RADIOIMMUNOASSAY OF BOVINE INSULIN AND CORTISOL LEVELS AS RELATED TO SERUM LIPID

by

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ABSTRACT

A sensitive radioimmunoassay for bovine serum insulin was developed. Further, three experiments under normal feeding conditions and one under starvation conditions were conducted using jugular catheterized steers. Also propionate and butyrate were infused into several steers for the production of high insulin serum.

The data indicated that unfrozen, Merthiolate-preserved serum lost insulin activity. Further, no meaningful correlations could be established between serum cortisol, insulin, and total serum lipids. However, after a 72-hr starvation period, serum cortisol concentrations rose drastically followed by a decrease in serum lipid levels, indicating a possible relationship between these two serum components. In addition insulin concentrations were elevated with the infusion of propionate or butyrate, indicating that serum insulin and lipid have an important metabolic relationship.

CHAPTER I

INTRODUCTION

The influence of serum insulin and cortisol on total serum lipids has not been studied with reference to the bovine. However, it has been indicated that insulin and cortisol play an important role in lipid metabolism of other species. Therefore, the relationships of both serum insulin and cortisol warranted investigation.

Not until recently have procedures, specifically radioimmunoassays, been developed that were sensitive enough to quantitatively measure hormones in physiological concentrations. Although the bovine serum cortisol assay had been developed and was working, there were extensive problems with the insulin radioimmunoassay.

For the most part, insulin radioimmunoassays that have been developed have been designed specifically for human insulin and are insensitive to bovine insulin because of slight but important differences in the amino acid sequence of the two insulin chains. Therefore, in order to quantitatively measure bovine insulin the radioimmunoassay must be specific for bovine insulin. Although other researchers have modified radioimmunoassays for bovine insulin, there are always chemical and mechanical problems encountered in the development of a radioimmunoassay for a specific laboratory.

The objectives of this research were to develop a sensitive radioimmunoassay for bovine insulin and to study the influence of bovine

serum insulin and cortisol on total serum lipid. Furthermore, it was hoped that a more thorough understanding of lipid metabolism in the bovine would lead to possible methods of influencing intramuscular lipids as well as increasing the knowledge of bovine subcutaneous fat deposition mechanisms.

CHAPTER II

LITERATURE REVIEW

Human Insulin Studies

Due to the medical significance of diabetes in man, there has been a great deal of research in the area of carbohydrate metabolism and its relationship to insulin. Not until recently has the area of lipids and lipid metabolism taken on a significant role in the insulin diabetes interaction.

Goodner, Conway, and Werrbach (1969) observed that plasma insulin in nonobese human subjects averaged 13 μU/ml of plasma, whereas in obese human subjects plasma insulin averaged 34 μU/ml. Goodner et al. (1969) further showed that in nonobese diabetics, plasma insulin was 39 μU/ml, a value more closely aligned with obese subjects than normal ones. These data support the widely accepted theory that the levels of blood insulin in humans are more closely related to body weight than to the degree of hyperglycemia.

The question as to whether fat and not simply body weight is the cause of high levels of insulin in some humans remains unanswered. Schalch and Kipnis (1965) reported that subjects ingesting a "fat meal mixture" showed a rise in nonessential fatty acids from 488 ± 45 μEq/L to 767 ± 11 μEq/L, but the blood glucose and insulin levels remained unchanged. Kahlenberg and Kalant (1964) concluded that in human incubated adipose tissue from nondiabetics insulin stimulated both the

transportation and phosphorylation of glucose. In human diabetics on the other hand, the glucose transport process in incubated adipose tissue had a decreased responsiveness to insulin. The same conclusions may possibly help explain the high insulin values that Goodner et al. (1969) found in obese subjects.

From these reports it seems reasonable to assume that, in humans, insulin levels and secretory responses of the pancreas are primarily a function of glucose metabolism. Pruett (1970) observed that, when healthy males were stressed in work experiments to 70% of their maximum oxygen uptake, insulin decreased significantly ($P < .01$) at the 50 and 70% level of the subject's maximum oxygen uptake. Furthermore, the tolerance to glucose was markedly increased after the exhausting work and insulin levels closely followed the glucose disappearance curves.

Randle et al. (1963) proposed that the interaction between glucose and fatty acid metabolism in muscle and adipose tissue took the form of a cycle which is fundamental to the control of blood glucose, fatty acid concentration, and insulin sensitivity. This is still further support for the theory that in humans insulin levels are primarily a function of glucose and its metabolites, but that there is a definite and important relationship with lipids.

Rat Adipose Tissue Studies

The glucose-fatty acid cycle that Randle et al. (1963) proposed becomes more evident when taken into account with rat adipose tissue studies. Leonards and Landon (1960) found that incubated rat

epididymal fat was stimulated by the addition of insulin to the medium. Glycogen, as well as oxidation of the #1 carbon atom of glycose to CO_2 and fatty acids, was stimulated. Conversely, increasing the amount of glycose in the medium stimulated the oxidation of the #1 carbon atom of glucose to CO_2 and the synthesis of glycerol. Leonards and Landon (1960) suggested that the action of insulin on glucose alone does not adequately explain the observed phenomena.

Goodman (1967), working with incubated epididymal adipose segments from rats, concluded that both insulin and growth hormone increased the incorporation of ^{14}C in the tissue from glucose, mannose, and fructose. Furthermore, insulin increased the incorporation of ^{14}C from glucose into glycogen. Goodman (1967) suggested that both insulin and growth hormone accelerated the entrance of hexoses into adipose tissue. Denton, Yorke, and Randle (1966) were able to increase the incorporation of glucose-6-phosphate into incubated adipose tissue and muscle with the addition of insulin to the incubation media.

In support of Goodman (1967), Sweeney and Ashmore (1965) showed that insulin deficient rats had a reduction in the incorporation of ^{14}C from labeled pyruvate or acetate into fatty acids in incubated liver slices. These workers further showed that the fatty acid synthesis was reduced in livers of hypophysectomized rats and that anti-insulin serum resulted in a further decrease in lipogenesis. Sweeney and Ashmore (1965) concluded that the fatty acid synthesis from pyruvate and acetate is dependent to some degree on insulin and, further, that the reduction in fatty acid synthesis from labeled pyruvate occurs

independently of any change in pyruvate utilization or incorporation of pyruvate carbons into glucose. The findings of Leonards and Landon (1960), Goodman (1967), and Sweeney and Ashmore (1965) support the hypothesis that insulin and lipid metabolism are closely related.

Fain, Kovaceu, and Scow (1966) reported that insulin reduced both fatty acid and glycerol release from incubated adipose tissue of starved rats. When glucose was added to the medium, the insulin again had an inhibitory effect. Fain et al. (1966) suggested that in adipose tissue cells, insulin has an anti-lipolytic effect whether or not glucose is present.

Gilmour and McKerns (1966) calculated a positive linear relationship between synthesis of ^{3}H labeled lipid and the log of the insulin concentration added to the incubation medium of rat epididymal fat pads. Gilmour and McKerns (1966) concluded that the pentose shunt plays a large role in glucose catabolism and the incorporation of ^{3}H labeled lipids from glucose and that the rate of glucose hydrogens incorporated into lipids might possibly be controlled by the amount of NADP present. However, lipid synthesis was not stimulated with the addition of $NADP^{+}$ or ATP to the medium.

In general support of the theory that insulin influences the synthesis of fatty acids in adipose tissue, Vassalli and Jeanrenaud (1970) showed that insulin decreased an induced lipolysis in incubated rat adipose tissue. Furthermore, Toomey et al. (1970) showed that proinsulin, a precursor of insulin, blocked induced lipolysis. Insulin was also shown to block lipolysis. Both insulin and proinsulin had a

stimulatory effect on glucose oxidation with insulin being the more potent. The findings of Toomey et al. (1970) supported the work of those who suggest that glucose oxidation is enhanced by insulin, and the resulting metabolites are shunted into lipid synthesis. Supporting Toomey et al. (1970), Chlorwerakis (1967) revealed that rat adipose tissue in a medium with insulin but no glucose resulted in a partial inhibition of the rate of lipolysis. However, Chlorwerakis (1967) further stated that at high lipolytic rates the hormone (insulin) not only failed to exhibit an inhibitory effect but had a mild stimulatory action. This work supports the theory that the action of insulin on lipid is independent of its action on glucose.

Insulin: Fatty Acid Synthesis and Release in Rats

Benjamin and Gallhorn (1964) found that lipid biosynthesis was depressed in the diabetic rat and was only partially repaired by insulin added in vitro compared to insulin administration in the live animal. Furthermore, in the diabetic rat a deficiency in oleic acid synthesis was corrected in time after insulin therapy. These researchers concluded that in the diabetic, an oxygenase is defective in lipid synthesis and is corrected only with time and insulin therapy. Further, insulin added in vitro increased the rate of total fatty acid synthesis by diabetic rat adipose tissue. This increase was reflected solely in saturated fatty acid synthesis as there was no concomitant increase in oleic or palmitoleic acid.

Malaisse and Lague (1968) reported that insulin secretion in incubated rat pancreatic tissue was stimulated by β-OH butyrate, palmitate, but not octanoate. This study suggested that the lipid and possibly some protein metabolites stimulate insulin secretion in a process comparable to that of glucose. The findings further suggested that the oxidation of substrate compounds through the Krebs cycle could possibly provide both the stimulus and the energy required to provoke the process of insulin secretion. Furthermore, the keto-acids, fatty acids, and amino acids might be involved in the feedback control mechanism of insulin secretion in vivo comparable to that existing for glucose. Contrary to the findings of Malaisse and Lague (1968), Sanbar and Martin (1967) reported that insulin secretion was significantly increased ($P < .001$) in incubated rat pancreatic tissue by the addition of 3.0 mM octanoate. Insulin levels increased over control levels by 4.9 ± 0.89 μU/mg pancreatic tissue. However with the further addition of glucose and no octanoate, Sanbar and Martin (1967) reported an increased insulin secretion of 2.96 ± 0.96 μU/mg tissue over control levels. According to these workers, octanoate is capable of directly stimulating additional release of insulin from the pancreas above that release caused by glucose.

Although the rat adipose tissue studies are not conclusive, it is reasonable to assume that lipids and insulin are closely related and quite possibly the action of insulin on lipid metabolism is independent of its action on glucose.

Sheep Infusion and Transplant Studies

The effect of short chain fatty acids on insulin in sheep is well documented. Manns and Boda (1967) reported that infusing propionate and butyrate increased plasma insulin, while infusion of glucose did not increase plasma insulin concentrations above the saline controls. Supporting Manns and Boda (1967), Hertelendy et al. (1968) demonstrated, whether glucose was present or not, that significantly more ($P < .001$) insulin was secreted in the presence of propionate or butyrate. The theory is now proposed that the pancreas of ruminants can be directly stimulated by volatile fatty acids independent of glucose utilization. Hertelendy et al. (1968) found that glucose and volatile fatty acids together had a stimulatory effect stronger than either one administered singly, suggesting that the volatile fatty acids and glucose together are additive. Boda (1964) established that glucose is a powerful pancreatic stimulator in ruminants. By infusing exogenous glucose into fasted wethers with an average serum insulin concentration of 126 μU/ml, this worker was able to produce a tenfold increase in 10 min in serum insulin levels. Conclusively, a positive relationship between amount of insulin secreted and utilization rate of glucose was established. Trenkle (1970a) established the same type of relationship as Boda (1964) with the exception that butyrate and propionate were used in place of glucose. Trenkle (1970a) noted a marked increase in serum insulin of sheep infused with butyrate, propionate, and glucose with butyrate giving the greatest increase and glucose the smallest.

By transplanting a portion of a sheep's exteriorized carotid artery into the jugular vein loop, Bell et al. (1970) was able to directly stimulate the pancreas by infusing glucose and butyrate into the transplanted tissue. Infusing butyrate at a rate of 0.2 mM/min for 60 min, serum insulin rose 5-15 μU/ml above controls.

