RADIO-IMMUNOASSAYS OF ANABOLIC HORMONES IN YOUNG RUMINANTS

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ABSTRACT

Four experiments with young calves and one with ewes and lambs are described, all of which involved taking blood samples via a jugular catheter. Plasma samples were all assayed for insulin and growth hormone by radio-immunoassays. In Experiments 1 - 4, prolactin assays were also carried out, and results of plasma glucose assays were presented for Experiments 2 - 4.

In Experiments 1 and 5, the effect of suckling on starved calves and lambs was investigated. Insulin rose from <1 ng/ml to 2 - 7 ng/ml after suckling. Growth hormone and prolactin did not change systematically in any way after suckling in calves, but lambs' growth hormone levels increased from <2 ng/ml to >10 ng/ml at the onset of suckling, as did the GH levels in plasma of their dams.

In Experiments 2 and 4, the effect of changing plasma energy substrate levels was investigated in calves. In Experiment 2, it was observed that a 30-second jugular infusion of either volatile fatty acids in an equimolecular mixture of acetate, propionate and butyrate, or glucose (both administered at 1.25 mM per kg body weight (bw)) resulted in a variable, but significant increase in insulin levels, but had no effect on growth hormone or prolactin. The results of 1.4 mM per kg bw glucose administration in Experiment 4, confirmed those of Experiment 2. Administration of 0.75 U per kg bw of protamine zinc insulin resulted in a prolonged hypoglycaemia, which was achieved more slowly in five-week-old calves than in week-old calves. Growth hormone and prolactin levels did not respond

immediately to insulin administration, but after hypoglycaemia had been maintained for 2 - 3 hours, growth hormone levels decreased from 4 - 8 ng/ml to <3 ng/ml in calves of both one week and five weeks of age, and prolactin levels increased slightly.

The effect of a 30-second infusion of 0.3 g per kg bw arginine on calves was tested in Experiment 3, and significant increases in all plasma measurements except growth hormone were observed. Increases of both insulin and glucose were higher in five-week-old milk-fed calves than they were in week-old calves or in five-week-old ruminant calves, and in all calves the glucose increase was quickly followed by a decrease in glucose levels to a deep hypoglycaemia. The prolactin response was smaller and more prolonged in week-old calves than in the older calves. Saline which was infused in an equi-osmolar solution to arginine, resulted in decreases in both growth hormone and prolactin. The results of Experiment 3 are contrasted with those of Experiment 5, in which a 0.5 g per kg bw arginine dose was infused over a 30-minute period into ewes and lambs. The insulin responses were smaller than those of calves, but the growth hormone levels increased significantly from <2 ng/ml to >4 ng/ml.

In addition to the experimental results described above, radio-immunoassays were described in detail, because all work utilised antibodies raised by the author during the course of study.

PREFACE

Plasma levels of insulin, growth hormone and prolactin have been measured in calves and lambs, with special reference to the change in energy substrate metabolism which occurs as a result of the transition from pre-ruminant to the ruminant mode of digestion. Whereas lambs' plasma hormone levels have been extensively studied in this respect, the literature contains very few references to those of young calves. Thus it was of interest to compare and contrast results obtained with calves and lambs, but to concentrate four out of the five experiments on calves.

Changes in plasma levels of the hormones and of glucose were measured in response to several acute stimuli, chosen for their significance either in relation to energy substrate metabolism, or the anabolic functions of the three hormones under investigation. The stimuli tested were as follows:

(i) The effect of suckling

(ii) The effect of rapid infusions of

- (a) Glucose
- (b) Volatile fatty acid mixture
- (c) Insulin
- (d) Arginine

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CHAPTER ONE: REVIEW OF THE LITERATURE

1.1 INTRODUCTION

An important factor in the development of the young ruminant is that it undergoes a change from typical non-ruminant metabolism in the pre-ruminant phase, to the ruminant condition. In the pre-ruminant phase, the major energy substrate is glucose, mainly derived from lactose in milk which is consumed in large meals resulting in sudden large increases in glucose absorption. After weaning, the ruminant condition involves the steady production of volatile fatty acids by rumen fermentation, and these largely replace glucose as the main energy substrate. Thus there is a change both in the nature of the main energy substrate, as well as in the rate of energy substrate absorption throughout the day.

Such a change is associated with profound biochemical alterations (for example Bauman (1976) has discussed the changes which occur in the pathways used for fatty acid synthesis before and after weaning in lambs), and is related to changes in the hormonal control of digestion and metabolism (Bassett, 1975). The endocrinological changes of this nature form the main focus of the present study. Plasma hormonal changes associated with suckling have been investigated in the pre-ruminant calf, and hormonal responses to intravenous infusions of arginine, glucose and insulin, have also been compared between calves of different ages and with

different degrees of rumen development.

The literature contains very few reports of the endocrinology of young calves, the present study being one of the first. There are several references to lambs and kids, which have been reviewed here in detail in order to provide a reference point for comparing and verifying the calf data. For the same reason, some data obtained from ewes and lambs while the author was working in collaboration with Dr. J.M. Gooden (Applied Biochemistry Division, D.S.I.R., Palmerston North) has also been included in Chapter Three.

The role of pituitary growth hormone in energy substrate metabolism of young ruminants is of central importance in the present study. Its actions are easily compared and contrasted with those of insulin. Their anabolic effects, and their similarity and complementary functions in the control of metabolism and growth, are such that either of them could equally well be given the name of 'growth hormone'.

In the present study, however, the emphasis is not on the role of these hormones in controlling or promoting growth, and studies in which growth hormone was measured over long periods of time and correlated with food intake and growth, have not been included in the review of the literature. Rather, the emphasis is on the hormonal responses of growth hormone and insulin, to short-term, acute changes in plasma levels of certain metabolites.

For the investigation of a further aspect of the factors which influence growth hormone secretion, the aspect of control of pituitary function as a whole, another pituitary hormone, prolactin, was also measured. Prolactin is known to have much in common with growth hormone, both in its chemical structure, and also in the fact that both have mammogenic and lactation stimulating effects (Cowie and Tindal, 1971). The two hormones are thought to share a common evolutionary history, and yet it seems that they are under opposing mechanisms of hypothalamic control of their secretion from the anterior pituitary (see pages 22 and 31). A study of the levels of the two hormones is therefore of great interest in shedding further light on their similarities and differences.

1.2 INSULIN, GROWTH HORMONE AND PROLACTIN LEVELS

1.2.1 Stimuli for secretion of insulin

(a) Plasma metabolites

The most potent stimulus for insulin secretion in non-ruminants is a high level of glucose reaching the β cells of the pancreas (reviewed by Field, 1964, and Mayhew et al, 1969). Recent evidence has suggested that the rate of increase in plasma glucose concentrations is more critical in controlling the insulin response than the actual levels of glucose reached (O'Connor et al, 1977). Other sugars, e.g. mannose and fructose, are less effective than glucose, and the majority of workers have found that galactose is not effective at all (Mayhew et al, 1969). In ruminants (only sheep have been studied), the relationship between glucose levels and insulin secretion is not as close as that between volatile fatty acids (VFAs) and insulin secretion (Manns and Boda, 1967: Horino et al, 1968: Ambo et al, 1973). VFAs produced by rumen fermentation largely replace glucose as the major energy substrate produced in ruminants by digestion. Thus the situation for both ruminants and non-ruminants, is a strong positive correlation between energy substrate levels and insulin secretion.

Insulin secretion is also stimulated by increased levels of certain amino acids in the plasma of non-ruminants (Mayhew et al, 1969) as well as ruminants (Hertelendy et al, 1970: Stern et al, 1971: Davis, 1972).

The physiological significance of both responses lies in the fact that insulin is the major anabolic hormone.

It promotes synthesis of proteins and nucleic acids, the materials necessary for growth, and also promotes storage of carbohydrates as liver glycogen and triglycerides as energy reserves (Krahl, 1972). Its initial short-term action is to increase the transport of amino acids and glucose into cells, thus leading to a dramatic decrease in the plasma levels of those metabolites which stimulated its secretion (Wool and Scharff, 1968). Administration of exogenous insulin leads to the development of hypoglycaemia, but in the normal animal the action of endogenous insulin is integrated with the actions of glucagon and growth hormone (GH) and GH-dependent peptides, so that homeostasis is maintained.

(b) Other hormones

(i) Enteric hormones

Following the ingestion of food, insulin is released in anticipation of increased blood metabolites, by a mechanism which involves the mediation of the enteric hormones, secretin, pancreozymin-cholecystokinin and gastrin (reviewed for non-ruminants by Mayhew et al, 1969). In ruminants, the situation with regard to enteric hormones has not been investigated in detail, but Chase et al (1977) have observed that since the increase in plasma insulin preceded the increase in plasma metabolite levels after spontaneous feeding in steers, a mechanism which is either neural, or involves enteric hormones, must be mediating the rapid response. A similar conclusion was reached by Bassett (1972) with sheep.

(ii) Glucagon

The importance of the opposing actions of insulin and glucagon in maintaining homeostasis is emphasized by the fact that insulin secretion is also stimulated by secretion of glucagon (reviewed for non-ruminants by Mayhew et al, 1969). In ruminants, insulin secretion was seen to follow very rapidly after glucagon infusion (Bassett, 1971). When VFAs were administered intravenously to sheep, the insulin response failed to produce hypoglycaemia (Manns and Boda, 1967: Horino et al, 1968) and sometimes the VFA administration has resulted in hyperglycaemia despite the insulin response (Phillips et al, 1969: Bassett, 1972). The authors have concluded that glucagon and insulin are secreted together, but the extent to which the insulin is responding to VFA levels, or to glucagon secretion is not clear.

(iii) Growth hormone

Both insulin and GH have been reported to be secreted in response to certain amino acids in plasma, in both ruminants and non-ruminants (see pages 4 and 9), but for insulin and possibly for GH, the relationship to plasma energy substrate levels may be overriding. Following food intake, insulin secretion is at a maximum, with low GH levels, while the situation is reversed after fasting (see page 12). Evidence is cited by Mayhew et al (1969) for an inverse relationship between the two hormones in man, and Bassett et al (1971) have observed a negative correlation between them ($r = -0.71$) in sheep.

High endogenous secretion of GH, as in acromegalic patients, or intravenous administration of exogenous GH to human subjects, have been reported to be accompanied by elevated insulin levels in plasma (Yalow and Berson, 1960). On a long-term basis, GH becomes diabetogenic, suggesting that continuous stimulation of the pancreas leads to β cell exhaustion (Young, 1968) and a direct stimulatory effect of GH on the pancreas *in vitro* has been observed by Bennett and Curry (1975). Recent discovery of the fact that many of the effects of GH are mediated by other peptides (see page 24) makes it seem likely that the effects of GH on the pancreas could also include long-term indirect effects.

(c) The adrenergic system

Observations that epinephrine inhibited the insulin release stimulated by hyperglycaemia, led to an examination of the role of the adrenergic system in insulin secretion (reviewed by Mayhew et al, 1969). It was found that α adrenergic stimulation had an inhibitory control, and β adrenergic stimulation enhanced insulin secretion by a mechanism which involved stimulation of the adenyl cyclase system in the β cells of the pancreatic islets. Since 1969, understanding of the system has not changed (Rossini and Buse, 1973) and the relative roles of β adrenergic stimulation and glucose metabolism within β cells is still not clear (Hermann and Deckert, 1977).

Certain hypothalamic extracts have been found to stimulate insulin release from rat pancreatic islets cultured

in vitro (Martin et al, 1973). There have also been some very interesting experiments which suggested that a hypothalamic factor released in dogs in response to glucose infusion, was responsible for producing enhanced insulin release from the pancreas, to a greater extent than the effect of the glucose alone reaching the pancreas (Chieri et al, 1976).

1.2.2 Stimuli for secretion of growth hormone

(a) Plasma metabolites and their relationship to growth hormone action

In determining the effect of plasma metabolites on plasma hormone levels, the development of radio-immunoassays has added a new dimension of precision and sensitivity. For a hormone with a well-defined biological action, such as insulin, it has been relatively easy to define the immunologically active molecule as being identical to the biologically active one (Dash and Lindsay, 1967). For GH, however, none of the bio-assays has been entirely satisfactory and many biological activities which are dependent upon GH are probably carried out by other molecules (see page 24). Thus the question has been raised: are all the studies based on radio-immunoassayable plasma GH, reliable in terms of the biologically active molecule?

In answer to the question, some severe discrepancies have arisen in the literature as a result of inadequate definition of the hormone being measured. For example, bioassayable GH (BA-GH) depletion from the pituitary was used by Schally et al (1968) during the investigation

into a possible GH-releasing hormone from the hypothalamus (see page 22), but the reliability of the results became suspect when it was observed that there was no agreement between BA-GH depletion from the pituitary, and radio-immunoassayable GH (RIA-GH) appearance in plasma (Frohman et al, 1971). Further suspicion has been aroused by Ellis et al (1975) whose results suggest that the ratio of BA-GH to RIA-GH in plasma was 200 - 300 : 1.

Although such discrepancies have arisen, Baetzner et al (1972) and also Stewart et al (1977) found very close correspondence between BA-GH and RIA-GH, the RIA having compared favourably with three different BA systems. Furthermore, where they do not compare well, there is evidence to suggest that the BA is at fault. Pena et al (1972) drew attention to the non-specificity of the tibia test BA, while it is well-established that radio-immunoassays are chemically highly specific (Skelley et al, 1973) and that although GH exists in at least two molecular forms, both of them are immunoreactive in their naturally occurring form in plasma (Bala et al, 1970: page 87 of the present study).

(i) Amino acids

GH bears a resemblance to insulin, in that it has been reported to be released in response to high levels of certain amino acids in the plasma. Intravenous infusion of arginine monohydrochloride stimulated secretion of both in primates (Rabinowitz et al, 1968) and dogs (Tsushima, 1971) as well as in all ruminants studied so far (Hertelendy et al, 1970: Stern et al, 1971: Davis, 1972). In cases where

simultaneous hormone measurements have been made (Hertelendy et al, 1970: Davis, 1972), the insulin peak has preceded the GH peak by 15 or 30 minutes, but sampling has not been frequent enough to determine whether the GH response is only a secondary one.

The physiological significance of the response probably lies in the fact that both GH and insulin have been found to cause enhanced transport of amino acids into cells of muscle and liver (reviewed by Snipes, 1968) and increased protein and nucleic acid synthesis, leading to growth. Thus the growth-promoting action of GH and insulin is enhanced in the presence of an adequate supply of dietary protein, the main substrate for growth.

(ii) Energy substrate levels

In relation to plasma metabolites other than amino acids, GH appears to be diametrically opposed to insulin. Whereas insulin is secreted in response to high levels of blood glucose (reviewed by Field, 1964), GH is often stimulated by hypoglycaemia, including that brought about by administration of insulin. The response is consistent in primates, and Roth et al (1964) noted that not only did hypoglycaemia stimulate GH secretion in man, but a rapid decrease in blood glucose was always effective, regardless of the absolute levels involved. The response has also been observed in ruminants (Stern et al, 1971: Wallace and Bassett, 1970) with the exception of one report with lambs (Trenkle, 1967) and one with goats (Tindal et al 1978) which did not respond.

The response has not been consistent in other mammals. Pigs (Machlin et al, 1968a) and dogs (Tsushima et al, 1971) only exhibited a sluggish GH response, and cats (Kokka et al, 1971), mice (Schindler et al, 1972) and rabbits (McIntyre and Odell, 1974) failed to respond at all. Depletion of BA-GH from the pituitary was observed in rats in response to insulin-induced hypoglycaemia (Krulich and McCann, 1966), but RIA work has repeatedly failed to substantiate it (Kokka et al, 1972: Takahashi et al, 1971). The discrepancy between BA-GH and RIA-GH has been discussed on page 8. Whatever the reason for the discrepancy between species, the GH secretion in response to hypoglycaemia can be said to be a well-established reliable response in primates, a response which usually occurs in ruminants but not in other mammals.

Conversely, hyperglycaemia has been found to be inhibitory to the secretion of GH in response to other stimuli, e.g. it inhibited the GH response to L-dopa ingestion in man (Mims et al, 1973) and Schalch and Reichlin (1966) observed that a microinfusion of glucose into the median eminence of the brain prevented the GH response to insulin-induced hypoglycaemia in monkeys. Hyperglycaemia also inhibited the GH response to arginine in ruminants (Hertelendy et al, 1970). However, under certian conditions hyperglycaemia has been seen to have the opposite effect, and is itself a stimulus for GH secretion. For example, hyperglycaemia was stimulatory to GH in human babies under six months of age (Cornblath et al, 1965) and the phenomenon has also been observed during acute bacterial infection or

starvation in primates (Rayfield et al, 1974). Among ruminants, Wallace and Bassett (1970) observed a GH increase in response to hyperglycaemia in lambs of 6 weeks of age, and the response decreased at 15 weeks of age, and was smaller still in lactating ewes. Stern et al (1971) also observed a GH response to infusion of glucose, but it was not related to age, and these authors related it to glucose utilisation rate rather than to the absolute levels of glucose reached.

There is some evidence that a similar inverse relationship exists between plasma levels of free fatty acids (FFAs) and GH, to that which exists generally between glucose and GH. The relationship has been shown to apply in man (Fineberg et al, 1972: Raptis et al, 1973: Quabbe et al, 1977). In ruminants, it has been demonstrated in sheep by Hertelendy and Kipnis (1973) and in cows by Reyneart et al (1975). Both groups of workers used several different lipolysis inhibitors in order to counteract specific side-effects of any one of them. Infused into fasted animals, they all resulted in a GH secretion that was inhibited by concomitant administration of FFAs or VFAs.

The evidence for increased GH secretion as a result of low energy substrate levels, is supported by the fact that GH levels are usually higher in the fasted animal, as seen in studies with man (Roth et al, 1964), pig (Machlin et al, 1968a) and rabbit (McIntyre and Odell, 1974). The situation in ruminants is questionable, since both Wallace and Bassett (1970) and Trenkle (1970) observed no effect on sheep fasted for four days. Also, Trenkle (1967) working

with sheep, Purchas et al (1971) with heifers and Hove and Blom (1973) with cows, all showed that a limited energy intake caused GH levels to be slightly higher than in control animals fed ad lib.

The main longer-term action of GH on energy substrates is to decrease the transport of glucose into cells, and to promote lipolysis. Thus plasma energy substrate levels are raised as a result of GH secretion occurring when they were low. The action of GH in this respect has been found to be biphasic: initially it exerts an insulin-like effect, prior to exerting its insulin-antagonistic effect. Thus Swislocki and Szego (1962) working with rats, and Altzuler et al (1968) with dogs, observed that the initial effect of administering GH to a hypophysectomised animal was a decrease in plasma glucose and FFAs, with a nadir at 30 - 60 minutes after the GH treatment, followed by a rise, from 2 - 6 hours after GH treatment.

The phenomenon has been studied in vitro in rat diaphragm by Ahren and Hjalmarsson (1968), who studied uptake of the non-utilisable sugar, xylose, which is thought to have the same transport characteristics as glucose. They found that GH administration to a hypophysectomised animal resulted in increased xylose transport for up to 3 hours, followed by decreased transport associated with a refractory period to further GH stimulation. Goodman (1968) observed the same pattern of events in uptake of l-arabinose by adipose cells.

The transport pattern may be of dubious significance, because it was observed only in hypophysectomised animals. However, the events must not be

considered as an all-or-none response, and the fact that the refractory period took 48 hours to disappear indicated that in the normal animal, the tissues are always at a varying degree of refractoriness to the insulin-like action of GH (Ahren et al, 1975). The small differences in refractoriness at different times relative to the last pulse of GH *in vivo* would be undetectable, but may be of great importance for allowing small amounts of metabolites into the cell, to provide the extra energy and amino acids necessary for GH to carry out its longer term effects in a controlled manner.

The other postulated action of GH in its maintenance of plasma energy substrate levels was thought to be a lipolytic effect, because intravenous administration of GH *in vivo* was observed to lead to an increase in plasma free fatty acid (FFA) levels (Bassett and Wallace, 1966: Luthman and Jonson, 1972). However, lipolysis stimulated directly by GH has not often been observed *in vitro*, so Nyberg and Smith (1977) have concluded from their results with cultured human adipose tissue that the *in vivo* lipolytic action of GH has been over-emphasized in the literature, and was possibly an artifact. Several other workers have observed GH-stimulated lipolysis *in vitro* under certain conditions, notably with the observation that it synergised with glucocorticoids to give rise to far more lipolysis than the additive effects of each alone (Caldwell and Fain, 1970: Goodman, 1970) and Hecht et al (1972) observed enhanced lipolysis *in vitro* by GH alone bound to sepharose beads.

Hotta and Sirek (1971) reported that the synergistic effect of GH on lipolysis was completely inhibited by 10 μU/ml of insulin in the medium (lower than the normal physiological level observed in fed human subjects) and there was a possibility that it was the inhibitory effect of insulin which accounted for observations that GH sometimes failed to stimulate lipolysis. The inability of GH to stimulate lipolysis except in the absence of insulin, reinforces the idea of a role of GH as the hormone of fasting, which mobilises fat in order to prevent energy substrates from declining, only when insulin has reached fasting levels.

1.2.2.(b) Different physiological states

(i) Age and reproductive status

In all mammals studied, it has been observed that plasma GH levels were high at birth, decreasing rapidly during the first 12 - 48 hours of neonatal life, and decreasing more slowly thereafter, to reach adult levels at about the time of weaning. The pattern has been found in non-ruminants, e.g. man (Cornblath et al, 1965), pigs (Machlin et al, 1968b), dogs (Tsushima et al, 1971) and mice (Sinha et al, 1972a), as well as ruminants e.g. calves (Tucker et al, 1974: Reyneart et al, 1976) and lambs (Bassett and Alexander, 1971: Hertelendy et al, 1969).

As the young animal reaches puberty, there is evidence that the GH secretion is again enhanced, and decreases again in adult-hood and further still during senescence (Finkelstein et al, 1972: Dudl et al, 1973).

In this connection, Aitken et al (1973) have emphasized that the occurrence of osteoporosis in post-menopausal women may be a result of decreasing oestrogen levels, when oestrogens possibly have an inhibitory effect on GH action. Thus, higher circulating GH levels in females of some species may be caused by oestrogenic inhibition of GH utilisation rather than by stimulation of secretion.

Pregnancy has been observed to cause a progressive increase in maternal plasma GH levels as the pregnancy advanced (Saunders et al, 1976, in rats: Koprowski and Tucker, 1973, in cows). Following the end of pregnancy, maternal plasma GH levels decreased gradually as lactation progressed (Koprowski and Tucker, 1973).

(ii) Lactation

It is probable that high maternal plasma GH levels immediately post-partum are associated with the important role of GH in the maintenance of lactation (Cowie and Tindal, 1971). Whereas prolactin is the major pituitary hormone required for the maintenance of lactation in non-ruminants (see page 29), it appears that GH is more important than prolactin in ruminants (Forsyth and Hart, 1975). The relative importance of GH in ruminant lactation is also emphasized by the comparison of cattle with high and low milk yields (Hart et al, 1978) in which it was observed that GH levels were higher in high-yielding cattle during the peak lactation period, whereas prolactin was highest in both groups during the dry period, with no difference between groups.

Both GH and prolactin have been seen to increase in maternal plasma in response to suckling or milking in ruminants (e.g. goats, Hart and Flux, 1973: sheep, Martal, 1975) and non-ruminants (e.g. rats, Saunders et al, 1976). However, the GH secretory response to milking is by no means universal, even in ruminants (e.g. cows, Reyneart and Peeters, 1972: Koprowski and Tucker, 1973: goats, Tindal et al, 1978).

(iii) Sleep and diurnal variation

The rise in plasma GH levels during sleep has been studied extensively in man (Finkelstein et al, 1972) but among ruminants it was not detectable in goats (Tindal et al, 1978) or bulls and steers (Anfinson et al, 1975). An increased number of GH peaks between 18.00 and 22.00 hours occurred in sheep (Davis and Borger, 1974) but was not clearly correlated with sleep.

More detailed studies with man (Alford et al, 1973) have shown that as the metabolic clearance rate was higher in supine subjects than those with upright posture, then the actual GH secretion rate was 2 - 5 times higher during sleep than during waking. GH levels have also been correlated with the electroencephalogram stages of sleep (Lucke et al, 1972). Whether or not the sleep response is entirely a central nervous system (CNS) effect, however, is still open to some doubt in view of the fact that Lucke et al (1972) were able to reduce nocturnal GH secretion in man to some extent by administering intravenous heparin before sleep, causing an elevation in plasma FFA levels. Such a result

raises the possibility that the GH secretion was merely a result of the nocturnal state of fasting (see page 12).

GH levels have been found to vary widely during the day, in ruminants (Davis and Borger, 1974: Anfinson et al, 1975) and in man (Glick and Goldsmith, 1968). One cause for variation was the change in plasma energy substrate levels following feeding and after digestion (see page 10), so that GH minima usually coincided with the end of a feeding period, during which time energy substrate levels and insulin were high (Blom et al, 1976, working with bulls). Even at these times, however, GH peaks still occurred, even though they were smaller than at times of fasting. Thus there were spontaneous GH peaks, seeming to occur at random throughout the day. Such an 'episodic' pattern has been found to be almost universal for GH secretion (Anfinson et al, 1975: Finkelstein et al, 1972).

The spontaneous GH episodes have been studied in detail in rats (Tannenbaum et al, 1976 a and b) and have been observed to occur with a cyclicity of 3.3 hr, independent of other physiological factors. Alternation of light and dark was observed to be unnecessary for the maintenance of the rhythm, although the 24-hr diurnal cycle did have a synchronising effect. However, the results of Tannenbaum et al (1976) are not in agreement with the study of Moberg et al (1975) in which rats' diurnal GH rhythm could be changed by enforcing a particular time of day for feeding.

1.2.2 (c) The adrenergic system

(i) Adverse environmental stimuli and epinephrine levels

The plasma GH levels of all mammals studied have been affected in some way by the infliction of adverse or 'stressful' stimuli. Ruminants have usually responded with an increase in plasma GH levels, for example as a result of inserting a jugular catheter in cattle (Eaton et al, 1968), of exposing cattle to sudden high temperatures (Mitra and Johnson, 1972) or of sustained low temperatures in sheep (Machlin et al, 1968a). One report to the contrary (Reyneart et al, 1976) in which cattle under transport stress had lower GH levels than controls, is of doubtful value in that only single blood samples were used for the GH measurement, and basal GH levels are known to vary widely (Davis and Borger, 1974). A GH increase is supported by studies with man, in which various stressful stimuli were effective (Baylis et al, 1968). An equally wide range of adverse stimuli, however, has brought about a decrease in GH secretion in all rodents studied (Takahashi et al, 1971: Schlindler et al, 1972).

Although the validity of the studies is questionable because the definition of a 'stressful' stimulus is rather subjective and several mechanisms which influence GH could be involved, it is noteworthy that some GH change has always been observed, indicating that 'stress' does play a part in GH control. The differences between species may be associated with the fact that in determining GH levels at any time, the metabolic clearance rate as well as the secretion rate are both involved, and may not be influenced in parallel. (See page 16 - 17).

The GH release observed in the majority of animals studied was probably not mediated by other hormones released into the circulation in response to 'stress'. An intravenous dose of ACTH did not affect GH levels in human subjects (Bruno et al, 1971) and results following an intravenous infusion of epinephrine have been very variable between studies. Wallace and Bassett (1970) observed that epinephrine caused a decrease in sheep GH levels, while it increased GH secretion in the monkey (Meyer and Knobil, 1967) and had no effect on man (Roth et al, 1963) or pig (Machlin et al, 1968a). Although dosage and length of infusion time were variable in these studies, a close inspection of the data has not revealed any relationship between dosage and type of response. Studies involving simultaneous application of an intravenous arginine load with epinephrine, show inhibition by epinephrine of the usual GH response to arginine, in sheep (Hertelendy et al, 1969) and in man (Fineberg et al, 1972). The infusion of epinephrine is of doubtful physiological significance (see page 22), but the results indicate that the GH secretory response to 'stress' in ruminants and primates is unlikely to be mediated by increases in plasma epinephrine levels.

The responses to noxious stimuli indicate that GH is probably under central nervous system (CNA) control to some extent. The spontaneous bursts of increased GH levels discussed on page 19 also indicate that CNS responses resulting from varying external conditions and changing emotions, could be influencing GH secretion and/or utilisation.

The fact that one of the most reliable stimuli

for GH secretion in man, is the ingestion of L-dopa (Imura et al, 1973) lends support to the probability that GH is under CNS control. Epinephrine infusion and application of noxious stimuli are both likely to stimulate the whole peripheral adrenergic system (Axelrod, 1965), bringing about numerous far-reaching secondary effects on metabolism and influencing GH secretion and utilisation in a variety of indirect ways. L-dopa, however, is able to cross the 'blood/brain barrier' (Axelrod, 1965) and its more reliable effect indicates that the ability to enter the brain may be important in bringing about GH release via the adrenergic system.

(ii) Hypothalamic influence on GH secretion

To ascertain the nature of the CNS control over GH secretion, pituitary-hypothalamic relationships have been studied. The work has been amply reviewed by Guillemin (1973), Burgus et al (1973) and Vale et al (1973) in three consecutive papers by members of the same team, so it is not necessary to review it again here. As a brief summary, secretion of GH is thought to be under dual hypothalamic control by means of neurohormones entering the hypophyseal portal system. The stimulatory hormone, a growth hormone releasing factor (GH-RF) has not yet been isolated, and its existence is inferred. The inhibiting hormone has been isolated, identified and synthesized, and has been found to be a tetradecapeptide which has been termed somatostatin. It is of further-reaching significance than simply being inhibitory to GH secretion: it has been found to suppress the release of thyrotropin (TSH) and possibly prolactin from the pituitary, as well as insulin

and glucagon from the pancreas (reviewed by Vale et al, 1973). The relative importance *in vivo* of its various effects has not yet been ascertained, especially in view of the fact that it is very rapidly inactivated in the circulation. There is some evidence that it is also produced in the pancreas as an additional pancreatic hormone (Efendic et al, 1975).

The neural mechanisms which control GH secretion via their influence on hypothalamic release of GH-RF and somatostatin, have been extensively investigated and have been reviewed by Muller (1973 a and b). To summarise the reviews, studies with electrolytic lesions and electrical stimulation of brain areas, have enabled specific neural areas to be implicated in GH control. Pharmacological studies with rats, monkeys and human subjects, lend support to the concept (already introduced in relation to L-dopa) that the adrenergic system plays an important role in the neurohormonal control of GH secretion, stimulation of α adrenergic receptors being stimulatory, and β adrenergic receptors being inhibitory. The exact identity of the adrenergic stimulant which is involved is not clear, in that dopamine and norepinephrine appear to have a mutually antagonistic role in rodents, while the evidence indicates that both amines possess a facilitatory function on GH release in the human subject. However, if the mechanism is slightly different between species, it could explain why 'stress' produces GH release in most species, but GH inhibition in rodents (see page 20).

Further pharmacological evidence (Muller, 1973 b) indicates that GH release is also stimulated by serotonin,

but there has also been evidence to the contrary (Pontiroli, 1976). In a situation where more than one neural pathway is involved, however, conflicting results would be likely to be obtained if the inhibition of one pathway causes an enhancement of the other pathway under some conditions. Recent evidence suggests that in man, a different mechanism is involved, depending on which stimulus is eliciting the GH response (Schwinn and Heckrodt, 1978).

The situation is far from having been conclusively worked out for any species. The only report of ruminant studies in this respect, complicates the issue still further, in that L-dopa infusion appeared to have no effect on basal GH levels, and an inhibitory effect on the response to arginine (Davis and Borger, 1973). The effect of an α adrenergic blocking drug, phenoxybenzamine, was ambiguous, in that in a study with only three animals, a GH release of doubtful significance ensued following the termination of infusion of the drug. When the drug was infused together with arginine, the normal GH response to arginine appeared to be prolonged, and levels were still elevated more than an hour after the infusion had finished. It is not possible to say whether the enhancement was caused by α blockade stimulating GH release, or by removal of α blockade stimulating it: more information is required before ruminants can be compared with primates and rats.

1.2.2 (d) Somatomedins and other peptides related to GH

A very interesting light has been shed on the mechanism of GH action, since the liver and various other

tissues (Uthne, 1973) have been shown to produce somatomedins as a result of GH action. Somatomedins, as the name implies, are a group of at least three different peptides (Fryklund et al, 1975) which mediate most of the actions which have been classically ascribed to GH itself. They not only stimulate incorporation of sulphate into cartilage, but also increase thymidine incorporation into DNA, effects which were observed to be mimicked by high insulin levels (Salmon et al, 1968). Further work (reviewed by Uthne, 1973) showed that they also stimulated glucose uptake and oxidation by adipose tissue, and stimulated amino acid uptake and protein synthesis in diaphragm. The similarity to insulin has also been noted because they are peptides of a similar size to insulin, and somatomedin A competes for the same receptor sites as insulin, on membranes of adipose tissue, liver and chondrocytes (Hintz et al, 1972).

Other growth-promoting peptides have also been found in plasma (reviewed by Megyesi et al, 1975 and Van Wyk et al, 1973). Of these, non-suppressible insulin-like activity (NSILA: the insulin-like activity left after plasma has been treated with excess antibodies to insulin itself) is noteworthy, because the soluble portion of the heterogeneous NSILA group of peptides is similar in all respects to Somatomedin A, and shows some GH dependency (Megyesi et al, 1975). The cross-reaction of the two in radio-receptor assays indicates that they are possibly identical, but more information is needed to clarify the situation. It is further complicated by the possible presence in plasma of (a) factor(s) which inhibits some of the actions of somatomedin (Salmon, 1973).

It is possible to form a unifying hypothesis from all the information concerning GH-dependent peptides. When somatomedins were first elucidated, it seemed likely that all the effects of GH were mediated via somatomedin production, the role of GH being therefore only that of a trophic hormone. Such a view was supported by observations that certain cases of familial dwarfism were found to have very high plasma GH, but no somatomedin (Daughaday et al, 1971). More recently, isolated somatomedin A and B have been seen to mimic GH effects in vivo (Fryklund et al, 1975).

The situation can be further simplified by postulating that all NSILA measured in plasma is simply due to the presence of the somatomedins (Megyesi et al, 1975), and the plasma factor which inhibits somatomedin activity (Salmon, 1973) could possibly be associated with the fact that in vitro studies show that tissues develop a pregressive refractoriness to the insulin-like actions of GH (Ahren et al, 1975, and see page 13).

Although it is always attractive to form a unifying hypothesis, there is ample evidence to indicate that here it is an over-simplification. For example, Underwood et al (1972) and Schwartz and Goodman (1975) studying adipose tissue metabolism, and Kostyo et al (1973) working with muscle, have provided evidence for striking differences between the effects of administering GH and somatomedins in vitro. The latter authors have also emphasized that exogenous GH does exert a direct influence on the rate of protein synthesis in muscle and liver in vivo, before any change occurs in somatomedin levels. Furthermore, somatomedin

production from the liver is not totally dependent upon GH stimulation, as it is also stimulated by insulin (Daughaday et al, 1975) and possibly by prolactin (Francis and Hill, 1975) although that finding was not repeatable (Daughaday et al, 1975).

