulin secretion but had no effect on glucagon secretion. Oleic acid stimulated insulin secretion but tended to inhibit glucagon secretion. The secretion of both insulin and glucagon was stimulated by butyrate. It is tempting to conclude from these observations that although glucagon stimulates insulin secretion, other factors which induce insulin secretion may do so without affecting glucagon secretion. However, because of the short

half life of glucagon and the dilution of pancreatic venous blood, a biologically significant increment of glucagon secretion may occur without a measurable change in peripheral glucagon level (Ketterer, Eisentraut and Unger, 1967). Therefore a better picture appears possible only when glucagon concentrations in pancreatic venous blood are measured.

From this study it is evident that nutritional factors are involved in the regulation of pancreatic endocrine secretions. Nutrients absorbed following feeding directly or indirectly stimulate insulin secretion which is necessary for the storage of excess nutrients in the form of glycogen, fat and protein.

SUMMARY

Experiments were conducted to study the relationship between dose of glucose, propionate and butyrate and plasma insulin levels in sheep. The effects of various diets, feeding, casein hydrolysate and oleic acid on insulin and glucagon secretion were also investigated. Increasing the dose of glucose, propionate or butyrate given intravenously resulted in a corresponding increase in insulin secretion; however, butyrate was more potent in stimulating insulin secretion than either glucose or propionate. Intravenous injection of butyrate was also found to stimulate glucagon secretion. This observation was considered important in view of the hyperglycemic effect of butyrate in sheep. In lambs fed restricted amounts of 0%, 50% or 80% grain diets no differences in rumen volatile fatty acid patterns or plasma insulin were observed. Feeding and casein hydrolysate infusion stimulated insulin secretion but had no effect on glucagon secretion. Oleic acid also stimulated insulin secretion but tended to inhibit glucagon secretion. The results of these studies indicate that nutritional factors are important in regulating insulin and glucagon secretion in the sheep.

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APPENDIX

Analysis of Variance Tables and Duncan's Multiple Range Test of the Means

Table 12. The effect of different doses of glucose on insulin secretion

Source of Variation	d.f.	M.S.	F
Time	4	2.33	2.09
Dose	2	8.33	7.50
Error	8	1.11	
Total	14		
Dose[a]	L	M	H
Mean[b]	1.42	1.66	3.77

[a]Dose: L = low (0.3 mM/kg); M = medium (0.6 mM/kg); H = high (1.0 mM/kg).

[b]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 13. The effect of different doses of glucose on plasma glucose levels

Source of Variation	d.f.	M.S.	F
Time	4	1528.08	9.67
Dose	2	190.94	1.2
Error	8	157.88	
Total	14		
Dose[a]	L	M	H
Mean[b]	112.82	123.20	123.82

[a]Dose: L = low (0.3 mM/kg); M = medium (0.6 mM/kg); H = high (1.0 mM/kg).

[b]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 14. The effect of different doses of propionic acid on insulin secretion

Source of Variation	d.f.	M.S.	F
Time	4	157.80	9.37
Dose	2	75.66	4.49
Error	8	16.82	
Total	14		

Dose[a]	L	M	H
Mean[b]	3.05	6.91	10.83

[a]Dose: L = low (0.5 mM/kg); M = medium (1.0 mM/kg); H = high (2.0 mM/kg).

[b]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 15. The effect of different doses of propionic acid on plasma glucose levels

Source of Variation	d.f.	M.S.	F
Time	4	1070.90	6.62
Dose	2	1202.35	7.43
Error	8	161.68	
Total	14		

Dose[a]	L	M	H
Mean[b]	79.94	91.10	110.58

[a]Dose: L = low (0.5 mM/kg); M = medium (1.0 mM/kg); H = high (2.0 mM/kg).

[b]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 16. The effect of different doses of butyric acid on insulin secretion

Source of Variation	d.f.	M.S.	F
Time	4	356.78	4.94
Dose	2	320.87	4.45
Error	8	72.08	
Total	14		

Dose[a]	L	M	H
Mean[b]	4.89	6.58	19.53

[a]Dose: L = low (0.25 mM/kg); M = medium (0.5 mM/kg); H = high (1.0 mM/kg).