Manns, Boda, and Willes (1967) developed a surgical technique which allowed the enrichment of the pancreatic arterial blood of sheep. By infusing butyrate, insulin secretion was stimulated by a direct effect on the pancreas which was not due to or associated with hyperglycemia. Insulin increased from 52 μU/ml to 120 μU/ml in 3 min in the portal vein plasma by infusing 0.2 mM of butyrate per minute, while blood glucose remained constant. It is thought that the liver may indirectly control pancreatic islet function in sheep because extraction of butyrate and propionate from the portal blood by the liver governs the concentration of substrates in the blood reaching the pancreas. This postulation is supported by the fact that the insulin concentrations reported by Manns et al. (1967) were low in jugular plasma but high in portal plasma due to the degradation in the liver and possible dilution in circulation.

Insulin Studies with Dogs

Madison et al. (1968), by injecting a triglyceride emulsion plus heparin into dogs, found an acute elevation in plasma free fatty acids comparable to that observed during prolonged starvation. Associated with the elevated free fatty acid level was a 40% decrease in plasma glucagon and a 35% decrease in plasma insulin. Madison et al.

(1968) concluded that the inhibitory feedback of free fatty acids on glucagon secretion and stimulatory feedback on insulin secretion may contribute to the smooth transition from carbohydrate to fat metabolism characteristic of prolonged starvation without the progressive danger of prolonged acidosis.

Crespin, Greenough, and Berg (1969) caused acute elevation of plasma free fatty acids by infusion of sodium oleate into conscious dogs. The infusion was accompanied by a rapid onset of a 2 to 12-fold increase in plasma insulin and a subsequent falling of plasma glucose. A significant ($P < .01$) rise in insulin levels accompanied by a decrease in glucose also occurred when glycerol was infused with oleate in order to stimulate lipolysis, whereas tri-olein produced only small changes in plasma insulin levels. The results indicated that under certain conditions, elevated free fatty acid levels may be a potent stimulus of insulin secretion and that oleate can stimulate the release of insulin directly from the pancreas.

Insulin Studies with Cattle

Young et al. (1970) reported that the levels of plasma insulin in milk-fed calves closely followed the same pattern as plasma glucose over a period of time. Plasma insulin concentrations were positively correlated with plasma glucose ($r = 0.78$) and total reducing sugars ($r = 0.72$). There was no significant relationship between age and plasma insulin concentrations. Young et al. (1970) stated that the role plasma insulin plays in decreasing glucose entry into ruminants is unknown.

Nathanielsz (1970), studying the newborn calf, theorized that amino acids play a similar role as glucose after the injestion of feed. By infusing 0.25 g Aminosol/kg in the newborn calf, this worker was able to increase the concentration of plasma insulin. On day 1 insulin concentrations averaged 0.60 ± 0.07 ng/ml after infusion. On days 2 through 6, 15 through 26, and 30 through 60, the increases were 0.83 ± 0.25, 1.10 ± 0.22, and 2.26 ± 0.37 ng/ml, respectively. These data indicated that, although basal levels of insulin remain nearly the same, the pancreas of newborn calves become more sensitive to amino acids with age.

Irvin and Trenkle (1971) found no significant differences in plasma insulin concentrations in steers and heifers at 18 and 204 days of age, indicating that insulin concentrations in the bovine do not increase with age. Increased levels of plasma insulin were observed during the finishing stages, but these researchers attributed this rise to feed composition rather than age and concluded that there were no significant differences due to growth.

Trenkle (1970b) noted differences in plasma insulin concentrations in steers and heifers fed a stilbestrol finishing ration. Levels of plasma insulin gradually increased while the animals were on feed. The correlation coefficient between plasma insulin and weight gain was 0.29, while the relationship between growth hormone and weight gain was 0.13.

Although insulin concentrations are apparently not a function of age in cattle, McAtee and Trenkle (1971) reported significantly

higher ($P < .01$) insulin levels immediately after feeding than before, and significantly ($P < .01$) lower concentrations 46 to 48 hours after feeding than the 6-hr period post feeding. Further, infusing propionate, butyrate, and glucose significantly increased ($P < .01$) plasma insulin levels in fasting steers. Insulin levels rose from 0.5 to 4.9 ng/ml after 30 min of propionate infusion and from 0.6 to 2.8 ng/ml after 15 min of butyrate infusion. Supporting Trenkle (1970b), McAtee and Trenkle (1971) reported that fasting caused a decrease in plasma insulin, while feeding increased insulin concentrations. McAtee and Trenkle (1971) suggested that changes in plasma insulin were caused by more than changes in plasma glucose because of the low correlations between plasma insulin and glucose. Possibly the volatile fatty acids have the ability to directly stimulate the pancreas of cattle.

Trenkle and Irvin (1970) found low correlations between insulin, growth hormone, and body weight in 18- and 198-day-old steers. However, the correlation coefficient between plasma insulin and yearling weight was 0.41 ($P < .01$). Furthermore, there was no significant correlation between plasma insulin and feedlot gain, but plasma insulin in young animals tended to be positively correlated with fat deposition in finished steers. Trenkle (1970b) suggested that a plausible explanation of why growth rate declines with age is that, with maturity, tissues become less responsive to the physiological levels of these hormones in biological fluids.

Cortisol Studies in Rats

Cortisol, like insulin, has received little attention with reference to the bovine species and especially beef cattle. However, the influence of cortisol and other glucocorticoids on lipid metabolism has been widely studied in the rat. Brady, Lukins, and Gurin (1951), studying the incorporation of ^{14}C acetate into long chain fatty acids in incubated rat liver slices, reported that pancreatotomy inhibited the process, whereas hypothysectomy produced a stimulatory effect. Furthermore, Brady et al. (1951) reported that cortisol injections resulted in a profound inhibition of fatty acid synthesis by liver slices of normal rats.

Jeanrenaud and Renold (1960) reported that the metabolism of pyruvate 2-^{14}C and glucose (uniformly labeled ^{14}C, 1-^{14}C, or 6-^{14}C) to CO_2 and fatty acids was stimulated in the tissues from adrenalectomized and normal rats following glucocorticoid administration. Adrenalectomy combined with alloxan diabetes did not modify the metabolic pattern characteristic of diabetes. Jeanrenaud and Renold (1960) further reported that cortisol failed to influence the oxidation of, or lipogenesis from, glucose or pyruvate when added in vitro at a concentration of 30 μg/ml. However, the presence of cortisol increased the net release of free fatty acids from adipose tissue. The effect of cortisol was greater at a concentration of 30 rather than that of 3 μg/ml.

It is apparent that cortisol is involved either directly or indirectly with lipid metabolism. Ashmore et al. (1956) stated that it was evident that cortisol decreased and insulin increased fatty acid

synthesis in the liver of rats. Ashmore et al. (1956) further reported that the activity of glucose-6-phosphate per gram of wet liver was increased over normal in fasting re-fed rats and alloxan diabetic rats. Diabetic adrenalectomized rats had a normal glucose-6-phosphate activity. Injection of insulin in vivo decreased liver glucose-6-phosphate while the adrenal corticoid hormones tended to increase liver glucose-6-phosphate. Ashmore et al. (1956) concluded that the role of cortisol and its effects on lipid metabolism is probably best explained by its effect on glucose-6-phosphatase, which of course would have a tremendous effect on glucose and lipid metabolism.

Goodman and Knobil (1961), studying fasting rats, reported adrenalectomy did not abolish the increase in plasma free fatty acid concentration during fasting, but the magnitude of the rise was greatly reduced. By treating the fasting rats with 1 mg of cortisol acetate for one week, Goodman and Knobil (1961) re-established the normal response to feeding. These workers further stated that, although the pituitary, thyroid, and adrenal glands did not initiate free fatty acid metabolism in response to fasting, they did appear to play a modifying role in this response.

Contrary to Goodman and Knobil (1961), Kabal and Ramey (1960) reported that adrenalectomy resulted in high plasma free fatty acid concentrations which could be depressed to normal levels by cortisol administration. There is no apparent reason for the aforementioned discrepancy.

Leboeuf, Renold, and Cahill (1962) observed no effect on the oxidation of glucose or lipogenesis from pyruvate with the addition of cortisol to the incubated rat adipose tissue at a concentration of 30 μg/ml. However, a significant ($P < .01$) increase in the rate of free fatty acid release into the incubation medium was observed with the addition of cortisol or corticosterone.

Slavin and Elias (1970) reported that explants of white and brown fat from young mice fasted for 48 hr responded differently to insulin and cortisol. In both brown and white adipose tissue, insulin acted to increase the cell size and amount of intracellular lipid. Cortisol administration had no influence on cell size or intracellular lipid. When cortisol and insulin were administered in combination, there was no significant increase in either cell size or intracellular lipid. The work of Slavin and Elias (1970) suggested that the role of cortisol in lipid metabolism is stronger than that of insulin due to the fact that when given together cortisol could cancel the increase in intracellular lipid caused when insulin was administered alone.

McGraw (1969) reported values for plasma free fatty acids of 0.04 μEq/ml in normal rats, 1.30 μEq/ml in diabetic rats, and 0.30 μEq/ml in adrenalectomized rats. Treatment with cortisol for 24 hr increased plasma free fatty acids in adrenalectomized rats to 2.24 μEq/ml. McGraw (1969) further reported that administration of cortisol to adrenalectomized diabetic rats returned the animals to a diabetic state manifested by high plasma free fatty acid concentrations and increased glycerol release.

Fain, Scow, and Chernick (1963) reported that the role of the adrenal cortex in fat metabolism is generally considered to be a permissive one, and further that glucose uptake and conversion of CO_2, total lipid, and fatty acids are decreased by glucocorticoids.

Cortisol Studies in Sheep

Paisey and Nathanielsz (1971) reported that plasma cortisol levels of ten newborn lambs were significantly higher ($P < .02$) on day 1 of postnatal life than on any other day postnatal. A value of 6.3 µg of cortisol/100 ml plasma on day 1 was reported and the cortisol levels remained between 1.0 and 2.0 µg/100 ml from 6 to 30 days of age with no further increase in plasma cortisol with age.

Panaretto and Vickery (1970) observed that plasma cortisol levels increased greatly when shorn sheep were exposed to cold, wet conditions for up to 70 hours. The increase continued until concentrations of 10 to 20 µg/100 ml were reached. These high levels of plasma cortisol are approximately ten times higher than those reported by Paisey and Nathanielsz (1971), indicating that under normal conditions plasma cortisol levels do not increase with age in sheep.

Bassett (1963) reported that ewes administered 25 mg of cortisol/day for a 21-day period had an increased feed intake. When levels of 50 to 75 mg/day were administered there was no additional response over the 25 mg/ day level. Furthermore, 150 mg of cortisol/day resulted in a marked decrease in voluntary feed intake. Only very small and nonsignificant changes in blood ketones and plasma free fatty acids during the cortisol administration were noted.

Spurlock and Clegg (1962) reported that 80 weaned ewes and wethers injected three times weekly with 25, 100, or 300 mg of cortisol had significantly higher ($P < .01$) gain, feed conversion, percent fat in the carcass, and fat depth over the Longissimus muscle at the 12th rib. Furthermore, carcass grade scores were increased with treatment. Spurlock and Clegg (1962) concluded by stating that cortisone shows promise for use in finishing lambs, providing they are old enough to have completed 75% of their muscular development before treatment.

Although the exact roles of both insulin and cortisol are not known with reference to lipids, it is clear that they are involved with lipid metabolism. Eisenstein (1967) reported that most of the effects of the corticoids are opposed by insulin. Homeostatis is therefore maintained by a counterbalance of the two hormones. It is therefore reasonable to assume that both insulin and cortisol play an important role in lipid metabolism, but as evidenced by the literature this role has yet to be established.

CHAPTER III

MATERIALS AND METHODS

Canulated Steers

Two steers of comparable weights (250 kg) were chosen for four experiments. Polythylene catheters (16.7 mm I.D.) were placed in the jugular veins of each steer using the method of C. B. Theurer (Dept. of Animal Science, The University of Arizona, personal communication, 1971). When not being used, the catheters were filled with 2.5% sodium citrate and closed with a tight fitting stainless steel brad. The animals were bled twice daily even when not on test. This aided in keeping the catheters clear for a longer period of time. Blood was taken with a 20-ml disposable syringe fitted with a sharp 18-gauge needle. If the blood was to be saved, it was immediately poured from the syringe into a 50-ml nalgene centrifuge tube, stored at 4 C for 24 hr to allow cotting, and then centrifuged at 10,000 rpm for 10 min. The serum was pipetted off and placed in nalgene bottles. One milliliter of of a 1% merthiolate solution was added per 100 ml of serum as a preservative and the samples were stored at 4 C until analysis.