If somatomedins were produced as cleavage products of GH, a definite relationship between somatomedin and GH levels would be likely. That this is not the case has been shown many times (Van den Brande, 1975): for example, very young animals have almost no somatomedin production, but high levels of GH (Stuart et al, 1976), and the two do not change in parallel in different nutritional status (Phillips and Young, 1976). It would also be expected that some immunological or receptor activity would cross-react between somatomedin and GH. Although somatomedin does cross-react with insulin receptors (Uthne, 1973), more recent work has shown that high molecular weight forms of somatomedin exist (Hall et al, 1975) which do not resemble GH in any way.

The importance to the present study, of the GH-dependent peptides, is that their existence raises the possibility that GH levels are directly controlled by the levels of the peptides (Van den Brande, 1975). Even though the extent of the importance of the trophic role of GH is not yet known, it is likely that many of the actions which have classically been ascribed to GH, are not direct effects of GH at all. Thus the metabolic significance of the GH responses, reviewed on pages 9 - 12, could be a misinterpretation of the true situation. At the same time, these discoveries also provide plausible explanations for the

discrepancies between species (see pages 10 - 12) in GH responsiveness. If somatomedin levels were measured as well as GH, it is possible that the GH results would become easier to interpret.

1.2.3 Stimuli for secretion of prolactin

(a) Plasma metabolites

Like GH, prolactin has been observed to be released in response to high levels of certain amino acids in the plasma (Forsyth and Edwards, 1972). In ruminants, a secretory response to arginine has been observed in heifers (McAtee and Trenkle, 1971) and to arginine, leucine and phenylalanine in sheep (Davis, 1972).

There is some confusion in the literature concerning the relationship between prolactin secretion and glucose levels. Horrobin (1974) cited evidence for a prolactin response to insulin-induced hypoglycaemia in human subjects, but among ruminants no relationship has been found in goats (Bryant et al, 1970) or heifers (McAtee and Trenkle, 1971).

The secretory response to increased levels of amino acids is typical of anabolic hormones (see pages 4 and 9). There is no doubt concerning its anabolic role in mammary growth and lactogenesis (reviewed by Horrobin, 1974) and its action as a general anabolic hormone has been shown in all classes of non-mammalian vertebrates (Bern and Nicoll, 1968). In mammals, however, its importance as a growth-promoting hormone is not well-established. Horrobin (1974) has reviewed the literature, which points to prolactin

having at least some growth-promoting activity, but he stated:

'There is still a great dearth of information about the action of prolactin on mammalian metabolism.'

Forsyth and Edwards (1972) have cited studies in which prolactin isolated by different methods of purification have differed greatly in their growth-promoting ability. They have drawn attention to the fact that human prolactin has several amino acid sequences in common with human GH, and it may be that the actions of the two only overlap when in certain molecular configurations determined by the medium.

In conclusion, its importance as a general anabolic hormone is secondary to its mammogenic and lactational role in mammals, and the fact that it is not intimately involved in homeostatic mechanisms is illustrated by the fact that it has been difficult to establish a relationship with plasma energy substrate levels.

1.2.3 (b) Different physiological states

(i) Lactation

Early work with rodents indicated that prolactin is of primary importance for the stimulation of mammogenesis leading to lactation, as well as for maintenance of lactation (reviewed by Cowie and Tindal, 1971), and it is this importance which gives prolactin its name. Although prolactin now appears to be less essential than GH in ruminant mammogenesis and lactation (Forsyth and Hart, 1975) it is widely established that plasma levels are high post-partum (Forsyth and Edwards, 1972), and that ruminant plasma

prolactin levels increase in response to the tactile stimulus of suckling (Arai and Lee, 1967: Bryant et al, 1970: Hart, 1974).

(ii) Age and reproductive status

During normal female reproductive cycles, many mammals including ruminants, have been shown to have increased prolactin secretion associated with oestrus (Forsyth and Edwards, 1972). In experimental administration of oestrogens, however, the results are not clear-cut, and the effect of oestrogens appears to be as potentiators of prolactin responses to other stimuli, rather than a direct stimulatory effect (Horrobin, 1974).

During pregnancy, maternal plasma prolactin levels are variable between species (Horrobin, 1974). There has been one report of foetal prolactin levels (Moger and Geschwind, 1971) which indicates that in foetal lambs, prolactin-secreting cells reach maturity early in the last third, as shown by the presence of prolactin in foetal plasma, and these authors also cited a histological study in which pituitary acidophils became detectable at the same age.

In the neonate the situation is also very variable between species, prolactin levels being high in young babies, but very low in young rats (reviewed by Horrobin, 1974). For the purpose of the present study, it is relevant to note that in heifers prepubertal values were consistently high (Swanson et al, 1972), although no data have been found for neonatal ruminants.

1.2.3 (c) The adrenergic system

(i) Adverse environmental stimuli

To quote Horrobin (1974) again:

'One of the best established facts about prolactin secretion is that it rises in response to stressful stimulation of almost any sort'.

Unlike GH secretion (see page 20), the response is consistent among all the species studied. It is possible that the prolactin response sometimes observed as a result of hypoglycaemia (see page 28) is a stress-mediated effect rather than a direct effect of decreasing blood glucose.

(ii) Hypothalamic influence on prolactin secretion

The mechanism of hypothalamic control of prolactin secretion has been reviewed by Horrobin (1974) and by Tolis and Friesen (1974). Prolactin secretion is apparently under the control of two hypothalamic influences, of which prolactin inhibiting factor(PIF) has been detected much more readily than prolactin releasing factor (PRF).

As in the control of GH secretion, the hypothalamic influences are in turn under the control of neurosecretions in the brain. The adrenergic control is opposite to that of GH, in that α adrenergic stimulation has an inhibitory effect on prolactin levels, an effect which has been accepted as being dopamine-mediated (Horrobin, 1974). However, the study of Davis and Borger (1973) is of interest, in that their results indicate that the inhibition is norepinephrine-mediated in sheep.

There is ample evidence which suggests that

prolactin release is under more than one neurosecretory control mechanism (Davis and Borger, 1973: Kulkarmi and Simpson, 1974), a suggestion which is supported by many reports of serotonin being stimulatory to prolactin release (Kamberi et al, 1971: Kordon et al, 1973), and of the cholinergic system also being involved (Grandison and Meties, 1976). There has also been a suggestion that dopamine in the circulation acts directly at the level of the pituitary to inhibit prolactin secretion, as well as at the hypothalamic level (MacLeod et al, 1975). However, more recent work indicates that hypothalamic PIF activity is not wholly accounted for by the inhibitory action of dopamine on the pituitary (Enjalbert et al, 1977).

The mechanism by which prolactin feeds back to inhibit its own secretion has been extensively reviewed by Tolis and Friesen (1974). Not only does it act at the pituitary level, but also prolactin itself has a profound influence on the levels of hypothalamic monoamines: it is suggested that it is that influence which also causes hyperprolactinaemic states to be associated with disrupted sexual functioning.

Finally, there is one other source of hypothalamic influence on prolactin secretion, and that is thyrotropin-releasing hormone (TRH), which stimulates prolactin secretion by a direct effect on the pituitary. The lack of evidence for a physiologically important role for the phenomenon has been discussed by Horrobin (1974).

1.3 DIGESTION AND METABOLISM IN YOUNG RUMINANTS

1.3.1 Anatomical development of the digestive system

At birth, the omasum and abomasum constitute more than 50% of the total stomach volume of the calf (Warner and Flatt, 1965). Following birth, there is a progressively greater increase in size of the reticulo-rumen relative to the abomasum, such that by four weeks of age, more than 50% of stomach volume is reticulo-rumen. In calves on 'normal' diets of milk, hay and grain, the most rapid period of reticulo-ruminal growth then follows between 4 and 8 weeks of age (Warner and Flatt, 1965). During that period of time, the reticulo-rumen was observed to grow at a rate of four times the body weight rate of gain, while the omasum/abomasum only grew at 2½ times the rate (Huber, 1968). However, calves limited to milk feeding during those weeks, showed an increase in size of all stomach portions, which was proportional to only 1 times the gain in body weight. Thus the consumption of solid feed is the stimulus for increased rumen development. By 12 - 16 weeks of age, a calf on 'normal' feed has obtained adult proportions, in which the reticulo-rumen constitutes 80 - 90% of the stomach volume (Huber, 1968).

Experiments in which calves have been fed wood shavings or plastic sponge, have indicated that merely the presence of solid matter entering the rumen is sufficient to stimulate increased rumen growth as well as increased musculature of the rumen wall. However, papillae on the rumen mucosa remain the same height as they were at birth (1 mm) (Warner and Flatt, 1965), and only grow to the height

of adult papillae, as a result of the presence of cellulose in a fermenting medium in the rumen. The volatile fatty acids (VFAs) are the end-products of such fermentation, and are the major stimulus for the finer anatomical and functional maturity of the rumen. They are also necessary for the maintenance of a mature rumen, since regression to small, non-functional papillae ensues rapidly if a calf is returned to a milk-only diet (Warner and Flatt, 1965).

1.3.2 Functional development of the digestive system

At birth, all the calf's nutrient requirements are supplied from milk, the major energy substrate being glucose, derived from lactose in milk. The digestive system is essentially monogastric, since the milk by-passes the reticulo-rumen and its gastric digestion is limited to the abomasum.

The mechanism whereby the fore-stomach is by-passed, is contraction of the reticular groove. A reflex of a Pavlovian type, associated with suckling from a teat, stimulates groove closure before milk reaches the stomach, thus enabling the milk to be shunted directly into the abomasum (Ørskov, 1972: Newhook, pers. comm.[1]). Drinking from a bucket in order to quench thirst, has a relaxing effect on the groove, so that only milk administered via a teat enters the abomasum directly. Solid food and saliva pass from the oesophagus into the rumen.

The rumen contains liquid (mainly from saliva)

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[1] Mr. J.C. Newhook, Department of Physiology and Anatomy, Massey University, Palmerston North.

from the time of birth (Newhook, pers. comm.), and the development of the ruminant mode of digestion begins almost immediately to some extent, in that Huber (1968) cites evidence that in a week-old calf, 25 - 40% of cellulose entering the rumen was able to be digested. The subsequent functional development of the rumen, however, is, in parallel to its anatomical development, dependent on the physical presence of solids entering the rumen, and upon the production of VFAs by rumen micro-organisms (Huber, 1968).

In calves fed a 'normal' diet of both milk and solids, VFA production in the rumen increased steadily from 1 week to 7 weeks of age (Huber, 1968). Restricting them to milk delayed the rise in rumen VFAs, but when returned to a cellulose-containing diet, they very rapidly attained a fermentation capacity which was equal to those which had been ruminating from soon after birth (Huber, 1968).

Following the entry of milk into the abomasum, the milk proteins form a casein clot or curd by the action of rennin and the low pH of the abomasal contents, although there is evidence to indicate that curd-formation is not necessary for the maintenance of a healthy digestive system (Huber, 1968). The lactose in the whey is digested more rapidly than the protein curd, and there is a high capacity for lactose digestion. Sucrose, on the other hand, is not able to be digested at all, and the presence of starch, or of disaccharides other than lactose results in diarrhoea (Huber, 1968). The milk-fat is also digested easily, with a utilisation rate of 97%: replacement by polyunsaturated fats has been observed to lead to neuromuscular disorders,

as does a fat-free diet (Huber, 1968).

1.3.3 Changes in post-prandial metabolism and endocrinology with development of the young ruminant

The ability of the developing rumen to absorb VFAs, has been extensively studied by Sutton et al, and the work reviewed by McGilliard et al (1965). The technique used was the capacity of the washed rumen to remove VFAs which had been added via a fistula. Two groups of calves were studied, one group limited to a milk-only diet (M), and the other on hay and grain with limited supply of milk (MHG). Group M showed no change in VFA absorbing capacity from birth to 34 weeks of age, while group MHG showed an initial abrupt increase, followed by a more steady increase. Changing a calf from M to MHG at 19 weeks, resulted in a sudden increase in absorptive capacity, to a level which was similar to the other MHG calves.

In the same review (McGilliard et al, 1965), absorption has been shown to be dependent on the ability of the rumen mucosa to metabolise the VFAs. Adult rumen tissue and liver metabolised butyrate extensively *in vitro*, to form ketone bodies, while the rumen of calves in group M was not able to do so. It was likely that the production of an inducible enzyme was responsible for the difference.

In contrast Khouri (1966) measured VFAs in portal blood, and used radioactive labelling to observe that there was no difference in VFA absorptive capacity of the rumen at different ages, or in VFA metabolism in the rumen mucosa

and liver. He suggested that the results cited by McGilliard et al (1965) could be suspect since the procedure of washing out the rumen before experiments, produced an abnormal situation.

Khouri (1966) also studied the subsequent metabolism of VFAs in calves, and observed that jugular blood VFA levels bore almost no relationship to VFA absorption by the rumen. The situation was complicated by the fact that both butyrate and propionate were rapidly metabolised in the liver (confirmed by evidence cited by Jarrett, 1968) and did not reach the peripheral circulation. Moreover, even in young calves which were fed on milk only, and which were not producing rumen VFAs at all, there was evidence that some VFAs were nevertheless present in the plasma from an endogenous source.

It has been repeatedly observed (Huber, 1968) that plasma glucose levels are high in the calf at birth (90 - 100 mg/100 ml), decline until the 6th or 7th week, and stabilise at the adult level of 50 - 60 mg/100 ml. Huber (1968) also cited evidence that decreases in blood glucose levels, and increases in plasma VFA levels, both occurred from weeks 1 - 6, regardless of the presence or absence of a functional rumen. It appeared that new-born ruminants did not only maintain higher levels of blood glucose, but also they had a higher capacity for glucose utilisation and turn-over than older ruminants, while older ruminants exhibited decreased glucose tolerance, as indicated by an increased time taken to remove a glucose load from the circulation.

The possible endocrinological changes associated with the differences between monogastric and ruminant digestion, have been discussed by Bassett (1975). Even though the adult ruminant maintained lower plasma levels of glucose, with a lower turn-over rate, it still utilised about ten times as much glucose as it obtained from food (Jarrett, 1968). Thus the adult ruminant was dependent upon gluconeogenic pathways to produce glucose, mainly from propionate and from amino acids (Bassett, 1975). Therefore, the development of the ruminant was likely to involve endocrine changes which favoured increasing rates of gluconeogenesis and greater conservation of glucose as the calf increased in age and ruminant status.

Bassett (1975) has stated that the role of the endocrine pancreas is central in ruminants, as it is in other animals, the sensitivity of ruminants to insulin being indicated by diabetic symptoms as severe as in other species. The role of insulin has been described by Bassett (1975) as follows:

> **'The role of insulin in the removal of large glucose loads is clearly less important in ruminants than in species absorbing large amounts of glucose, but rates of glucose turnover in sheep fed a variety of diets are closely related to plasma insulin concentrations. Since hepatic uptake of glucose in ruminants is unlikely, the principal effect of insulin on glucose metabolism in ruminants may be to increase its peripheral utilisation...'**

whereas in non-ruminants, it promotes both hepatic uptake and peripheral utilisation.

In young milk-fed lambs, the pattern of insulin secretion following feeding, was almost identical to that of non-ruminants. As the ruminant mode of digestion developed, there was a prolongation of the digestive process, and a parallel prolongation of the period of insulin secretion (Bassett, 1975). It was not clear whether insulin secretion after feeding in adult ruminants was caused primarily by enteric hormones, by vagal stimulation, by amino acids being absorbed from the small intestine, or by the absorption of VFAs. While jugular VFA infusion had a greater insulin-stimulatory effect in ruminants than glucose infusion (reviewed on page 4), the significance of the insulin response to VFAs is doubtful because an increased rumen content of VFAs was found not to have the same effect (Bassett, 1972).

In adult ruminants, because of the necessity to maintain blood glucose levels by gluconeogenesis, the action of glucagon in opposing insulin and promoting gluconeogenesis is of great importance to the glucose economy. While little work has yet been carried out on control of glucagon secretion, Bassett (1975) observed that glucagon and insulin are generally secreted together, and that small changes in the ratio of insulin to glucagon may be more important than absolute levels of either.

The secretion and role of GH in ruminants have been reviewed on pages 8 - 19 of the present study, and as with insulin and glucagon, there is no evidence to indicate that adult ruminants require special endocrine mechanisms to regulate their rather specialised mode of digestion.

In conclusion, the changes accompanying the

development of the ruminant mode of digestion fall into two categories. In one category, the anatomical changes and some aspects of ruminant physiology, are only able to develop as a result of the change in diet associated with weaning. Other changes, particularly those governing glucose and VFA levels in the blood of fasted animals, appear to be constitutional to ruminants reaching a certain age, regardless of diet. However, the endocrinological control of such changes is not clear at present, and the change in dietary habit could have a more profound influence on all aspects of carbohydrate metabolism, than has so far been observed.

1.4 RATIONALE OF THE PRESENT STUDY

Because some of the developmental changes which occur in the growth of the ruminant digestive system are dependent on changes in diet, while others are not, the present study was carried out with the aim of separating as far as possible, the effects of increasing age from the effects of rumen development. The technique used by Sutton (McGilliard et al, 1965) was adopted in later experiments here, in which one group of calves was fed on milk only throughout the experiment, while another was fed on hay and meal, with limited supply of milk. The hormonal responses of the two groups of calves were compared with each other, and with the same responses when the calves were new-born, in order to ascertain what changes may have taken place with an increase in age but no rumen feeding, or with the same increase in age plus a change to the ruminant mode of digestion.

In a preliminary experiment, very young suckling

calves were studied in order to measure basal plasma levels of insulin, growth hormone (GH) and prolactin, and to observe variations both associated with, and after, milk-feeding, and also to observe changes throughout the hours of daylight. Subsequently, a pilot experiment was carried out in which four milk-fed calves were given intravenous doses of metabolites which have been observed to have an influence on plasma levels of insulin and/or GH in several other species. Because of the involvement of insulin and GH with carbohydrate metabolism, glucose was tested first, followed by a mixture of the three VFAs (acetate, propionate and butyrate) which are produced as a result of rumen fermentation. Finally, intravenous salicylate was tested, because this had been observed to cause a decrease in free fatty acid levels, resulting in a large increase in GH secretion in sheep (Hertelendy and Kipnis, 1973).

In the third experiment, five-week-old milk-fed calves (group M5) and five-week-old ruminant calves (group R5) were compared with new-born calves (group M1), in their response to intravenous arginine. Because arginine has been shown to elicit secretion in sheep, of all three of the hormones studied here (Davis, 1972), it seemed to be a useful tool for assessing changes in responsiveness of the hormones, associated with age and/or diet.

In the fourth experiment, a more detailed investigation of carbohydrate metabolism was made, again using calves which had been treated as M5 and R5, and comparing them with M1. Two different treatments were given on different days of each sampling week: one was an intravenous

dose of glucose, and the other was intravenous insulin.

Finally, a report is presented of some work done by the author in collaboration with Dr. J.M. Gooden[1] with sheep. Plasma samples from lambs, together with their respective ewes, were studied before and after the time of weaning. Sampling took place before and after the lambs and ewes were given access to feed, or to each other for the purpose of suckling, and, on another day, the effect of intravenous arginine was tested. It was of interest to compare the results with results of Experiment 1 and 3 with calves.

In all experiments involving intravenous administration of metabolites to calves, there were control days when either nothing was given, or physiological saline, or stronger saline to control for stronger ionic solutions being administered during the treatment days. In addition to the three hormones, glucose levels were also assayed in all blood samples from Experiments 2 - 4. The hormones were measured by double antibody radio-immunoassays for which antibodies were raised during the course of study.

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[1] Applied Biochemistry Division, D.S.I.R., Palmerston North.

CHAPTER TWO:

MATERIALS, METHODS AND DEVELOPMENT OF METHODS

2.1 ANIMALS, CATHETERISATION AND BLOOD SAMPLING

2.1.1 Animals and catheterisation

The calves were female Jersey-Friesian cross-breds obtained at 2 - 4 days of age from the Massey University No.1 Dairy Unit. For the duration of the experiments, they were housed indoors in individual pens.

Calves were catheterised with 60-cm, 14-gauge polyethylene catheters in the jugular vein. In Experiment 1, catheters of different materials were used with varying degrees of success: the flexibility properties of polyvinyl and teflon were found to be inferior. From the end of Experiment 1 onwards, 'Bardic-I' catheters (C.R. Bard International Ltd., Sunderland, U.K.) were used consistently. During the procedure of catheterisation, a local anaesthetic of xylocaine* alone (Astra Pharmaceuticals, Australia, Ltd.) was found to be unsatisfactory, and from the end of Experiment 1 onwards, calves were immobilised with 0.25 ml of Rompun* (Bayer Leverkusen, Germany) given intramuscularly. The immobilising effect of Rompun lasted for 20 minutes, an appropriate length of time to complete the catheterisation procedure. On the rare occasions when the catheterisation did not go smoothly, it was necessary to give a second 0.25 ml injection of Rompun after the first 20-minute period was over. On no occasion did blood sampling commence until 24 hours after the Rompun injection, so that the animals had recovered from relatively long-term effects of Rompun.

*Xylocaine: lignocaine, Rompun: xylazine.

Patency of catheters was maintained by filling them with 1 ml of sterile heparinised saline (200 I.U. heparin/ml, Evans Medical Supplies Ltd., U.K.), whenever sampling was not in progress. While Bardic-I catheters were used, patency was maintained for as long as desired in 90% of cases. In Experiment 2, some catheters remained patent for a three-week period.

The most satisfactory method of preventing kinking of catheters was for the catheters to be inserted as follows. The catheter was inserted in a posterior direction into the jugular vein in order to minimise kinking caused by movement of the neck of the calf. At the point of entry into the skin above the vein, a 2-cm by 5-cm piece of surgical sticking plaster was wrapped around the catheter in such a way as to form a 'tab' of plaster, which was stitched on to the skin of the calf, preventing the catheter from slipping out, but also allowing flexibility of movement. The remainder of the tubing outside the calf, was attached over the shoulder and back of the animal, by means of a series of 4 - 6 'mattress stitches'. The catheter was thus given free movement through the stitches so that movements of the calf did not subject the catheter to any strain.

2.1.2 Health and maintenance of calves

Because of the risk of infection, either through the catheter or through the incision where the catheter was passed through the skin, 2 ml Triplopen (Glaxo Laboratories, Palmerston North) was injected intramuscularly on alternate days. In order to check for possible introduction of

pyrogens or infections into the blood, the calves' rectal temperatures were monitored four times (at approximately hourly intervals) throughout the sampling days in Experiment 3.

From the beginning of Experiment 2, care was taken to ensure that calves did not become anaemic. Blood samples were taken so that the volume of blood removed did not exceed 10% of the total blood volume of the smallest calf (Swenson, 1970, gives 57 ml blood/kg body weight as the blood volume of calves). The additional precaution of supplementing milk with 100 mg $FeSO_4.7H_2O$ per calf per day, was also taken.

Calves in Experiment 1 suckled twice each day (between 8.45 and 9.15 hr and between 15.30 and 16.30 hr) from a lactating cow. They were allowed access to solid feed in the form of meal (NZ Farm Products Ltd., Ration 102, see Table I) with 10% by weight of hay mixed with it.

Table I. Composition of meal fed to calves.
Ration 102. NZ Farm Products Ltd.

Ingredient	% weight
Barley	43
Maize	20.5
Meat meal	17
Pollard	5.5
Molasses	5
Malt culmings	4.5
Sugar	3
Salt (NaCl)	1
Lime	0.5
Flavouring	0.025

In all other experiments, milk was given only once a day, after the blood sampling for that day was complete, at about 14.00 hours. Therefore the milk-fed calves had always fasted for 20 - 22 hours at the time of blood sampling, which usually commenced between 9.00 and 10.00 hours. All calves were given free access to drinking water.

In Experiments 3 and 4, milk-fed calves were given milk through a teat in order to stimulate oesophageal groove closure and prevent rumination (Ørskov, 1972). Ruminant calves were given free access at all times to meal (see Table I) and were fed milk once a day from a bucket. In both diet groups, the daily energy intake was adjusted to that required for a body weight gain of 0.3 kg per day in Experiment 3, and 0.5 kg per day in Experiment 4. The ruminant calves' milk supply was dependent upon the weight of meal eaten during the previous 24 hours. Feed requirements were calculated according to the figures in Table II, which were obtained from Dr. A.W.F. Davey[1], pers. comm.

Table II Daily energy requirements of calves, and digestible energy of milk and meal

Daily energy requirements of calves	
For maintenance, per kg body weight	0.205 MJ
For each kg body weight gain per day	14.016 MJ
Digestible energy of milk and meal	
Milk	3.138 MJ digestible energy/kg whole milk
Meal	13.389 MJ digestible energy/kg dry matter of meal

[1] Department of Dairy Husbandry, Massey University.

2.1.3 Blood sampling

Each blood sample was withdrawn by means of a 5-ml syringe. The first 1.5 ml was discarded since it was mainly heparinised saline, and also the catheter was flushed out with blood of the same composition as the sample, as a result of discarding a greater volume than the volume of the catheter (1 ml). A 3-ml sample was then withdrawn, and 1 ml of sterile heparinised saline (200 IU/ml) was immediately flushed into the catheter from another syringe, taking care not to let any air into the catheter. In Experiment 1, heparin at a final concentration of 60 IU/ml blood, was used as anticoagulant. For all other experiments, samples were transferred from the syringe into 5-ml tubes ('Vacutainer', Becton-Dickinson Co., USA) in ice. The tubes contained 7.2 mg disodium ethylene diamine tetra-acetate (EDTA) dissolved in 0.048 ml of water, as anticoagulant. Samples were centrifuged, and the plasma (except for a 50 µl aliquot which was used for the glucose assay) was frozen within 30 minutes of taking each sample.

2.2 PLASMA GLUCOSE ASSAY

2.2.1 Method

The protein in the 50-µl aliquot which had been removed from each plasma sample before freezing, was precipitated with 0.3N $BaSO_4$ (Caraway, 1970). Glucose in the supernatant was assayed on the same day as blood sampling, by the glucose oxidase method described by Caraway (1970) using glucose oxidase (Fermcozyme, Hughes and Hughes Ltd., England), horseradish peroxidase (Sigma Chemical Co., Type II)

and o-dianisidine dihydrochloride as the oxygen acceptor. Five levels of a glucose standard (0, 40, 80, 120, and 160 mg/100 ml, May and Baker Ltd.) were included in each assay, and the absorbance of all standards and samples at 490 nm was measured on a Unicam SP 500 spectrophotometer. The glucose concentrations of samples were calculated from the average linear relationship produced when all the standards within one assay were taken into account.

An analysis of variance was carried out on the results of all the glucose assays performed during Experiment 3.

2.2.2 Results

For Experiment 3, a total of 22 assays were carried out. There was a linear relationship between absorbance and glucose concentration within the range of glucose concentrations observed. The mean absorbance ± the standard error for the 80 mg/100 ml standard, was 0.099 ± 0.000937. Of the variance components contributing to the standard error, only 1.76% was contributed by the difference between replicates within each assay, the remainder being contributed by the difference between samples.

2.3 RADIO-IMMUNOASSAY PROCEDURE

2.3.1 Hormones used in radio-immunoassays

The hormones used for radioactive tracers, for standards in the radio-immunoassays, and for raising antibodies, were as follows:-

Insulin: crystalline bovine pancreatic insulin from Sigma Chemical Co., Lot No. 121C-1350: 26.4 international units (IU)/mg.

Growth hormone: bovine GH, NIH-GH-B8[1]

Prolactin: bovine prolactin, NIH-P-B1[1]

Further purification of both GH and prolactin was carried out by ion exchange chromatography on DEAE-cellulose according to the method described by Hart et al, (1975).

2.3.2 Procedure for raising antibodies and isolating carrier protein

Antibodies to all three hormones were raised in guinea pigs immunised with hormone conjugated to bovine thyroglobulin or to bovine serum albumin, or in the case of insulin, with polymerised insulin. In each case, the bovine hormone was conjugated to bovine thyroglobulin or bovine serum albumin (both from Sigma Chemical Co., Lot No. 83C-8160 and 24C-1740 respectively) by the method of Frohman et al (1970), or insulin was polymerised using diethyl pyrocarbonate, by the method of Wolf et al (1970). The hormone preparation, 0.25 mg dissolved in 0.5 ml sterile saline and emulsified with an equal volume of Freunds

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[1] The author wishes to thank Professor A.E. Wilhelmi of the National Institute of Health, USA, for the generous gift of growth hormone and prolactin to Professor D.S.Flux.

complete adjuvant (Bacto Adjuvant, Difco Laboratories), was injected into guinea pigs at multiple subcutaneous sites, and injections were repeated at approximately monthly intervals for 4 to 6 months. 'Booster' injections of the free hormone in sterile physiological saline were then given at monthly intervals, and plasma containing antibodies was collected by heart puncture, 7 to 10 days after each 'booster'.

For the raising of second antibodies, crude preparations of guinea pig gamma globulin (GPγ) were made by 'salting out' plasma from normal guinea pigs, with 33% $(NH_4)_2SO_4$. Larger molecules had previously been precipitated with 18% $(NH_4)_2SO_4$, and the 33% precipitate was then washed twice in 40% $(NH_4)_2SO_4$, dialysed against 0.1 M NH_4HCO_3 and lyophilised. The GPγ was injected into sheep and rabbits, following the same procedure described above for the hormones, only without any prior conjugation or polymerisation.

The GPγ prepared by the same 'salting out' process described above, was also used as the carrier protein (Skelley et al, 1973) added to assay tubes in order to increase the amount of antigen to the second antibodies.

2.3.3 ^{125}I-labelled hormones

(a) Preparation

Radioactively labelled hormones were prepared using ^{125}-I-sodium iodide in sodium hydroxide solution from the Radiochemical Centre, Amersham, U.K., by a modification of the chloramine-T method of Greenwood et al (1963). It

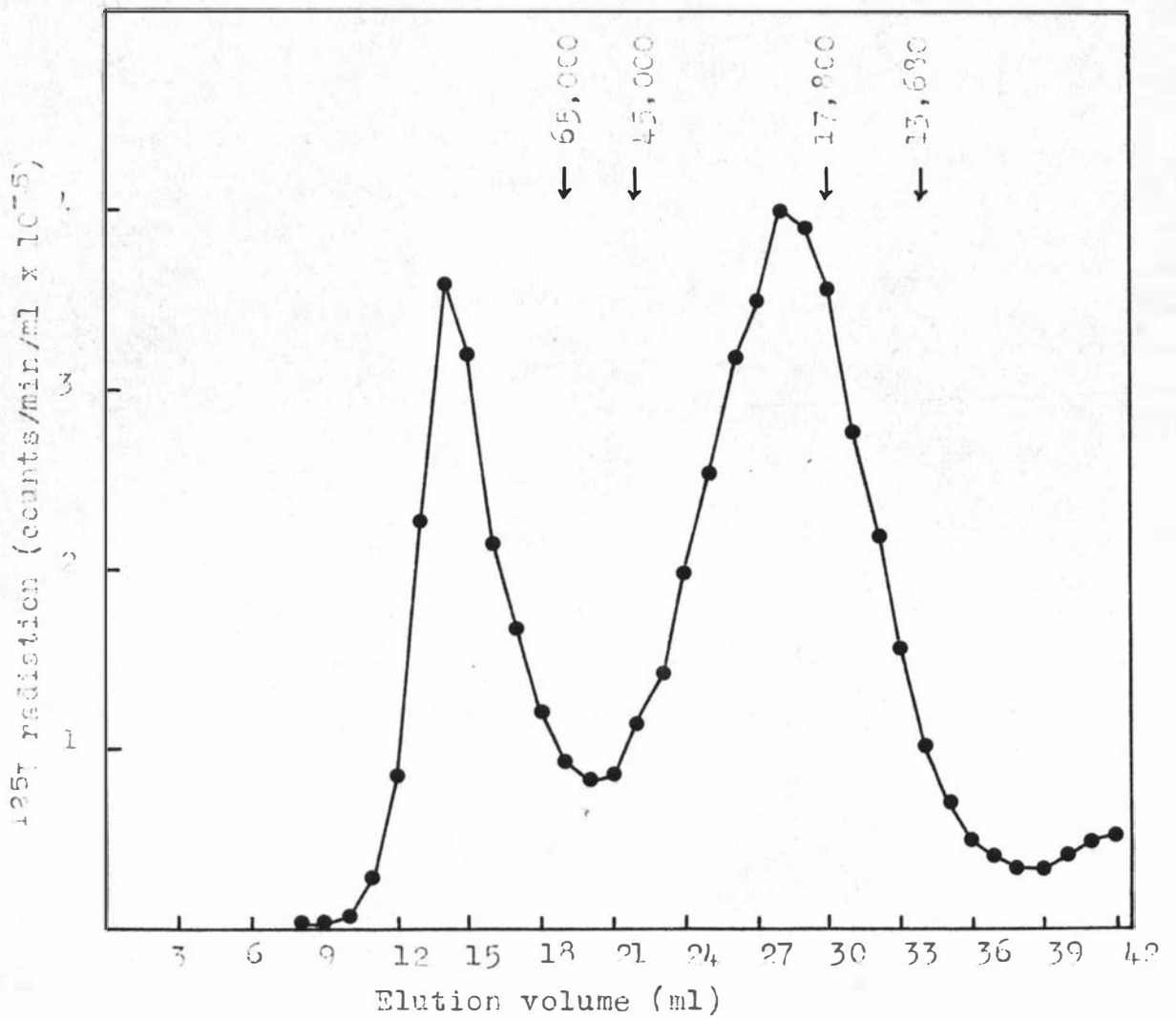

Figure 1 Gel filtration of ^{125}I-bovine GH after 1 month's storage at -20°C, on Sephadex G-100 equilibrated with 0.025 M borate buffer at pH 8.4. The vertical arrows represent elution peaks of column markers with their molecular weights.

was found to be necessary to dissolve GH in 2 M urea at room temperature for 1 - 2 hours before iodination (Hart et al, 1975). Sephadex G-50 (Pharmacia) columns were used to separate protein-bound from free iodine after iodinating GH and prolactin, and cellulose acetate powder was used for insulin (Berson and Yalow, 1961) according to a method obtained by personal communication from Dr. R.A. Donald[1]. Effluent fractions were collected in diluent plasma (see page 54) tc a final concentration of 10% plasma (the plasma has been shown to retard aggregation of hormones in solution (Van Orden, 1972)), and stored at 4°C or -20°C until use.

(b) Purification

It was observed that replication between duplicate tubes and also the level of binding of labelled hormones in the radio-immunoassay (RIA), were both severely impaired if a preparation of labelled hormone was used more than 3 - 4 weeks after iodination. A 1 cm x 24 cm Sephadex G-100 (Pharmacia) column was set up and molecular weight markers (bovine serum albumin, ovalbumin, myoglobin and ribonuclease) were run through it in 0.05 M phosphate buffer at pH 7.4, to ascertain the elution volume for each molecular weight. A preparation of labelled GH from an iodination carried out 1 month earlier, was run through the same column in 0.025 M borate buffer at pH 8.4, and the elution pattern is shown in Figure 1. A large peak of radioactivity was eluted in the void volume, indicating the presence of GH which was either aggregated or bound to

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[1] Princess Margaret Hospital, Christchurch, N.Z.