[b]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 17. The effect of different doses of butyric acid on plasma glucose levels

Source of Variation	d.f.	M.S.	F
Time	4	1508.92	4.15
Dose	2	1994.85	5.48
Error	8	363.43	
Total	14		

Dose[a]	L	M	H
Mean[b]	80.34	95.6	119.94

[a]Dose: L = low (0.25 mM/kg); M = medium (0.5 mM/kg); H = high (1.0 mM/kg).

[b]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 18. The effect of different diets on plasma insulin levels

Source of Variation	d.f.	M.S.	F
Animals	5	.0777	2.15
Periods	2	.0043	0.119
Treatments	2	.0354	0.98
Error	8	.0361	
Total	17		

Treatments[a]	C	A	B
Mean[b]	.38	.49	.53

[a]Treatments: A = hay; B = 50% grain; and C = 80% grain.

[b]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 19. The effect of different diets on plasma glucose levels

Source of Variation	d.f.	M.S.	F
Animals	5	65.36	1.67
Periods	2	26.72	0.68
Treatments	2	5.24	0.13
Error	8	38.99	
Total	17		

Treatments[a]	A	C	B
Mean[b]	70.6	70.7	72.6

[a]Treatments: A = hay; B = 50% grain; and C = 80% grain.

[b]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 20. The effect of different diets on total rumen VFA concentration

Source of Variation	d.f.	M.S.	F
Animals	5	126.50	0.76
Periods	2	132.54	0.80
Treatments	2	1326.65	8.02
Error	8	165.22	
Total	17		

Treatments[a]	B	C	A
Mean[b]	57.98	65.83	86.75

[a]Treatments: A = hay; B = 50% grain; and C = 80% grain.

[b]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 21. The effect of different diets on the concentration of acetic acid in the rumen

Source of Variation	d.f.	M.S.	F
Animals	5	44.97	.55
Periods	2	70.33	.86
Treatments	2	970.00	11.97
Error	8	80.99	
Total	17		

Treatments[a]	B	C	A
Mean[b]	42.05	43.4	64.72

[a]Treatments: A = hay; B = 50% grain; and C = 80% grain.

[b]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 22. The effect of different diets on the concentration of propionic acid in the rumen

Source of Variation	d.f.	M.S.	F
Animals	5	16.281	0.937
Periods	2	2.734	0.157
Treatments	2	72.794	4.189
Error	8	17.375	
Total	17		

Treatments[a]	B	C	A
Mean[b]	10.28	14.73	17.15

[a]Treatments: A = hay; B = 50% grain; and C = 80% grain.

[b]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 23. The effect of different diets on the concentration of butyric acid in the rumen

Source of Variation	d.f.	M.S.	F
Animals	5	6.3626	0.8266
Periods	2	2.0266	0.2633
Treatments	2	12.4800	1.6214
Error	8	7.6966	
Total	17		

Treatments[a]	A	B	C
Mean[b]	4.9	5.7	7.7

[a]Treatments: A = hay; B = 50% grain; and C = 80% grain.

[b]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 24. The effect of feeding and 24 hrs. fasting on plasma glucose levels in lambs

Source of Variation	d.f.	M.S.	F
Animals	3	106.6148	0.8847
Time	8	132.0530	1.0958
Error	20	120.5138	
Total	31		

Time (hrs.)	3	24	5	0	7	1.5	12
Mean[a]	76.3	77.8	79.8	83.0	84.3	88.8	88.9

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 25. The effect of feeding and 24 hrs. fasting on insulin secretion in lambs

Source of Variation	d.f.	M.S.	F
Animal	3	9.2006	7.20
Time	8	2.2178	1.74
Error	20	1.2776	
Total	31		

Time (hrs.)	24	0	7	12	5	3	1.5
Mean[a]	0.74	0.95	1.65	1.72	1.90	2.59	2.75

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 26. The effect of feeding and 24 hrs. fasting on plasma FFA levels in lambs

Source of Variation	d.f.	M.S.	F
Animals	3	414625.58	12.82
Time	8	57703.92	1.78
Error	20	32330.97	
Total	31		