Experiments I and II

During the first and second experiments, the animals were maintained on a 60% concentrate ration, each receiving 3.6 kg twice daily. While on feed, the animals were bled every 4 hr for a 48-hr period.

The first bleeding was at 7 am on day 1 and the last was at 7 am on day 3. At each bleeding, approximately 50 ml of blood was taken.

Experiment III

During the third experiment, the animals were bled every 4 hr for a 72-hr period. However, 24 hr before the experiment began, the animals were taken off feed and were not fed again until the 48th hour of the experiment, when normal feeding resumed.

Experiment IV

Five days after the completion of the third experiment, one of the steers died. Consequently, the catheter was removed from the remaining steer. Four weeks later, a new steer of comparable weight was obtained, and a catheter was again placed in both animals. At this point the steers were maintained on a 75% concentrate ration; both received feed twice daily. During this experiment, the animals were bled at 3 pm daily for a period of four weeks.

Production of High Insulin Serum

In general, the procedure of McAtee and Trenkle (1971) was followed for the production of high insulin serum. Four crossbred steers of comparable weights (200 kg) were chosen. All of the steers had the jugular previously catheterized.

To obtain the high insulin serum, two steers were infused with propionate (1 mM/ml) and two with butyrate (1 mM/ml). Before the infusion began, approximately 50 ml of blood were taken from each animal with a 20-ml syringe. Following this, 40 ml of substrate were infused

over a 5-min period. Five minutes post infusion, blood was removed from the catheter with a syringe until clotting within the catheter occurred. Each syringe full of blood was poured into a separate 50-ml nalgene centrifuge tube. A total of approximately 500 ml of blood was taken from each steer.

The blood was allowed to clot for 24 hr at 4 C and then centrifuged at 10,000 rpm for 10 min. The serum was extracted, pooled, and frozen. Table 1 shows the scheme for the collection of blood and how the serum was pooled for each steer.

Production of Insulin Free Serum

An alloxan diabetic sheep was produced by injecting intravenously 40 mg of alloxan/kg body weight in a yearling ewe lamb (Reid and Hinks 1963). The alloxan was dissolved in 20 ml of sterile water and injected into the jugular over a period of 2 to 3 min.

The injected sheep was observed daily for any abnormal conditions. On the 7th day post injection, labored respiration and a general lack of alertness was noticed. Consequently, 700 ml of blood were taken by jugular puncture. On the 8th day post injection, the animal was down and nearly dead. Again, 700 ml of blood was taken. The sheep died less than 5 hr after the second bleeding.

The blood from both days was stored at 4 C for 24 hr to allow clotting. It was then centrifuged at 10,000 rpm for 10 min and the serum was extracted and frozen.

Table 1. Protocol for the collection of high insulin serum.

Propionate infusion		Butyrate infusion	
Animal 1		Animal 3	
Tube no.		Tube no.	
*1	preinfusion	*1	preinfusion
2	preinfusion	2	preinfusion
	5 min infusion period		5 min infusion period
*3	9.5 min post infusion	*3	9.5 min post infusion
4	12 min post infusion	4	12 min post infusion
*5	13 min post infusion	*5	13 min post infusion
6	14 min post infusion	6	14 min post infusion
7	15 min post infusion	7	15 min post infusion
8	15.5 min post infusion		
*9	16 min post infusion		
10	19 min post infusion		
Animal 2		Animal 4	
*1	preinfusion	*1	preinfusion
2	preinfusion	2	preinfusion
	5 min infusion period		5 min infusion period
*3	8.5 min post infusion	*3	8 min post infusion
4	14 min post infusion	4	10 min post infusion
*5	15 min post infusion	*5	12 min post infusion
6	17 min post infusion	6	13 min post infusion

* The serum in the tube with the asterisk (*) was pooled with the serum from the tube(s) immediately below without an asterisk.

Chemical Analyses

Preparation of Reagents for Radioimmunoassay of Bovine Insulin

Unless otherwise stated, all reagents were stored at 4 C in capped nalgene bottles. The charcoal solution was made fresh every three days or when needed. The other reagents were made only when needed.

pH 2.5 Distilled Water. Five hundred ml of water were brought to a pH of 2.5 by adding 10 N HCl. The solution was stored at room temperature.

pH 8.6 Buffer. [Barbitol buffers and charcoal solution according to the procedure of A. Trenkle (Dept. of Animal Science, Iowa State University, personal communication, 1972)]

1. Add 2.76 g of diethyl barbituric acid, 15.40 g of sodium diethyl barbiturate, and 650 ml of distilled water to a 1000-ml beaker

2. Add 75 mg of Merthiolate and 13.96 g of disodium EDTA

3. Stir on magnetic stirrer and adjust the pH to 8.6 with 45% NaOH (a higher pH may be required to get the EDTA into solution. if so, bring the pH back to 8.6 with 10 N HCl)

4. Bring the solution to a final volume of 750 ml with distilled water and store

Protein Buffer. Ten ml of normal guinea pig serum was added to 990 ml of the pH 8.6 buffer and mixed well.

Stock A Insulin Solution.

1. Add exactly 10 mg of bovine crystalline insulin (Sigma Chemical Company, St. Louis, Mo.) into a 10-ml volumetric flask

2. Dissolve with 2 to 3 ml of pH 2.5 water

3. Bring to a final volume of 10 ml with protein buffer

4. Final concentration is 1 mg/ml

5. Divide between two polystyrene tubes; cap and store

Stock B Insulin Solution.

1. Ten µl of stock A insulin solution diluted to 10 ml with protein buffer

2. Divide between two polystyrene tubes; cap and store

pH 7.4 Buffer.

1. Add 1.47 g of sodium barbitol, 0.97 g of sodium acetate, and 7.65 g of sodium chloride to a 1000-ml beaker with 900 ml of distilled water

2. Stir on magnetic stirrer and adjust pH to 7.4 with 10 N HCl

3. Bring to a final volume of 1000 ml with distilled water and store at room temperature

Charcoal Solution.

1. Add 7.5 g of Norite-A charcoal (Amend Drug and Chemical Co., Inc., New York, N.Y.), 0.75 g of dextran T-80 (Pharmacia Fine Chemicals, Piscataway, N.J.), and 3 g bovine serum albumin V fraction to a 100-ml beaker

2. Add 100 ml of pH 7.4 buffer

3. Stir on magnetic stirrer until the charcoal is suspended in the solution

4. Store in a parafilm-covered beaker at 4 C

0.5 M Sodium Phosphate Buffer (Monobasic). [Phosphate buffers according to the method of Niswender et al. (1969)]

1. Weigh 69.005 g of sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$)

2. Transfer to a 1 L volumetric flask and add approximately 500 ml of distilled water

3. Place on magnetic stirrer until completely dissolved

4. Bring to a final volume of 1000 ml with distilled water and store

0.5 M Sodium Phosphate Buffer (Diabasic).

1. Weigh exactly 70.98 g of sodium phosphate diabasic (Na_2HPO_4)

2. Transfer to a 1 L volumetric flask and add approximately 500 ml of lukewarm water

3. Place on magnetic stirrer until completely dissolved

(A solid cake will form which takes time to dissolve.)

4. When the solution is at room temperature, bring to a final volume of 1000 ml with distilled water and store

0.5 M Buffer. Add the monobasic solution to the desired volume of diabasic solution until the pH reaches 7.5 (the quantities in question depend entirely on the volume of desired 0.5 M buffer); add 0.1 g of Merthiolate per 100 ml of buffer and store

0.05 M Buffer. Dilute the 0.5 M buffer 1:10 with distilled water

Column Preparation

The column was constructed from a 10-ml disposable polystyrene pipette, which was shortened by removing the portion above the zero ml

mark. A small, glass-wool plug was inserted into the delivery end, and a suitable stopcock was made by fitting the delivery end with a 5-cm piece of rubber tubing and closing with a clamp.

Ten to 15 g of Sephadex #G-10-120 (Sigma Chemical Company, St. Louis, Mo.) was mixed with 40 to 45 ml of 0.05 M buffer. The mixture was slurried gently but thoroughly and allowed to set overnight at 4 C. After 15 hr at 4 C, the mixture was reslurried and evacuated with 75 additional ml of 0.05 M buffer and 5 ml of 1% egg white. The three solutions were evacuated for a minimum of one hour.

Following evacuation, approximately two-thirds of the buffer above the Sephadex was aspirated off and discarded. Before pouring the Sephadex, the column was prepared by placing 4 to 5 ml of 0.05 M buffer in the closed column, opening the clamp and allowing 2 to 3 ml to drain out. The column was closed, the Sephadex gently reslurried, and enough was poured to give a final packed length of 10 to 12 cm. When the Sephadex had begun to settle, the clamp was opened and 20 to 30 ml of evacuated 0.05 M buffer was passed through the settling bed. When the last of the buffer was about to flow into the Sephadex, 1 ml of the 1% egg white was added to the column. After the egg white had nearly passed into the bed, an additional 10 to 15 ml of 0.05 M buffer was added. The column was closed and considered ready for use when 0.5 ml of the buffer remained above the Sephadex bed.

Iodination of Insulin

The conical 0.5-ml iodine shipment vial containing 1 mCi ^{131}I (Industrial Nuclear, St. Louis, Mo.) served as the reaction vial.

Fifty µl of 0.5 M buffer, followed by 2 µl of stock A insulin were transferred to the vial. Immediately following, 25 µl of chloramine T (6 mg/10 ml 0.05 M buffer) were delivered to the vial and allowed to react for one minute. During the reaction time, the vial was tilted from side to side to mix the reactants. The reaction was stopped by adding 50 µl of sodium metabisulfite (18 mg/10 ml 0.05 M buffer). The contents of the vial were then transferred to the previously prepared column with a transfer pipette that had been coated with 1% egg white solution. When the contents from the reaction vial had passed into the Sephadex, the column was filled with the evacuated 0.05 M buffer, and 0.5-ml aliquots of eluate were collected in disposable polystyrene tubes containing 0.5 ml of protein buffer.

As each 0.5 ml aliquot was eluted from the column, it was counted for 0.5 min, and the first tube on the descending side of the first peak was selected for the assay (Figure 1). This tube was usually number 5, 6, or 7, depending on the column length. The contents of the selected tube were diluted with sufficient protein buffer to give a final dilution containing 20,000 to 30,000 cpm/100 µl. The diluted ^{131}I was stored in nalgene bottles at 4 C until needed.

First Antibody Titration

One ml of antibody to bovine insulin produced in guinea pigs was purchased from Miles Laboratories, Inc., Kankakee, Illinois. The entire one ml was diluted to 100 ml with pH 8.6 buffer, divided into 5-ml aliquots, and frozen.