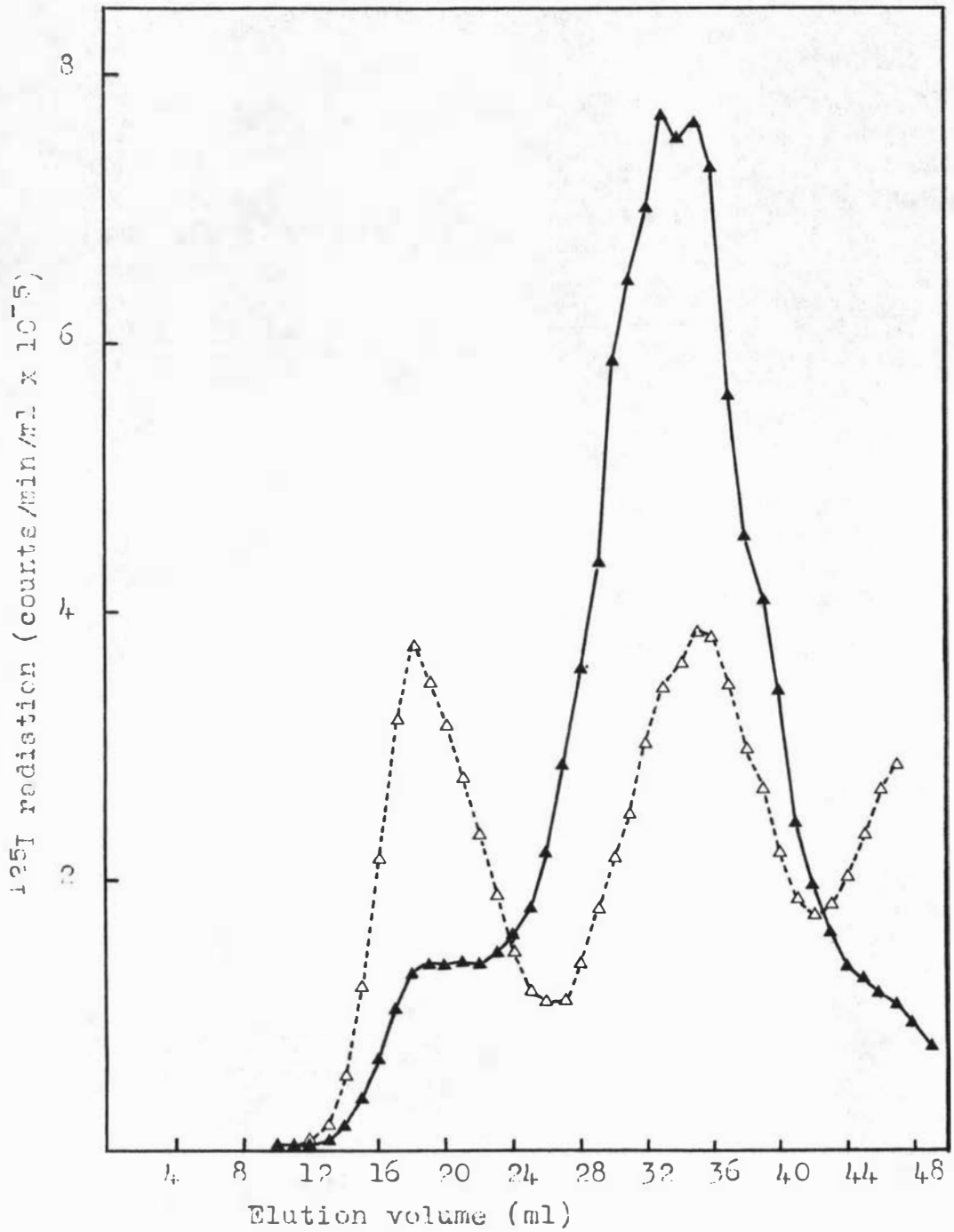

Figure 2 Gel filtration of ^{125}I-bovine GH after five days' storage (solid line) and 3½ months' storage (broken line) at -20°C, on Sephadex G-100 equilibrated with 0.025 M borate buffer at pH 8.4.

one of the proteins in the diluent plasma. The second large peak corresponded to a molecular weight of about 20,000 daltons, and was probably GH in monomeric form (Andrews and Folley, 1963). A further study, comparing freshly iodinated with 3-month-old labelled GH on a larger column (Figure 2) indicated a third peak of very small molecular size, presumably corresponding to break-down products of GH. In the freshly-iodinated preparation, both aggregate and fragments were very much less evident, the hormone apparently being mainly present in monomeric form.

For routine assays, fractionation on Sephadex G-100 was carried out every three weeks and only those fractions which corresponded to monomer were used: great improvement in assay quality was observed as a result.

2.3.4 Diluent plasma

Evidence from studies of binding labelled hormones in the presence of varying concentrations of bovine serum albumin suggested that protein concentration influenced the level of binding, and would thus be important in the relationship between the binding of standard hormones and of hormones in plasma samples. In order to equalise the protein concentration, it was necessary to make up the standards in a solution which was the same as the plasma samples in its composition: for GH and prolactin assays, human plasma, with citrate as anticoagulant, was used, since human and bovine GH and prolactin do not cross-react immunologically (Hayashida and Li, 1959). The similarity of insulin structure between species precluded the use of

untreated plasma from other species in the insulin assay. Therefore, insulin was removed from plasma according to the following procedure.

Several litres of bovine blood were collected from the Longburn freezing works, using disodium ethylene diamine tetra-acetate (EDTA) at 0.01 M as anticoagulant. The plasma was separated by centrifugation, and stirred for 1 hour at 4°C, with 10% by weight of decolourising charcoal (May and Baker Ltd.). The charcoal was separated first by passing through Whatman No. 1 filter paper, and then finer particles were removed by centrifugation at 12,000 r.p.m. for 45 minutes in an MSE centrifuge. It was frequently found at this stage that a clot formed in the plasma. Clot formation was of great assistance in the separation of charcoal fines, as the fines were incorporated in the clot, leaving the plasma charcoal-free. The charcoal-treated plasma was stored frozen in 10-ml aliquots. Before use in the assay, all diluent plasma was passed through a 0.45 μm filter (Millipore, Type HA).

2.3.5 Procedure for each assay

The assay procedure was the same for all three hormones and is shown in Table III. Assays were carried out in 3-ml plastic LP3 tubes (Luckham Co., England).

Standards were made up in diluent plasma, and 100 μl of each standard was assayed in triplicate. Preparation of stock standard solutions was as follows: a small amount (< 50 μg) of hormone was accurately weighed using a Cahn Electrobalance, and dissolved in an appropriate

Table III Reagents and procedures for radio-immunoassays

Day of assay	Volume of reagent	Reagent
1	100 µl	Plasma[1]
	100 µl	2% diluent plasma[2] in PBS[3] + EDTA[4] containing first antibodies at a final dilution of 1:40,000 - 80,000
	100 µl	2% diluent plasma in PBS + EDTA containing GPγ at 0.6 - 1.2 µg/100 µl
	Each tube mixed on a cyclo-mixer	
	Incubated at room temperature.	
2	50 µl	^{125}I-labelled bovine hormone diluted in 2% diluent plasma in PBS + EDTA to 5,000 counts per minute (cpm)
	Each tube mixed on a cyclo-mixer	
	Incubated at room temperature	
3	50 µl	Antibodies against GPγ at a plasma dilution of 1:2 - 1:50 in PBS + EDTA
	Each tube mixed on a cyclo-mixer	
	Placed in a refrigerator at 4°C	
5	All tubes centrifuged at 2,000 rpm for 30 minutes at 4°C, and supernatant aspirated. Precipitates counted in a Packard Auto γ Counter Model 5285.	

[1] 100 µl plasma was any one of the following:
100 µl plasma sample, hormone to be measured
50 µl plasma sample + 50 µl diluent plasma
100 µl diluent plasma containing standard hormone

[2] Human plasma or charcoal-treated bovine plasma.

[3] Phosphate buffered 9% saline at pH 7.4, 0.05 M phosphate.

[4] Disodium ethylene diamine tetra-acetate at 0.01 M

volume of 10% diluent plasma in phosphate buffered saline (0.05 M phosphate in 0.9% NaCl at pH 7.4, abbreviated to PBS) containing disodium ethylene diamine tetra-acetate at 0.01 M (EDTA), to make a stock standard solution at 1 µg/ml. The stock solution was stored in 200 µl aliquots at -20°C. On the day of the assay, a single aliquot was thawed, and diluted with diluent plasma to standard solutions serially diluted from 12.8 ng/ml to 50 pg/ml for insulin, and from 128 ng/ml to 0.5 ng/ml for GH and prolactin.

Two dilutions of each 'unknown plasma' sample (100 µl and 50 µl) were assayed in duplicate, with addition of 50 µl of diluent plasma where necessary, to equalise the plasma concentration. Each assay also contained 2 to 4 'standard plasma' samples: plasma from large pools with previously estimated hormone levels, which provided an additional means of reading hormone levels from the 'unknown plasma' samples. The standard plasma samples provided a valuable check and a means of assessing variance between assays.

As seen in Table III, the final stage of the assay procedure was the counting of the ^{125}I radiation in the precipitate in each tube. The results, expressed as counts per minute, were processed on a computer (IBM Model 1620) using a programme developed by Professor R.E. Munford[1] (pers. comm.) based on Burger et al,(1972). The untransformed standard curve data were represented by the following equation.

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[1]Department of Physiology and Anatomy, Massey University.

$$Y = \frac{A}{C + X^E} + e$$

where Y was the amount of radioactive hormone bound by first antibody, and X was the amount of non-radioactive hormone present in the tube. A, C and E were constants which were specific for each assay, and e was the random error. Determination of A, C and E made use of an iterative technique, and once the values were established, the amount of hormone present in each unknown sample ($\hat{X}$) was calculated from the equation:

$$\hat{X} = \left(\frac{A}{Y} - C\right)^{\frac{1}{E}}$$

The programme included calculations of the precision throughout the range of the standard curve (see page 93) and also the 95% confidence limits, based on that precision, of the estimates of hormone levels in each unknown plasma sample.

2.4 VALIDATION OF RADIO-IMMUNOASSAYS

2.4.1 Investigation into parallelism

(a) Introduction

When results of a radio-immunoassay (RIA) were plotted as in Figure 3, it was observed during the course of study, that there was sometimes a significant difference between the slope of the standard curve, and the slope produced by plotting the two dilutions of the unknown plasma sample, so that they were the same distance apart as a doubling of hormone concentration on the X axis. The importance to the assay of such a deviation from parallelism, has been concisely expressed by Midgley et al (1969):

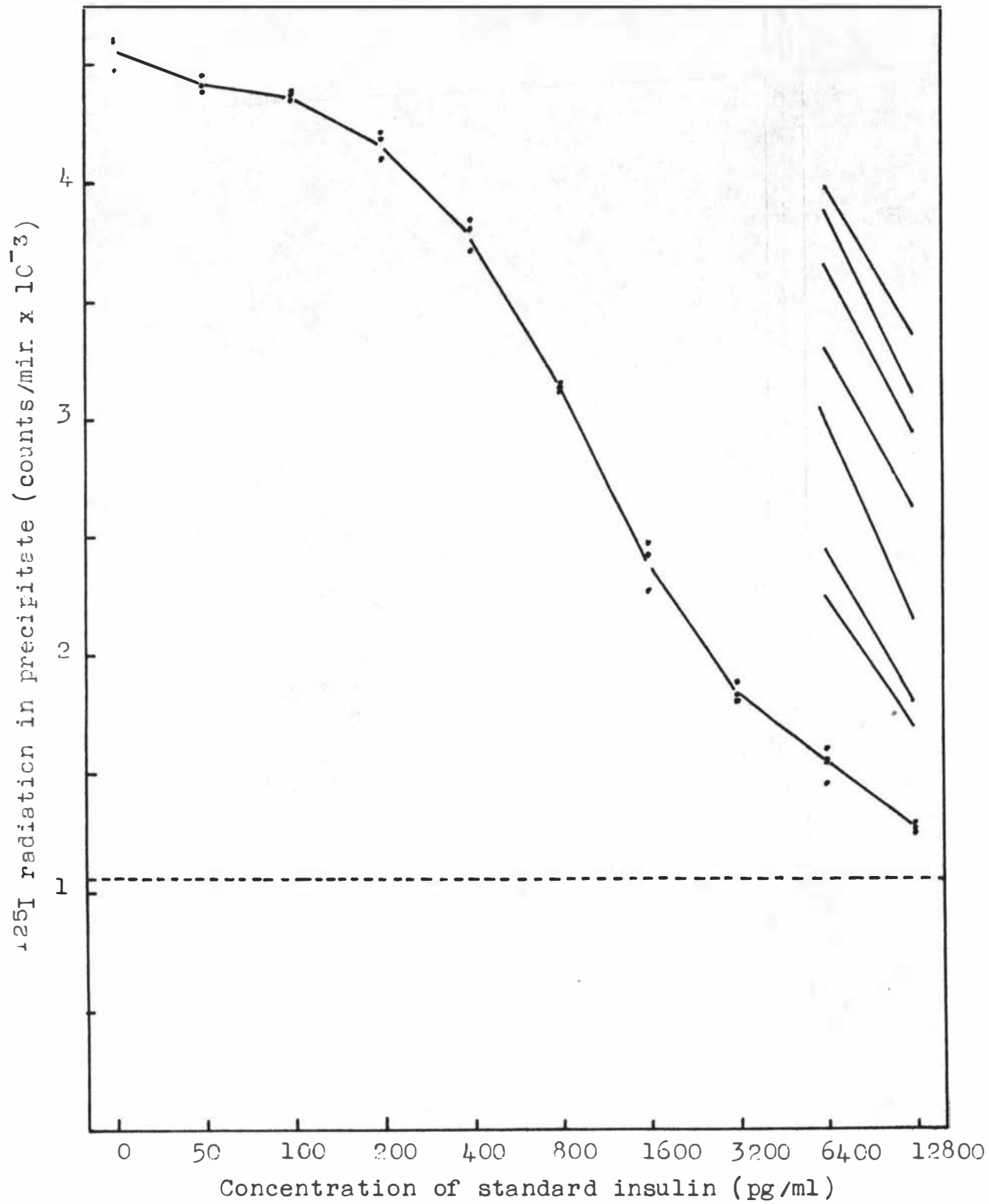

Figure 3 Standard curve of an insulin assay (solid line). The broken line represents the level of non-specific binding of ^{125}I-insulin in each assay tube. The short, solid lines are lines joining the ^{125}I radiation level in precipitates of two dilutions of each plasma sample. First antibodies were added at a final dilution of 1:72,000.

> 'While the presence of parallelism does not prove similarity of unknown and standard, lack of parallelism proves dissimilarity between hormones or incubation media'.

Thus the difference in slopes can indicate:-

(1) That the active material in the standard hormone preparation differs from the hormone in naturally occurring form in the plasma.

(2) Alternatively that another factor in the assay tubes, e.g. protein concentration, or an interfering effect caused by anticoagulant, is promoting or inhibiting binding in unknown tubes, by comparison with standards.

In the present study, all the RIAs gave slopes of standards and unknowns that did not differ in at least a proportion of the assays. For prolactin assays, there was no serious parallelism problem. For GH assays, 25% of assays with calf plasma gave a significant difference between slopes, but differences were not observed for GH assays of sheep and cow plasma. For insulin assays, 44% of all calf and sheep assays showed some significant differences between slopes.

Attempts were made to find the cause(s) of interference, by purifying hormone preparations, adjusting protein concentrations, etc. The results of the attempts are shown on pages 70 to 93, and the degree of success was such that the parallelism problem was able to be reduced in subsequent assays.

Many assays were repeated after the parallelism problem had been reduced. Because time was not unlimited,

however, it was not always possible to obtain adequate parallelism in every assay. Thus the figure of 25% for GH and 44% for insulin assays, is the proportion of assays which were actually used for obtaining results, even though there was still a deviation from parallelism. The use of such assays could be justified as follows.

(1) There was no evidence other than the lack of parallelism in some assays, that the assays were not measuring the appropriate hormone.

(2) The absolute plasma levels of hormones were of less relevance to the present study, than relative changes in levels in response to treatments.

(3) A mathematical procedure was adopted which gave a quantitative measure of the deviation from parallelism, together with a procedure for adjusting the slopes of the unknowns to make them parallel with the standards. The results thus obtained were verified by observing the effect of the procedure on the results of standard plasma samples of which aliquots were included in every assay. Where the standard plasma results were in agreement with those of assays which had no parallelism problem, the unknown plasma results were calculated using the adjusted slopes, in order to assess the effectiveness of the treatments. Although the procedure did not define or remove the cause of deviations from parallelism, it was a practical method for reducing the need to repeat several assays.

2.4.1(b) Assessment of parallelism

(i) Methods

1. Criteria for routine assessment of parallelism

For routine assays, parallelism was assessed for each assay by plotting the standards and unknowns as in Figure 3, and hormone levels for 10 to 20 of the unknowns were read from the curve for each of the two dilutions. If the hormone levels obtained from the 50 µl tubes multiplied by two, were consistently more than 20% different from the levels obtained from the 100 µl tubes, it was concluded that the assay was faulty, and further investigation was required. If the difference was less than 20%, the assay was used for obtaining plasma hormone level data according to the computer method described on pages 57 - 58. Because equal weight was given to 50 µl tubes as to 100 µl tubes in assessing the hormone level of each sample, a difference of 20% between the two dilutions could give rise to a final assessment which was 10% more or less than the estimated hormone level in the sample. When assays were consistently successful, the difference between dilutions was much less than 20%, and only a glance at the raw data was necessary to see that there was no consistent discrepancy.

2. Mathematical investigation of two insulin assays

A further investigation into the parallelism of the data was made in two insulin assays, one of which was acceptable, and the other had an unacceptable deviation from parallelism in one consistent direction for all samples. For these two assays, three assessment procedures were adopted.

(A) The percentage difference between the two levels as read from the standard curve plotted on logarithmic graph paper, was assessed for 20 unknown samples.

(B) An analysis of variance was carried out on the results as read from the plotted standard curve (as in (A)). The results obtained for each individual tube were used, so that for each of the 20 samples there were two dilutions, and two replicate tubes within each dilution.

(C) The data for the points on the standard curve were transformed so that the counts per minute were expressed as the logit of bound counts (B) divided by counts per minute bound in the absence of any non-radioactive hormone (B_o), and the hormone levels were expressed as $\log_{10}$ pg/ml.

$$X = \log_{10} \text{ pg/ml}$$

$$Y = \text{logit } \frac{B}{B_o}$$

The transformed curve formed a straight line, which was used to calculate the slope in a simple linear regression analysis of X on Y. A test to assess the suitability of the logit transformation was performed by correlating the points on the regression line, with the transformed points obtained from the standard curve data of the assay (Midgley et al, 1969).

The 20 unknown samples used in (A) and (B) were transformed in the same way as the standard curve. Each individual sample was used to produce a regression line, and the 20 lines were averaged to obtain a mean 'unknown' slope. Because the 100 µl unknown tube had exactly twice as much plasma, and therefore twice as much

of the hormone to be measured, as the 50 µl tube, calculation of the slope of each unknown involved the use of X values which had a 2:1 ratio. Each X value was therefore read from a graph, as in (A) above, and then for each unknown an average was obtained by giving equal weight to 50 µl as to 100 µl samples. The average so produced was used as the 100 µl sample, and then divided by two to give the correct relationship between it and the 50 µl sample.

The resulting curves for standards and unknowns were weighted before the regression calculations, according to Finney (1964) and Midgley et al (1969), using the variance of Y obtained from 5 different standard curves. The average slope for the 20 unknown samples was compared with the slope of the standard curve. The appropriate 'counts per minute' data required to give results of 100, 500, 1,000 and 2,000 pg/ml when using the transformed standard curve, were used to obtain pg/ml data from the transformed 'unknown' curve. The results were compared as an additional estimation of the deviation from parallelism.

(ii) Results

(A) The hormone levels read from a standard curve are shown in the left-hand columns of Table XLIV a and b, (page 207-8). Assay 1 had an average percentage discrepancy between results obtained from 100 µl tubes and 50 µl tubes, of 7.2%, and Assay 2 of 31.6%: It can be seen that even though the discrepancy of individual samples of Assay 1 was rather variable, the problem was less severe than in Assay 2, where all the deviations from parallelism were in the same direction.

Table IV Variance components from the analysis of two insulin assays, read from raw standard curves

Assay	Source of variance		Mean square	Degrees freedom	% of total variance
1	between dilutions (d)	+	0.00047	1	0.45
	between samples (s)	+++	0.10013	19	96.54
	d x s interaction	NS	0.0	19	0.0
	residual		0.00311	40	3.003
2	d	+++	0.61072	1	11.054
	s	+++	0.47003	19	84.696
	d x s interaction	+++	0.00905	19	2.399
	residual		0.00252	40	1.85

NS = not significant, + = $p<0.05$, ++ = $p<0.01$, +++ = $p<0.005$

(B) Table IV shows that the proportion of total variance contributed by differences between dilutions, was 24 times greater in Assay 2 than in Assay 1. The significance of the difference between dilutions was at the 5% level for Assay 1, and at the 0.5% level for Assay 2, indicating that there was a real difference between the two assays.

Table V 95% fiducial limits of hormone concentration assessments from raw standard curves, for two insulin assays: based on residual variance.

Insulin level (pg/ml)	95% fiducial limits Assay 1	Assay 2
100	87.8 - 113.9	89.0 - 112.4
500	439.1 - 569.4	444.9 - 561.9
1,000	878.1 - 1,138.8	889.8 - 1.123.8
2,000	1,756.3 - 2,277.4	1,779.5 - 2,247.5

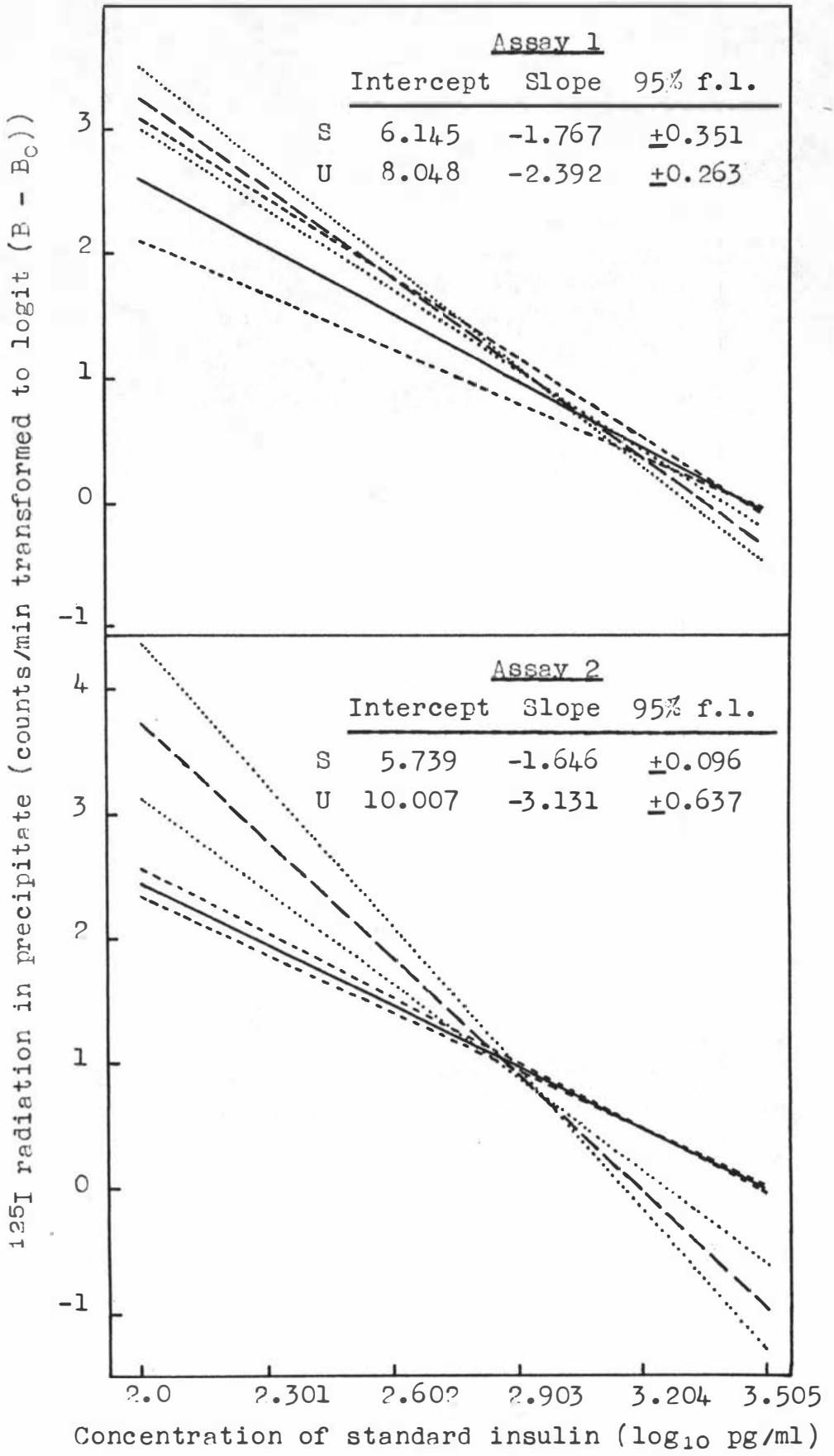

Figure 4 Regression slopes of transformed data from two insulin assays. ——— Standard curve (S) with ---------- 95% fiducial limits, and — — — — — unknowns (U) with ····· 95% fiducial limits.

Table V illustrates that replication between tubes within each assay, based on the residual variance given in Table IV, was almost identical for the two assays.

(C) From the slopes shown in Figure 4, it can be seen that the 95% fiducial limits of each slope were such that they overlapped over nearly the entire length of the standard and unknown slopes in Assay 1, but the overlap only extended for one doubling of the value of X, in Assay 2. The overlap indicates that the deviation from parallelism was not significant for Assay 1, and could have occurred by chance. The deviation from parallelism was consistent, and not a chance occurrence in Assay 2.

Table VI shows the effect which the deviations from parallelism had on the hormone data if the different slopes were used for converting the same 'counts per minute' data to pg/ml.

Table VI Discrepancy between results obtained from standard curve slope, and from unknown sample slope, as measured after transforming slopes to a straight line in two insulin assays. 95% fiducial limits in parenthesis.

Insulin level (pg/ml) read from standard slope	Insulin level read from unknown slopes	
	Assay 1	Assay 2
100	187 (155-218)	260 (184-327)
500	616 (590-637)	606 (531-661)
1,000	1,027 (1,010-1,049)	872 (840-894)
2,000	1,714 (1,602-1,865)	1,256 (1,211-1,327)

(iii) Discussion

(A) When the parallelism situation was at its worst, as in Assay 2, there was a difference of about 30% between the two dilutions, which was consistent throughout the entire range of samples. Thus, if all samples were simply read from the raw standard curve, with equal weight given to both dilutions, the results would simply read 15% out, in one or other direction. While such an error could have serious consequences if the absolute hormone levels were being compared between different assays, in situations where the most important aspects to be compared were included within one assay, the relative results would not be influenced, because the same deviation would apply to all samples.

(B) The finding of a significant difference between hormone levels estimated by different dilutions depends on both the mean square for 'dilutions', the numerator in the F test, and the residual mean square, caused by differences between replicate tubes within dilutions, or the dilution x sample interaction mean square, used as the denominator in the F test. If the latter is unusually small as in an assay with very good precision, a deviation from parallelism which would go undetected (not significant) in a rougher assay, would be significant.

Tables IV and V provide evidence to show that in Assay 1, a typical insulin assay with parallelism within the acceptable range defined on page 62, there was no significant deviation from parallelism even though the residual variance was of the same order in both assays.

(C) Figure 4 and Table VI illustrate that if a simple regression analysis is used, the deviations from parallelism had a cumulative effect. There were parts of the curve where the error caused by the deviation was very small, increasing towards the extremes of the range of hormone levels. The situation here may be contrasted with the procedure of using raw standard curve data, as in (A), in which the % error was relatively consistent over the entire range of hormone levels.

If (C) is used as a routine procedure, provided that the basal hormone levels fell within the range where the error was smallest (in practice, repeated measurements of standard plasma samples included in every assay were important for confirming that the error was smallest in the basal range), an increasing error away from the basal range would make very little difference to the results. One reason why this is so, is that, for pituitary hormones especially, secretion occurs in an episodic pattern (see page 19) and the frequency of high bursts of secretion is of more interest than the size of the hormone peaks. Moreover, if an acute treatment was used to elicit a burst of hormone secretion, the highest hormone level would probably not coincide with the collection of a blood sample, and so the blood samples showing the highest levels only gave an approximation of the extent of the response. Log_{10} transformations of the hormone data were used, in order to minimise the effect of differences at very high hormone levels, and a larger assay error in the high range is tolerable.

Similarly, where the inaccuracy became large at very low hormone levels, the error was considerable only when exceptionally low individual samples were involved. Errors in the range of 100 - 200 pg/ml were not a source of concern in interpreting the biological importance of hormone changes. It is of interest to note that the insulin assay produced more assays with a consistent deviation from parallelism than the other two assays (see page 60), and there is probably a connection between that, and the fact that the insulin assay was used in such a way as to be sensitive in a lower range than the other two hormones.

As a consequence of the observations described here, investigations were made into ways of producing assay results which did not deviate from parallelism (pages 70 to 93). In addition, precautions were taken during routine assays, so that the effects would be minimised if assays did happen to be obtained with poor parallelism. The precautions included assaying all the samples requiring comparison within the same assay, so that inter-assay comparisons were avoided, and including standard plasma samples in every assay as an additional reference point as well as purified hormone preparations.

2.4.1 (c) Concentration of reagents

(i) Methods

During the course of carrying out numerous assays for each hormone, investigations were made into the effects of changing the concentrations of each of the assay components, one at a time in a controlled manner. The

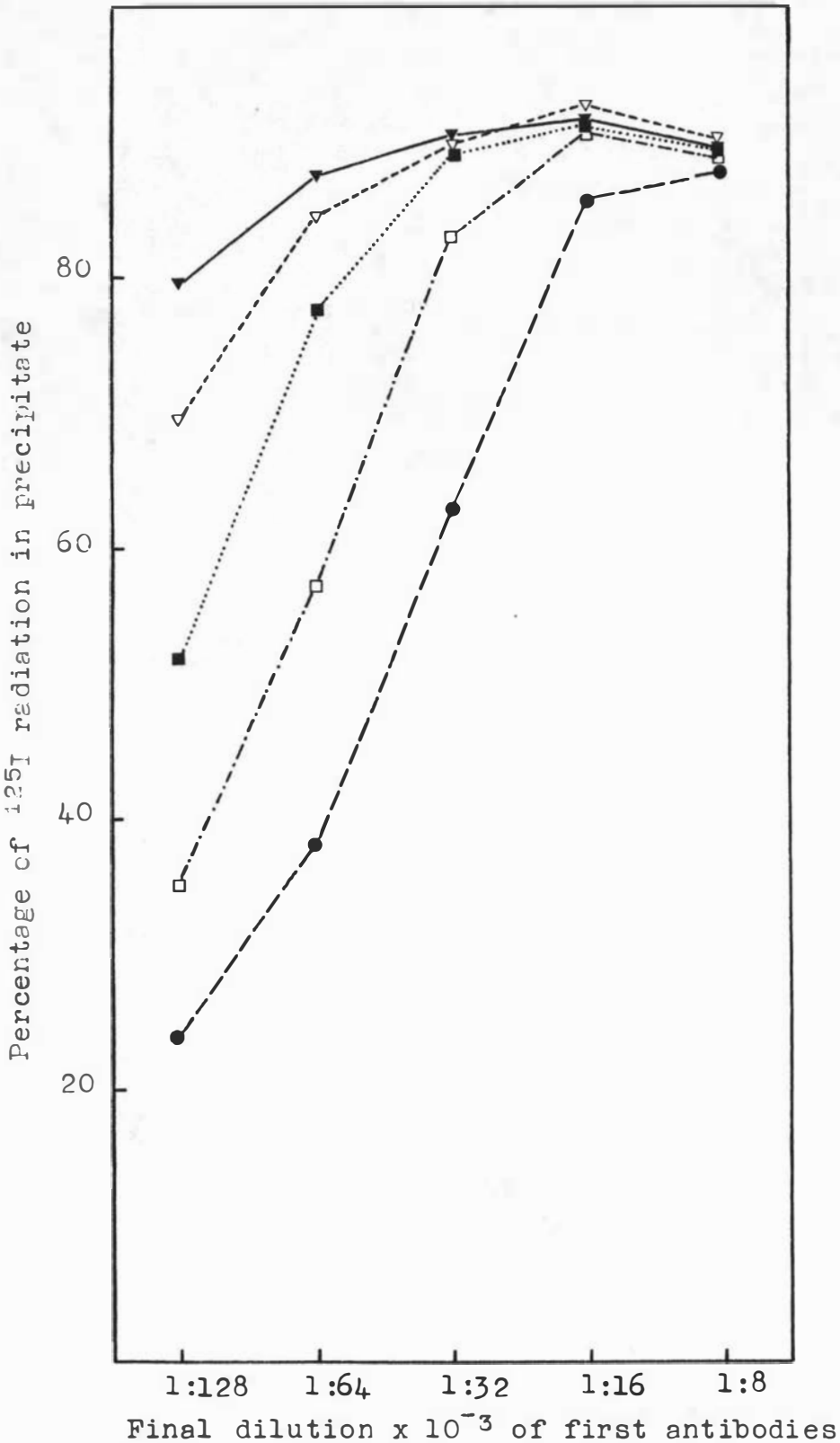

Figure 5 Percentage of ^{125}I-bovine GH bound, with varying dilutions of antibodies and varying amounts of ^{125}I-bovine GH.