Time (hrs.)	5	3	1.5	7	12	0	24
Mean[a]	394	395	465	469	521	543	784

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 27. The effect of feeding and 24 hrs. fasting on glucagon secretion in lambs

Source of Variation	d.f.	M.S.	F
Animal	2	0.02954	11.318
Time	8	0.00244	0.935
Error	16	0.00261	
Total	26		

Time (hrs.)	0	12	3	5	1.5	7	24
Mean[a]	0.52	0.53	0.54	0.55	0.57	0.58	0.58

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 28. The effect of feeding and 24 hrs. fasting on total rumen VFA concentrations in lambs

Source of Variation	d.f.	M.S.	F
Animals	3	263.137	2.219
Time	8	1603.802	13.530
Error	20	118.533	
Total	31		

Time (hrs.)	0	24	7	12	1.5	5	3
Mean[a]	38.30	49.43	70.71	70.83	75.96	79.24	88.59

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 29. The effect of feeding and 24 hrs. fasting on the rumen acetic acid concentrations in lambs

Source of Variation	d.f.	M.S.	F
Animals	23	127.193	2.48
Time	8	730.728	14.25
Error	20	51.280	
Total	31		

Time (hrs.)	24	0	12	7	1.5	5	3
Mean[a]	35.99	36.61	49.51	49.59	54.12	55.69	62.87

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 30. The effect of feeding and 24 hrs. fasting on the rumen propionic acid concentrations in lambs

Source of Variation	d.f.	M.S.	F
Animals	3	15.187	1.571
Time	8	97.208	10.061
Error	20	9.661	
Total	31		

Time (hrs.)	0	24	12	7	5	1.5	3
Mean[a]	5.73	7.62	12.40	12.91	15.30	15.95	18.05

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 31. The effect of feeding and 24 hrs. fasting on the rumen butyric acid concentrations in lambs

Source of Variation	d.f.	M.S.	F
Animals	3	11.3293	6.70
Time	8	14.8785	8.80
Error	20	1.6910	
Total	31		

Time (hrs.)	0	24	1.5	3	7	5	12
Mean[a]	4.21	5.80	5.88	7.66	8.20	8.24	8.91

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 32. The effect of feeding and 24 hrs. fasting on plasma FFA levels in milk-fed calves

Source of Variation	d.f.	M.S.	F
Animals	2	80067.39	3.65
Time	10	107154.09	4.89
Error	19	21890.38	
Total	31		

Time (hrs.)	12	1.5	25	2.5	1	1.5	3	2	0	6	24
Mean[a]	556	608	730	754	768	772	773	871	910	910	1273

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 33. The effect of feeding and 24 hrs. fasting on insulin secretion in milk-fed calves

Source of Variation	d.f.	M.S.	F
Animals	2	36.5410	2.12
Time	10	72.2055	4.20
Error	19	17.2122	
Total	31		

Time (hrs.)	24	12	0	6	.5	1	25	1.5	2	3	2.5
Mean[a]	0.15	0.39	0.32	1.2	2.49	3.57	4.26	6.02	9.15	12.83	13.39

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 34. The effect of feeding and 24 hrs. fasting on plasma glucose levels in milk-fed calves

Source of Variation	d.f.	M.S.	F
Animals	2	403.46	1.09
Time	10	12119.35	32.94
Error	19	367.86	
Total	31		

Time (hrs.)	0	6	24	12	25	.5	1	3	1.5	2.5	2
Mean[a]	53.9	56.8	60.6	74.6	163.4	163.7	187.6	189.7	192.9	195.0	200.6

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 35. The effect of the infusion of 5 percent casein hydrolysate solution on plasma glucose levels in sheep

Source of Variation	d.f.	M.S.	F
Period	1	3070.24	72.77
Sample time	9	84.64	1.98
Error	9	42.19	
Total	19		

Time (min.)	90	75	120	45	15	105	30	0	60
Mean[a]	62.4	66.7	68.2	70.2	72.2	74.2	74.8	78.6	81.4