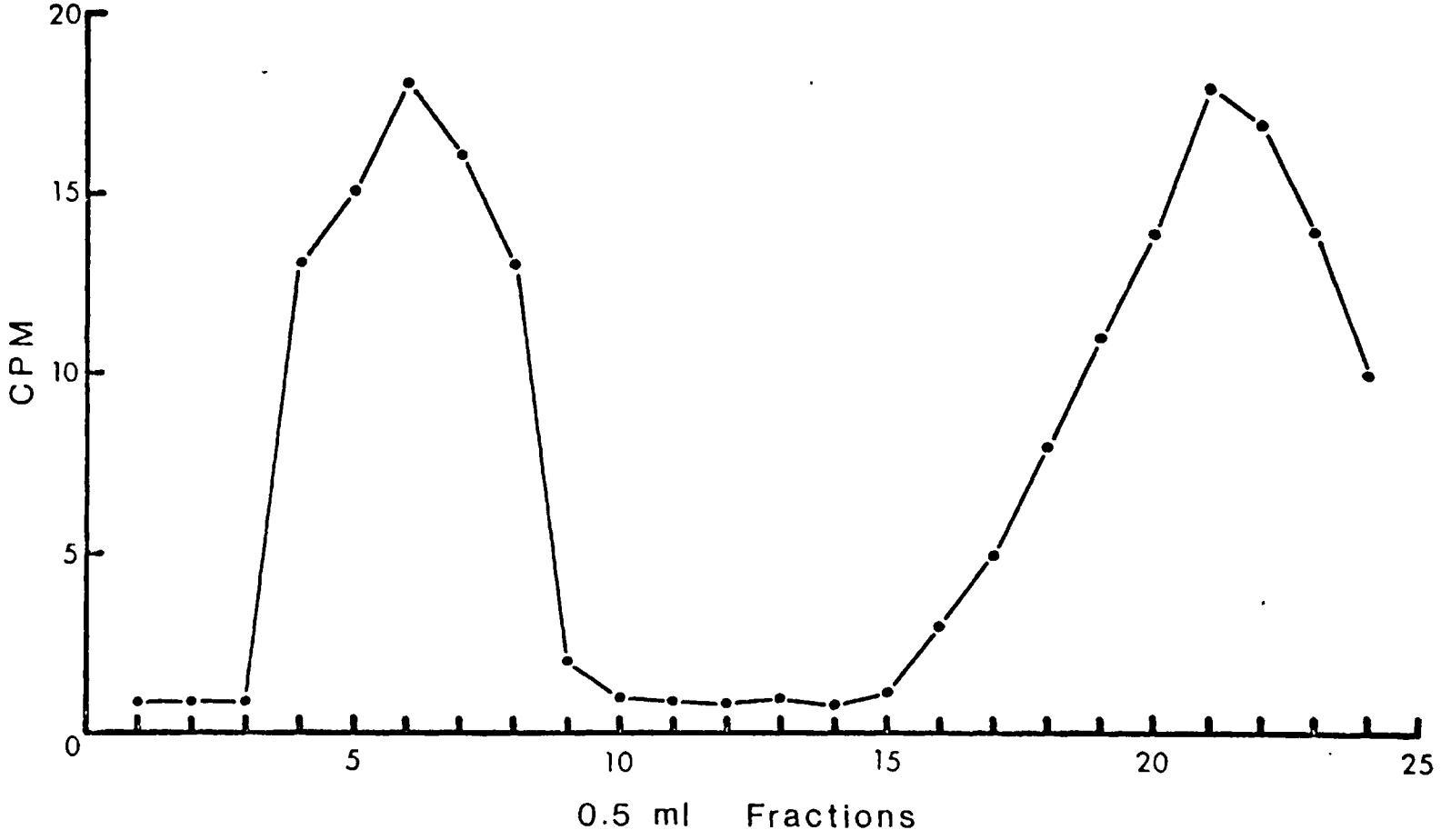


Figure 1. ^{131}I Iodination curve for bovine insulin.

The dilutions of the first antibody for the titration curve were made with protein buffer in 12 mm x 75 mm polystyrene tubes. The following illustration shows schematically how the dilutions were made:

a. stock antibody 1:100 . . . . . 10^2

b. 100 µl of 10^2 plus 900 µl protein buffer . . . . . 10^3

c. 100 µl of 10^3 plus 900 µl protein buffer . . . . . 10^4

d. 100 µl of 10^4 plus 900 µl protein buffer . . . . . 10^5

e. 100 µl of 10^5 plus 900 µl protein buffer . . . . . 10^6

f. 100 µl of 10^6 plus 900 µl protein buffer . . . . . 10^7

The titration curve consisted of six points run in triplicate. The tubes were first numbered, and the correct amounts of protein buffer, first antibody, and ^{131}I insulin to give a final volume of 1 ml were added (Table 2). The tubes were mixed and incubated at 37 C for 2 hr after which 1.5 ml of constantly stirred charcoal was added. Again the tubes were mixed and allowed to incubate at room temperature for 1 hr. The tubes were then centrifuged at 4500 rpm for 40 min, and the supernatant was aspirated and discarded. The tubes were counted for 0.5 min and the titration curve constructed on semi-log paper, plotting antibody dilution logarithmically and the percent binding linearly (Figure 2). The dilution that bound approximately 50% of the ^{131}I insulin was chosen for the assay. A titration curve was run after every iodination to check the antibody dilution. Table 2 shows the protocol used for the first antibody titration assay, and the formula needed for calculations is as follows:

Table 2. Protocol for first antibody titration.

Tube no.	First antibody dilution 200 µl	µl ^{131}I Insulin	µl Protein buffer
1	10^2	200	600
2	10^2	200	600
3	10^2	200	600
4	10^3	200	600
5	10^3	200	600
6	10^3	200	600
7	10^4	200	600
8	10^4	200	600
9	10^4	200	600
10	10^5	200	600
11	10^5	200	600
12	10^5	200	600
13	10^6	200	600
14	10^6	200	600
15	10^6	200	600
16	10^7	200	600
17	10^7	200	600
18	10^7	200	600
Total count tubes (5)	0	200	800

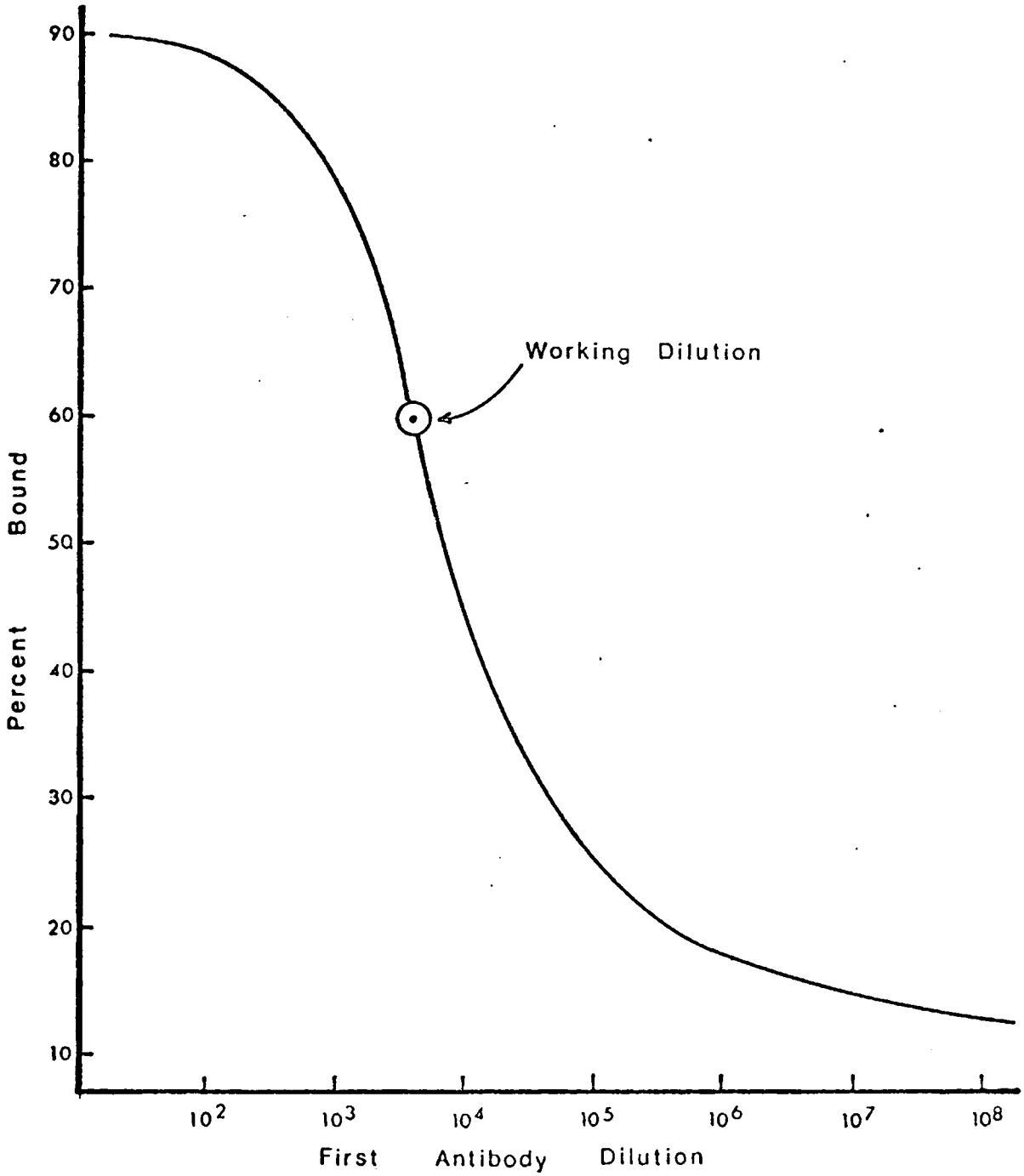


Figure 2. First antibody titration curve for antibovine insulin.

$$\% \text{ Binding} = \frac{(\text{total counts} - \text{charcoal bound counts})}{\text{total counts}}$$

Insulin Dilutions for Standard Curve

The insulin standards for the standard curve were made from the stock B insulin solution. The standards were made in 10-ml volumetric flasks containing 5 ml of protein buffer. The necessary volume of stock B insulin solution was added, and the flask brought to volume with protein buffer following the protocol of Table 3. The standards were then transferred into polystyrene tubes, capped, and stored at 4 C.

Protocol for the Insulin Radioimmunoassay

The procedure for the radioimmunoassay generally follows that of Yallow and Berson (1964). A sample protocol sheet is shown in Table 4. All tubes (10 mm x 75 mm disposable polystyrene) had a final volume of 1 ml making the dilutions of first antibody and ^{131}I insulin the same throughout the assay.

The tubes were first numbered and the correct amount of protein buffer was added. After the addition of the standards or unknowns and first antibody, the tubes were mixed gently to avoid foaming and incubated at 4 C for 5 days. The ^{131}I insulin was added following the first incubation, and again the tubes were mixed and incubated at 4 C for 48 hr. Following the second incubation, 1.5 ml of constantly stirred charcoal solution were added, and the tubes were mixed and incubated at 4 C for 2 hr. The tubes were centrifuged at 4500 rpm for 40 min, and the supernatant was aspirated off and discarded.

Table 3. Standard insulin dilutions.

Desired ng of insulin/ 100 µl	Volume of stock B insulin
0.05*	5.0 µl
0.10*	10.0 µl
0.20*	20.0 µl
0.30	30.0 µl
0.40*	40.0 µl
0.60*	60.0 µl
1.0*	100.0 µl
2.0*	200.0 µl
2.5	250.0 µl
4.0*	400.0 µl
9.0	900.0 µl
10.0*	1.0 ml
25.0	2.5 ml
30.0*	3.0 ml
40.0	4.0 ml
50.0*	5.0 ml

* Indicates those solutions actually used in the standard curve.

Table 4. Protocol for radioimmunoassay of bovine insulin.

Tube no.	µl of unk. or conc. of std. (ng/100 µl)	µl First antibody	µl ^{131}I insulin	µl Protein buffer
Tubes with unknown	250 µl	200	200	350
Control tubes (8)	0	200	200	600
Total count tubes (5)	0	0	200	800
Standard curve tubes				
1.	0.05	200	200	500
2.	0.05	200	200	500
3.	0.1	200	200	500
4.	0.1	200	200	500
5.	0.2	200	200	500
6.	0.2	200	200	500
7.	0.4	200	200	500
8.	0.4	200	200	500
9.	0.6	200	200	500
10.	0.6	200	200	500
11.	1.0	200	200	500
12.	1.0	200	200	500
13.	2.0	200	200	500
14.	2.0	200	200	500
15.	4.0	200	200	500
16.	4.0	200	200	500
17.	10.0	200	200	500
18.	10.0	200	200	500
19.	30.0	200	200	500
20.	30.0	200	200	500
21.	50.0	200	200	500
22.	50.0	200	200	500

After the standards had been counted, a standard curve was constructed on four-cycle semilog paper (Figure 3). The standard concentrations were plotted logarithmically and the percent binding linearly. From the resulting curve, the concentrations of the unknowns were determined. Formulae for the calculations were as follows:

$$\text{\% Binding in control} = \frac{\text{(total counts-control counts)}}{\text{total counts}} \times 100$$

$$\text{\% Binding in standards or unknowns} =$$

$$\frac{\text{(total counts-control counts)-(unknown or standard counts)}}{\text{total counts - control counts}} \times 100$$

Serum Lipid Extraction

Total serum lipid was extracted using the method of Folch, Lees, and Sloane Stanley (1957). Two ml aliquots of serum were extracted in duplicate with chloroform-methanol (2:1 v/v) and the weight of the lipid was obtained following evaporation of the solvent and drying of the lipid under vacuum at 50 C for 12 to 14 hr.