Amount of ^{125}I-GH added to each tube (cpm):

8,000 — 64,000

16,000 — 128,000

32,000

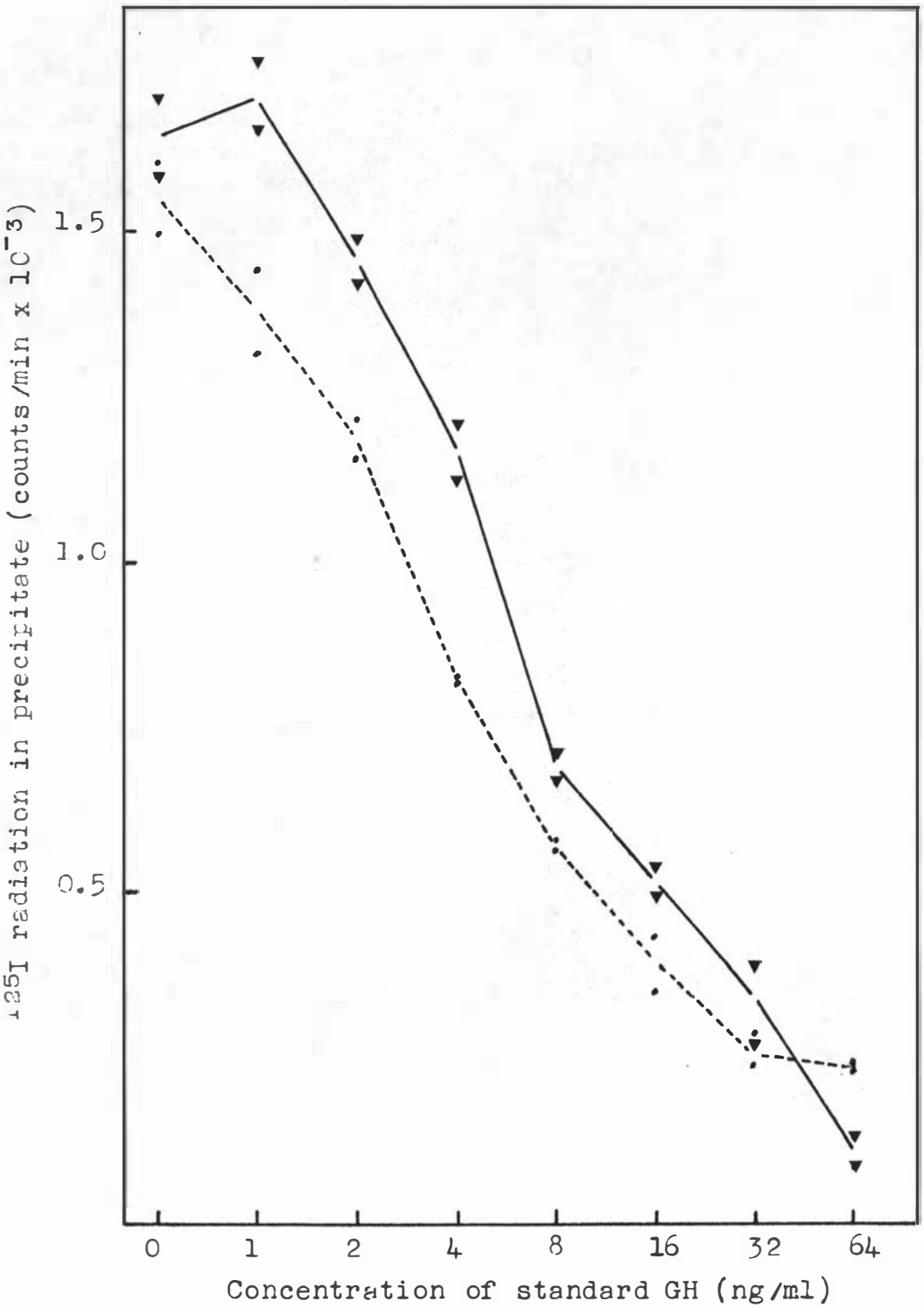

Figure 6 Standard curves of GH assays with two different dilutions of first antibodies.

▼——▼ 1:48,000

•----• 1:60,000

effects were examined in relation to percentage binding, possible changes in the shape of the standard curve, the degree of parallelism and the results given by repeated assay of standard plasma samples.

(ii) Results

The effect on percentage binding, of changing both the concentration of first antibody and the concentration of labelled hormone, is shown in Figure 5. For any given level of first antibodies, maximum binding was achieved if the minimum amount of labelled hormone was added.

The effect on the shape of the standard curve, of changing the concentration of first antibodies, is shown in Figure 6. Although increasing the amount of first antibodies in the tubes increased the binding of labelled hormone in the absence of unlabelled hormone, giving a higher value for counts per minute (cpm) at 0 ng/ml, there was a decrease in sensitivity at very low levels of standards.

(iii) Discussion

The observation that decreasing concentrations of first antibodies increased the sensitivity of the assay at lower hormone levels (see Figure 6) has been noted by all other workers in the field (e.g. Yalow and Berson, 1968). In the present study, low concentrations of antibodies were frequently used so that an assay was sensitive to as little as 50 pg/ml of insulin. At such low concentrations, the percentage of labelled hormone which can be bound may be much reduced, because a condition of antigen excess is

reached. Therefore the correct balance between first antibodies and labelled hormone should be achieved, depending on the range of hormone concentrations which it is desired to measure.

2.4.1 (d) Precipitation reaction

(i) Method

It is possible that deviations from parallelism could have been brought about by substances in the plasma which interfered with the ability of the second antibodies to precipitate the first antibody/hormone complex. In addition to testing higher concentrations of second antibodies in order to overcome such interference, several alternative procedures were attempted, including:

(1) The use of polyethylene glycol (as used by Desbuquois and Aurbach, 1971) instead of second antibodies.

(2) The refrigeration time, between adding second antibodies to tubes, and centrifuging them (Table III) was varied.

(3) The addition of ammonium sulphate together with second antibodies (a procedure suggested by Professor J. Landon[1], pers. comm.).

(4) The pre-reaction of antibodies, by mixing solutions of first antibodies, GPγ and second antibodies, before the addition of hormones or plasma (Hales and Randle, 1963).

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[1] St. Bartholemew's Hospital, London, U.K.

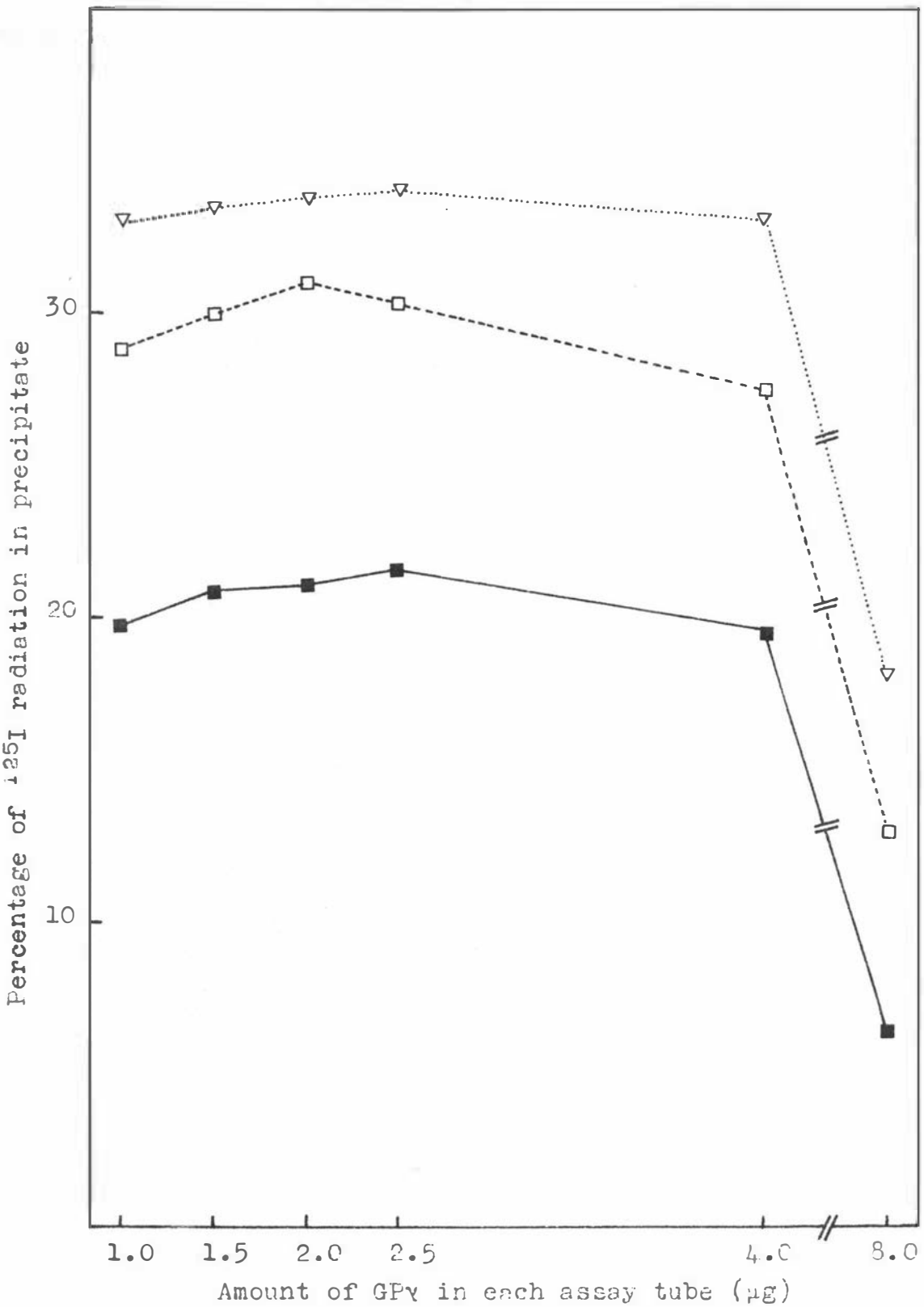

Figure 7 Percentage of ^{125}I-insulin bound, with varying amounts of GPγ in the assay tubes, and in the presence of different plasma or protein. First antibodies added at a **final** dilution of 1:72,000.

■——■ Calf plasma containing 610 pg/ml insulin

□- - - - - - -□ Charcoal treated porcine plasma

▽·····▽ 0.1% bovine serum albumin

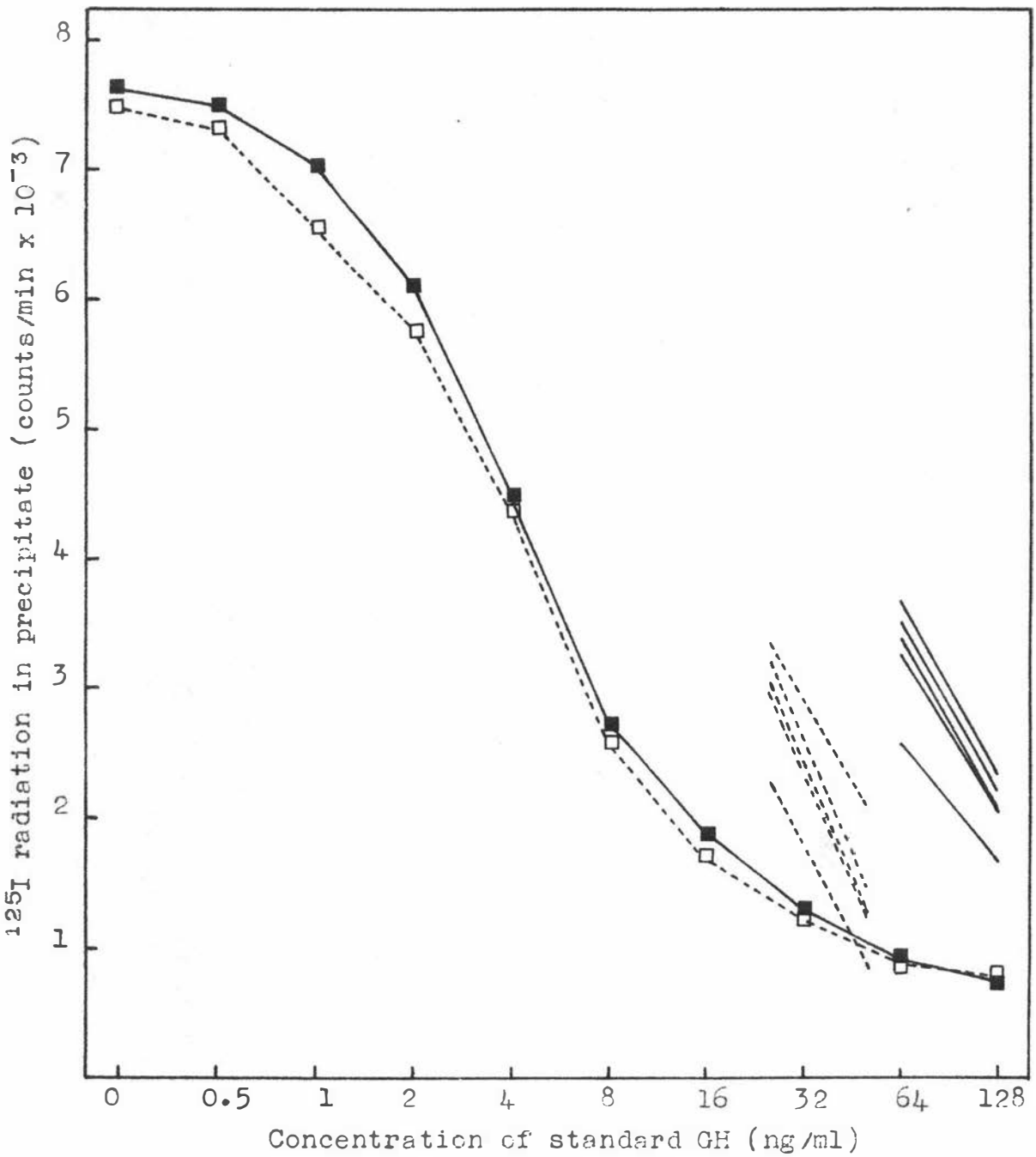

Figure 8 Standard curves of GH assays, to show the effect on parallelism of changing the dilution of second antibodies. Final dilutions of second antibodies were ■——■ 1:16 and □--------□ 1:32. First antibodies added at a final dilution of 1:60,000. The significance of the difference in slope between standards and unknowns was not significant at 1:16 and $p<0.05$ at 1:32.

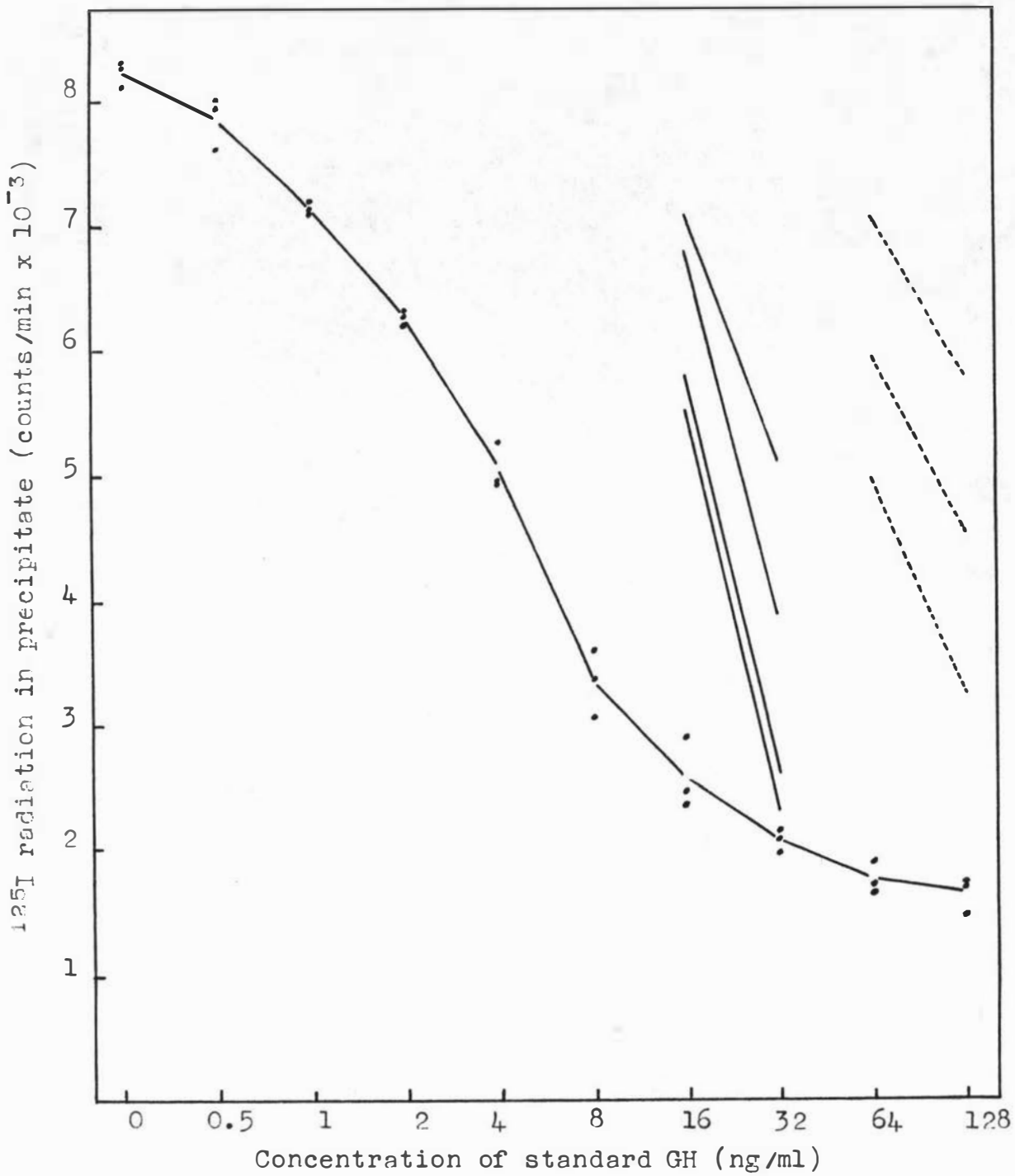

Figure 9 Standard curve of GH assay with second antibodies at a final dilution of 1:64, assaying hormone levels in calf plasma (solid lines) and cow plasma (broken lines). First antibodies at a final dilution of 1:60,000. The significance of the difference in slope between standards and unknowns was not significant for cow plasma, and $p<0.01$ for calf plasma.

(ii) Results

Figure 7 shows the effect on percentage binding of labelled hormone, of changing the concentration of carrier protein, GPγ. It can be seen that the optimum binding reached a plateau, with respect to GPγ concentration. Binding generally was low because of the use of first antibodies at 1:72,000, a dilution which gave low binding but very good sensitivity at low insulin levels.

Figure 8 shows the effect on parallelism of changing the concentration of 'Phil' plasma ('Phil' was the sheep used to produce antibodies to GPγ) from 1:32 to 1:16 (using plasma from the November 1973 bleeding of 'Phil'). Although both dilutions gave the same percentage binding in the absence of calf plasma, when 'Phil' was used at 1:32, there was an inappropriately great inhibition of binding by calf plasma, resulting in unknown slopes which were too steep (the significance of the difference between the unknown plasma slopes at different dilutions of 'Phil' was $p<0.05$). The situation was rectified by using 'Phil' at 1:16. In Figure 9, the lack of parallelism brought about by using calf plasma together with an insufficient amount of 'Phil' (from the November 1973 bleeding of 'Phil'), can be seen to be a specific effect of calf plasma unknowns, since cow plasma in the same proportions gave near-perfect parallelism ($p<0.01$ for calf plasma compared with slope of standard curve, and not significant for cow plasma).[1]

The results of the other alternative precipitation reactions were as follows:

(1) Precipitation of the first antibody-hormone

[1] See also results obtained with December 1975 bleeding of 'Phil', included as Post Script on pages 218 - 220.

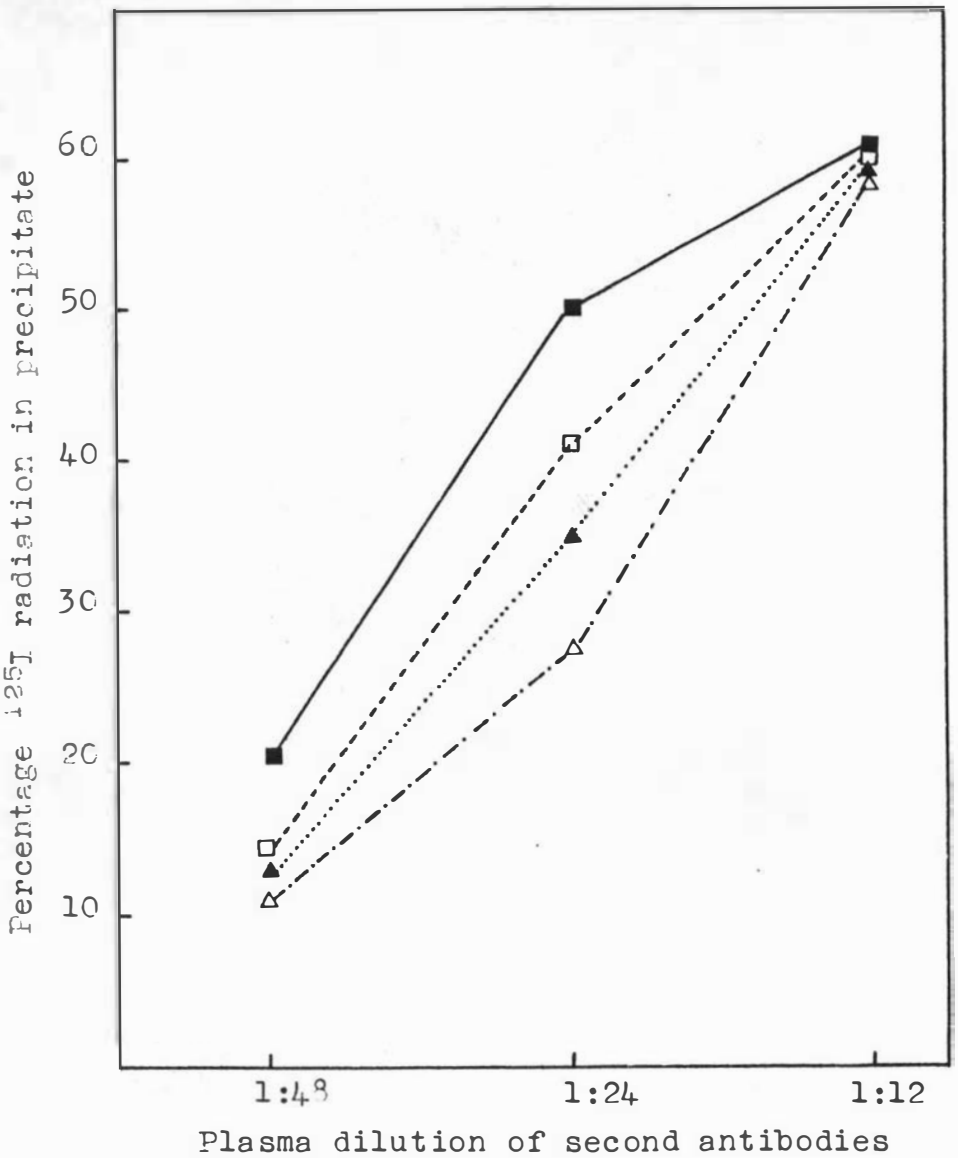

Figure 10 Percentage of ^{125}I-GH bound, with varying dilutions of second antibodies added with varying concentrations of ammonium sulphate, as follows: ■——■ no ammonium sulphate, □---------□ 5%, ▲·····▲ 10%, △—·—·—·—△ 20%. First antibodies added at a final dilution of 1:60,000.

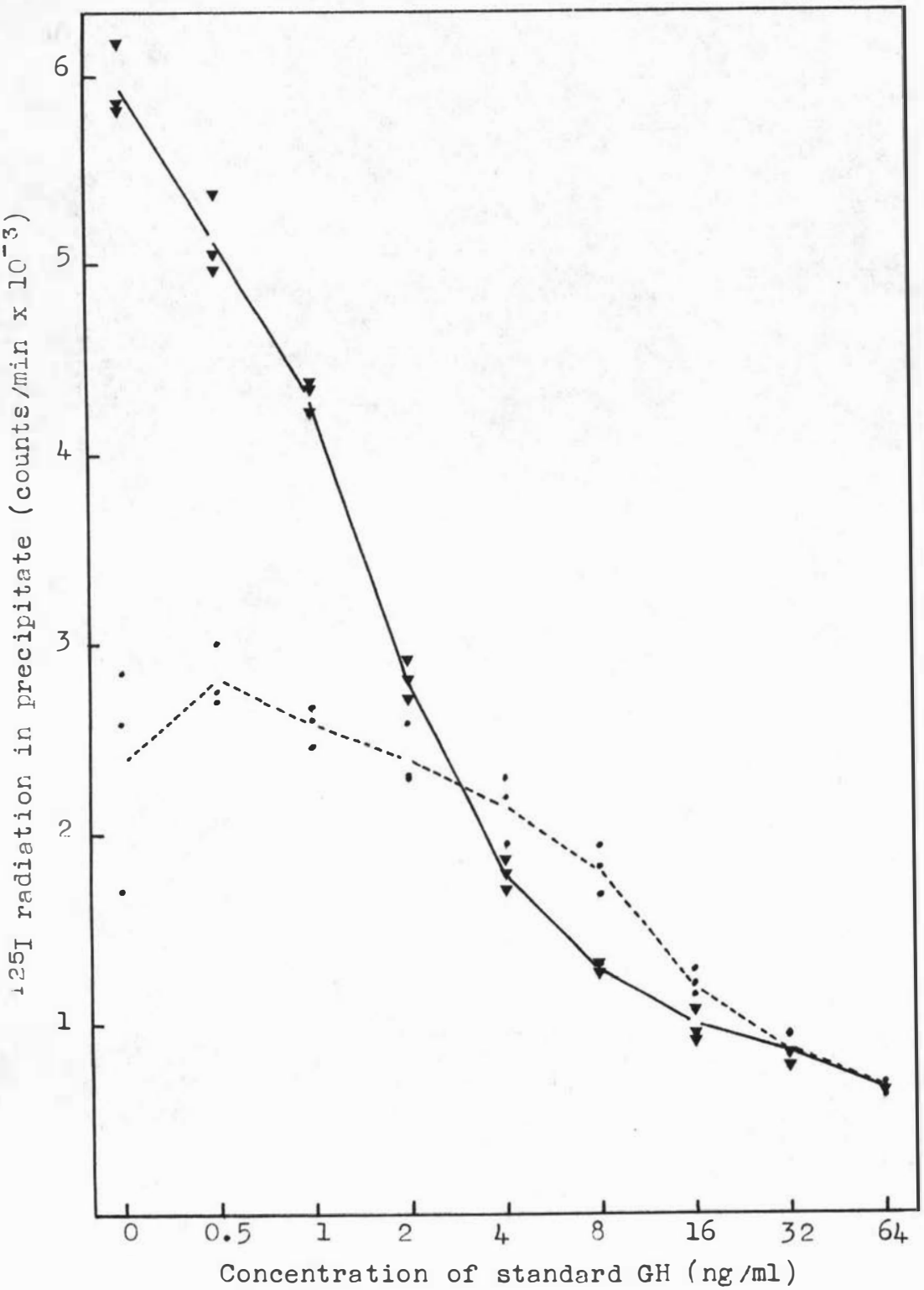

Figure 11 Standard curves of GH assays with second antibodies added according to Table III (solid line) or with antibodies pre-reacted (broken line). First antibodies were added at a final dilution of 1:60,000 and second antibodies at 1:16.

complex by polyethylene glycol (PEG) was found to yield a very bulky precipitate. After subtracting the non-specific binding of radioactive hormone adhering to the bulk, the actual binding was found to be very similar to that obtained with second antibodies (Table VII).

Table VII Percentage binding achieved by double antibody RIA compared with the use of PEG

	% bound (total)	Non-specific binding	actual % bound
Phil at 1:16 for 48 hr.	35.9	4.9	31.0
Phil at 1:16 for 72 hr.	36.1	5.5	30.6
12% PEG	55.9	25.0	30.9

(2) Table VII also indicates that a 48 hour refrigeration period allowed precipitation to the same extent as a 72 hour refrigeration period.

(3) The effect of precipitation with different concentrations of Phil dissolved in four different concentrations of $(NH_4)_2SO_4$, is shown in Figure 10. The presence of $(NH_4)_2SO_4$ consistently inhibited binding.

(4) The effect of pre-reacting the first and second antibodies is shown in Figure 11. An insensitive standard curve with poor duplication between tubes was obtained, by comparison with the usual assay procedure.

(iii) Discussion

Although both the precipitation reaction and the first antibody/hormone reaction may both be antibody/antigen reactions, in a RIA they are rather different in function and

extent. The dilution of first antibodies used is determined by the range of hormone which it is required to measure, and the binding of labelled hormone by first antibodies is never complete, but is usually near 40 - 50% for assaying hormones at physiological plasma concentrations. Because it is the displacement with unlabelled hormone of the 40 - 50% binding by first antibodies that produces the results in terms of hormone levels, the radioactivity of the precipitate as counted in the final step of the assay, should be an exact and direct reflection of the binding of labelled hormone by first antibodies. Therefore, it is necessary for the reaction of the precipitation step, to go as far towards completion as practicable.

It is generally accepted that the incubation procedure allows an equilibrium to be achieved (Skelley et al, 1973). It may not therefore be possible for 100% precipitation to occur, but the reaction time should be sufficient to allow precipitation to reach a stable maximum. It is possible for an assay to reach equilibrium at a different rate for standards and unknowns, giving a temporary lack of parallelism not seen if the assay reaches completion. Therefore it was of interest to observe that there was no enhancement of binding (Table VII) or change in parallelism when refrigeration time with 'Phil' (November 1973 bleeding) was extended beyond 48 hours.[1]

It can be seen from Figure 7 that the precipitation reaction was not fully efficient if there was too much or too little GPγ in each tube. The amount of GPγ added in

[1] More recent work with December 1975 bleeding of 'Phil' has been included on pages 218 - 220 as a Post Script, and it indicates that the time factor was more important than had previously been thought.

routine assays was well in the plateau range of precipitation, so that minor variations between tubes did not interfere with the completion of the precipitation reaction.

Figure 8 also indicates that under certain conditions, the concentration of second antibodies needed to be increased to ensure complete precipitation in all tubes. If either the unknown tubes or the standard tubes contained an interfering substance which prevented the second antibody precipitation from being complete, the result was inevitably a deviation from parallelism. Aubert (1970) stated that where binding was too weak in the presence of plasma,

> '... it is probably due to a bad proportion of the concentrations of first and second antibodies.'

Other workers have observed a similar situation, in that plasma from certain individuals interfered more than others. Some human subjects have been found to produce plasma which completely inhibited precipitation: in assays, their plasma always appeared to have excessively high levels of the hormone, regardless of the actual levels measured by other means (Dr. R. Kirk[1], pers. comm.). The difference between calf plasma and cow plasma in this respect (Figure 9) is probably a reflection of the fact that the proteins in calf plasma are considerably different from those in the adult cow. For example, at the time of birth, the plasma proteins of the calf consist of 50% foetuin, a protein which decreases rapidly after birth, and is very low in adult plasma (Hsu et al, 1973). Such a protein may have a non-specific interfering effect on the precipitation reaction,

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[1] Princess Margaret Hospital, Christchurch, N.Z.

or could even interfere with the assay because of GH-like properties. Further investigation into the immunological properties of foetuin is warranted.

It has been observed by Welborn and Fraser (1965) that plasma from many individuals may have lesser interfering properties, leading to artificially elevated or 'negative' values for the assay. Many of them may be overcome if the plateau of complete precipitation by second antibodies is rigidly achieved, because such plasma may simply have a property of giving greater sensitivity to the precipitation reaction, and narrowing the plateau. The effect (termed the 'prozone effect' by Welborn and Fraser, 1965) has not been observed in Figure 7 in the present investigation, but its possibility was taken into account by always using the correct balance between second antibody dilution and GPγ concentration, to give the peak of precipitation.

The use of PEG, $(NH_4)_2SO_4$, or the pre-reaction of antibodies, were not effective as replacements for the technique of adding second antibodies as the final inclusion in assay tubes.

2.4.1 (e) Molecular structure of the hormone

(i) Method

A likely cause of deviation from parallelism is that the hormone being measured in the standard tubes is different from the naturally occurring hormone in the plasma samples under investigation. The ^{125}I-labelled hormone could also interfere with the parallelism if its immunoreactivity were different from the hormone in plasma. The molecular

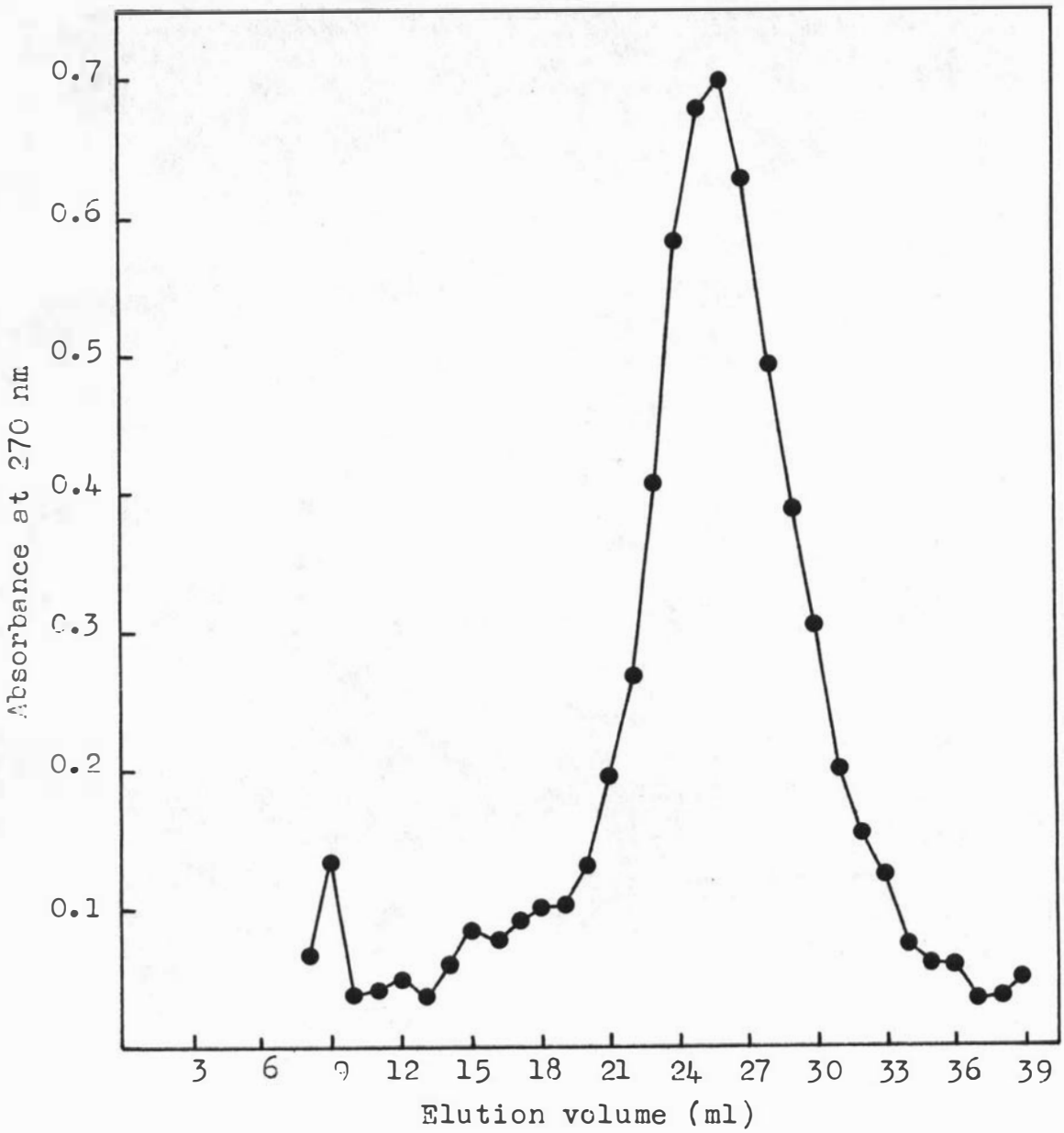

Figure 12 Gel filtration of lyophilised bovine GH, after 10 months' storage in a dessiccator at 4°C, on Sephadex G-100 equilibrated with 0.025 M borate buffer.