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 36. The effect of the infusion of 5 percent casein hydrolysate solution on plasma alpha amino nitrogen concentration in sheep

Source of Variation	d.f.	M.S.	F
Period	1	12.01	148.089
Sample time	9	0.99	12.207
Error	9	0.0811	
Total	19		

Time (min.)									
Mean[a]	2.3	2.7	2.8	3.2	3.2	4.0	4.1	4.3	4.6

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 37. The effect of the infusion of 5 percent casein hydrolysate solution on insulin secretion in sheep

Source of Variation	d.f.	M.S.	F
Period	1	1.44	1.22
Sample time	9	.87	0.737
Error	9	1.18	
Total	19		

Time (min.)	120	105	90	75	45	0	30	60	15
Mean[a]	0.99	1.00	1.11	1.16	1.31	1.5	1.87	2.50	2.79

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 38. The effect of the infusion of 10 percent casein hydrolysate solution on plasma alpha amino nitrogen concentrations in sheep

Source of Variation	d.f.	M.S.	F
Animals	3	22.183	126.039
Time	8	12.712	72.227
Error	24	0.176	
Total	35		

Time (min.)	0	120	90	15	30	45	60
mean[a]	2.04	2.86	3.67	4.20	5.18	6.12	6.56

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 39. The effect of the infusion of 10 percent casein hydrolysate solution on plasma glucose levels in sheep

Source of Variation	d.f.	M.S.	F
Animals	3	3682.04	41.95
Time	8	228.81	2.60
Error	24	87.76	
Total	35		

Time (min.)	120	0	15	90	30	45	60
Mean[a]	64.7	69.7	77.9	79.7	79.8	82.6	84.3

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 40. The effect of the infusion of 10 percent casein hydrolysate solution on insulin secretion in sheep

Source of Variation	d.f.	M.S.	F
Animals	3	29.141	2.519
Time	8	27.478	2.375
Error	24	11.568	
Total	35		

Time (min.)	0	15	30	45	120	60	90
mean[a]	0.59	0.82	1.13	1.94	2.78	4.19	8.52

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 41. The effect of the infusion of 10 percent casein hydrolysate solution on glucagon secretion in sheep

Source of Variation	d.f.	M.S.	F
Animals	3	0.04267	3.727
Time	8	0.00514	0.449
Error	24	0.01145	
Total	35		

Time (min.)	15	45	30	0	90	120	60
Mean[a]	0.64	0.68	0.68	0.68	0.69	0.74	0.75

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 42. The effect of plasma emulsion of oleic acid on plasma FFA

Source of Variation	d.f.	M.S.	F
Animals	1	42437.56	28.83
Time	8	11333.62	7.70
Error	7	1471.92	
Total	16		

Time (min.)	0	60	90	120	15	30	45
Mean[a]	288	338	366	403	410	459	468

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 43. The effect of plasma emulsion of oleic acid on plasma glucose levels in sheep

Source of Variation	d.f.	M.S.	F
Animals	1	3068.578	15.082
Time	8	113.502	0.557
Error	7	203.451	
Total	16		

Time (min.)	120	45	0	15	60	90	30
Mean[a]	77.9	81.6	83.0	86.6	87.0	96.4	98.2

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 44. The effect of plasma emulsion of oleic acid on glucagon secretion in sheep

Source of Variation	d.f.	M.S.	F
Animals	1	0.060	7.50
Time	8	0.011	1.38
Error	7	0.008	
Total	16		

Time (min.)	45	120	15	30	60	0	90
Mean[a]	0.59	0.60	0.63	0.65	0.71	0.73	0.79

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 45. The effect of plasma emulsion of oleic acid on insulin secretion in sheep

Source of Variation	d.f.	M.S.	F
Animals	1	0.024	12
Time	8	0.236	118.0
Error	7	0.002	
Total	16		

Time (min.)	0	90	120	60	45	30	15
Mean[a]	0.56	0.57	0.66	0.84	0.94	1.17	1.54

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 46. The effect of butyrate on plasma glucose levels in sheep (Experiment 7a)

Source of Variation	d.f.	M.S.	F
Animals	3	636.11	4.14
Time	8	3254.54	21.22
Error	24	153.32	
Total	35		