Competitive Protein Binding Method for Bovine Serum Cortisol

The following cortisol assay generally follows the procedure of Murphy (1967) with modifications by G. Stott (Dept. of Dairy Science, The University of Arizona, personal communication, 1972).

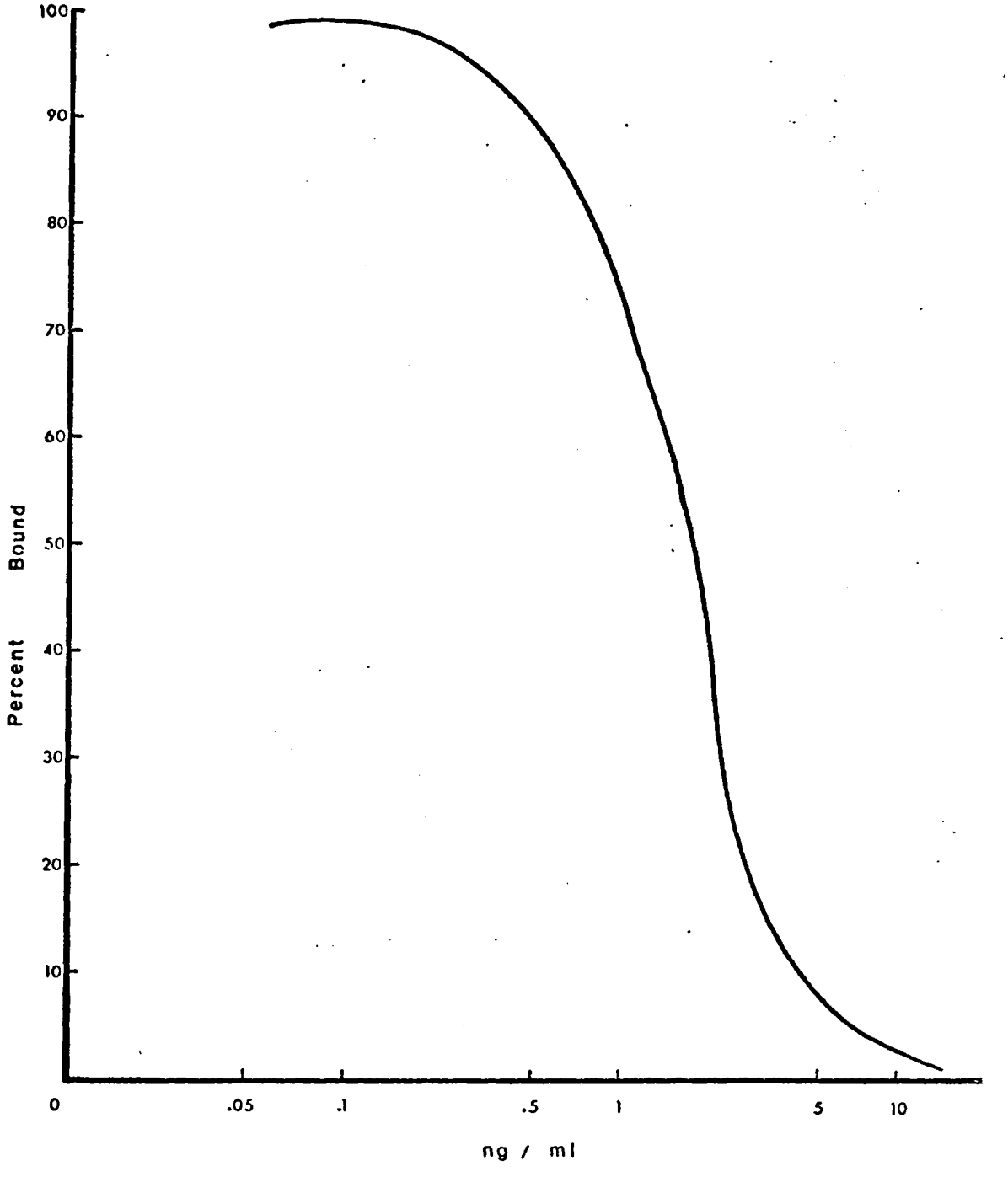


Figure 3. Standard curve for the radioimmunoassay of bovine insulin.

Corticosteroid Binding Globulin (CBG).

1. Place 0.25 mCi of Corticosterone (New England Nuclear Corporation, Boston, Mass.) into a 25-ml volumetric flask, fill to volume with absolute ethanol, and store in individual glass vials (0.5 ml/vial) at -20 C

2. Place 5 μCi Corticosterone-1,2-H^3 (1 vial) in a 100-ml volumetric flask

3. Add 75 ml PO_4 buffer (0.1 M KH_2PO_4 and K_2HPO_4 in distilled water)

4. Add 2.5 ml dog serum (frozen and stored in 2.5-ml aliquots)

5. Fill to volume with PO_4 buffer and store at 4 C in a dark glass bottle

Cortisol Assay.

1. Pipette 50 μl of serum into a 12-ml conical centrifuge tube

2. Add 500 μl of ethanol and mix for 30 sec

3. Centrifuge at 2000 RPM for 15 min

4. With a disposable pipette extract the ethanol and transfer to a 15 x 85 mm culture tube

5. Repeat the ethanol extraction (tubes may be covered and temporarily stored at 4 C at this time if necessary)

6. Evaporate to dryness under N_2 at 40 to 50 C

7. Add 1 ml cold CBG (in ice bath) to each culture tube; shake each tube for 10 sec and incubate at 45 C for 5 min

8. Cool in ice bath for 10 min

9. Using a premeasured plastic spoon, add approximately 40 mg of Florisil (60/100 mesh, Floridin Company, Tallahassee, Florida) to the tube and shake for 15 sec; wait 15 sec and add Florisil to the next sample, repeating this procedure until all samples are finished.

10. At the same time that Florisil is being added to the fifth sample, another person should transfer 500 µl of the CBG mixture, avoiding any Florisil, to a vial containing 10 ml of scintillation fluid.

11. Keeping 3 samples apart, the two people repeat this procedure until all samples have been exposed to Florisil for 2 min 30 sec, and 500 ul of CBG taken off.

12. Count each vial and compare to 0, 0.5, 1, 2, 3, 4, 5, 6, 8, and 10 ng.

Scintillation Fluid

1. Place 0.3 g of POPOP (Matheson, Coleman, and Bell, Norwood, Ohio), and 5.0 of PPO (MC&B) in a 1-liter flask.

2. Fill to volume with toluene and mix well.

3. Add 100 ml of BIO-SOLV (Beckman Instruments, Fullerton, Calif.) and store in a dark bottle.

Statistical Procedure

Simple correlation coefficients were calculated using the method of Steel and Torrie (1960).

CHAPTER IV

RESULTS AND DISCUSSION

General

The serum cortisol and lipid data from Experiments I, II, III, and IV as well as the serum insulin and lipid data from the infusion of propionate and butyrate are presented and discussed in this chapter. In addition to the presentation of numerical data, the problems encountered with the development of the insulin assay and their solutions are discussed in detail. In view of the fact that radioimmunoassay of bovine insulin is relatively new, it is hoped that this work will be of considerable aid to other researchers in adapting the insulin radioimmunoassay to their laboratories and specific needs.

Radioimmunoassay of Bovine Insulin

Iodination of Bovine Insulin. The proper iodination of the hormone was one of the most critical aspects of the assay. Yallow and Berson (1964) stated that the labeled insulin should be prepared at the highest possible specific activity consistent with the maintenance of the molecule.

After several less than satisfactory iodinations of insulin, following the procedure of Greenwood, Hunter, and Glover (1963), it became apparent that the concentration of chloramine T used in the reaction was not giving a suitable antigen. Yallow and Berson (1964) used 10 to 20 µl of a 35 mg/100 ml solution, while Greenwood et al. (1963)

suggested the use of 24 µl of a 40 mg/10 ml solution. The two aforementioned concentrations, at their extremes, differ by as much as 50 µg of chloramine T actually delivered. However, the quantity of ^{131}I used by these researchers was similar.

By varying the amount of chloramine T in the iodination reaction, the responses obtained with the standard curve varied. After using the published concentrations and obtaining standard curves with various slopes and sensitivities, which could not be repeated, the concentration of chloramine T was varied from as much as 20 mg/10 ml to as little as 2 mg/10 ml. Again, unusable standard curves resulted from the extreme concentrations because of the production of unsuitable labeled antigen. At the higher concentration, the integrity of the molecule was most likely altered due to over-oxidation, while at the low concentrations there was little oxidation of the tyrosine residues resulting in a low specific activity. It was therefore determined, through trial and error, that 25 µl of a 6 mg/10 ml solution of chloramine T was the most desirable concentration to use. Using this concentration of chloramine T resulted in the production of a labeled antigen which could be used in developing a standard curve with a steep slope, a less than one ng insulin sensitivity (Figure 3), and a specific activity of approximately 175 µCi/µg insulin.

Although the concentration of chloramine T was found to be a critical factor in the iodination procedure, the concentration of sodium metabisulfite was not nearly as critical. The concentration of sodium metabisulfite used (18 mg/10 ml) was very similar to that used by Greenwood et al. (1963) and Yallow and Berson (1964).

The iodine was specifically purchased in 1-mCi quantities in 0.5-ml conical vials. Further the iodine was shipped in a minimum volume with an activity of between 500 and 600 mCi/ml. The conical vial was used as the reaction vial, eliminating the need to transfer the iodine, thus preventing the evaporation of the iodine solvent that occurred when attempts were made to transfer the iodine to a polystyrene reaction vial. After preparation, the labeled antigen was diluted with protein buffer to a volume giving approximately 30,000 cpm/100 µl. However, it should be noted that a dilution of the labeled antigen, giving 25 times fewer cpm/100 µl, did not alter the sensitivity of the standard curve or first antibody required to give approximately 60% binding.

The described modifications of the iodination procedure worked well in this laboratory but it must be emphasized that in another location these changes may not be desirable. Moreover, before the assay can be developed further the iodination procedure must yield an antigen which will give repeatable data.

First Antibody. A working first antibody of 1:5000, which bound approximately 60% of the labeled antigen, was selected for use in the assay (Figure 2). A. Trenkle (Dept. of Animal Science, Iowa State University, 1972), also using antibovine insulin produced in guinea pigs (purchased from the same source), reported a working dilution of 1:7000 which bound 50% of the labeled antigen. The working dilution used and that used by A. Trenkle were very similar, indicating that the antibody was responding to a similar degree in both assays. Since

the first antibody dilutions were alike in both assays, this indicated that the labeled antigens of both assays must have been comparable.

The antibody was stored at -20 C in 5-ml aliquots at a dilution of 1:100. These dilutions were thawed and refrozen only once. The working dilution was stored at 4 C for as many as three weeks with no apparent adverse effects on the assay.

Insulin Standards and Standard Curve. The pH of the standards was found to be an important factor in the sensitivity of the assay. Originally the standards were made with pH 2.5 water which resulted in nonsensitive standard curves. If a minimal amount of pH 2.5 water was used to solubilize the insulin and protein buffer was used to make the dilutions, sensitive and repeatable standard curves were obtained.