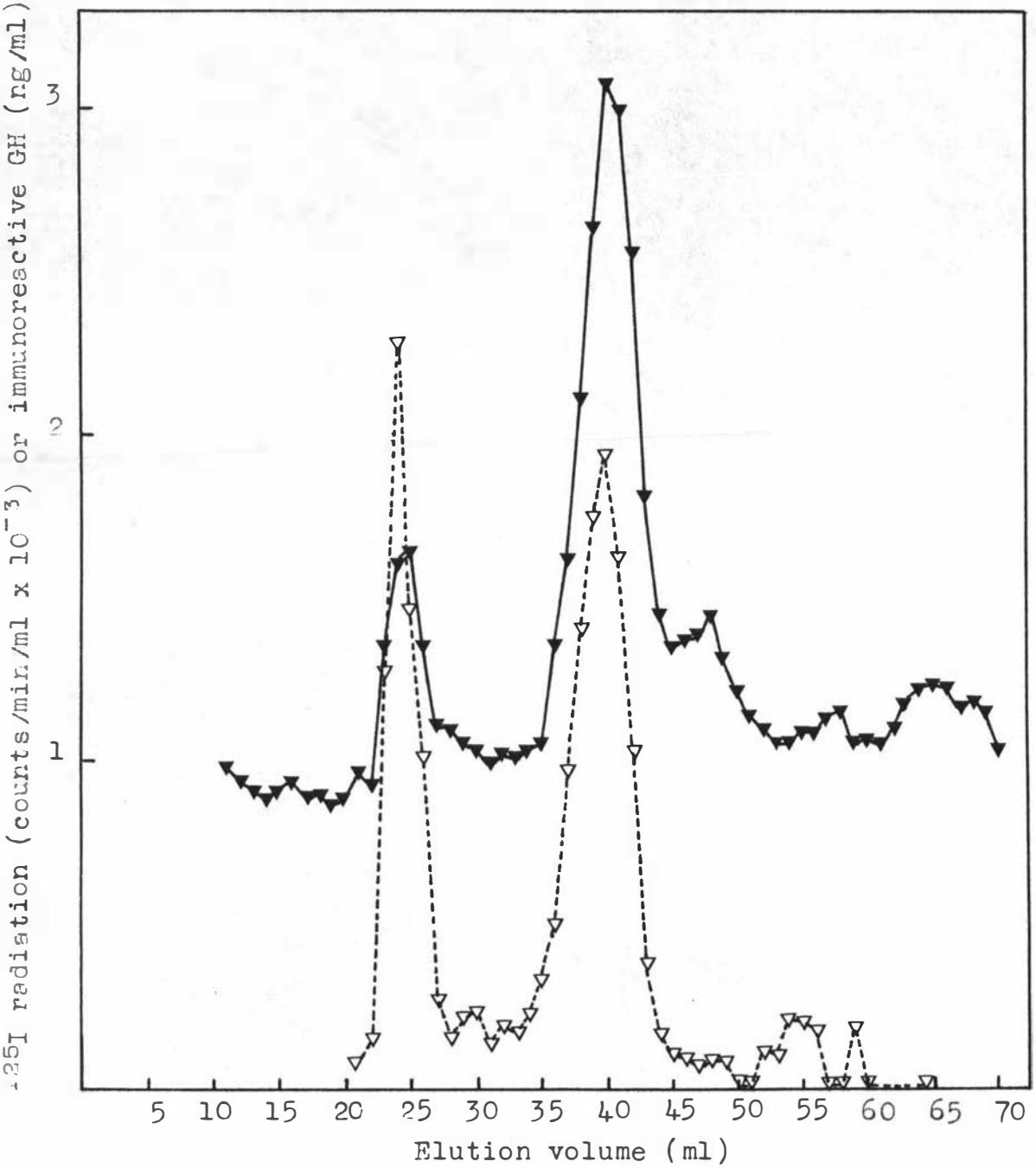

Figure 13 Gel filtration of ^{125}I-bovine GH (solid line) or of immunoreactive GH in whole calf plasma (broken line) on Sephadex G-100 equilibrated with 0.05 M phosphate buffer + 0.01 M EDTA at pH 7.4.

composition of all three was therefore investigated after fractionation of Sephadex G-100 columns (see page 52).

(1) A sample of bovine GH purified on a DEAE-cellulose column was lyophilised and stored at 4°C in a dessiccator. Ten months later, its elution pattern from a Sephadex G-100 column was investigated by measuring the absorbance at 270 nm of the eluant fractions. The same preparation of lyophilised, purified GH was used consistently for the standard curve in all GH assays.

(2) A large, pooled bovine plasma sample was passed through a 2.5 cm x 58 cm Sephadex G-100 column, with a small amount of labelled GH as a marker. Eluant fractions were counted in a Packard Auto γ Counter (model 5285) in order to localise the aggregate and monomer portions of the radioactive hormone (see page 52) and then tested as the unknown samples in a RIA, to detect the quantity of immuno-reactive GH from the plasma, in each fraction.

(3) The ^{125}I-labelled GH which had been fractionated on Sephadex G-100 as described on page 52, was assessed for the ability of all fractions to be bound by antibodies to bovine GH.

(ii) Results

(1) It can be seen from Figure 12, that almost all of the lyophilised GH was eluted as a single molecular species.

(2) Figure 13 shows that, as in Figure 1, the radio-active tracer GH was eluted as a double peak (solid line), the main peak representing GH in the molecular size range

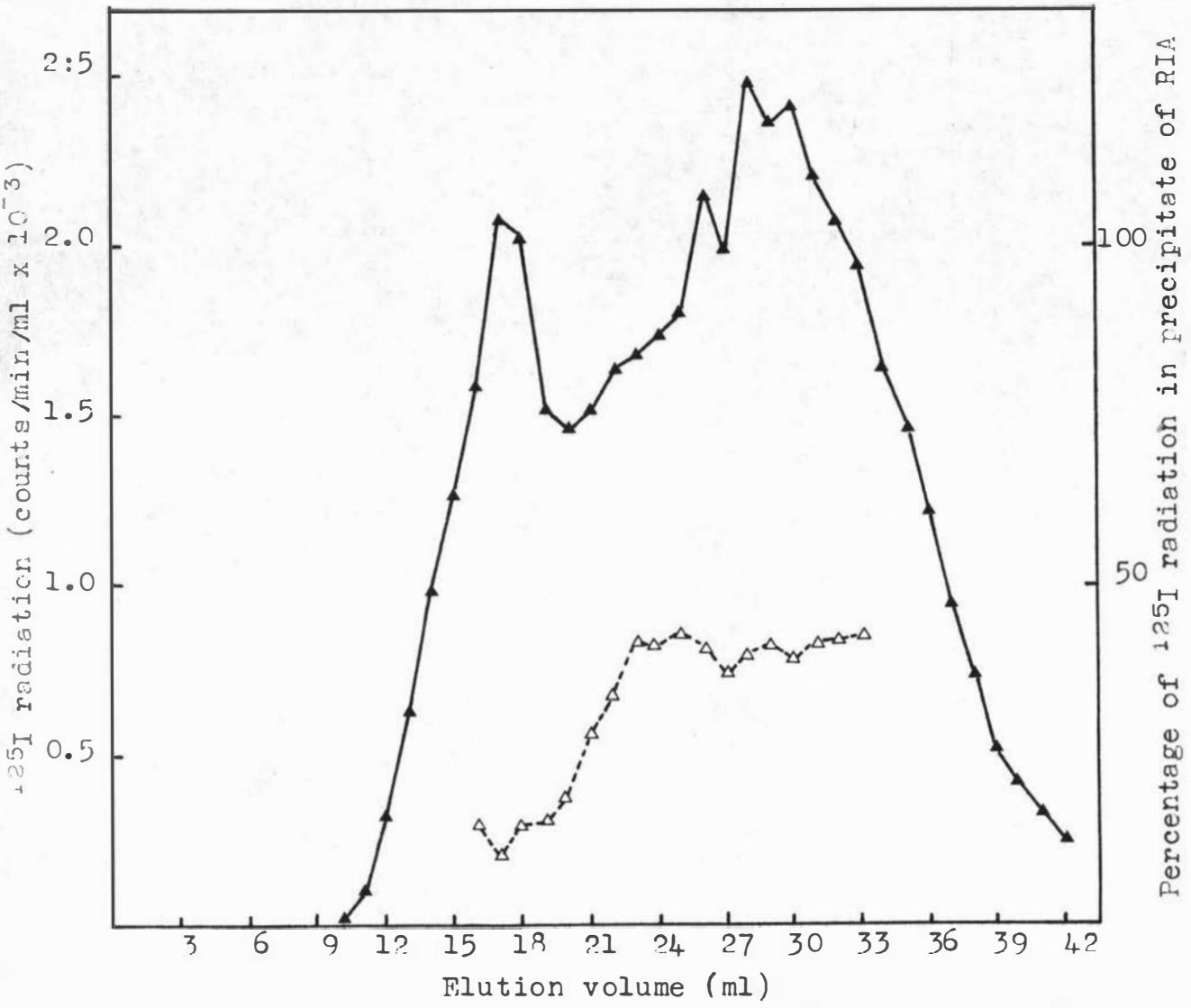

Figure 14 Gel filtration of ^{125}I-bovine GH on Sephadex G-100 equilibrated with 0.025 M borate buffer at pH 8.4. The solid line indicates the ^{125}I radiation in each fraction, and the broken line expresses the ability of each fraction to be bound in a RIA. The RIA was carried out with first antibodies at a final dilution of 1:64,000.

of 20,000, with a smaller peak of a larger molecular species. The dotted line, representing immunoreactivity of the naturally occurring plasma proteins, shows that molecules of both sizes occurred in calf plasma, and both were immunoreactive.

(3) Figure 14 again shows a double peak of labelled GH (solid line) but the dotted line indicates that immunoreactivity of labelled GH was confined mainly to the monomer GH with very little immunoreactivity in the larger molecular size.

(iii) Discussion

Because the standard GH used consisted of a single molecular species (Figure 12), no further purification was considered necessary. However, the fact that calf plasma contained two immunoreactive species of GH molecule (Figure 13) indicated that there was a difference between the hormone being measured in standard and unknown tubes. The difference warrants further investigation: for example by examining the behaviour of storing standard GH in solution in plasma.

When labelled GH was added to assay tubes, it was repeatedly observed that sensitivity and precision of the assay were decreased by the presence of labelled GH of any molecular size other than 20,000. The observation here that the other molecular sizes were not immunoreactive (Figure 14) drew attention to the need for ^{125}I-GH to be fractionated at regular intervals of once every 3 or 4 weeks. The relatively rapid deterioration of labelled hormone was

in close agreement with observations of other workers (Schwartz and Batt, 1973), and was probably caused by a number of factors, e.g.:

(A) The iodinated molecule could have a different secondary and tertiary structure from the natural GH, simply because of the presence of extra iodine atoms.

(B) The hormone is being constantly bombarded with γ radiation.

(C) The treatment with chloramine-T during the iodination procedure is known to have a disordering effect on protein molecules (Berson and Yalow, 1968).

It is interesting to speculate on the possible reasons why the large molecular size GH was not immunoreactive in the labelled hormone preparation, while the naturally occurring large GH in plasma was detectable by RIA. Since they were eluted in almost exactly the same fractions on Sephadex G-100 (Figure 13), it seems likely that the two large GH molecules were of the same molecular size. It may have been that in both cases, it was only 20% immunoreactive, but that there was such a large amount of it in calf plasma, that even at reduced immunoreactivity, it produced a peak which was equal in size to that of the monomer. A more likely explanation is that it represented a form of GH which was bound to a plasma protein (Stachura and Frohman, 1973). When the radioactive molecule was stored in diluent human plasma, the binding was not a biologically natural binding and was irreversible, while native hormone in calf plasma was bound in a form which was reversible, and enabled the hormone to become released so that it reacted with the antibodies in the RIA.

2.4.1 (f) Diluent plasma and anticoagulants

(i) Method

Because substances in the plasma, other than the hormone being assayed, may have an effect on the amount of binding of the labelled hormone, it was necessary to include the same volume of plasma in the tubes containing standards, so that the interfering effect on binding was of the same magnitude for both standards and unknowns (see page 54). In addition to the method described on page 54 for removal of insulin from the diluent plasma, other techniques were tested, for example molecular sieving through a Diaflow untrafiltration membrane PM 10 (Amicon Corporation, USA), or high pH treatment (according to Grodsky and Forsham, 1960).

An alternative technique which was investigated was the use of protein dissolved in buffered saline, at approximately the same concentration as the protein in plasma. For example, bovine serum albumin (Sigma Chemical Co., Lot No. 24C-1740) was tested at varying concentrations in order to ascertain whether it interfered in the insulin assay.

For unknown samples, plasma was used rather than serum, because plasma samples could be centrifuged and frozen rapidly, without having to wait at room temperature for a clot to form. The use of plasma necessitated the presence of an anticoagulant in the samples. EDTA was used as anticoagulant in the majority of experiments, and its presence, as well as the presence of heparin, were tested for their possible influence on assay results and parallelism.

(ii) Results

Neither molecular sieving nor high pH treatment yielded insulin-free plasma which was as suitable for use as a diluent in insulin assays, as was charcoal-treated plasma. Plasma treated with either of the first two methods significantly inhibited the binding of labelled hormone to antiserum, and because the amount of diluent plasma varied between standard tubes and between the different plasma levels in unknown tubes, its binding inhibitory effect caused a serious deviation from parallelism. Bovine serum albumin was not satisfactory as a diluent, because at low concentrations, it actually had an enhancing effect on binding of labelled insulin. The enhancing effect increased with increasing concentrations of bovine serum albumin, up to about 3 mg/ml, and increasing the concentration further brought about an inhibitory effect on binding because of the non-specific effect of increasing protein concentration.

The presence or absence of heparin, even up to amounts as high as 50 IU per assay tube, had no effect on the GH assay. EDTA was found to be essential for assays to proceed. In its absence, there was no binding at all of labelled hormone, and its presence at concentrations ranging from 0.01 M to 0.05 M enabled the assays to proceed, with identical results.

(iii) Discussion

A preparation of charcoal treated bovine plasma was produced, which neither enhanced nor inhibited binding, and had the same precipitation profile in relation to GH

concentrations, as did all the calf plasma samples (Figure 7).

EDTA was used as anticoagulant in the majority of experiments, because it was required to be present in assays, so it was convenient to have it present in plasma at the same concentration as it was used in assay buffer solutions (0.01 M). The reason for its importance in assays was thought to be that the C'1 component of complement in plasma becomes dissociated as a result of chelating the divalent cations with EDTA. Complement becomes involved in all antigen/antibody reactions if it is present, and it appeared that it had an inhibitory effect if it was intact in the RIA system (Aubert, 1970). More detailed investigation of the nature of the role of complement in RIA is warranted, especially in relation to the 'prozone effect' and other cases of unexplained inhibition described on pages 89 - 94.

2.4.2 Precision, sensitivity and specificity

(a) Precision

(i) Method

1. Individual assays

Midgley et al (1969) have defined precision as 'the extent to which a given set of measurements of the same sample agrees with the mean'. The values used as measures of precision for any one assay, were calculated routinely as part of the computer programme described on page 57 - 58 (Burger et al, 1972), according to the formula:

$$\text{Precision} = \frac{SD}{\bar{X}}$$

where SD is the standard deviation of the calculated value of X in pg/ml. This value is high at very low and very high concentrations of X, and the region of maximum assay precision (where the value is lowest) in the standard curve of each assay was also assessed.

2. Combined assays

Ten assays each of insulin and GH were selected at random, and the results of the four (for insulin) or three (for GH) standard plasma samples were analysed for variance components. For prolactin, many routine assays had been carried out before it was realised that in assays where a large amount of ^{125}I-prolactin (about 20,000 cpm or more per tube) was added, the hormone in the plasma samples was giving elevated prolactin values compared with those assays containing about 5,000 - 10,000 cpm per tube. Because time did not allow a repetition of the assays, the existing assays were divided into two groups, those with 20,000 cpm per tube and those with 5,000 - 10,000 cpm. Ten assays were selected at random from each of the two groups, and results of the two standard plasma samples were analysed for variance components within each group of ten. The variance components within each group were added to those of the other groups, in order to compare the values obtained with those of ten assays of insulin or of GH.

The variance component contributed by 'between replicate tubes' was used to obtain 95% fiducial limits for the assessment of hormone levels in each standard plasma sample. The fiducial limits, based on the degree of repeatability of two tubes treated in identical manner in

assays, gave a good indication of the precision of each group of assays.

(ii) Results

1. Individual assays

Table VIII shows the estimates of precision for given values of X, for two individual assays of each hormone.

Table VIII Precision (standard deviation of hormone estimates ÷ mean hormone estimate (X)), at given hormone levels, region of maximum precision, and sensitivity, of two assays for each hormone.

Insulin			Growth hormone			Prolactin		
X (pg/ml)	Assay 1	Assay 2	X (ng/ml)	Assay 1	Assay 2	X (ng/ml)	Assay 1	Assay 2
100	0.199	0.277	1	0.097	0.098	1	1.454	0.194
500	0.048	0.079	5	0.033	0.045	5	0.218	0.052
1,000	0.033	0.056	10	0.034	0.051	10	0.074	0.043
2,000	0.030	0.050	15	0.041	0.058	15	0.043	0.059
P	1,700	1,767		6.83	5.52		16.00	8.00
S	100	40		0.37	0.25		7.16	0.997

P = zone of maximum precision, in pg/ml for insulin and in ng/ml for GH and prolactin.

S = sensitivity (lowest detectable hormone level) in pg/ml for insulin and in ng/ml for GH and prolactin.

2. Combined assays

Tables IX -XII show the variance components from ten assays each of GH and insulin, and the fiducial limits of the estimates of the standard plasma samples which were measured in all assays. Table XIII gives the total within group variance components, after those within each group of

Table IX Variance components from insulin levels of four plasma samples assayed repeatedly in ten insulin assays. + = $p<0.05$, ++ = $p<0.01$

Source of variance		Variance component	Degrees of freedom	% of total
Between assays (a)		0.0054115	9	3.934
between samples (s)	++	0.1223109	3	88.923
s x a interaction	++	0.004674	27	3.393
between dilutions (d)		0.000624	1	0.454
d x a interaction		0.0005555	9	0.404
d x s interaction	+	0.00076	3	0.552
a x s x d	+	0.001022	27	0.743
between replicates		0.0021887	80	1.591

Table X Estimates of insulin levels in four standard plasma samples measured in ten assays

Sample no.	Mean estimate (pg/ml)	95% fiducial limits
2	389	349 - 433
3	2,576	2,314 - 2,867
4	985	895 - 1,096
5	576	517 - 641

Table XI Variance components from GH levels of three plasma samples assayed repeatedly in ten GH assays. + = $p<0.05$, ++ = $p<0.01$

Source of variance		Variance component	Degrees of freedom	% of total
Between assays (a)		0.0538127	9	2.581
between samples (s)	++	2.025002	2	97.137
s x a interaction	++	0.0025389	18	0.122
between dilutions (d)		0.00	0	0.0
d x a interaction		0.0005464	9	0.026
d x s interaction	+	0.0003182	2	0.015
a x s x d	+	0.0008213	18	0.039
between replicates		0.0016555	60	0.079

Table XII Estimates of GH levels in three standard plasma samples measured in ten assays

Sample no.	Mean estimate (ng/ml)	95% fiducial limits
1	4.98	4.53 - 5.47
2	8.85	8.06 - 9.72
3	3.25	2.96 - 3.57

Table XIII Variance components from prolactin levels of two plasma samples assayed in two groups of ten prolactin assays. ++ = $p<0.01$

Source of variance		Variance component	Degrees of freedom	% of total
Between assays[1](a)		0.0006812	18	9.85
between samples (s)	++	0.0046563	2	67.33
s x a interaction		0.00011217	18	1.62
between dilutions (d)	++	0.0000875	2	1.26
d x a interaction		0.0002275	18	3.29
d x s interaction	++	0.0007649	2	11.06
a x s x d	++	0.0002251	18	3.25
between replicates		0.0001605	20	2.32

[1] **Within groups of assays.**

Table XIV Estimates of prolactin levels in two standard plasma samples measured in two groups of ten assays.
(a) Assays with 20,000 cpm ^{125}I-prolactin per tube
(b) Assays with 5,000 cpm ^{125}I-prolactin per tube.

Sample no.	Mean estimate (ng/ml)	95% fiducial limits
1(a)	12.29	11.72 - 12.90
2(a)	8.78	8.37 - 9.21
1(b)	7.00	6.77 - 7.24
2(b)	5.06	4.89 - 5.24

ten prolactin assays had been added to those of the other. The estimates given in Table XIV indicate the extent of the difference in estimates between the two groups.

(iii) Discussion

1. Individual assays

The precision of an individual assay was affected adversely by the use of preparations of labelled hormone which were not recently purified, as described on page 87. In situations where the labelled hormone was not ideal, the statistical nature of the calculation allowed the situation to be overcome if larger numbers of replicate tubes were used.

2. Combined assays

It can be seen from Tables IX and XI that the difference between standard plasma samples of insulin and GH was highly significant, as was to be expected because the samples covered a wide range of levels. The differences between assays were not significant, and the very small amount of variance caused by differences between the two dilutions of each sample, indicated that in these assays the parallelism problem had been overcome.

For prolactin assays, the lack of significant differences between assays within each group of assays (Table XIII) indicates that estimates of prolactin were reliable provided that the amount of ^{125}I-prolactin was kept relatively constant. There was a further problem, however, in that the differences between dilutions indicated a deviation from parallelism. Inspection of plots of

standard curves showed that the curve fitted by the computer programme (see pages 57 - 58) was probably responsible for the apparent lack of parallelism. Because the deviations were very small and unimportant, the data and programme were used to make estimates of prolactin levels. If they had been larger, one of the methods described on pages 62 - 64 would have been used to transform the data before feeding it into the computer.

2.4.2(b) Sensitivity

(i) Method

Sensitivity is defined as 'the smallest amount of unlabelled hormone which can be distinguished from no hormone' (Midgley et al, 1969). Again, the computer programme which was used to assess the hormone level of each plasma sample, also printed out the values for variance between replicates of each point on the standard curve. From the variance, the standard error of the difference between tubes containing no unlabelled hormone, and tubes containing the lowest amount of unlabelled hormone, was calculated, multiplied by t, to give the smallest difference which could be detected at 95% confidence limits in counts per minute (values of t were given in Snedecor and Cochran, 1967) and the difference was subtracted from the mean counts per minute of the tubes with no unlabelled hormone. The resulting value was then converted to pg/ml using the sensitivity curve of the computer programme, and the result was the smallest amount of detectable hormone.

(ii) Results

The estimates of sensitivity for two assays of each hormone are given in Table VIII on page 95.

(iii) Discussion

The sensitivity of an assay was able to be changed at will by altering the amount of first antibody added to the tubes (see page 73). For example, prolactin Assay 1 (Table VIII) was relatively insensitive, the lowest detectable level of hormone being 7.16 ng/ml. The reason for the insensitivity at low levels was that the assay was used for measuring prolactin in samples from lactating cows, and very high prolactin levels were anticipated. For routine calf work, a higher dilution of first antibodies was used, and the sensitivity was usually well below 1 ng/ml and comparable to the values obtained for GH. Insulin assays were similarly adjusted, so that levels of less than 100 pg/ml were detectable. On no occasions were assays used at the limits of their sensitivity, because repetition of assays with a higher dilution of first antibodies was always available as a technique for decreasing the lower limit of sensitivity.

2.4.2(c) Specificity

(i) Method

The specificity of the insulin assay was investigated by Montgomery (1976) using the same first and second antibody preparations as in the present investigation. Porcine glucagon (Eli Lilly, Lot No. TLF 599A) and

chemically denatured porcine insulin were tested for their ability to inhibit binding of ^{125}I-insulin by first antibodies.

The specificity of the GH and prolactin assays was assessed by testing the ability of bovine prolactin standards to inhibit binding of ^{125}I-GH, and also by testing the ability of the first antibody to GH to bind ^{125}I-prolactin.

(ii) Results and discussion

Neither porcine glucagon nor chemically denatured porcine insulin, showed any inhibition of binding of ^{125}I-insulin (Montgomery, 1976). No cross-reaction between GH and prolactin was observed. Thus it seemed likely that the antibodies raised during the course of the present study were highly specific to the hormone against which they had been raised.

2.5 STATISTICAL METHODS

For comparing unstimulated hormone levels, raw data were plotted for individual calves on each day, without any mathematical analysis or grouping. Where grouping was required, a non-parametric method was used, in order to avoid giving undue weight to samples which had been taken during an episode of peak hormone secretion (see pages 18 - 19 for discussion of the literature on episodic release of GH). Thus comparison was made between groups of calves in Experiments 3 and 4, by analysing the median and range of samples taken on the control day or on saline treatment days,

for each individual calf. These values for each group of calves were compared with the other two groups combined, by means of the Mann-Whitney U-test (Sokal and Rohlf, 1969).

Before further analyses were carried out, data were transformed according to Table XV, in order to make the variance between calves for each sampling time as constant as possible over the entire range of levels.

Table XV Techniques used to transform data in order to standardise the variance at all hormone levels

Measurement	Expression of untransformed data	Transformation
Glucose	mg/100 ml	None
Insulin	pg/ml or ng/ml	$\log_{10}$ pg/ml
Growth hormone	pg/ml or ng/ml	$\log_{10}$ pg/ml
Prolactin	ng/ml	square root ng/ml

Analyses of covariance were carried out on the transformed data using the pre-treatment mean (mean of samples 1 and 2) as the independent variable, and one or two sampling periods post-treatment as the dependent variable (Snedecor and Cochran, 1967). The post-treatment sampling periods chosen as the dependent variable were selected for their ability to characterise a particular pattern of response i.e. the samples with the highest levels were chosen in defining a hormonal peak, or with the lowest in defining a nadir. Although such a technique contained an element of subjectivity, within any one analysis of covariance calculation, the post-treatment samples selected were the same for all calves.

CHAPTER THREE: EXPERIMENTAL SECTION

3.1 EXPERIMENT 1

3.1.1 Experimental Design

The aim of the first experiment was to gain experience in handling calves, to test different techniques of catheterisation, (see page 43) and to provide some blood samples for use while radio-immunoassays were being developed. Therefore, calves were catheterised as they became available over a two-month period, and no attempt was made to follow a design or to control the experiment.

Blood samples were taken with two questions in mind concerning the hormone results:

(a) What are the basal levels of insulin, GH and prolactin in plasma of calves? What are their ranges and diurnal variation in levels?

(b) Do the plasma levels of GH and prolactin undergo changes during suckling, which are in any way similar to or complementary to those which take place in the maternal plasma (reviewed on pages 17 and 29)?

Blood sampling was carried out at intervals of 20 minutes or 30 minutes from 8.30 hours until 17.00 or 20.00 hours. During suckling, samples were taken at the rate of one per minute for the 9-minute duration of suckling. Blood sampling was carried out as described on page 47, except that no plasma glucose assays were carried out.

Table XVI Calves and blood sampling in Experiment 1

Calf	Sex	Birth date	Weight (Kg)	Suckling samples Date	Suckling samples Time	Daily range samples From	Daily range samples To
7	F	22 Mar	17.7	27 Mar	15.45		
				28 Mar	9.20		
9	F	22 Mar	21.3	27 Mar	16.00		
				28 Mar	9.00		
				28 Mar	16.00		
X	M	28 Mar		2 Apr	16.13		
				3 Apr	9.02	9.00	19.00
				4 Apr	9.02	9.00	17.00
				5 Apr	8.54		
8a	F	28 Mar		2 Apr	16.30		
				3 Apr	9.14	9.00	19.00
				4 Apr	9.12	9.00	17.00
				5 Apr	9.05		
A	F	8 Apr	34.0	17 Apr	8.47	8.40	20.00
				19 Apr	9.02		
B	M	8 Apr	28.1	17 Apr	8.58	8.40	20.00
C	M	8 May	24.0	15 May		19.20	21.20
				16 May	9.15	9.00	16.35
				17 May	9.08	9.00	10.40
				18 May		9.00	13.30
D	M	9 May	28.1	15 May		19.20	21.20
				16 May	9.22	9.00	16.35
				17 May	9.08	9.00	10.40
				18 May		9.00	13.30
8b	F	18 May	33.1	21 May		18.40	20.20
				22 May	10.35	9.10	12.30
				23 May	9.26	9.00	11.30
				24 May		9.00	17.30
				25 May	10.40	9.25	11.15
				28 May		9.35	12.45

Table XVI (continued) Calves and blood sampling in Experiment 1.

Calf	Sex	Birth date	Weight (Kg)	Suckling samples Date	Suckling samples Time	Daily range samples From	Daily range samples To
11	F	20 May	34.9	21 May		16.40 -	20.20
				22 May	9.13	9.10 -	12.30
				23 May	9.06	9.00 -	11.30
				24 May		9.00 -	1 .30
				25 May	10.50	9.25 -	11.15
				28 May		9.35 -	12.45

For the purpose of examination of results, either raw data were plotted for each individual calf, or combined results with transformed data (see page 103) from just one sampling session for each individual calf were used. The combined results were therefore not influenced by the varying number of sampling days for each calf. Where there was a choice of days, the one chosen was the first day in which sampling was complete.

3.1.2 Results

(a) Diurnal pattern of hormone secretion

Raw data from individual calves are shown in Figures 15 - 17. Plasma insulin basal levels were uniformly < 1 ng/ml, with definite peaks of secretion to 2 - 6 ng/ml following immediately after suckling. The post-suckling peaks were very often bi-modal, with an initial peak within the first hour, and a second peak at 2 - 3 hr after suckling. One striking feature of the results is that although the post-suckling peak always occurred to some extent, it was

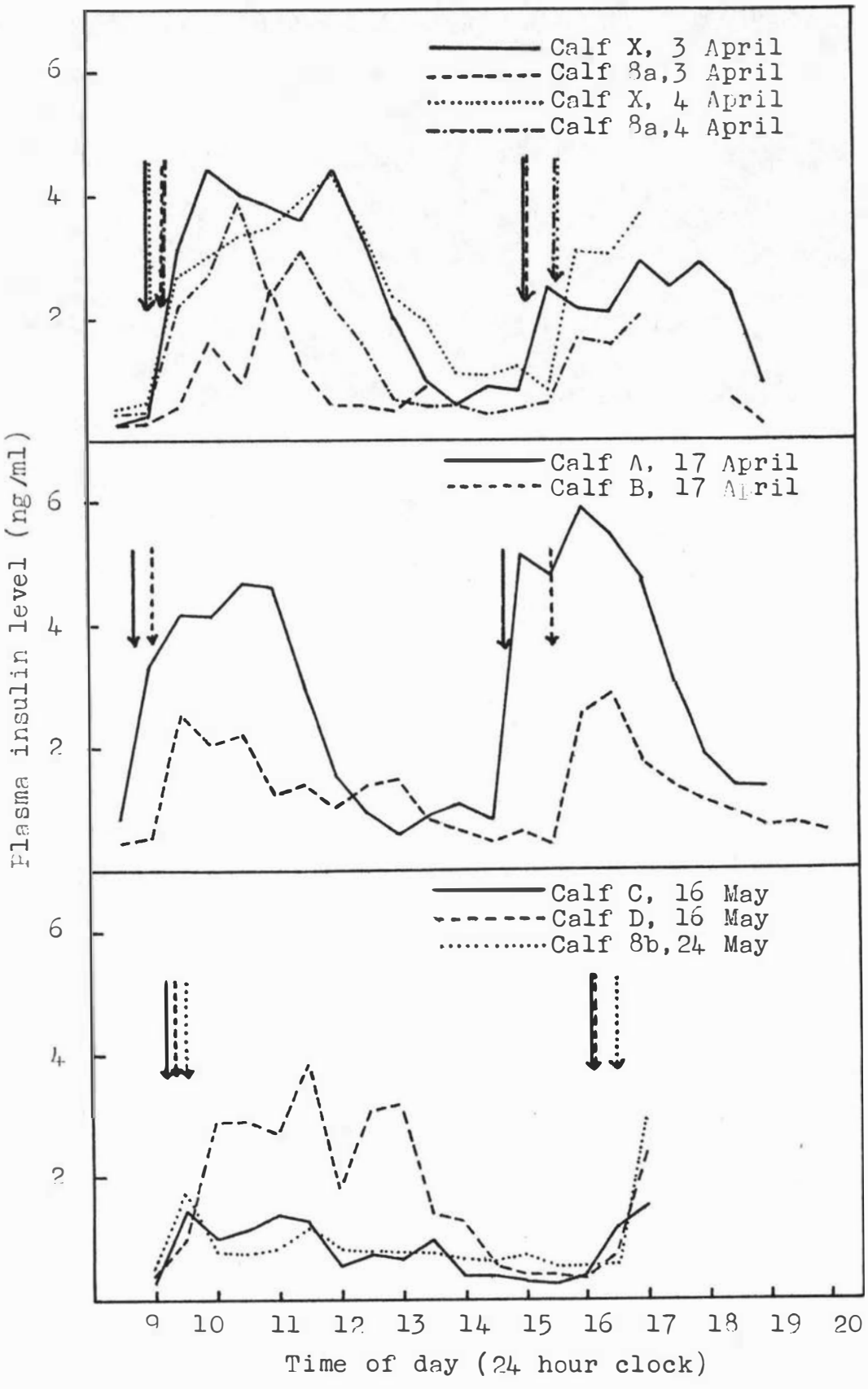

Figure 15 Plasma insulin level in individual calves sampled at 30-minute intervals. Suckling from a cow occurred between 8.45 and 9.30 hours, and between 15.00 and 16.30, and is indicated by vertical arrows.