Time (min.)	90	120	0	60	45	30	15
Mean[a]	80.4	91.5	95.2	99.6	114.9	141.1	169.4

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 47. The effect of butyrate on insulin secretion in sheep (Experiment 7a)

Source of Variation	d.f.	M.S.	F
Animals	3	11.05	2.93
Time	8	71.64	19.05
Error	24	3.76	
Total	35		

Time (min.)	120	90	0	60	45	30	15
Mean[a]	0.22	0.30	0.35	0.80	1.62	4.80	13.00

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 48. The effect of butyrate on glucagon secretion in sheep (Experiment 7a)

Source of Variation	d.f.	M.S.	F
Animal	3	0.69	72.63
Time	8	0.0725	7.63
Error	24	0.0095	
Total	35		

Time (min.)	60	30	45	90	0	120	15
Mean[a]	0.88	0.89	0.89	0.89	0.93	0.96	1.31

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 49. The effect of butyrate on plasma glucose levels in sheep (Experiment 7b)

Source of Variation	d.f.	M.S.	F
Animal	1	789.37	12.43
Time	8	1476.50	23.25
Error	8	63.48	
Total	17		

Time (min.)	0	30	5	25	20	15	10
Mean[a]	100.7	114.2	130.0	137.2	146.7	164.5	169.0

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 50. The effect of butyrate on glucagon secretion in sheep (Experiment 7b)

Source of Variation	d.f.	M.S.	F
Animals	1	0.6651	214.55
Time	8	0.0913	29.45
Error	8	0.0031	
Total	17		

Time (min.)	30	25	0	20	15	10	5
Mean[a]	0.58	0.68	0.69	0.76	0.82	1.05	1.24

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 51. The effect of butyrate on insulin secretion in sheep (Experiment 7b)

Source of Variation	d.f.	M.S.	F
Animals	1	3.1584	0.98
Time	8	160.4775	49.81
Error	8	3.2218	
Total	17		

Time (min.)	0	30	25	20	5	15	10
Mean[a]	0.73	4.24	6.35	10.68	17.64	17.68	24.96

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 52. The effect of glucagon on insulin secretion in sheep

Source of Variation	d.f.	M.S.	F
Animal	1	5.67	7.46
Time	9	2.47	3.25
Error	9	0.76	
Total	19		

Time (min.)	0	20	5	10	40	20	30	25
Mean[a]	0.81	2.90	3.11	4.25	5.50	5.66	5.88	6.25

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 53. The effect of glucagon on plasma glucose levels in sheep

Source of Variation	d.f.	M.S.	F
Animals	1	152.90	4.16
Time	9	4260.93	116.16
Error	9	36.68	
Total	19		

Time (min.)	0	5	10	15	20	30	25	40
Mean[a]	94.7	153.4	168.5	182.0	192.0	196.0	202.0	202.0

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 54. The effect of glucagon on plasma urea nitrogen levels in sheep

Source of Variation	d.f.	M.S.	F
Animals	1	122.51	41.52
Time	9	4.90	1.66
Error	9	2.95	
Total	19		

Time (min.)	25	0	15	5	40	30	20	10
Mean[a]	18.0	18.1	18.2	19.0	19.0	21.0	21.2	21.8

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 55. The effect of glucagon on plasma alpha amino nitrogen levels in sheep

Source of Variation	d.f.	M.S.	F
Animals	1	3.66	117.68
Time	9	0.25	8.04
Error	9	0.0311	
Total	19		

Time (min.)	40	30	25	20	0	10	15	5
Mean[a]	2.42	2.58	2.75	2.95	3.16	3.28	3.30	3.52

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.

Table 56. The effect of glucagon on plasma FFA levels in sheep

Source of Variation	d.f.	M.S.	F
Animals	1	16704.20	2.38
Time	9	111123.31	15.87
Error	9	7000.42	
Total	19		

Time (min.)	40	30	25	20	15	0	10	5
Mean[a]	475	506	610	746	844	938	1025	1187

[a]Any two means not underscored by the same line are significantly different at 0.05 level of probability.