The insulin standards stored at 4 C were held as long as five months with no detectable loss or change in the insulin molecule. Preliminary work indicated that if glass tubes were used in the assay the standard curve was less sensitive. This was apparently caused by a loss of insulin due to its adsorption to the glass. Yallow and Berson (1964) prepared insulin standards with serum albumin to prevent loss due to adsorption. Furthermore, Schwarz Mann Co. (1971) recommended the use of polystyrene tubes when working with insulin because of the low affinity of insulin for this material. The longevity of the insulin standards was apparently due to the protein buffer with which they were made and the polystyrene tubes in which they were stored.

Originally it was thought that, in order for the standards to be comparable with the unknown serum samples, noninsulin serum should

be added. Greenwood et al. (1963) have reported alloxanized serum in the standard curves for insulin, while Yallow and Berson (1964) make no mention of the use of noninsulin serum. Consequently, alloxanized sheep serum was added to the standards, control tubes, and total count tubes, thus eliminating possible competing factors from the serum and making the standards comparable to the unknowns. However, there was little or no change in the slope or sensitivity of the standard curve when differing amounts of alloxan serum were added to the standards solutions. Furthermore, the alloxan serum had no effect on the control tubes or total count tubes and thus was not used in the assay.

Finally, the insulin standards required accurate volumetric handling in order to obtain a true sigmoid-shaped standard curve. The insulin standards were made with lambda pipettes to obtain the desired accuracy. The standards used were convenient to prepare, had a broad range (0.05 ng to 50.0 ng), were easily stored for long periods of time, and most important could be prepared to have a concentration that was very repeatable.

Sample Collection

Heparin was not used to prevent clotting in the polyethylene catheters in the experimental animals. This was done to avoid the introduction of excessive quantities of heparin into the blood and making it available to react with lipoprotein lipase as well as other enzymes and other metabolic pathways which are influenced by heparin. This eliminated any possible alteration in the levels of serum insulin which may have been caused by heparin. Consequently, a 2.5% solution

of sodium citrate was used as an anticoagulant in the catheters. Furthermore, the serum was extracted and assayed rather than plasma. Henderson (1970) reported that serum should be used when using the radioimmunoassay to determine insulin. This worker found heparin to cross-react with some unidentified factor in the assay, resulting in low insulin values.

Loss of Serum Insulin

As mentioned previously, the serum samples were held at 4 C and preserved with Merthiolate ($1/10^4$, w/v). Furthermore, the samples were stored for nearly a year while the assay was being developed. Once the assay was working it became apparent that the insulin molecule had been degraded or had undergone a structural change because essentially no serum insulin could be detected in the samples from Experiments I, II, or III and 80% of the samples from Experiment IV. However the preinfusion serum samples from the steers infused with propionate and butyrate were stored frozen and did have detectable serum insulin, indicating that the loss of insulin was a function of storage temperature and not of the assay.

Henderson (1970) reported that serum should be frozen if analysis for insulin was not immediate. Furthermore, Hales and Randle (1963) reported that freezing resulted in no loss of insulin activity.

It was possible that the serum insulin levels of the experimental animals were less than the sensitivity of the radioimmunoassay. However, the literature reviewed (Trenkle 1970b, McAtee and Trenkle 1971) did not indicate that such a problem existed. Furthermore, A.

Trenkle (Dept. of Animal Science, Iowa State University, personal communication, 1972) reported that 100 µl of serum was used in the radioimmunoassay of beef insulin. For this assay, unknowns of 250 µl were used and no serum insulin was detected, indicating that the serum insulin had been lost. It was therefore reasonable to assume that the animals from Experiment I, II, III, and IV did have higher serum insulin levels than the results indicated.

It has been established that the animals in Experiment I, II, III, and IV most likely had higher serum insulin levels than the assay indicated. Furthermore, it is recognized that serum should be frozen when stored for long periods of time. However, the question as to why the serum insulin was lost in the unfrozen samples remains unanswered. It was possible that the Merthiolate, used as a preservative, cross-reacted with some component of the unfrozen serum (possibly the insulin itself) resulting in nondetectable insulin concentrations. On the other hand, a proteolytic enzyme system may have remained functional in the unfrozen serum and over a period of time destroyed the integrity of the insulin molecule. The long period of time that the samples were stored could have also been an important factor in the loss of the serum insulin.

It seems reasonable to assume that the Merthiolate is not a contributing factor in the insulin loss because Merthiolate was used in the insulin standards with no apparent adverse effect on the insulin molecule. Also the length of time in storage is not a plausible explanation for the loss because the insulin standards were stored

unfrozen for as long as 5 months with no detectable change in the insulin molecule. Therefore, at this time it is reasonable to assume that some enzyme system remained functional in the unfrozen serum and destroyed the insulin molecule. However before this question can be totally resolved, more study in this area is needed.

Insulin and Lipid Data

The high serum insulin was produced to obtain a sample which could be used routinely in the assay as a quantitative evaluation of the insulin assay. The amount of insulin in this serum, as judged from two separate radioimmunoassays and lipid data, is presented in Table 5. Although the absolute values between the two assays were not exactly the same, the detected changes in serum insulin concentration were definitely similar and of the same magnitude for both assays.

The greatest response was seen in the serum from tubes three and four (approximately 10 min post infusion) for both propionate and butyrate. Averaging tubes three and four (Table 5) from both assays, Animal I increased 3.5 ng/ml over the preinfusion insulin levels. It was observed that butyrate caused a greater response in serum insulin than did propionate. McAtee and Trenkle (1971) reported that propionate increased serum insulin levels to a greater degree than butyrate (approximately 3.5 ng/ml), while Horino et al. (1968) and Trenkle (1970a), both working with sheep, reported that butyrate caused the greatest response in serum insulin. Nevertheless, the insulin values reported by McAtee and Trenkle (1971) throughout the infusion of both propionate and butyrate and those reported here were very similar.

Table 5. Effect of propionate and butyrate infusion on serum insulin.

Tube no.[a]	Propionate infusion			Butyrate infusion		
	Assay I	Assay II	Total serum lipid	Assay I	Assay II	Total serum lipid
	Animal I			Animal III		
1,2	1.1[b]	-	275[c]	2.1	1.5	285
3,4	4.6	4.6	217.5	6.4	7.2	267.5
5,6,7,8	3.4	2.9	230	4.8	3.4	315
9,10	2.1	0.9	265	-	-	-
	Animal II			Animal IV		
1,2	2.5	1.8	320	1.8	0.6	275
3,4	3.2	3.9	320	5.8	5.2	300
5,6	2.7	1.5	275	4.7	4.2	310

[a]Pooled serum. Table 1 gives original bleed scheme.

[b]ng insulin/ml serum.

[c]mg/100 ml serum.

Differences in infusion rates and amounts were most likely the cause for the apparent discrepancies with some of the literature.

There were no observed relationships between serum insulin and total serum lipids when butyrate or propionate was infused in the catheterized steers. Furthermore, correlation coefficients between serum insulin and total serum lipid were not meaningful (Assay I $r = -.20$, Assay II $r = -.10$). However the infusion of propionate and butyrate in the two crossbred steers and the subsequent rise in serum insulin support the premise that lipids and insulin are in fact related. Whether propionate and butyrate can directly stimulate the β cells of the pancreas is not known, but the results of this infusion trial indicated that this might be possible.

The serum insulin and lipid data collected from the alloxan diabetic sheep lends further support to the theory that insulin and lipids are related. Serum collected before alloxan injection contained 502.5 mg lipid/100 ml. On the seventh day post injection, total serum lipid was 1632.5 mg/100 ml, while on the eighth day post injection serum lipid rose to 2445 mg/100 ml. The serum from the seventh and eighth days post injection contained less than 0.05 ng insulin/ml. Although the exact relationship between lipid insulin cannot be determined from this limited study, the evidence suggests that a metabolic relationship does exist.

Cortisol and Lipid Data

It should be noted that, although the term serum cortisol was used, the reported cortisol concentrations are actually total

corticoids. Garvernick et al. (1971), using the corticoid assay of Murphy (1967), reported that cortisol was approximately 70% of the total corticoid concentration in the serum from pregnant cows. The corticoid assay is not sensitive enough to detect only cortisol due to small structural differences in the corticoid hormones. Because cortisol accounts for the majority of the corticoids in bovine serum, the term cortisol was used. This portion of the study was designed as a preliminary investigation to determine whether total serum lipid levels may in part be a function of the level of circulating serum cortisol. Furthermore, it was thought necessary to determine if serum cortisol or total serum lipid concentrations varied consistently under conditions of normal feed intake. A starvation period of 72 hr followed by refeeding was used as an experimental means of influencing the total serum lipids and serum cortisol. If the 72-hr fast did influence serum lipid or cortisol levels, further support might be given to any trends established under normal feeding conditions. The literature contains no information in these particular areas with reference to the bovine.

Experiments I and II were for 48-hr periods, beginning at 7 am with normal feeding conditions. The data from Experiment I (Table 6) and Experiment II (Table 7) revealed essentially no trends between the circulating serum cortisol and total serum lipid concentrations. The serum from the animals in each experiment varied tremendously in cortisol and total lipid concentration. Furthermore, the levels of these two serum components often demonstrated changes that were completely opposite. For example, in Experiment I, bleed period 11, Animal I had

Table 6. Serum lipid and cortisol data by bleed period for Experiment I.

Bleed period[a]	Animal I		Animal II	
	Lipid[b]	Cortisol[c]	Lipid	Cortisol
1	393.5	10.0	453.5	20.4
2	333.5	20.0	443.5	22.6
3	531.0	14.4	430.0	25.1
4	468.5	18.0	388.5	23.6
5	443.5	13.4	368.5	18.3
6	431.0	11.8	383.5	28.7
7	490.0	22.2	295.0	15.0
8	467.0	4.7	260.0	9.7
9	385.0	6.5	293.0	8.4
10	385.0	2.4	286.5	18.9
11	375.0	8.6	462.5	20.5
12	355.0	18.5	431.0	17.4
13	380.0	8.0	441.0	16.9
Mean	418.3 ± 58.4	12.1 ± 6.2	379.7 ± 72.6	18.8 ± 5.7

[a]4-hr periods (period 1 - 7 am).

[b]mg lipid/100 ml serum.

[c]ng cortisol/ml serum.

Table 7. Serum lipid and cortisol data by bleed period for Experiment II.

Bleed period[a]	Animal I		Animal II	
	Lipid[b]	Cortisol[c]	Lipid	Cortisol
1	270.0	7.0	345.0	7.1
2	291.5	8.9	328.5	3.3
3	287.5	4.3	338.0	22.2
4	287.5	7.9	320.0	3.7
5	293.5	5.9	385.0	10.5
6	280.0	6.2	288.5	46.4
7	258.5	6.3	323.5	16.6
8	282.5	6.7	277.5	24.4
9	273.5	7.6	315.0	7.0
10	310.0	5.9	310.0	16.3
11	297.5	6.9	333.0	27.2
12	310.0	6.5	305.0	15.0
13	302.5	13.6	291.0	11.6
Mean	288.0 ± 15.3	7.2 ± 2.2	320.0 ± 28.0	16.2 ± 11.8

[a] 4-hr periods (period 1 - 7 am).

[b] mg lipid/100 ml serum.

[c] ng cortisol/ml serum.

375 mg lipid/100 ml serum and 8.6 ng cortisol/ml serum, while Animal II had 462 mg lipid/100 ml serum and 20.5 ng cortisol/ml serum. The correlation coefficients calculated between cortisol and serum lipids were 0.17 (Animal I, Experiment I), 0.60 (Animal II, Experiment I), 0.19 (Animal I, Experiment II), and -.39 (Animal II, Experiment II). No concrete conclusions could be made from either Experiments I or II because of the tremendous variation of the data which was indicated by the low correlation coefficients that were calculated.