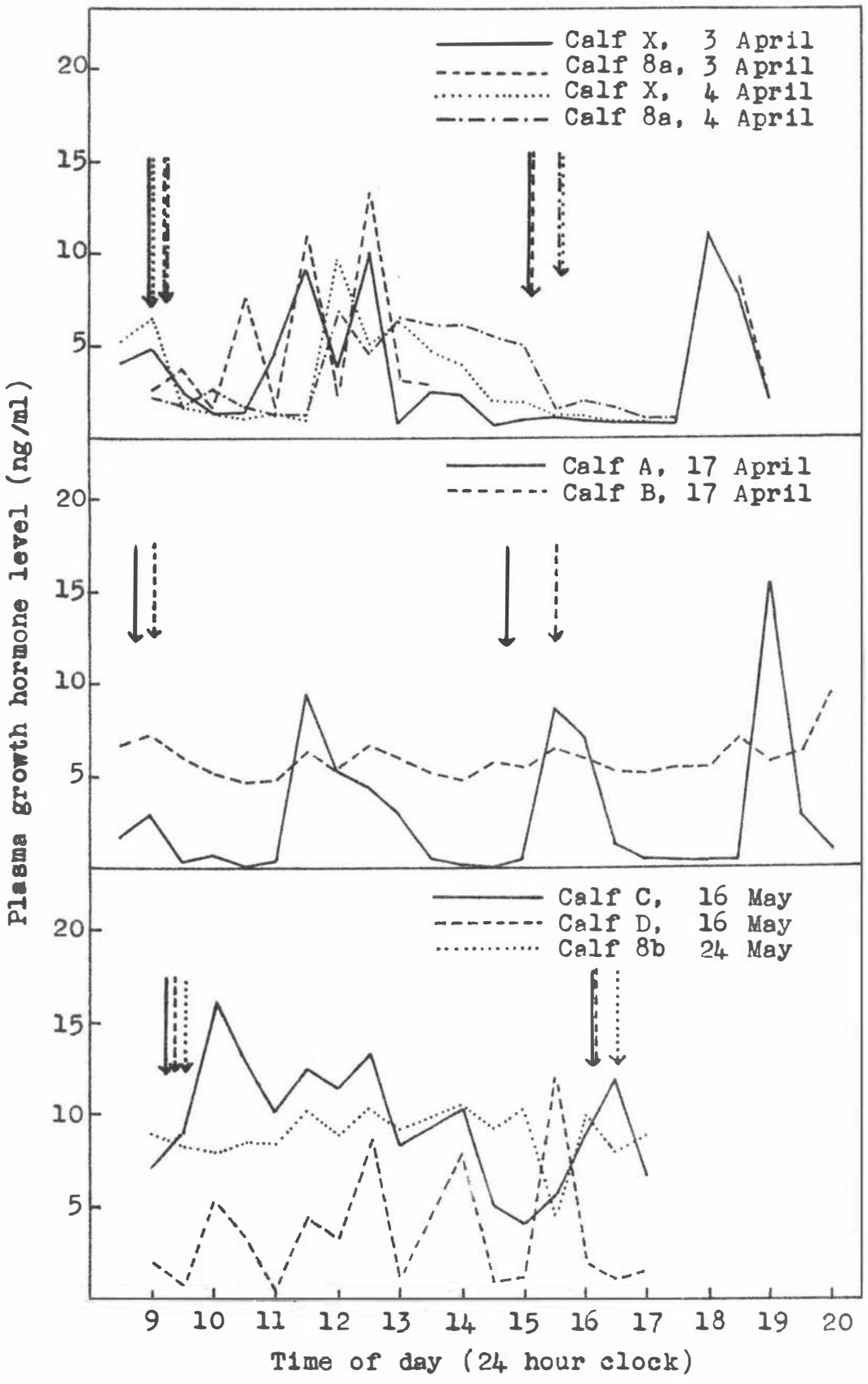

Figure 16 Plasma growth hormone level in individual calves sampled at 30-minute intervals. Suckling from a cow occurred between 8.45 and 9.30 hours, and between 15.00 and 16.30, and is indicated by vertical arrows.

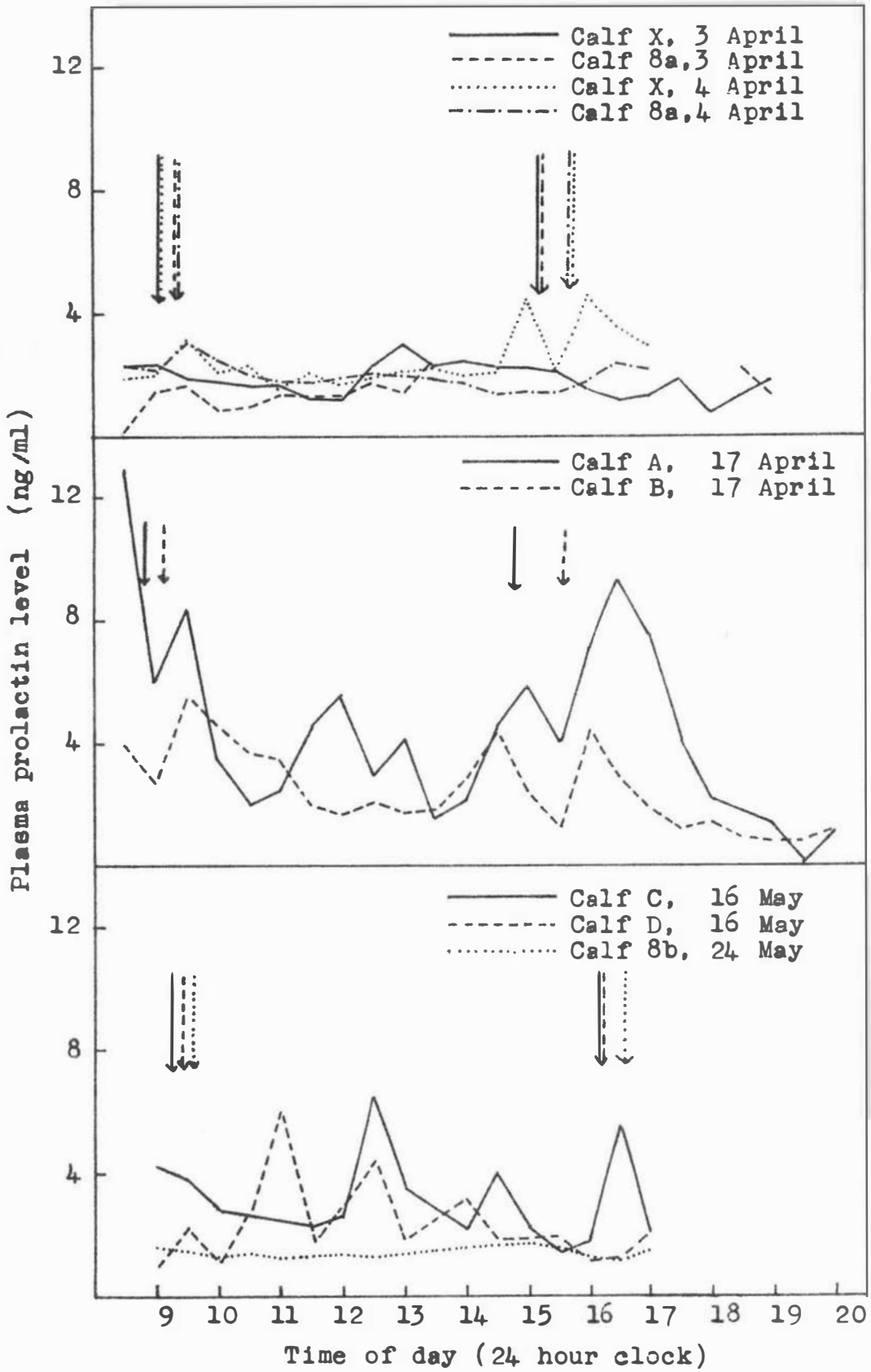

Figure 17 Plasma prolactin level in individual calves sampled at 30-minute intervals. Suckling from a cow occurred between 8.45 and 9.30 hours, and between 15.00 and 16.30, and is indicated by vertical arrows.

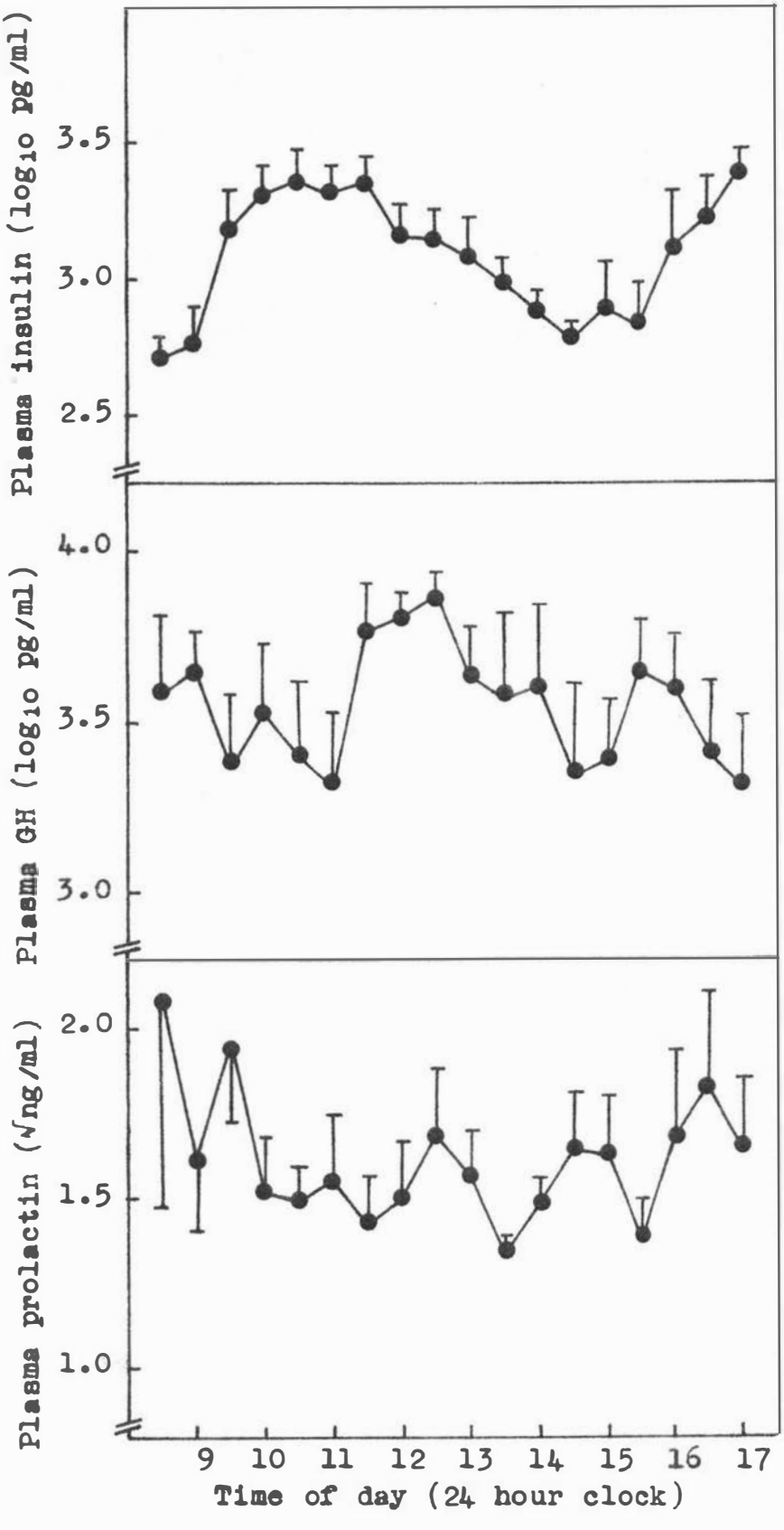

Figure 18 Mean values of plasma insulin, GH and prolactin levels of seven calves sampled at 30-minute intervals. Results are expressed as $\log_{10}$ pg/ml for insulin and GH, and $\sqrt{}$ng/ml for prolactin. Vertical bars represent standard error of the mean.

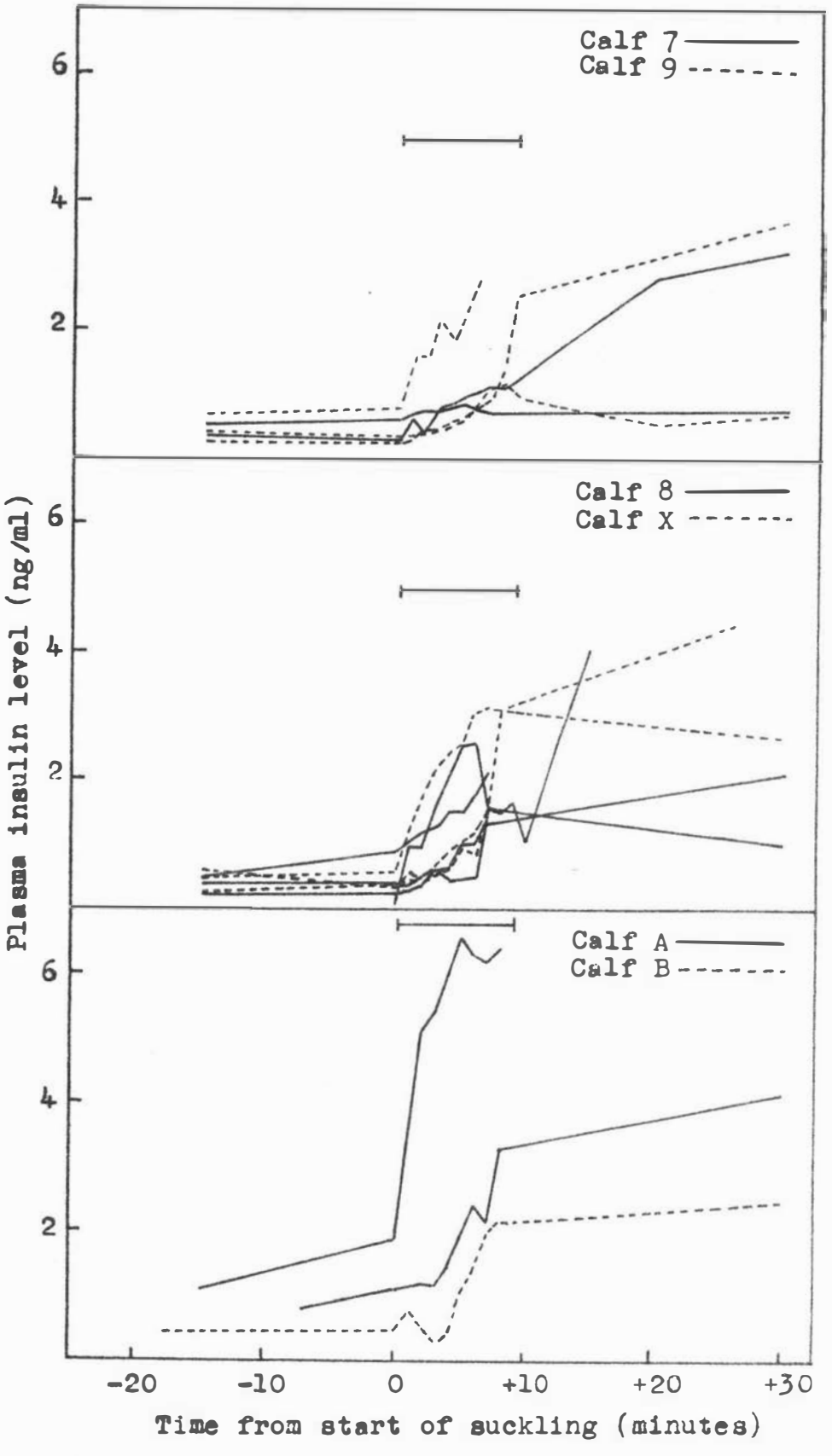

Figure 19 Plasma insulin levels of individual calves sampled before, during and after suckling from a cow. The horizontal bars represent the duration of suckling.

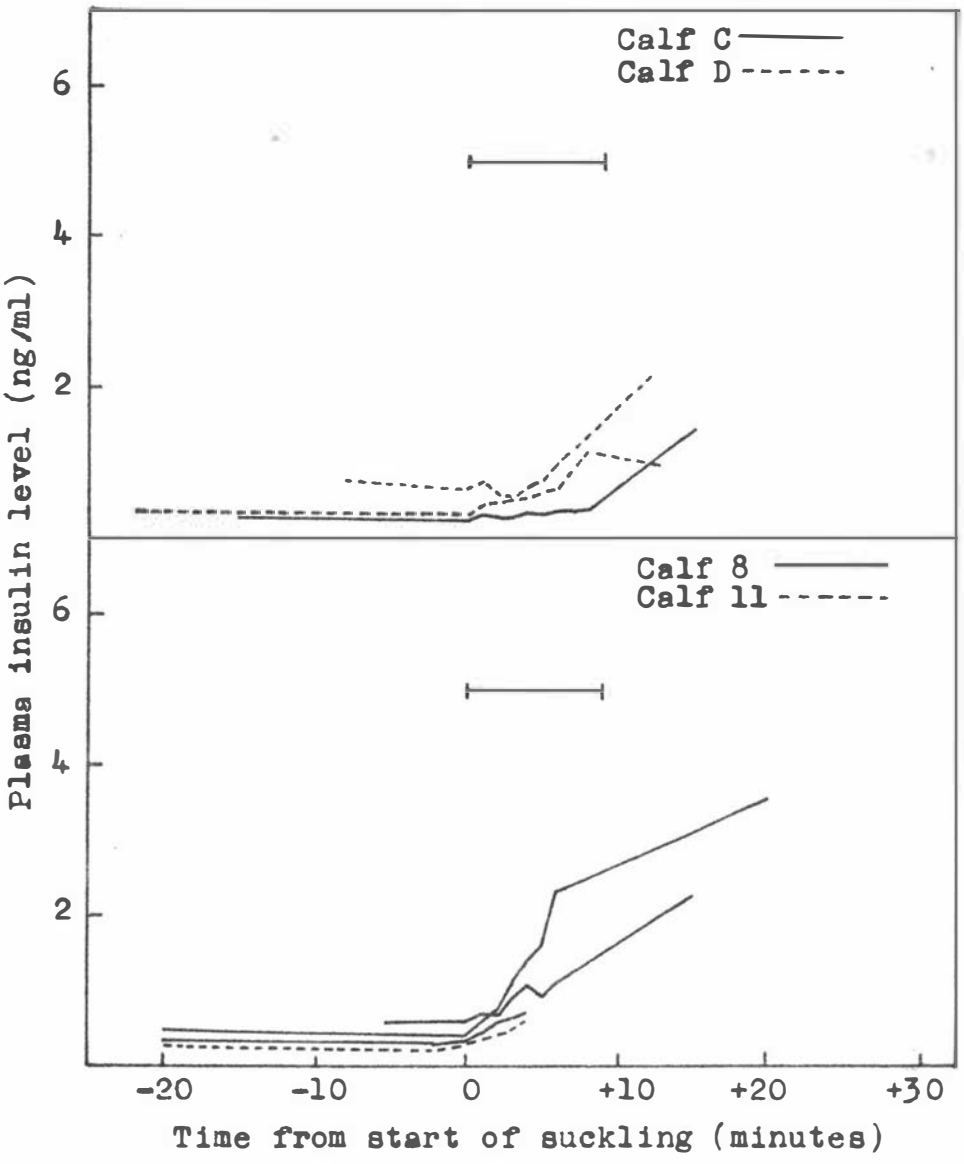

Figure 19 (continued)

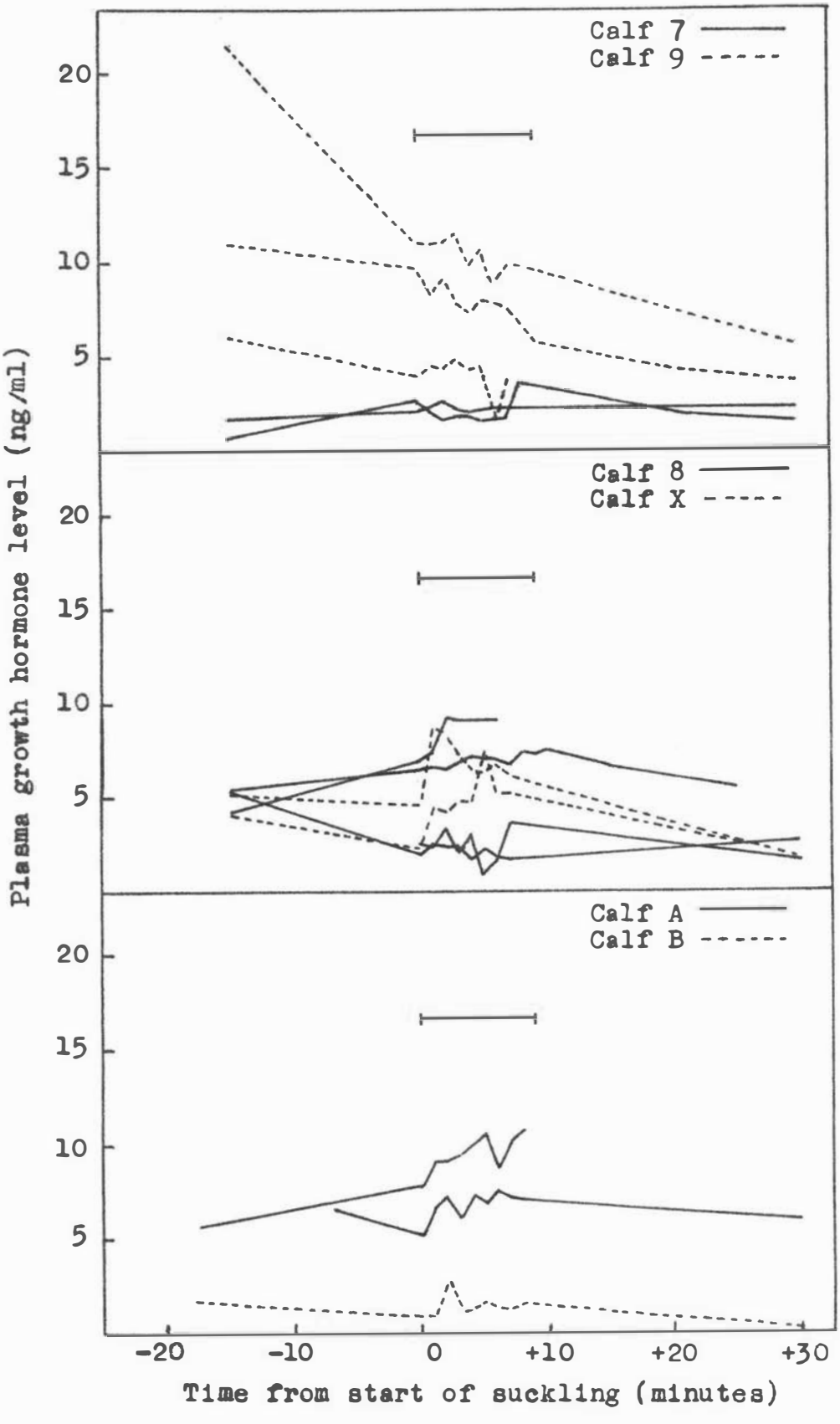

Figure 20 Plasma GH levels of individual calves sampled before, during and after suckling from a cow. The horizontal bars represent the duration of suckling.

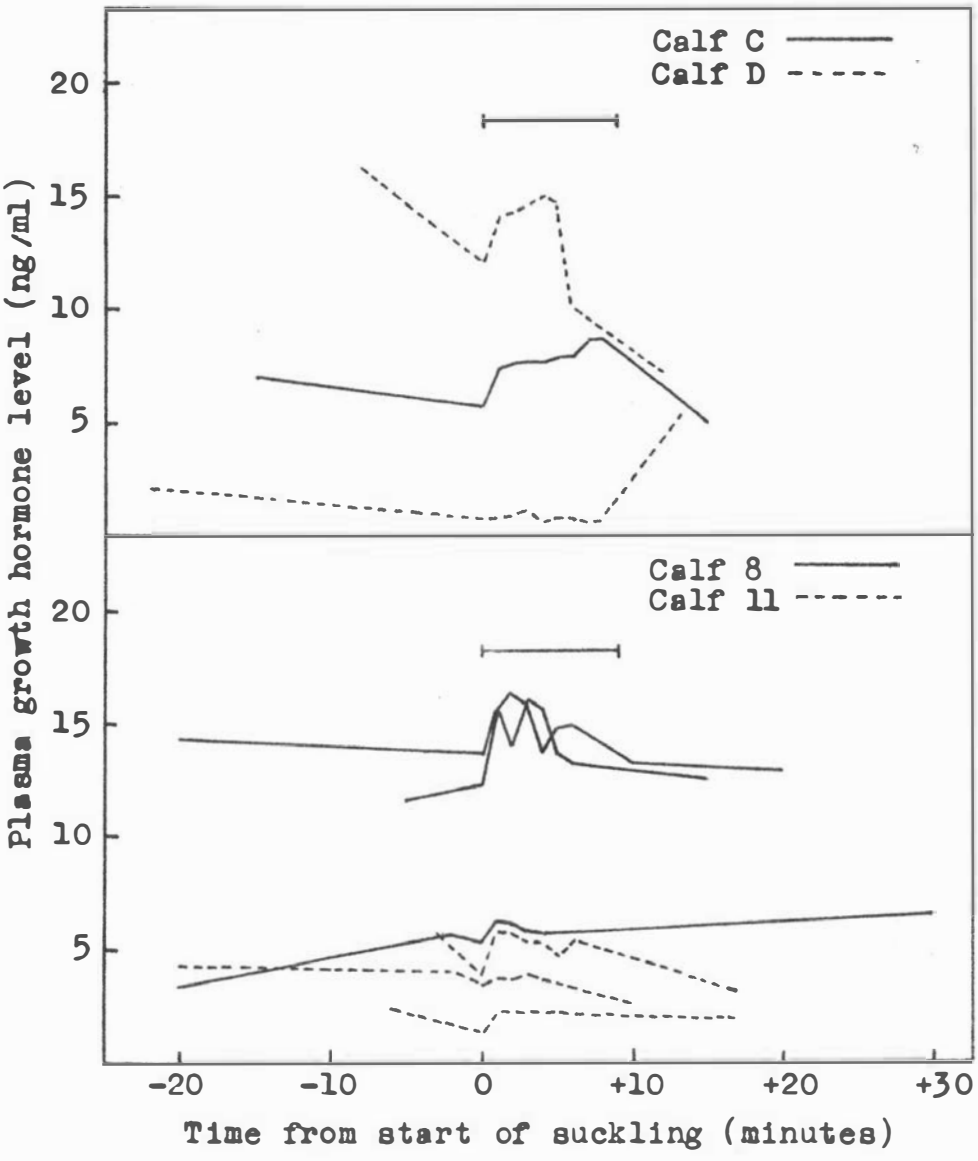

Figure 20 (continued)

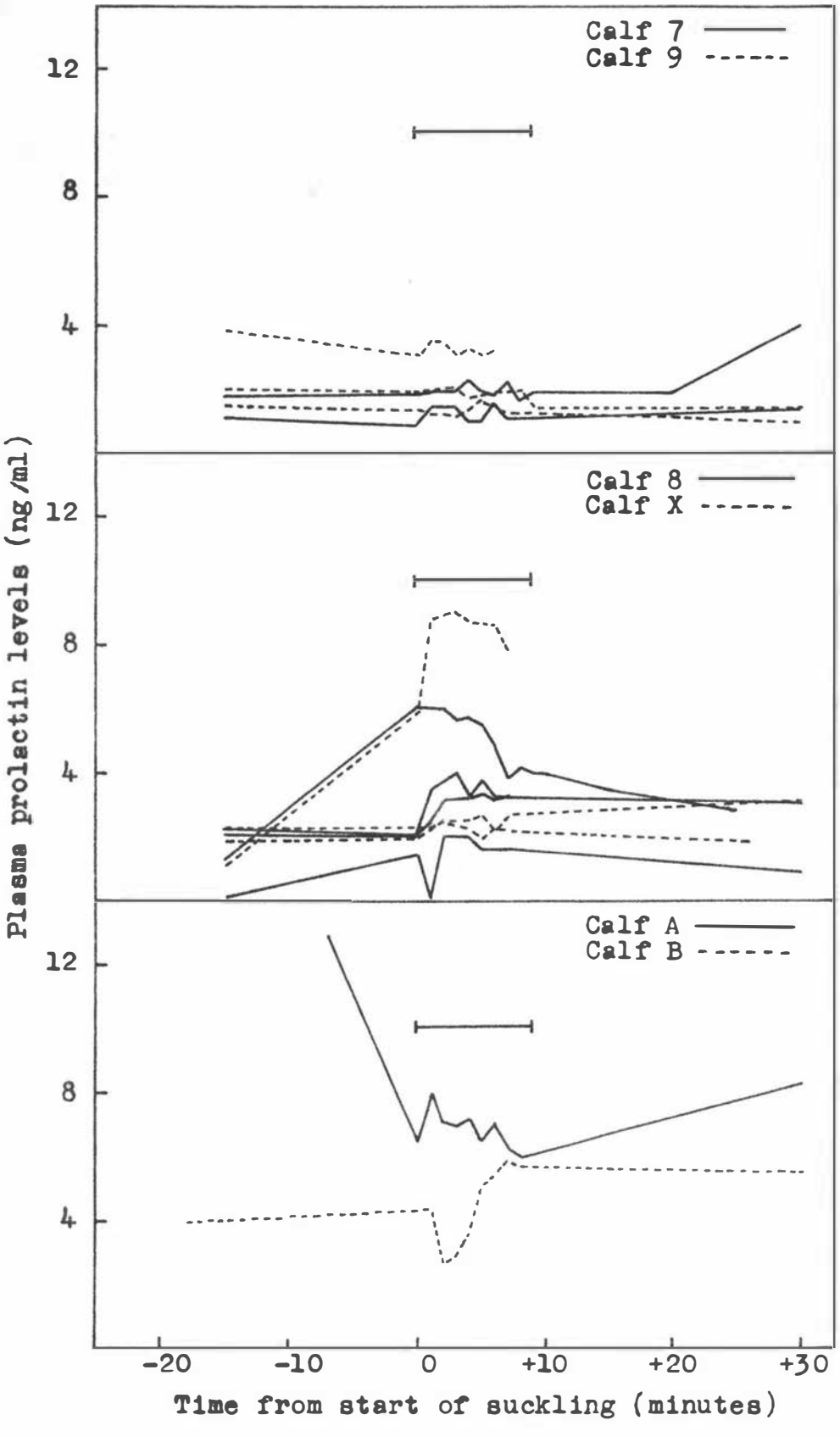

Figure 21 Plasma prolactin levels of individual calves sampled before, during and after suckling from a cow. The horizontal bars represent the duration of suckling.

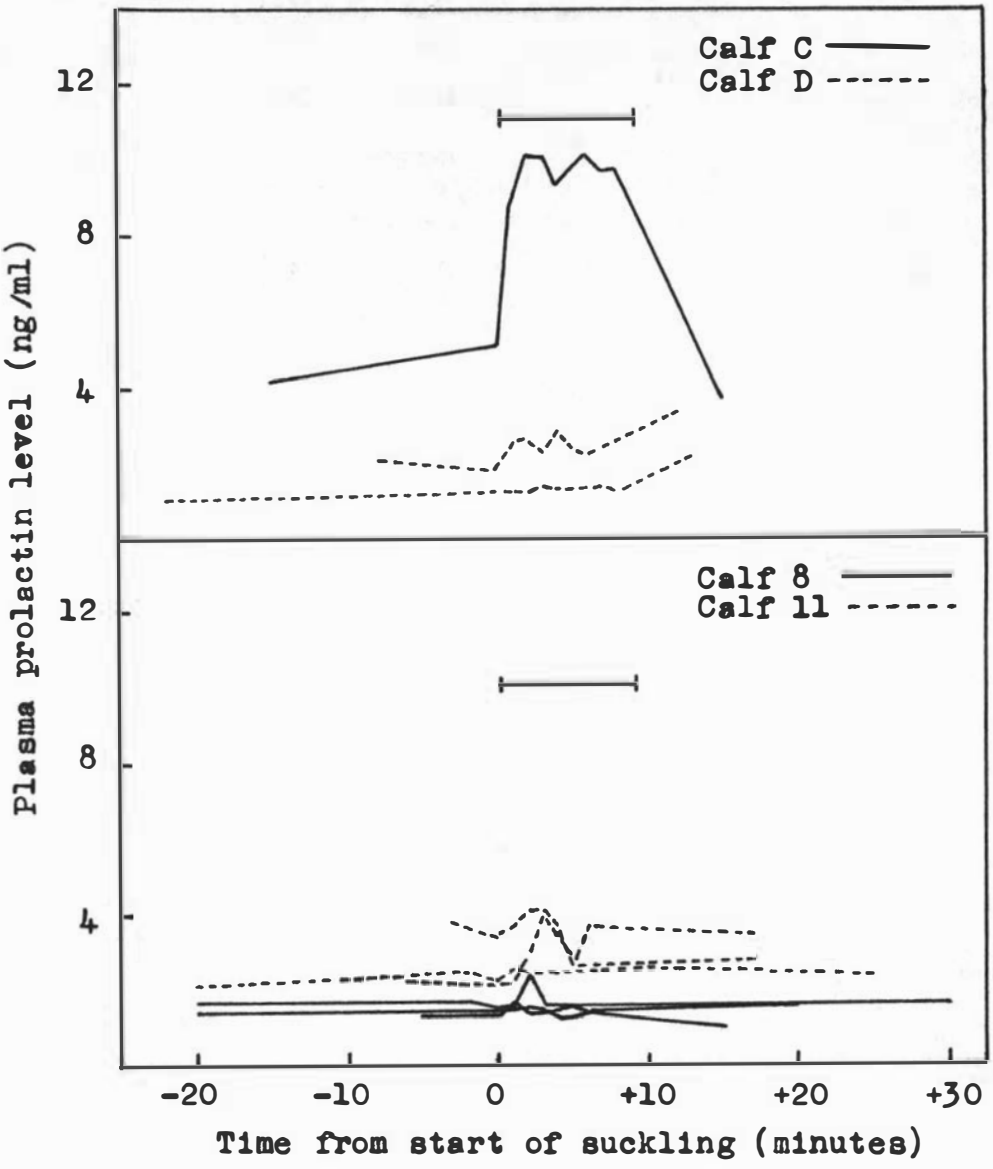

Figure 21 (continued)

variable in its size. Calf B, C and 8b showed small responses, and can be termed 'low responders'. Averaged transformed data (Figure 18) shows how effective the transformation was, for minimising the high degree of scatter at high plasma insulin levels.

The GH results showed a basal level of < 3 ng/ml for all calves except B, C and 8b. Peak concentrations of approximately 10 ng/ml were measured frequently, but were short-lived and their frequency varied between calves. Figure 18, showing bulked transformed data, exphasizes that despite the variability, GH levels were lower for two hours following the morning feed, and for a short period at approximately the time of the afternoon feeding, and there were peaks in GH levels of most calves at about mid-day.

All calves showed a basal prolactin of 1 - 3 ng/ml. As with GH data, peaks of secretion were spontaneous and variable. Averaging transformed data (Figure 18) did not cause a pattern in prolactin peaks to become apparent.

(b) Suckling and plasma hormone levels

Raw data of samples taken immediately before, during and after suckling are shown in Figures 19 - 21. The uniformity of plasma insulin basal levels was evident again, as was the effectiveness of feeding as a stimulus for insulin secretion.

It was hard to detect any specific trend in the GH levels during suckling, as shown in Figure 20. If GH levels were dropping or rising before suckling, they continued to do so after suckling. The 95% fiducial limits

for repeat measurements of the same sample were approximately ± 0.5 ng/ml for a sample containing 5 ng/ml GH, increasing to ± 1.0 ng/ml for a sample containing 10 ng/ml GH (see Table XII, page 97), and the small variations within calves during suckling could easily be accounted for by this variation.

In some calves, more acute changes in trend could be seen in the prolactin levels at the time of suckling (Figure 21). However, these were so variable between suckling occasions that no relationship to suckling was detectable.

3.1.3 Discussion

Following suckling, the rapid increase in insulin levels, followed by sustained high levels for a period of more than three hours, was probably a reflection of the formation of a milk clot in the abomasum (Radostits and Bell, 1970). Initially the milk is coagulated by rennin to form a clot and whey. The latter is digested rapidly, and begins to appear in the duodenum within 5 minutes of feeding. The immediate insulin secretion may have been caused by the stimulation of milk entering the abomasum and of whey entering the duodenum, a stimulation which was possibly mediated by a direct neural pathway or by cholecystokinin-pancreozymin (CCK) secretion. Baile et al (1969) observed that in the goat, CCK stimulated insulin secretion less well than it did in monogastric animals, and secretin did not stimulate it at all. However, they did not rule out the possibility that in ruminants, the hormones of the duodenum may, nevertheless, be of importance in allowing a rapid

insulin secretion in anticipation of increased absorption of glucose. Chase et al (1977) also observed a very rapid insulin response to feeding in steers: since the response preceeded changes in portal metabolite levels, a direct neural stimulation of the β cells of the pancreas was thought to be responsible for immediate insulin secretion.

Where the insulin secretion appeared to reach a double peak, it could reflect the fact that as whey digestion begins to be reduced, digestive products from the casein clot begin to enter the duodenum. The first peak would be maintained by the digestion and absorption of lactose in the whey. Increased glucose concentration is of primary importance in stimulating insulin secretion in all animals which have been studied (Mayhew et al, 1969) and there is some evidence (Stern et al, 1971) that young ruminants show a similar response. Whether the galactose moiety of the lactose digestion is effective as a stimulator of insulin secretion is controversial: Pozza et al (1958) stated that it was potent, while Field (1964) cited results where it was observed to be without effect. The second peak could reflect the peak of absorption of casein digestive products; the products would include amino acids, some of which are effective, especially in the presence of glucose, in stimulating insulin secretion, in both isolated rat pancreas (Gerich et al, 1974) and <u>in vivo</u> in ruminants (Davis, 1972: Hertelendy et al, 1970).

The situation regarding insulin secretion was relatively simple, all calves showing a uniform increase following feeding, and a return to very uniform basal

levels after 3 - 4 hours. Thus it appears that the feeding stimulus is of over-riding importance in stimulating insulin secretion. The situation in which calf 8a (3 April), calves B, C and 8b responded very much less than the other sampling days, could well be related to some adverse environmental condition causing high rates of epinephrine secretion. Epinephrine has been shown to inhibit the insulin response to glucose by means of an α-adrenergically-controlled mechanism in isolated islets of mice (Hermann and Deckert, 1977) and the inhibition has been observed *in vivo* in ruminants (Hertelendy et al, 1969). However, a close study of the observations made during blood sampling did not reveal any reasons or evidence for discomfort in these particular calves at this time.

The factors controlling secretion of GH are more complex, and have been discussed at length in Chapter 1. Because of the great variation between calves, the number of animals used was insufficient for firm conclusions to be drawn. However, the results appear to some extent to support the view that GH release decreased after food intake, and increased under conditions of low energy substrate levels: Figure 16 shows that the relationship held more completely after the morning feed, when feeding time was more constant than in the afternoon. Such a pattern was in agreement with other studies with ruminants (Bassett, 1972).

The results were also in accordance with the observation that spontaneous peaks of GH secretion occurred at approximately regular intervals throughout the day (Tannenbaum and Martin, working with rats, 1976). These authors

observed a frequency of 3.3 hours in the GH secretion cycles, a time that can be seen to coincide with the major peaks present in Figure 18.

It is of interest that calves B, C and 8b showed increased basal GH levels, since these are three of the four calves which had reduced insulin responses. It has been mentioned on page 120 that inhibition of the insulin response could have been related to these calves having higher epinephrine levels than the other calves. Elevated basal levels of GH could also be a result of high epinephrine levels, because stimuli described as 'non-physical excitement or disturbance', i.e. stimuli which are associated with epinephrine secretion, have been shown to result in a ten-fold increase in GH levels in plasma of yearling steers (Eaton et al, 1968). Alternatively, the simultaneous observation of high basal GH levels and low insulin secretion, serves to support the negative correlation between the two hormones, observed by Bassett et al (1971) and could simply be associated with reduced milk intake in these calves. Milk intake was not measured, as the calves were suckling from cows.

The lack of a detectable regularity or causal relationship in the plasma prolactin levels, has also been found in other investigations into prolactin levels of young ruminants (Bryant et al, 1968: Hart, 1974). Prolactin levels have been observed to be unresponsive to changes in plasma energy substrate levels in ruminants (McAtee and Trenkle, 1971: Bryant et al, 1970), so it is to be expected that they would not be related to feeding times. The levels

in the present study were very much lower than those cited for heifers (McAtee and Trenkle, 1971) anoestrus virgin goats (Hart, 1974) or kids (Bryant et al, 1968) and they could be a reflection of the assay conditions (see page 94).

Despite the large number of investigations into hormonal changes during suckling in the lactating mother animal (Bryant et al, 1968; Grosvenor et al, 1968: Hart and Flux, 1973: Saunders et al, 1976), there are no parallel investigations in the young while they are being suckled. Although the present study was a pilot investigation in this respect, and further work should be carried out in which mother and young are sampled simultaneously, it appears at this stage that prolactin and GH are not involved in the suckling response of the young. The only repeatable response to feeding was the insulin secretion, thought to be a direct response to the presence of milk in the abomasum or of whey entering the duodenum. It would be of interest to conduct further studies of pituitary and adrenal hormones and adrenergic receptor mechanisms, to ascertain whether the suckling response involved parallel changes between mother and young.

3.1.4 Conclusions

1. As with other animals studied, insulin levels were uniformly below 1 ng/ml in the plasma of these calves, except when under the stimulus of either feeding or digestion, when levels rose to 2 - 6 ng/ml.

2. GH secretion took place episodically in a 'random' manner throughout the day, but with a possible tendency to reach a nadir after feeding.

3. Prolactin secretion also showed episodic behaviour, but with no relationship to feeding.

4. Apart from the insulin response to the feeding stimulus, the suckling stimulus did not have a repeatable influence on the hormone levels studied in these young calves.

3.2 EXPERIMENT 2

3.2.1 Experimental design

The purpose of Experiment 2 was to carry out a pilot investigation into the effects of acute intravenous infusion through the catheter, of certain agents. Two of the treatments tested were glucose and volatile fatty acids (VFAs), chosen in order to investigate the relationship between energy substrate levels, insulin and GH. It was hoped that further light would be thrown on the same relationship, by testing the effects of sodium salicylate, an agent known to reduce levels of plasma free fatty acids (FFAs) in ruminants (Hertelendy and Kipnis, 1973).

Table XVII Calves used in Experiment 2

Calf no.	Sex	Date of birth	Weight on 30 July
7	M	23 July	25.0 kg
28	F	26 July	25.5 kg
29	F	26 July	20.0 kg
30	F	26 July	22.5 kg

Table XVIII Overall experimental design

Week beginning	Treatment	Dosage (mM/kg)	Volume (ml/kg)
30 July	Glucose	1.25	0.5
7 August	VFA[1]	1.25	0.5
13 August	Sodium salicylate	40 mg/kg	0.5

[1]VFA = an equimolar mixture of sodium acetate, sodium propionate and sodium butyrate, brought to pH 7.0 with sodium hydroxide.

Table XIX Weekly treatment procedure of Experiment 2

Calf no.	Tuesday	Wednesday	Thursday	Friday
7	control	treatment	treatment	control
28	treatment	control	control	treatment
29	control	treatment	control	treatment
30	treatment	control	treatment	control

Four calves were used (Table XVII) and a three-week experimental design was used in which each of the three treatments was given for one week only (Table XVIII). Within each week, the Monday was the time for catheterisation, or replacing catheters if necessary, and Tuesday to Friday were four days of blood sampling. The experiment was a reversal type, with treatment and control days alternating for each calf through two cycles. On each day, two calves were treated, and two were in the control phase (Table XIX). Controls were given 0.5 ml/kg body weight of sterile physiological saline in a single intravenous dose through the catheter.

On each sampling day, sampling commenced between 9.00 and 9.30 hours. Two pre-treatment samples were taken 15 minutes apart. The first two post-treatment samples were also at 15-minute intervals, and five more samples were taken at 30-minute intervals thereafter. The blood sampling and separation procedure are described on page 47.

Each calf was fed 12% of its body weight of milk, administered through a teat, within an hour after the last sample was taken. The weekly body weights of the calves were measured.

3.2.2 Results

The weekly body weights of the calves are plotted in Figure 43 and recorded in Table XLV.

Intravenous doses of glucose and salicylate had no observable effect on the condition of the calves. However, the VFA mixture caused an immediate response of muscular spasms lasting for about 5 minutes after the infusion in some calves. Table XX indicates the extent of the reaction in the different calves. Although it was very variable between calves, each calf was consistent over the two treatment periods.

Table XX Observations after infusions of VFAs

Calf no.	Side-effects
7	No effects
28	Coughing, abdominal convulsions, collapse
29	Defecation
30	Coughing, violent defecation

Injections of both glucose and VFAs increased insulin levels (Figures 22 and 23), the levels reached being significantly higher than after saline injection (Tables XXI and XXII). Insulin levels reached were higher following VFA treatment than following glucose, but the response to each was variable (Table XXIV), and the difference between them was significant at only the 10% level.

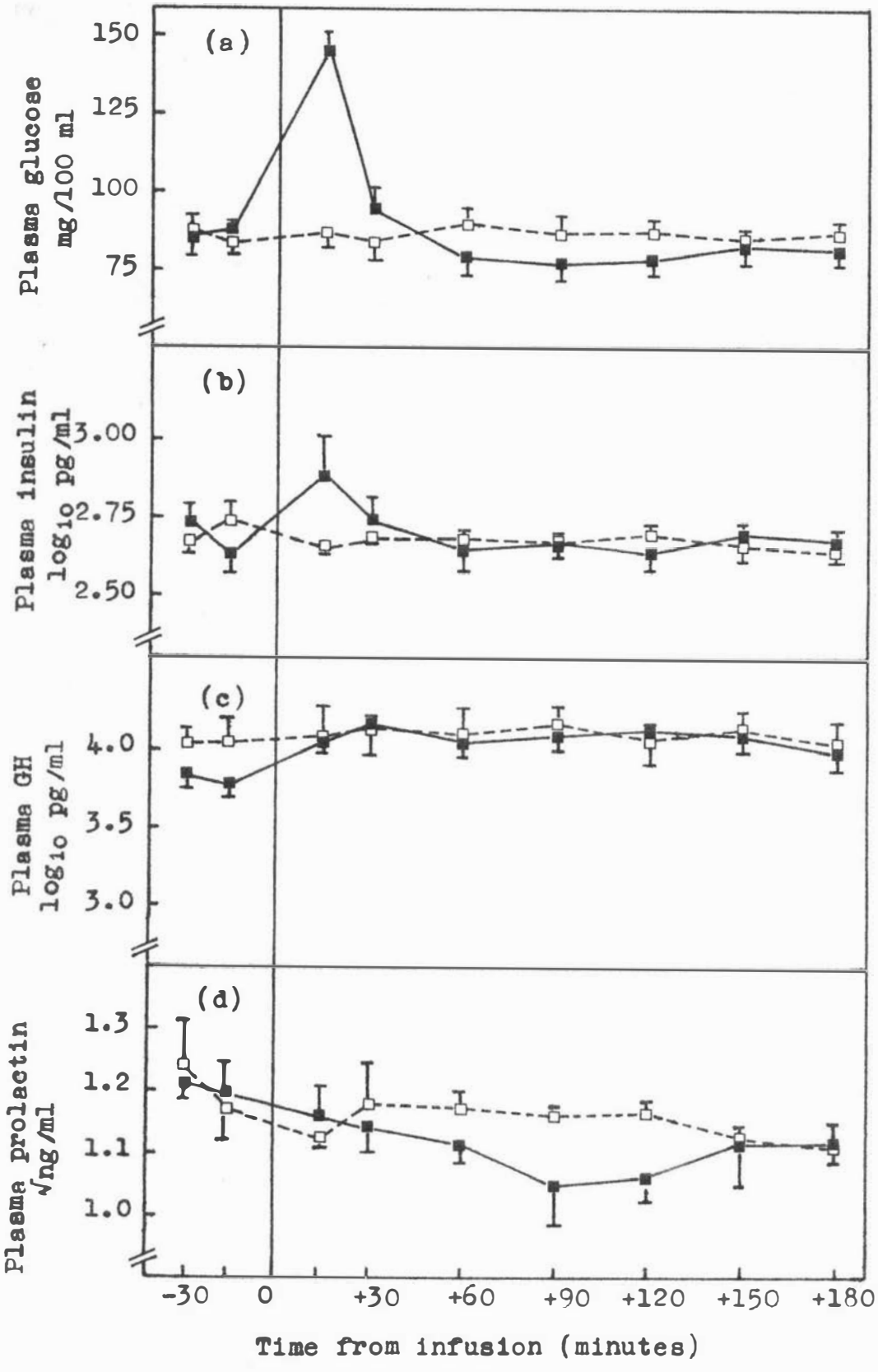

Figure 22 Effect of glucose treatment. Plasma levels of (a) glucose, (b) insulin, (c) growth hormone and (d) prolactin. Mean for values obtained on glucose treatment days (solid lines) and on control days (broken lines). Vertical lines represent standard error of the mean based on variance between calves within different sampling days.

Table XXI Glucose treatment: mean values of glucose, insulin, GH and prolactin in plasma for different sample times on days of glucose and saline treatment:

NS = not significant: + = $p<0.1$: ++ = $p<0.05$: +++ = $p<0.01$: difference between post-treatment samples adjusted by covariance for differences in pre-treatment levels.

	Samples	Glucose	Saline	T	P	C
Glucose mg/100 ml	1-2	86.25	83.16			
	3	145.25	84.07	+++	NS	NS
Insulin $\log_{10}$ pg/ml: pg/ml in parentheses	1-2	2.6591 (456.1)	2.6926 (492.7)			
	3	2.8823 (762.6)	2.6526 (449.8)	++	NS	NS
GH $\log_{10}$ pg/ml: ng/ml in parentheses	1-2	3.7934 (6.209)	4.0012 (10.027)			
	3	4.1112 (12.919)	4.0596 (11.471)	NS	NS	NS
Prolactin $\sqrt{}$ng/ml: ng/ml in parentheses	1-2	1.20441 (1.451)	1.2287 (1.509)			
	3-4	1.1508 (1.324)	1.1693 (1.367)	NS	NS	NS

T = between treatments (glucose versus saline)

P = between periods (Tuesday and Wednesday versus Thursday and Friday)

C = between calves

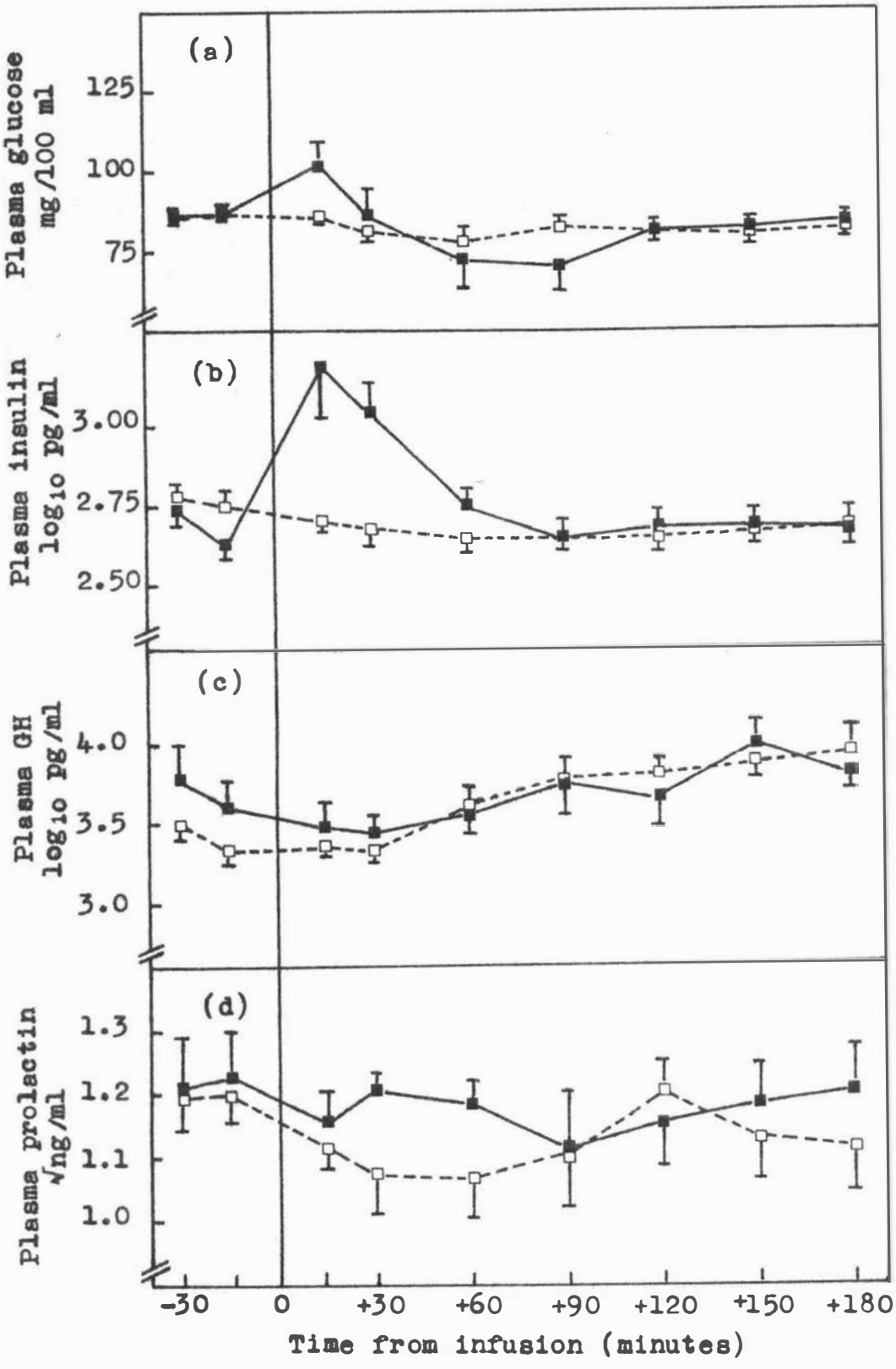

Figure 23 Effect of VFA treatment. Plasma levels of (a) glucose, (b) insulin, (c) growth hormone and (d) prolactin. Mean for values obtained on VFA treatment days (solid lines) and on control days (broken lines). Vertical lines represent standard error of the mean based on variance between calves within different sampling days.

Table XXII VFA treatment: mean values of glucose, insulin, GH and prolactin in plasma for different sample times on days of VFA and saline treatment.
NS = not significant: + = $p<0.1$: ++ = $p<0.05$: +++ = $p<0.01$: difference between post-treatment samples adjusted by covariance for varying pretreatment levels.

	Samples	VFAs	Saline	T	P	C
Glucose mg/100 ml	1-2	86.8	85.6			
	3	102.1	85.6	++	NS	NS
Insulin $\log_{10}$ pg/ml: pg/ml in parentheses	1-2	2.6837 (482.7)	2.7654 (582.7)			
	3	3.1169 (1,308)	2.6918 (491.8)	++	NS	NS
GH $\log_{10}$ pg/ml: ng/ml in parentheses	1-2	3.7009 (5.022)	3.422 (2.642)			
	3	3.4604 (2.887)	3.3491 (2.234)	NS	NS	NS
Prolactin $\sqrt{}$ng/ml: ng/ml in parentheses	1-2	1.2334 (1.521)	1.1994 (1.439)			
	3-5	1.1816 (1.396)	1.0864 (1.180)	NS	NS	NS

T = between treatments (VFAs versus saline)

P = between periods (Tuesday and Wednesday versus Thursday and Friday)

C = between calves

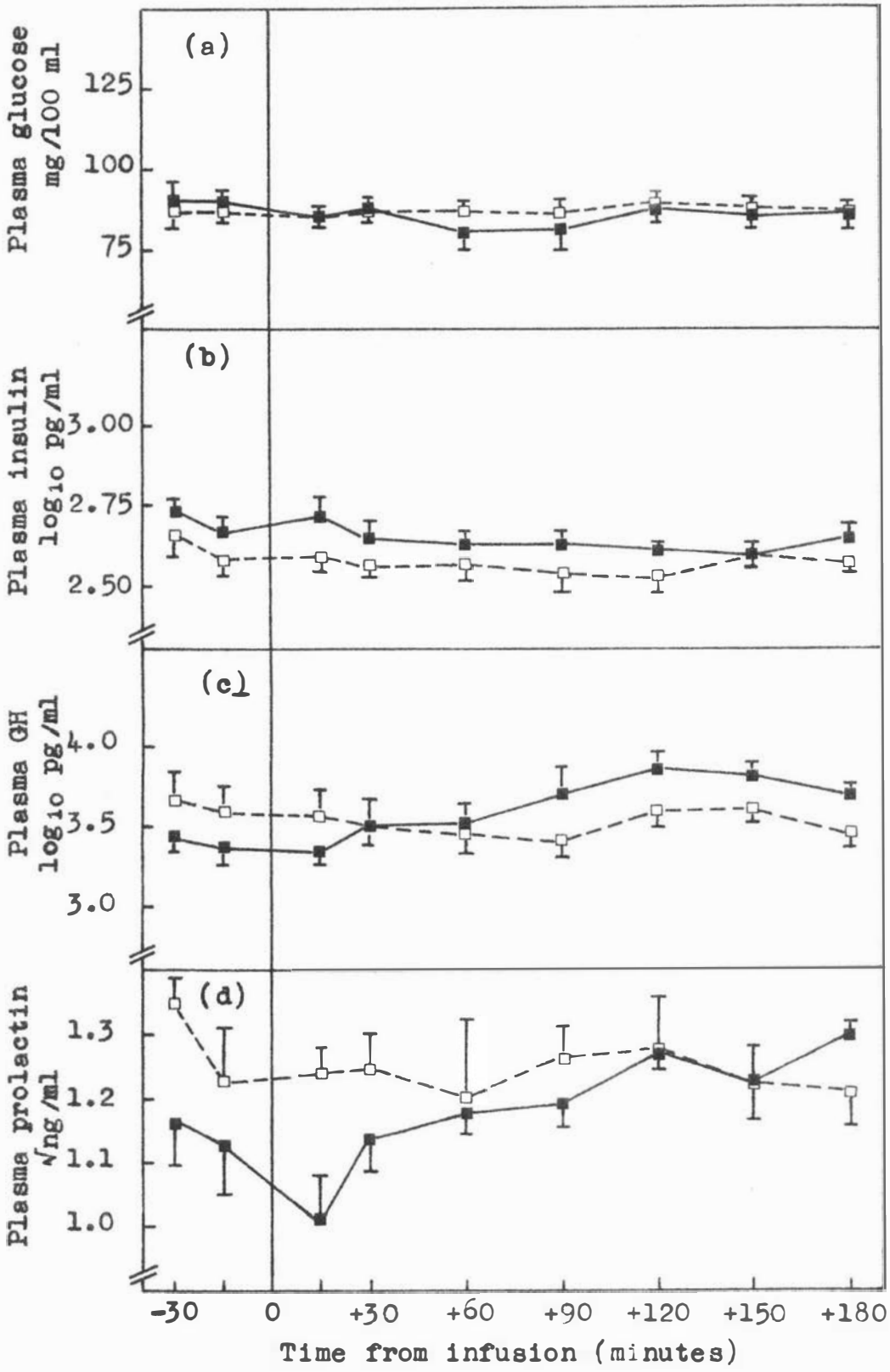

Figure 24 Effect of salicylate treatment. Plasma levels of (a) Glucose, (b) insulin, (c) growth hormone and (d) prolactin. Mean for values obtained on salicylate treatment days (solid lines) and on control days (broken lines). Vertical lines represent standard error of the mean, based on variance between calves within different sampling days.

Table XXIII Salicylate treatment: mean values of glucose, insulin, GH and prolactin in plasma for different sample times on days of salicylate and saline treatment.
NS = not significant: + = $p<0.1$: ++ = $p<0.05$: +++ = $p<0.01$: difference between post-treatment samples adjusted by covariance for varying pretreatment levels.

Samples	Samples	Salicylate	Saline	T	P	C
Glucose mg/100 ml	1-2	90.2	85.1			
	3	85.4	84.9	NS	NS	NS
Insulin $\log_{10}$ pg/ml: pg/ml in parentheses	1-2	2.6901 (489.9)	2.6198 (416.7)			
	3	2.7160 (520.0)	2.5966 (395.1)	NS	NS	NS
GH $\log_{10}$ pg/ml: ng/ml in parentheses	1-2	3.3888 (2.447)	3.6282 (4.248)			
	3-4	3.4271 (2.673)	3.5377 (3.449)	NS	NS	+
Prolactin $\sqrt{}$ng/ml: ng/ml in parentheses	1-2	1.1470 (1.316)	1.2841 (1.649)			
	3	1.0636 (1.131)	1.2366 (1.529)	NS	NS	NS

T = between treatments (salicylate versus saline)
P = between periods (Tues and Wed versus Thurs and Fri)
C = between calves

Neither GH nor prolactin levels changed in any significant manner following glucose or VFA treatments (Figures 22 and 23, Tables XXI and XXII. The salicylate treatment influenced none of the three hormones measured, nor glucose levels (Figure 24 and Table XXIII).

Table XXIV Plasma insulin levels (ng/ml) in individual calves in the first sample after glucose and VFA treatments.

	Glucose treatment		VFA treatment	
Calf no.	Period 1	Period 2	Period 1	Period 2
7	0.61	1.04	0.35	0.97
28	0.86	0.73	3.26	0.93
29	1.08	3.39	3.87	5.29
30	0.23	0.33	2.94	5.83

Table XXV Relationship between insulin responsiveness and basal GH levels.
Data represent number of calf sampling occasions falling into each category.
Insulin low responders = levels < 1,00 ng/ml
High basal GH levels = minimum > 4.00 ng/ml
Glucose treatment data not in parentheses
VFA treatment data in parentheses

	Insulin low responders		Insulin responders	
High basal GH	4	(0)	2	(2)
Low basal GH	1	(3)	1	(3)

3.2.3 Discussion

The insulin response to VFA treatment was generally greater than that to glucose. As glucose did not reach such high plasma levels after VFA treatment as it did after glucose treatment, the insulin secretion was probably occurring as a direct response to VFAs as well as to glucose. The finding that a closer relationship may exist between VFA levels and insulin secretion, than between glucose levels and insulin secretion, is in agreement with findings for sheep (Manns and Boda, 1967: Horino et al, 1968). It seems that in the ruminant pancreas, the role of glucose may be different from the situation in *in vitro* studies with non-ruminant pancreas, where the presence of glucose was found to be of primary importance as a potentiator of the insulin release (Gerich et al, 1974: Hermann and Deckert (1977). The study of Ambo et al (1973) is noteworthy because the same dosages of glucose and VFAs as were used in the present study, were employed, and the insulin and glucose results obtained in sheep bore a very striking resemblance to the order of magnitude of the responses obtained here (making allowance for the fact that the VFAs were injected as an equimolar mixture here).

The relatively strong insulin response to VFAs is a response which is constitutional for ruminants, as the calves used in this experiment had had no access to solid feed, and therefore would not have been previously exposed to rumen production of VFAs. The same conclusion was reached by Hertelendy et al (1969), working with lambs.

Although the response seemed to be of physiolcgical significance in relation to the absorption of VFAs as the main energy substrates in the adult ruminant, its role is not fully understood, in view of the fact that when VFAs were administered intra-ruminally, the insulin response was not observed (Bassett, 1972). Moreover, initial insulin secretion in response to food intake, was observed to occur before any increase in absorption of digestion products, and was therefore thought to be elicited by means of neural stimuli, rather than by metabolites (Chase et al, 1977).

The extreme variability of the insulin response (Table XXIV) was also observed for insulin levels after feeding in Experiment 1. As in Experiment 1, it is again of interest to attempt to correlate the phenomenon of low responsiveness to the possible adrenergic status of the calves. If it is to be expected that high endogenous levels of epinephrine are the cause of low responsiveness of β cells of the pancreas, the low responders should be the calves which showed the most adverse reaction to the VFA infusion. Comparison of VFA treatment data of Table XX with insulin responses of Table XXIV, indicates that if any relationship exists, however, it is the reverse, with the low responsive calf being the one with no side-effects.

In Experiment 1, it was also observed that the insulin low responsive calves were, in three cases out of four, the only calves with GH basal levels of more than 4 ng/ml. Table XXV shows results of an investigation into the same relationship here. There is no evidence that insulin low responsiveness correlated with high basal levels of GH in the present experiment.

The lack of responsiveness of GH to any of the treatments, in either direction, means that this experiment has not shed any light on relationships between energy substrate levels and GH secretion in young calves. The lack of responsiveness is unusual for ruminants (Wallace and Bassett, 1970: Stern et al, 1971: Hertelendy and Kipnis, 1973), but there are many examples of GH being unresponsive to the stimuli which cause its secretion in man, in other species, e.g. cat (Kokka et al, 1971), rabbit (McIntyre and Odell, 1974) and mouse (Schindler et al, 1972), and the literature is discussed on pages 8 - 15.

The lack of responsiveness of prolactin to any of the treatments is to be expected in view of the fact that other workers have not found any relationship between energy substrate levels and prolactin (McAtee and Trenkle, 1971: Bryant et al, 1970).

3.2.4 Conclusions

1. The present study is in agreement with other studies with ruminants, in that circulating VFA (administered at 1.25 mM/kg body weight) appear to stimulate an increase in plasma insulin levels more strongly than hyperglycaemia.

2. GH and prolactin levels in plasma were not affected in any way by treatment with 1.25 mM/kg body weight glucose or VFA.

3. None of the three hormones was influenced by treatment with 40 mg/kg body weight salicylate, an agent

which is known to decrease plasma free fatty acid levels. The lack of GH responsiveness to any of the treatments used, was unexpected in view of other studies with ruminants.

3.3 EXPERIMENT 3

3.3.1 Experimental design

Intravenous arginine infusion has been shown to elicit an increase in plasma GH and insulin levels in almost all animals studied, and it also elicits a prolactin response in sheep (reviewed on pages 4, 9 and 28). In the present experiment, the stimulus of an arginine infusion through a jugular catheter, was used to investigate the responses of all three hormones in calves of different ages and with different degrees of rumen development.

Table XXVI Experimental groups of calves used in Experiment 3

Group	No.	Age	Diet		Digestion
			Milk	Meal	
M1	5	1 week	fed by teat	none	pre-ruminant
M5	5	5 weeks	fed by teat	none	pre-ruminant
R5	4	5 weeks	fed by bucket	meal + hay	ruminant

The experimental groups of calves were as described in Table XXVI. Calves were all female Jersey-Friesian cross-bred. The same calves were used at different ages for group M1 and M5, and milk was fed through a teat in order to stimulate oesophageal groove closure and prevent rumination (Orskov, 1972). R5 calves were fed milk from a bucket and also given solid feed from the day they were obtained (at 4 - 5 days of age) in order to encourage early development of the ruminant

mode of digestion, but otherwise their treatment (e.g. housing etc.) was identical and has been described, together with details of catheterisation and composition of meal, on pages 43 - 46.

Table XXVII Experimental procedure for each five-day treatment period in Experiment 3.

Period	Day	Treatment	Dosage g/kg bw	Molarity	Volume ml/kg bw
	1	Control	No solutions administered		
Period 1	(2	Arginine	0.3	1.72	1.0
	(3	NaCl	0.1	1.72	1.0
Period 2	(4	Arginine	0.3	1.72	1.0
	(5	NaCl	0.1	1.72	1.0

All treatments were administered in a 30-second pulse through the catheter.

The experimental design involved a five-day regime which was identical for all three groups of calves (Table XXVII). Because the 1.72 molar arginine monohydrochloride solution entering the circulation would cause an immediate increase in plasma osmolality of approximately 18%, the saline treatments were given at the same volume and osmolality as the 1.72 M arginine. Solutions were made up to the appropriate volume and pH and then sterilised by autoclaving at 1.1 kg/cm^2 for 15 minutes.

As in Experiment 2, each calf acted as its own control within each of period 1 and period 2. However, the present experiment did not involve a

reversal design and the order of treatment days was the same for all calves.

The blood sampling technique has been described on page 47. Sampling began between 8.30 and 9.15 hours each day, and was completed by 14.00, at which time feeding took place. On Day 1, ten 3-ml blood samples were taken at regular 30-minute intervals. On days 2 - 5, the first four samples were 10 minutes apart for two samples before and two after each infusion, and at 30-minute intervals for samples 5 - 10.

Calves were weighed weekly, and their rectal temperatures were measured approximately once each hour on sampling days.

3.3.2 Results

The growth pattern of individual calves is shown in Figure 44 and average growth rates in Table XLVI. The growth rates were higher for group R, reaching the 0.3 kg body weight gain per day, which their feed rations predicted (page 46). Their solid feed consumption increased steadily throughout the experimental period (Figure 45). Growth rates for group M were comparable with those obtained in Experiment 2.

The rectal temperatures are plotted in Figures 46 - 48 and show that for both experimental weeks of group M, temperatures were uniform between 38.0 and 39.5°C, with no detectable trend within each week or within each day. Group R was less homogeneous, temperatures varying between 37°C and 40°C. Each individual

calf was relatively constant in temperature, the wide variation being caused by differences between calves.

The calves showed marked behavioural differences between groups. Group M5 showed indications of over-excitability, with suckling responses made at any object within reach at times of blood sampling. Calves of the same age on solid feed remained still and placid at sampling times.

Plasma levels of glucose and hormones in untreated calves are shown in Figures 25 - 28 for individual calves. Figures 26 - 28 and Table XXVIII indicate that plasma levels of all three hormones were higher in group M1 than in the other groups, indicating an age-related change in secretion or utilisation rates. The glucose median levels were significantly lower in group R5 than in group M5 and M1 combined.

Following the arginine stimulus there was a rapid increase in levels of insulin (Figure 30) and prolactin (Figure 32) in all calves. The prolactin increase was slower and less significant (Table XXXII) in week-old calves, whose prolactin levels were already higher than those of the other groups, but it was nonetheless consistent. There was a concurrent increase in glucose levels which was observed in all calves, but it was smaller and less significant in group R5 (Figure 29 and Table XXIX). It was followed by a deep, highly significant hypoglycaemia.