The two animals in Experiment III were taken off-feed 24 hr before the experiment began and were not refed until the 48th hr (bleed period 13) of the experiment (Table 8). Both animals had approximately the same concentration of total serum lipid at the beginning of the experiment (Animal I 342.5 mg/100 ml, Animal II 350 mg/100 ml). Throughout the experiment the two animals varied greatly in total serum lipid levels with Animal I reaching a maximum concentration (406.5 mg/100 ml) during bleed period 6, while Animal II reached a maximum concentration (490 mg/100 ml) during bleed period 16. However, at the end of the 48-hr experiment both animals were again at similar serum lipid concentrations (Animal I 263.5 mg/100 ml and Animal II 275 mg/100 ml). These data indicated that both animals, because of the 72-hr starvation periods, were mobilizing body depot fat for necessary energy as evidenced by the high serum lipid concentrations. After the animals were fed (bleed period 13), it took between 12 and 16 hr for the serum lipid concentrations to drop, indicating that the depletion of the depot fat had stopped and the animals were again mobilizing energy from the

Table 8. Serum lipid and cortisol data by bleed period for Experiment III.

Bleed period[a]	Animal I		Animal II	
	Lipid[b]	Cortisol[c]	Lipid	Cortisol
1	342.5	4.2	350.0	14.0
2	336.5	11.4	410.0	23.8
3	333.5	8.3	393.5	27.6
4	308.5	25.1	351.5	15.1
5	393.5	15.2	371.5	27.6
6	406.5	11.0	293.0	25.7
7	392.5	17.1	412.5	16.0
8	348.0	15.8	418.0	39.3
9	343.0	10.6	348.5	18.8
10	315.0	17.8	413.0	16.2
11	326.0	6.1	373.0	20.2
12	306.0	24.5	468.0	14.2
13	348.5	26.2	417.5	26.2
14	315.0	5.6	410.0	14.4
15	300.0	41.4	356.5	56.4
16	252.5	9.4	490.0	3.6
17	267.5	23.4	318.5	20.4
18	261.0	5.6	286.5	7.3
19	263.5	16.8	275.0	4.8
Mean	324.2 ± 44.7	15.5 ± 9.4	376.8 ± 58.5	20.7 ± 12.3

[a]4-hr periods [(period 1 - 7 am) (off feed 24 hr before period 1, refeed period 13 - 7 am)].

[b]mg lipid/110 ml serum.

[c]ng cortisol/ml serum.

ration. During the 72-hr starvation period the homeostatis of the rumen microorganisms most likely suffered and the population probably declined sharply. The long time period (12 to 16 hr) before the serum lipid concentrations fell was most likely a function of two things. First, a certain amount of time was needed for the rumen microorganisms to return to a balanced condition and, secondly, part of the 12 to 16 hr was simply a function of the rate of passage of the feed.

The highest serum cortisol concentrations for both animals was 8 hr (bleed period 15) after the animals were refed (Animal I 41 ng cortisol/ml serum and Animal II 56 ng cortisol/ml serum). Ashmore et al. (1956) reported high glucose-6-phosphate levels in fasted rats, accompanied by an increased serum cortisol concentration. Possibly 8 hr after the animals were refed, the glucose-6-phosphate levels increased as one of the first steps in the mobilization of the nutrients from the feed. The increased glucose-6-phosphate possibly was the stimulus to the adrenal glands causing the increased concentrations in serum cortisol. Correlating serum cortisol to total serum lipids again resulted in varied coefficients (Animal I $r = -.10$, Animal II $r = 0.02$).

The total serum lipid and cortisol concentrations of the two animals bled at 3 pm for 27 consecutive days (Experiment IV) are given in Table 9. These data did not indicate any trends which could be established between serum cortisol and total serum lipid. Furthermore, graphing the serum cortisol concentrations for both animals (Figure 4) revealed a gradual upward trend in cortisol concentrations during the first 8 days of the experiment. However, as the experiment progressed

Table 9. Serum lipid and cortisol data by bleed period for Experiment IV.

Bleed period[a]	Animal I		Animal II	
	Lipid[b]	Cortisol[c]	Lipid	Cortisol
1	337.5	17.9	348.5	3.9
2	405.0	21.4	413.0	4.7
3	346.0	12.1	328.5	7.7
4	330.0	20.8	325.0	4.8
5	292.5	29.3	353.5	3.1
6	313.5	8.0	350.0	5.6
7	295.0	11.3	385.0	4.1
8	312.5	19.1	362.5	15.8
9	343.5	12.6	331.0	7.6
10	336.5	12.8	361.5	8.4
11	298.5	11.7	338.5	11.1
12	293.0	13.6	333.0	15.6
13	253.0	8.5	348.5	9.6
14	313.5	7.3	301.0	5.1
15	283.5	6.0	293.0	3.1
16	278.5	6.8	298.0	5.3
17	288.0	3.7	321.0	7.0
18	351.5	13.8	351.5	10.0
19	273.5	5.1	297.5	31.9
20	222.5	6.6	308.5	13.7
21	221.5	9.0	340.0	9.9
22	256.0	7.3	288.5	10.6
23	338.0	7.6	318.0	10.1
24	290.0	14.6	303.0	15.3
25	236.0	19.3	322.5	5.8
26	310.0	16.8	313.5	19.4
27	306.0	9.5	360.0	5.5
Mean	300.9 ± 41.8	12.3 ± 6.05	333.1 ± 29.5	9.4 ± 6.2

[a] 3 pm for 27 consecutive days.

[b] mg lipid/100 ml serum.

[c] ng cortisol/ml serum.

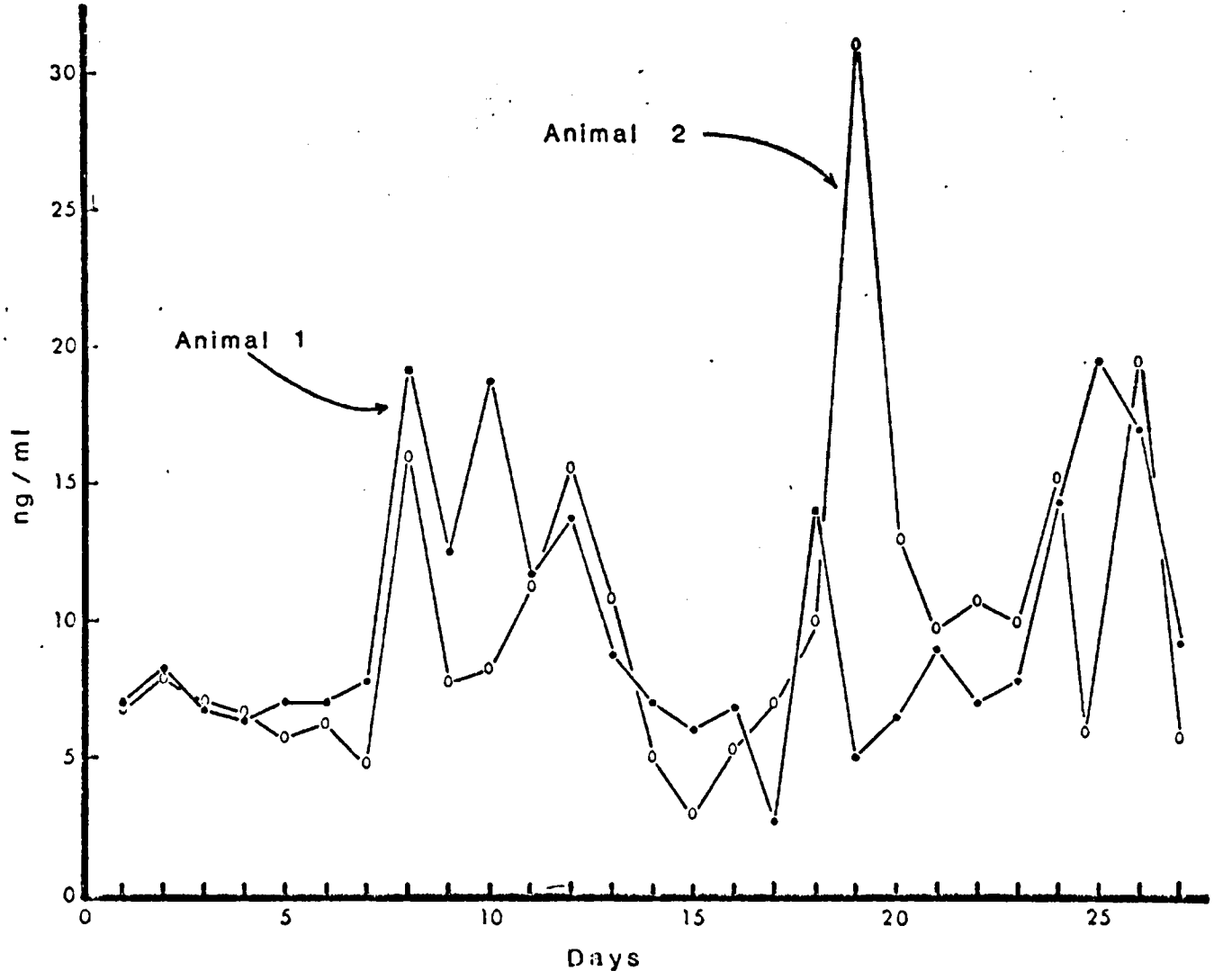


Figure 4. Changes in serum cortisol concentrations over a 27-day period.

the trend became less apparent and at the half-way point of the experiment the cortisol concentrations were varying tremendously. The variation might possibly have been due to temperature of some type of physiological stress. Correlating serum cortisol to total serum lipids gave coefficients of 0.35 and -.32 for Animals I and II, respectively. It should be noted that when the total serum lipid and serum cortisol data from Experiments I, II, and III were graphed in such a manner as to compare the animals over the three experiments, no meaningful trends could be established.

Perhaps if there had been more experimental animals, the bleeding periods had been longer, or the samples had been taken at another time of the day, more meaningful results could have been obtained. Furthermore, if the serum lipids had been fractionated and quantitated along with the corresponding fatty acid composition, more meaningful results may have possibly been established between serum cortisol and the serum lipids.

CHAPTER V

SUMMARY

The objectives of this research were to develop a sensitive radioimmunoassay for bovine insulin and to study the relationships between serum insulin, cortisol, and total serum lipids. Three experiments under normal feeding conditions and one under starvation conditions were conducted using jugular catheterized crossbred steers. As an extension of the research, several steers were infused with either propionate or butyrate in order to produce high insulin serum.

After the assay had been developed, it became apparent that the serum insulin had been degraded, and the data suggested that some enzyme system had remained functional in the unfrozen serum, causing the degradation of the insulin molecule.

The results of the infusion trial indicated a definite relationship between serum insulin and propionate or butyrate. Although there were no meaningful correlations between serum cortisol and total lipid, after a 72-hr fast there was a sizable increase in serum cortisol followed by a decrease in total serum lipids. These data indicated a possible relationship between serum cortisol and lipid with a possible involvement of glucose-6-phosphate.

From this limited study, it was impossible to make definite conclusions concerning the relationship of serum cortisol and insulin

to total serum lipid. However, the data indicated a possible relationship between these serum components and certainly this area warrants further investigation.

LITERATURE CITED

Ashmore, J., A. B. Hastings, F. B. Nesbett, and A. E. Renold. 1956. Studies on carbohydrate metabolism in rat liver slices; VI Hormonal factors influencing glucose-6-phosphate. J. Biol. Chem. 218:77.

Bassett, J. M. 1963. The influence of cortisol on feed intake and glucose metabolism in sheep. J. Endocrinol. 26:539.

Bell, J. P., L. A. Salamonsen, G. W. Holland, E. A. Espiner, D. W. Beaven, and D. S. Hart. 1970. Autotransplantation of the pancreas in sheep: insulin secretion from the transplant. J. Endocrinol. 48:511.

Benjamin, W., and A. Gallhorn. 1964. The effect of diabetes and insulin on the biosynthesis of individual fatty acids in adipose tissue. J. Biol. Chem. 239:64.

Boda, J. M. 1964. Effect of fast and hexose injection on serum insulin concentrations of sheep. Amer. J. Physiol. 206:419.

Brady, R. O., F. W. Lukens, and S. Gurin. 1951. Synthesis of radioactive fatty acids in vitro and its hormonal control. J. Biol. Chem. 193:459.

Chlorwerakis, C. 1967. Factors affecting the inhibitory action of insulin on lipolysis in a glucose free medium. Endocrinol. 81:521.

Crespin, S. R., W. B. Greenough, and D. S. Berg. 1969. Stimulation of insulin secretion by infusion of free fatty acids. J. Clin. Invest. 48:1934.

Denton, R. M., R. E. Yorke, and P. J. Randle. 1966. Measurements of concentrations of metabolites in adipose tissue and effects of insulin alloxan diabetes and adrenaline. Biochem. J. 100:407.

Eisenstein, A. B. (Ed.). 1967. The Adrenal Cortex. Little, Brown, and Company, Boston.

Fain, J. N., R. O. Scow, and S. S. Chernick. 1963. Effect of glucocorticoids on metabolism of adipose tissue in vitro. J. Biol. Chem. 238:54.

Fain, J. N., V. P. Kovaceu, and R. W. Scow. 1966. Antilipolytic effect of insulin in isolated fat cells of the rat. Endocrinol. 78:773.

Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissue. J. Biol. Chem. 266:497.

Garvernick, H. A., R. E. Erb, G. D. Niswender, and C. J. Callahan. 1971. Reproductive steroids in the bovine. III. Changes during the estrous cycle. J. Anim. Si . 32:946.

Gilmour, K. E., and K. W. McKerns. 1966. Insulin and estrogen regulation of lipid synthesis in adipose tissue. Biochem. Biophys. Acta 116:220.

Goodman, H. M. 1967. A comparative study of the effects of insulin and growth hormone on hexose metabolism in adipose tissue. Endocrinol. 80:45.

Goodman, H. M., and E. Knobil. 1961. Some endocrine factors in regulation of fatty acid mobilization during fasting. Amer. J. Physiol. 201:1.

Goodner, M., J. Conway, and J. Werrbach. 1969. Control of insulin secretion during fasting hyperglycemia in adult diabetics and in non-diabetic subjects during infusion of glucose. J. Clin. Invest. 48:1878.

Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of I-labeled human growth hormone of high specific radioactivity. Biochem. J. 89:114.

Hales, C. N., and P. J. Randle. 1963. Immunoassay of insulin with insulin antibody precipitate. Biochem. J. 88:137.

Henderson, J. R. 1970. Serum insulin or plasma insulin? Lancet. 2:545.

Hertelendy, F., L. J. Machlin, Y. Takahashi, and D. M. Kipnis. 1968. Insulin release from sheep pancreas in vitro. J. Endocrinol. 41: 605.

Horino, M., L. J. Machlin, F. Hertelendy, and D. M. Kipnis. 1968. Effect of short chain fatty acids on plasma insulin in ruminant and non-ruminant species. Endocrinol. 83:118.

Irvin, R., and A. Trenkle. 1971. Influence of age, breed, and sex on plasma hormones in cattle. J. Anim. Sci. 32:292.

Jeanrenaud, B., and A. Renold. 1960. Studies on rat adipose tissue in vitro VII: Effects of adrenal cortical hormones. J. Biol. Chem. 235:2217.

Kabal, J., and E. Ramey. 1960. Effect of guanylic acid on plasma NEFA response to stress. Proc. Soc. Exptl. Biol. Med. 104:95.

Kahlenberg, A., and N. Kalant. 1964. The effect of insulin on human adipose tissue. Can. J. Biochem. 42:1623.

Leboeuf, B., A. Renold, and G. Cahill. 1962. Studies on rat adipose tissue in vitro. J. Biol. Chem. 237:988.

Leonards, J. R., and B. R. Landon. 1960. A study on the equivalence of metabolic patterns in rat adipose tissue: Insulin vs. glucose concentrations. Arch. Biochem. 91:194.

Madison, L. L., W. A. Seyffert, R. H. Unger, and B. Baker. 1968. Effect of plasma free fatty acids on plasma glucagon and serum insulin concentrations. Metabolism. 17:301.

Malaisse, W. J., and F. Lague. 1968. Stimulation of insulin secretion by non-carbohydrate metabolites. J. Lab. Clin. Med. 72:438.

Manns, J. G., and J. M. Boda. 1967. Insulin release by acetate, propionate, butyrate and glucose in lambs and adult sheep. Amer. J. Physiol. 212:747.

Manns, J. G., J. M. Boda, and R. F. Willes. 1967. Probable role of propionate and butyrate in control of insulin secretion in sheep. Amer. J. Physiol. 212:756.

McAtee, J. W., and A. Trenkle. 1971. Metabolic regulation of plasma insulin levels in cattle. J. Anim. Sci. 33:438.

McGraw, E. F. 1969. Cortisol induction of lipolysis in adrenalectomized diabetic rats. Life Sci. 8:805.

Murphy, B. E. 1967. Some studies on the protein binding steroids and their application to the routine micro and ultra micro measurements of various steroids in body fluids by competitive protein binding radioassay. J. Clin. Endocrinol. and Metabolism 27:973.

Nathanielsz, P. W. 1970. Amino acid stimulation of insulin release in the newborn calf. J. Endocrinol. 48:141.

Niswender, G. D., L. E. Reichert, A. R. Midgley, and A. V. Nallandov. 1969. Radioimmunoassay for bovine and ovine luteinizing hormone. Endocrinol. 84:1166.

Paisey, R. B., and P. W. Nathanielsz. 1971. Plasma cortisol levels in the newborn lamb from birth to 30 days. J. Endocrinol. 50:701.

Panaretto, B. A., and M. R. Vickery. 1970. The rates of plasma cortisol entry and clearance in sheep before and during their exposure to a cold wet climate. J. Endocrinol. 47:273.

Pruett, E. D. 1970. Glucose and insulin during prolonged work stress in man living on different diets. J. Appl. Physiol. 28:199.

Randle, P. J., P. B. Garland, C. N. Hales, and E. A. Newshaline. 1963. The glucose fatty acid cycle: its role in insulin sensitivity and metabolic disturbance of diabetes mellitus. Lancet. 1:785.

Reid, R. L., and N. T. Hinks. 1963. Effects of insulin, glucose, and glycerol on fat metabolism in alloxan diabetic sheep. J. Endocrinol. 27:21.

Sanbar, S. S., and J. M. Martin. 1967. Stimulation of octanoate insulin release from the isolated rat pancreas. Metabolism 16: 482.

Schalch, D. S., and D. M. Kipnis. 1965. Abnormalities in carbohydrate tolerance associated with elevated plasma non esterified fatty acids. J. Clin. Invest. 44:2010.

Schwarz Mann. 1971. Procedures for the radioimmunoassay of insulin levels in serum or plasma using iodinated insulin as tracer: New Product Bulletin. Schwarz Mann Company, Orangeburg, New York.

Slavin, B. G., and J. Elias. 1970. Metabolic changes in white and brown adipose tissue by insulin, thyroxin, and cortisol in organ culture. Anat. Record. 167:213.

Spurlock, G. M., and M. T. Clegg. 1962. Effect of cortisone acetate on carcass composition and wool characteristics of weaned lambs. J. Anim. Sci. 21:494.

Steel, R. G., and J. H. Torrie. 1960. Principles and Procedures of Statistics. McGraw-Hill Book Co., New York.

Sweeney, M. J., and J. Ashmore. 1965. Effects of acute insulin insufficiency on liver and adipose tissue fatty acid synthesis. Metabolism 14:516.

Toomey, R. E., W. N. Shaw, L. R. Reid, and W. K. Young. 1970. Comparative study of the effects of porcine proinsulin and insulin on lipolysis and glucose oxidation in rat adipocytes. Diabetes 19:209.

Trenkle, A. 1970a. Effects of short chain fatty acids, fasting and type of diet on plasma insulin levels in sheep. J. Nutr. 100: 1323.

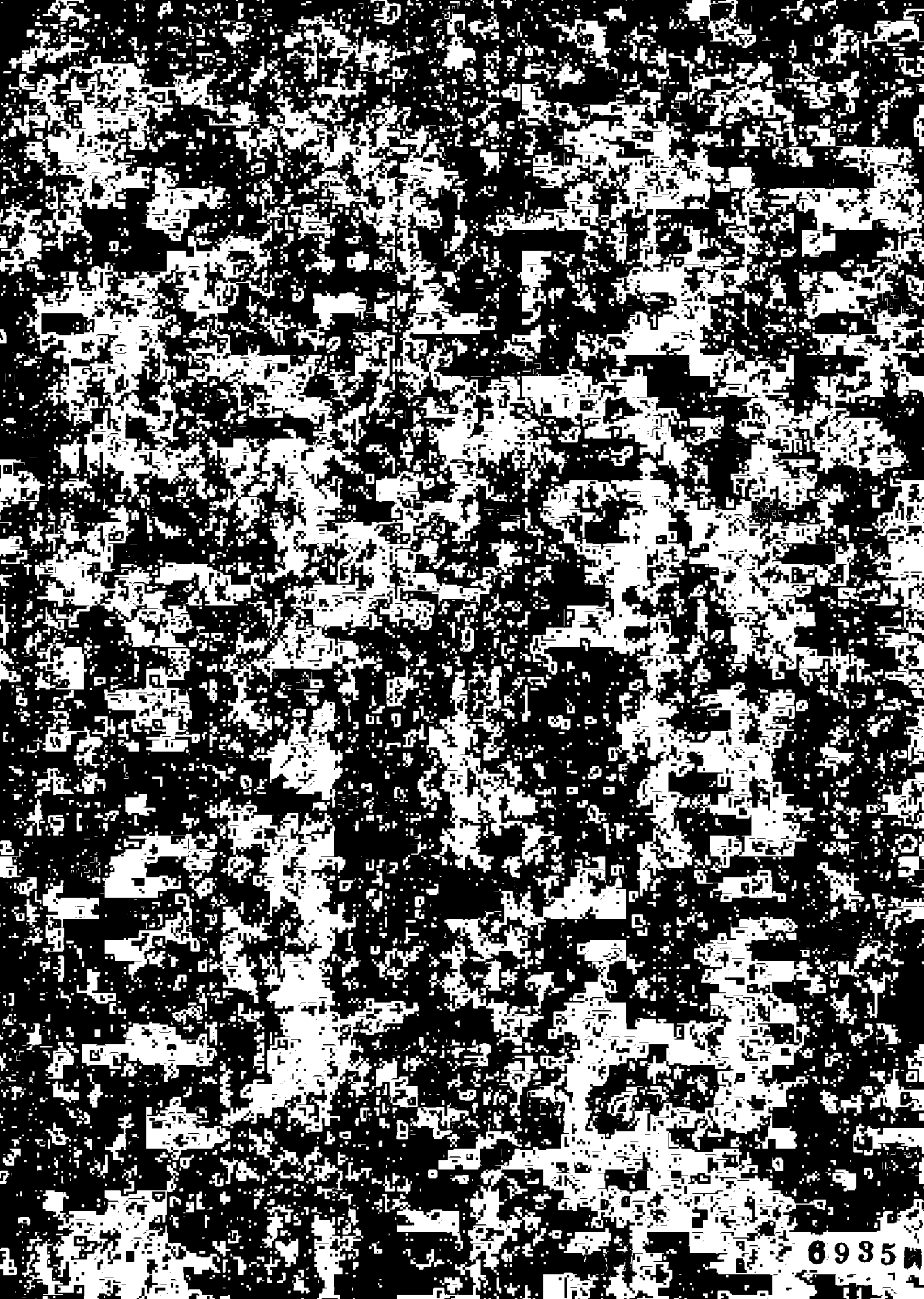
Trenkle, A. 1970b. Plasma levels of growth hormone, insulin, and plasma bound iodine in finished cattle. J. Anim. Sci. 31:389.

Trenkle, A., and R. Irvin. 1970. Correlation of plasma growth hormone levels with growth and carcass characteristics of cattle. Growth 34:313.

Vassalli, J. D., and B. Jeanrenaud. 1970. Lipolysis and α-aminoisobutyric acid uptake in isolated fat cells. Biochem. Biophys. Acta 202:477.

Yallow, R. S., and S. A. Berson. 1964. Immunoassay of insulin. Methods of Biochemical Analysis 12:69.

Young, J. W., E. W. Otchere, A. Trenkle, and N. L. Jaconson. 1970. Effect of age on glucose, reducing sugars and plasma insulin in blood of milk fed calves. J. Nutr. 100:1267.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