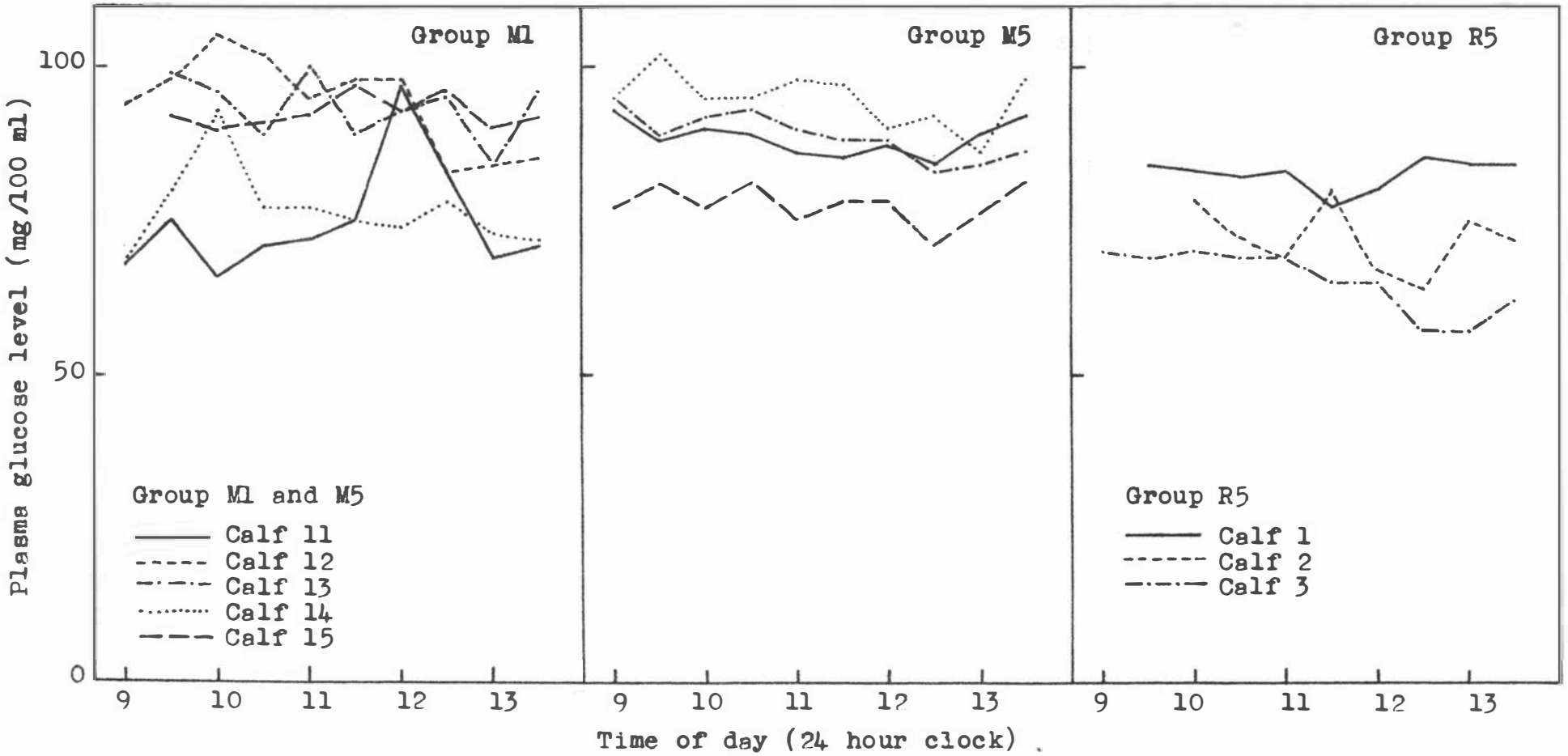

Figure 25 Plasma levels of glucose in individual calves on control day: samples taken at 30-minute intervals with no treatment.

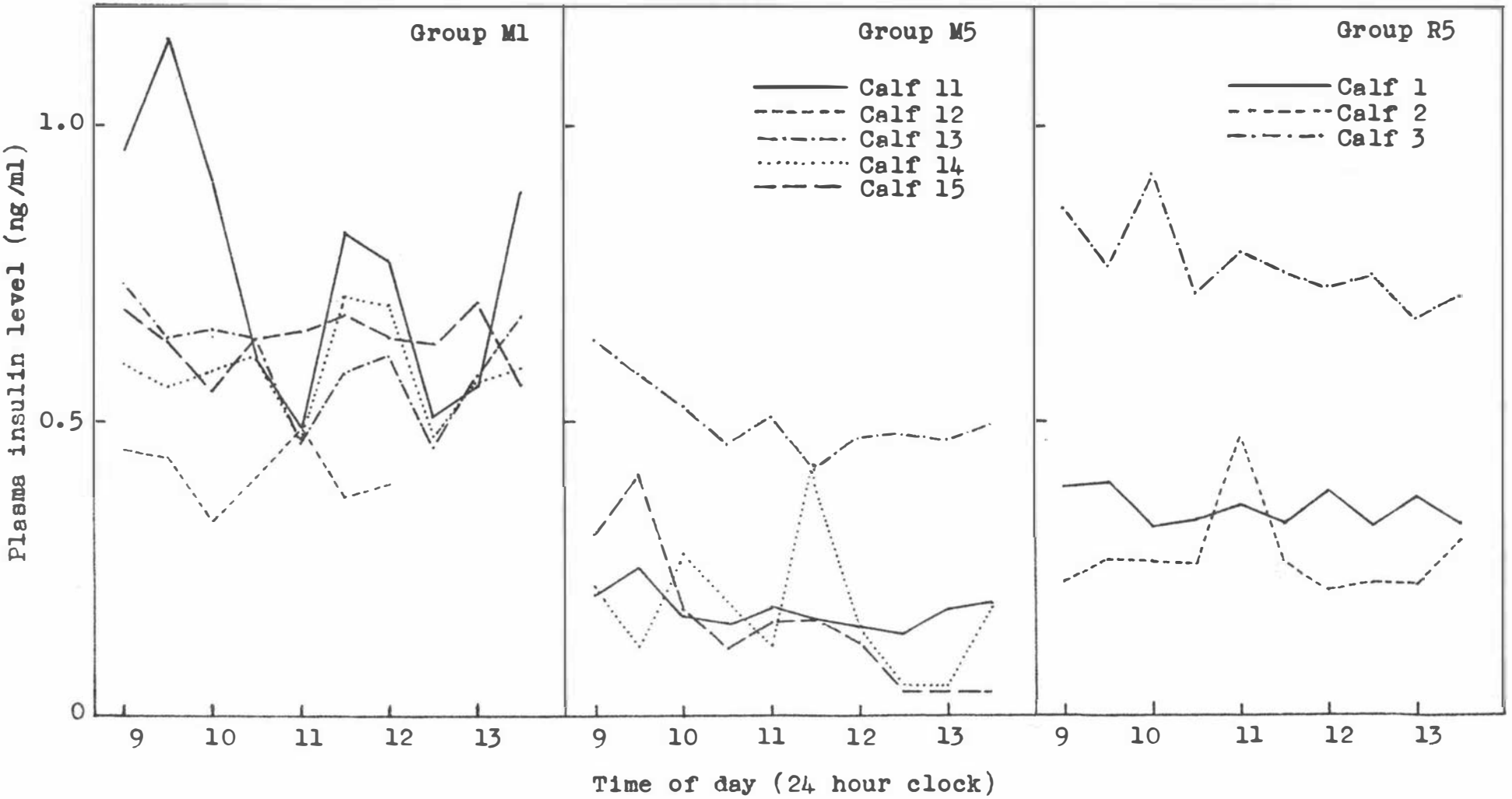

Figure 26 Plasma levels of insulin in individual calves on control day: samples taken at 30-minute intervals with no treatment.

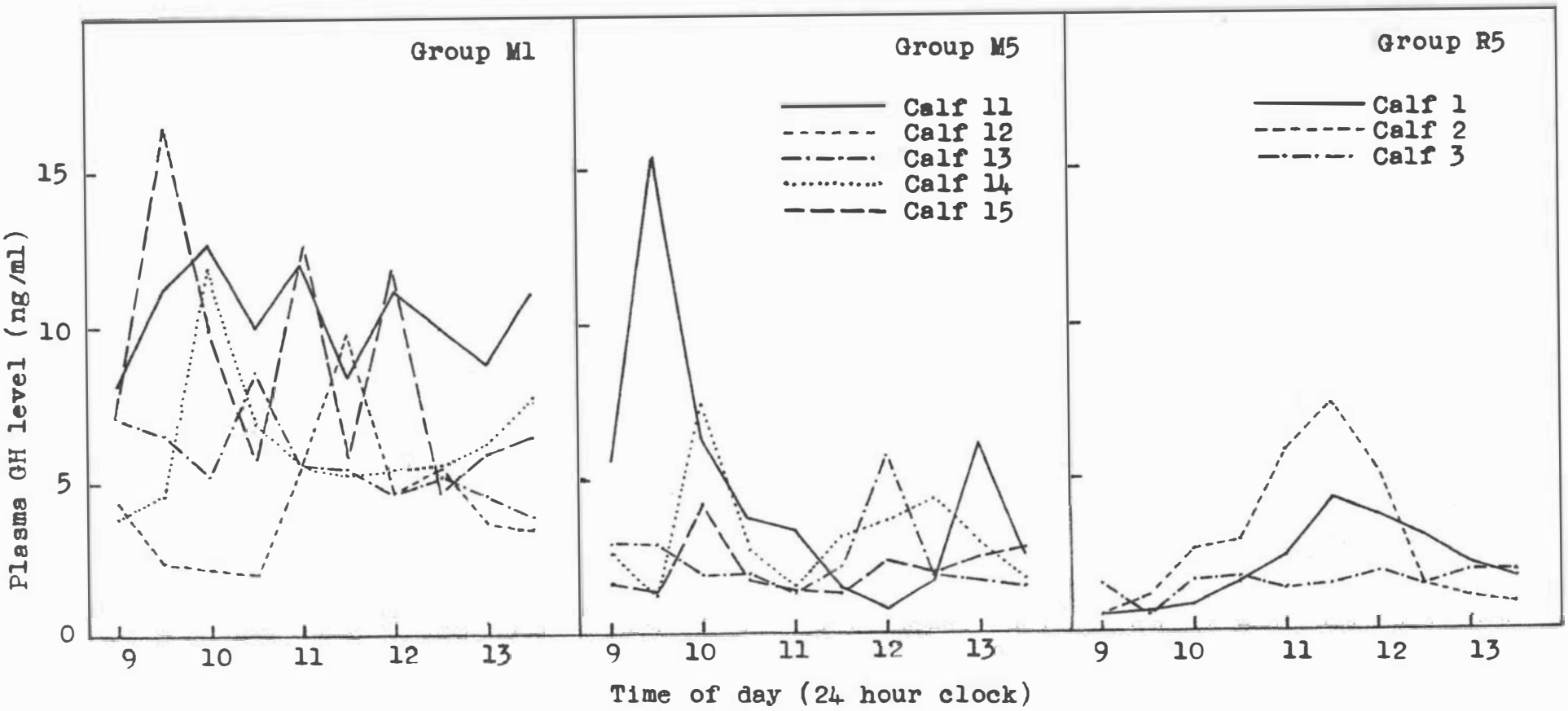

Figure 27 Plasma levels of GH in individual calves on control day: samples taken at 30-minute intervals with no treatment.

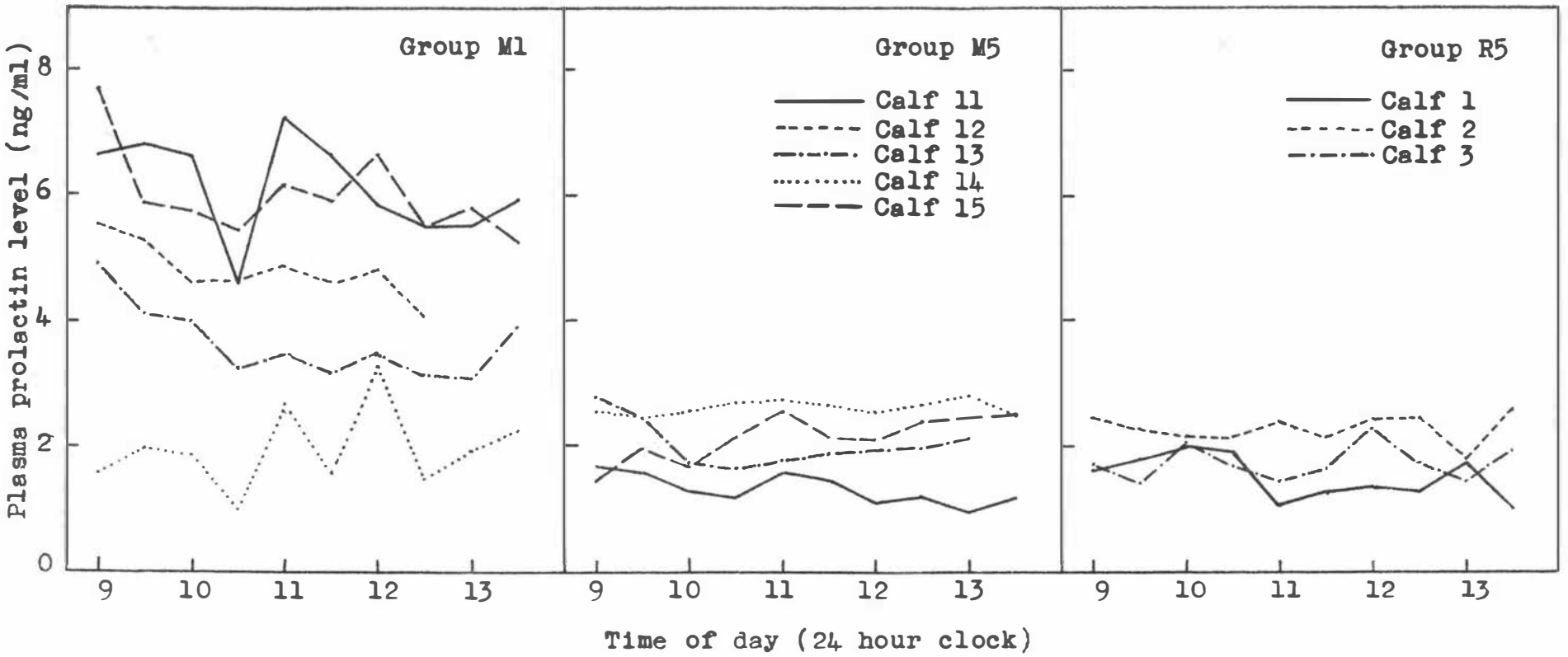

Figure 28 Plasma levels of prolactin in individual calves on control day: samples taken at 30-minute intervals with no treatment.

Table XXVIII Average of medians and ranges for individual calves of samples taken on control day: untransformed data.
+ = $p<0.1$: ++ = $p<0.05$: +++ = $p<0.01$:
each group compared with other two groups combined by Mann-Whitney U test.

	Median			Range		
	M1	M5	R5	M1	M5	R5
Glucose mg/100 ml	86.5	87.7	72.7^{++}	19.8	11.7	13.0
Insulin ng/ml	0.61^{++}	0.24^{+}	0.45	0.30	0.24	0.19
GH ng/ml	6.51^{+++}	2.5	2.0	7.4	7.1	3.3^{++}
Prolactin ng/ml	4.45^{++}	1.95	1.92	2.34^{++}	0.82^{++}	1.30

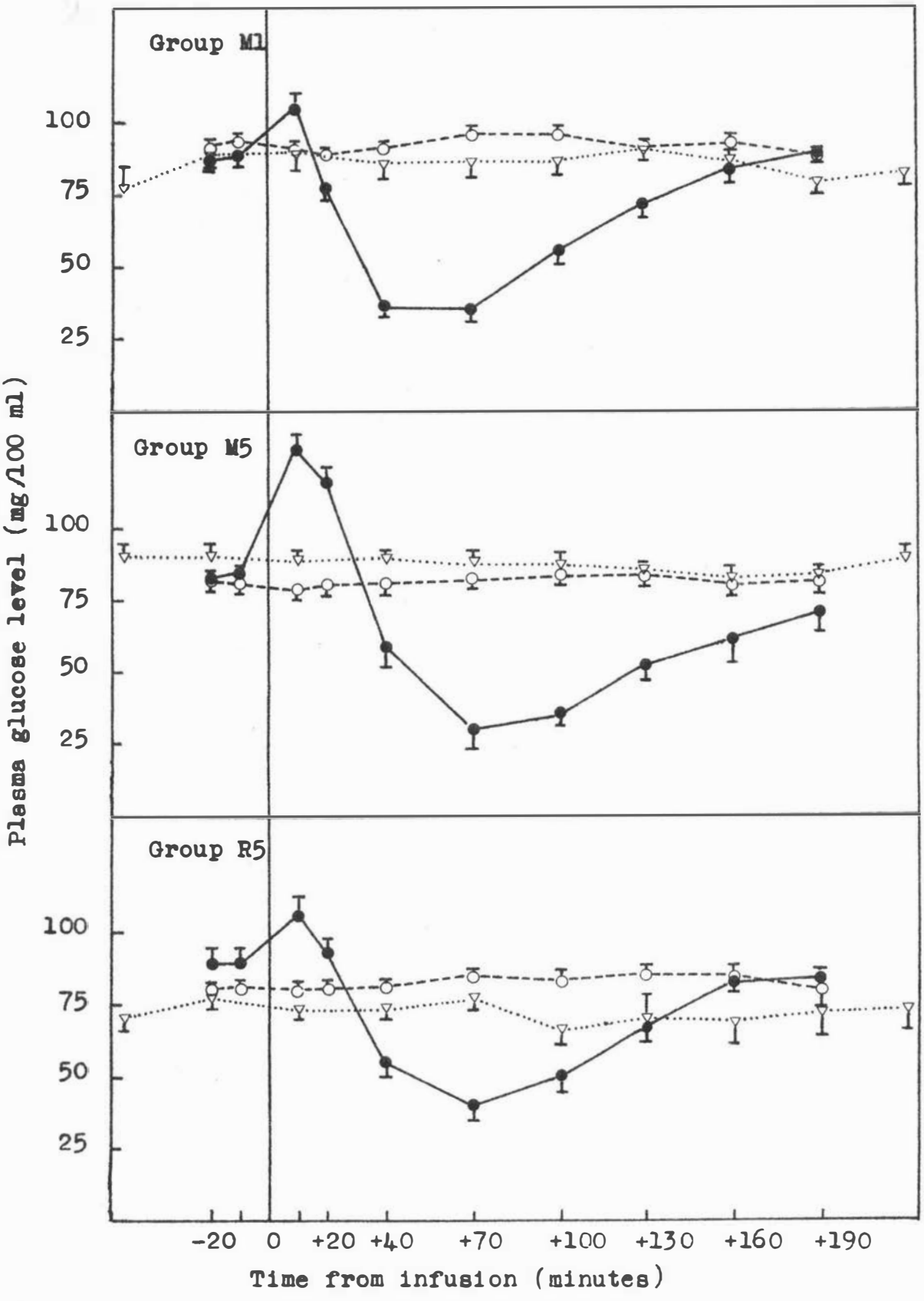

Figure 29 Plasma levels of glucose. Mean for values obtained on arginine treatment days (solid lines), on saline treatment days (broken lines) and on control days (dotted lines). Vertical bars represent standard error of the mean (SEM) based on variance between calves within different sampling days.

Table XXIXa Plasma glucose levels: mean values for different sample times on days of three different treatments.

NS = not significant: + = $p<0.1$: ++ = $p<0.05$: +++ = $p<0.01$: difference between post-treatment samples adjusted by covariance for differences in pre-treatment levels.

Treatments: C=control. A=arginine, S=saline.

Group	Samples	Treatment			Significance		
		C	A	S	A vs C	A vs S	S vs C
M1	1-2	86.5	88.0	92.9			
	3	90.0	105.9	90.9	++	+++	NS
	6	86.8	35.7	96.5	+++	+++	NS
M5	1-2	90.0	83.8	81.7			
	3	88.5	126.4	79.0	+++	+++	NS
	6	87.0	31.4	82.5	+++	+++	NS
R5	1-2	75.2	89.5	81.4			
	3	73.0	106.6	80.4	++	++	NS
	6	68.7	39.9	85.5	++	+++	NS

Table XXIXb Plasma glucose levels: significance of differences between groups of calves after arginine treatment, adjusted for pre-treatment means.

	Sample 3	Sample 6
M1 vs M5	+++	NS
M5 vs R5	+++	NS
M1 vs R5	NS	NS

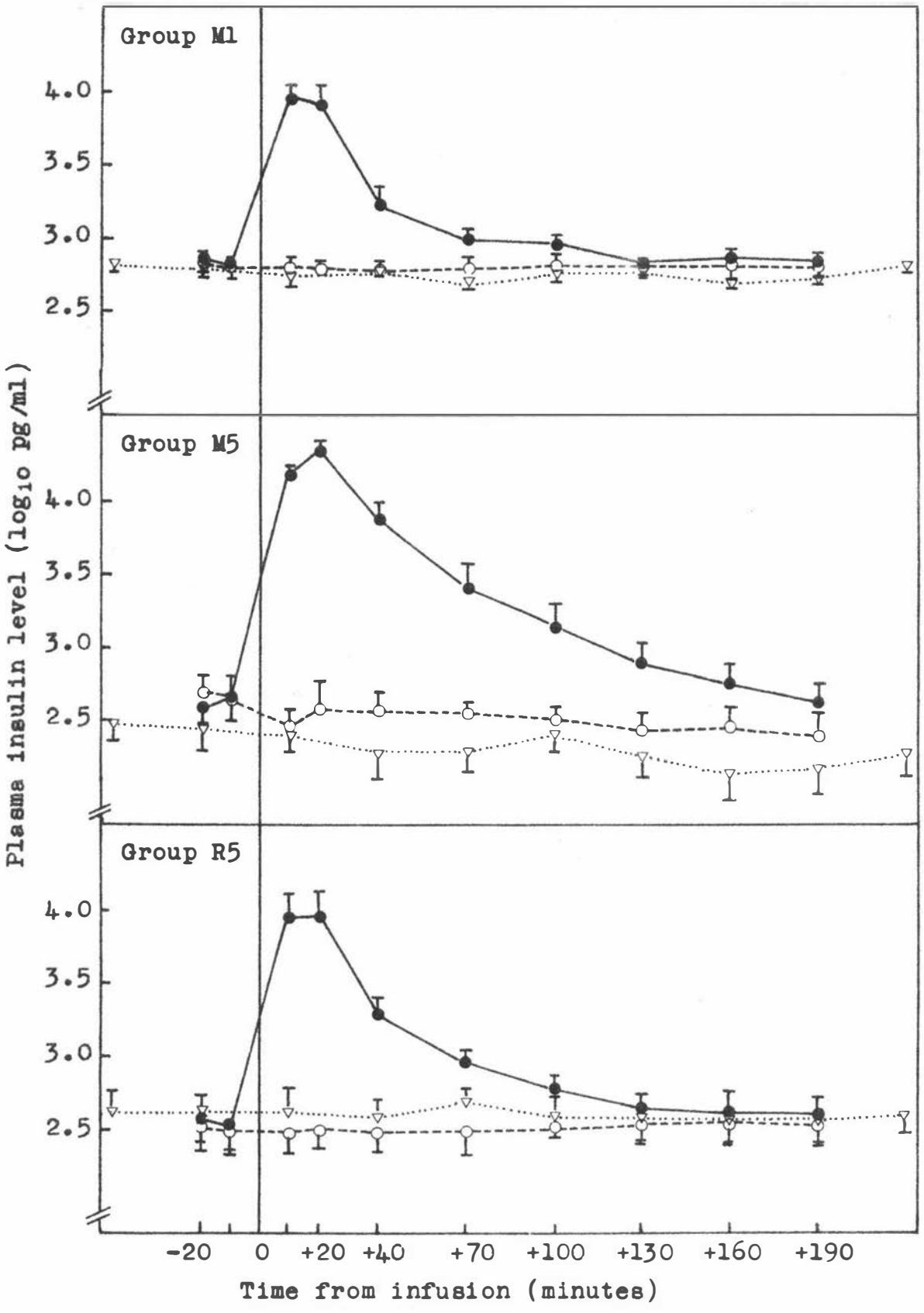

Figure 30 Plasma levels of insulin. Mean for values obtained on arginine treatment days (solid lines), on saline treatment days (broken lines) and on control days (dotted lines). Vertical bars represent SEM based on variance between calves within different sampling days.

Table XXXa Plasma insulin levels: mean values for different sample times on days of three different treatments. Data expressed as $\log_{10}$ pg/ml with untransformed data (ng/ml) in parentheses.

NS = not significant: + = $p<0.1$: ++ = $p<0.05$: +++ = $p<0.01$: difference between post-treatment samples adjusted by covariance for differences in pre-treatment levels.

Treatments: C=control, A=arginine, S=saline.

Group	Samples	Treatment			Significance		
		C	A	S	A vs C	A vs S	S vs C
M1	1-2	2.818 (0.66)	2.846 (0.70)	2.825 (0.67)			
	3-4	2.750 (0.56)	3.955 (9.01)	2.815 (0.65)	+++	+++	NS
M5	1-2	2.471 (0.30)	2.675 (0.47)	2.709 (0.51)			
	3-4	2.37 (0.23)	4.27 (18.6)	2.57 (0.37)	+++	+++	NS
R5	1-2	2.63 (0.43)	2.55 (0.36)	2.51 (0.33)			
	3-4	2.61 (0.41)	3.97 (9.25)	2.51 (0.32)	+++	+++	NS

Table XXXb Plasma insulin levels: significance of differences between groups of calves after arginine treatment, adjusted for pre-treatment means.

	Samples 3-4
M1 vs M5	+++
M5 vs R5	+++
M1 vs R5	NS

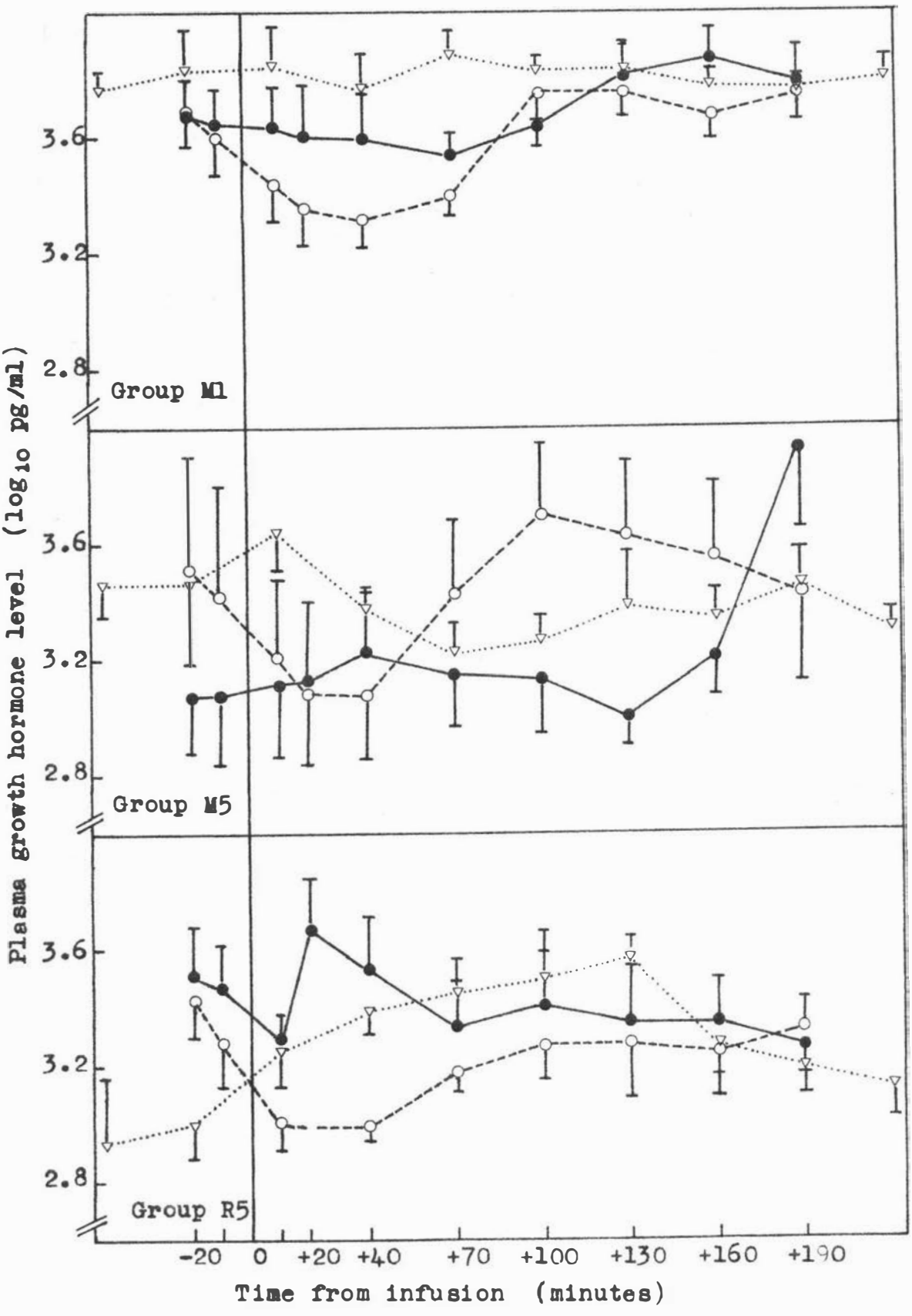

Figure 31 Plasma levels of growth hormone. Mean for values obtained on arginine treatment days (solid lines), on saline treatment days (broken lines) and on control days (dotted lines). Vertical bars represent SEM based on variance between calves within different sampling days.

Table XXXI Plasma GH levels: mean values for different sample times on days of three different treatments. Data expressed as $\log_{10}$ pg/ml with untransformed data (ng/ml) in parentheses

NS = not significant: + = $p<0.1$
++ = $p<0.05$: +++ = $p<0.01$: difference between post-treatment samples adjusted by covariance for differences in pre-treatment levels

Treatments: C=control, A=arginine, S=saline

Group	Samples	Treatment C	Treatment A	Treatment S	Significance A vs C	Significance A vs S	Significance S vs C
M1	1-2	3.79 (6.24)	3.66 (4.57)	3.64 (4.39)			
	3-5	3.83 (6.82)	3.61 (4.13)	3.38 (2.38)	NS	+++	+++
M5	1-2	3.46 (2.88)	3.14 (1.37)	3.47 (2.97)			
	3-5	3.41 (2.59)	3.25 (1.77)	3.12 (1.33)	NS	++	+
R5	1-2	2.96 (0.91)	3.49 (3.08)	3.35 (2.25)			
	3-5	3.36 (2.30)	3.49 (3.12)	2.92 0.83)	NS	+++	+++

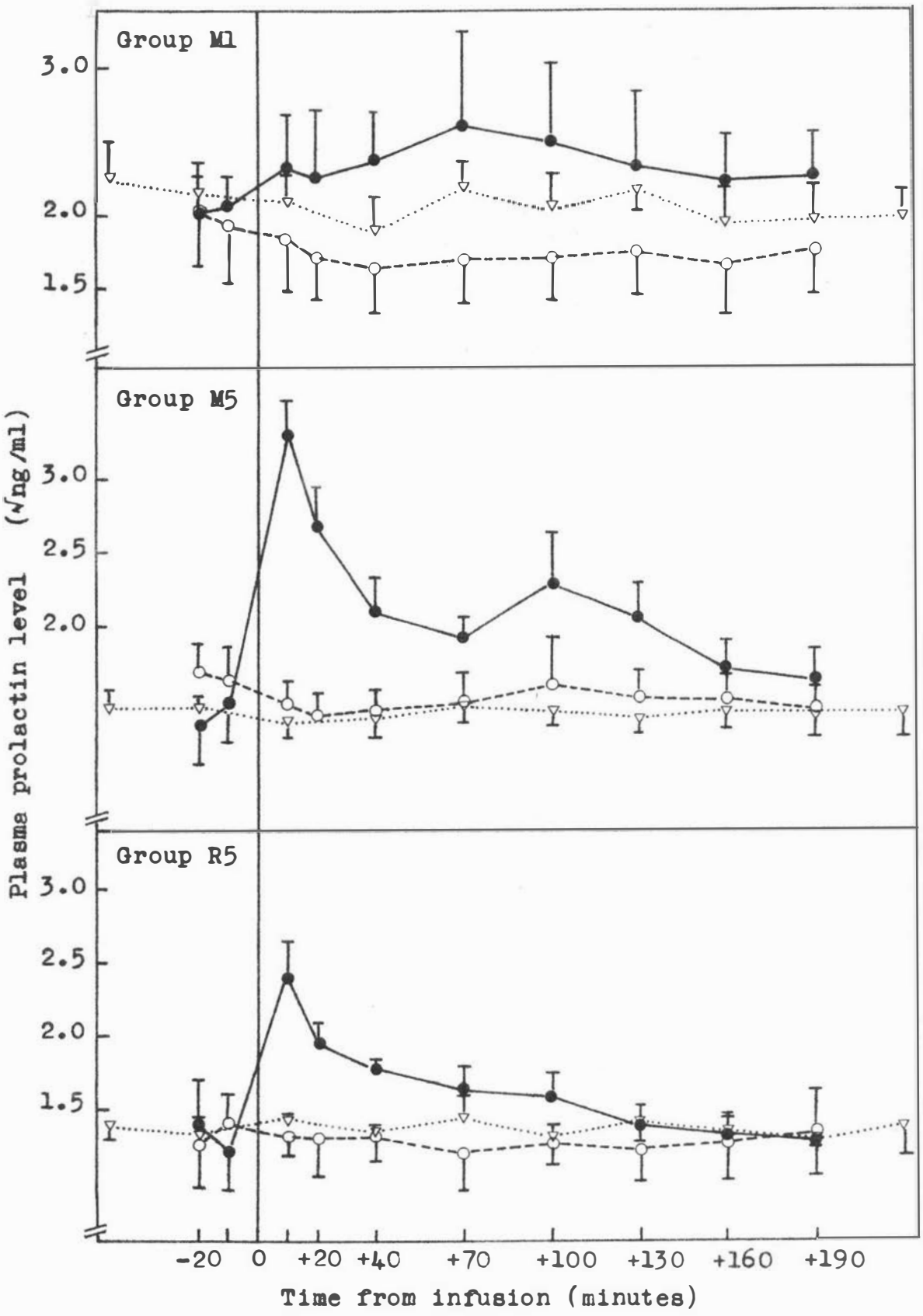

Figure 32 Plasma levels of prolactin. Mean for values obtained on arginine treatment days (solid lines), on saline treatment days (broken lines) and on control days (dotted lines). Vertical bars represent SEM based on variance between calves within different sampling days.

Table XXXIIa Plasma prolactin levels: mean values for different sample times on days of three different treatments. Data expressed as √ng/ml with untransformed data (ng/ml) in parentheses.

NS = not significant: + = $p<0.1$: ++ = $p<0.05$: +++ = $p<0.01$: difference between post-treatment samples adjusted by covariance for differences in pre-treatment levels.

Treatments: C=control, A=arginine, S=saline.

Group	Samples	Treatment			Significance		
		C	A	S	A vs C	A vs S	S vs C
M1	1-2	2.19 (4.82)	2.04 (4.18)	1.97 (3.90)			
	3	2.09 (4.39)	2.32 (5.39)	1.83 (3.37)	NS	NS	NS
	7	2.17 (4.72)	2.50 (6.26)	1.71 (2.92)	NS	++	NS
M5	1-2	1.44 (2.07)	1.38 (1.90)	1.69 (2.85)			
	3	1.33 (1.77)	3.16 (10.0)	1.52 (2.32)	+++	+++	NS
	7	1.36 (1.86)	2.14 (4.60)	1.62 (2.64)	+++	+++	NS
R5	1-2	1.37 (1.87)	1.31 (1.71)	1.33 (1.78)			
	3	1.44 (2.08)	2.40 (5.76)	1.32 (1.75)	++	++	NS
	7	1.41 (2.00)	1.59 (2.52)	1.27 (1.61)	+	++	NS

Table XXXIIb Plasma prolactin levels: significance of differences between groups of calves after arginine treatment, adjusted for pre-treatment means.

	Sample 3	Sample 7
M1 vs M5	+++	NS
M5 vs R5	++	NS
M1 vs R5	NS	NS

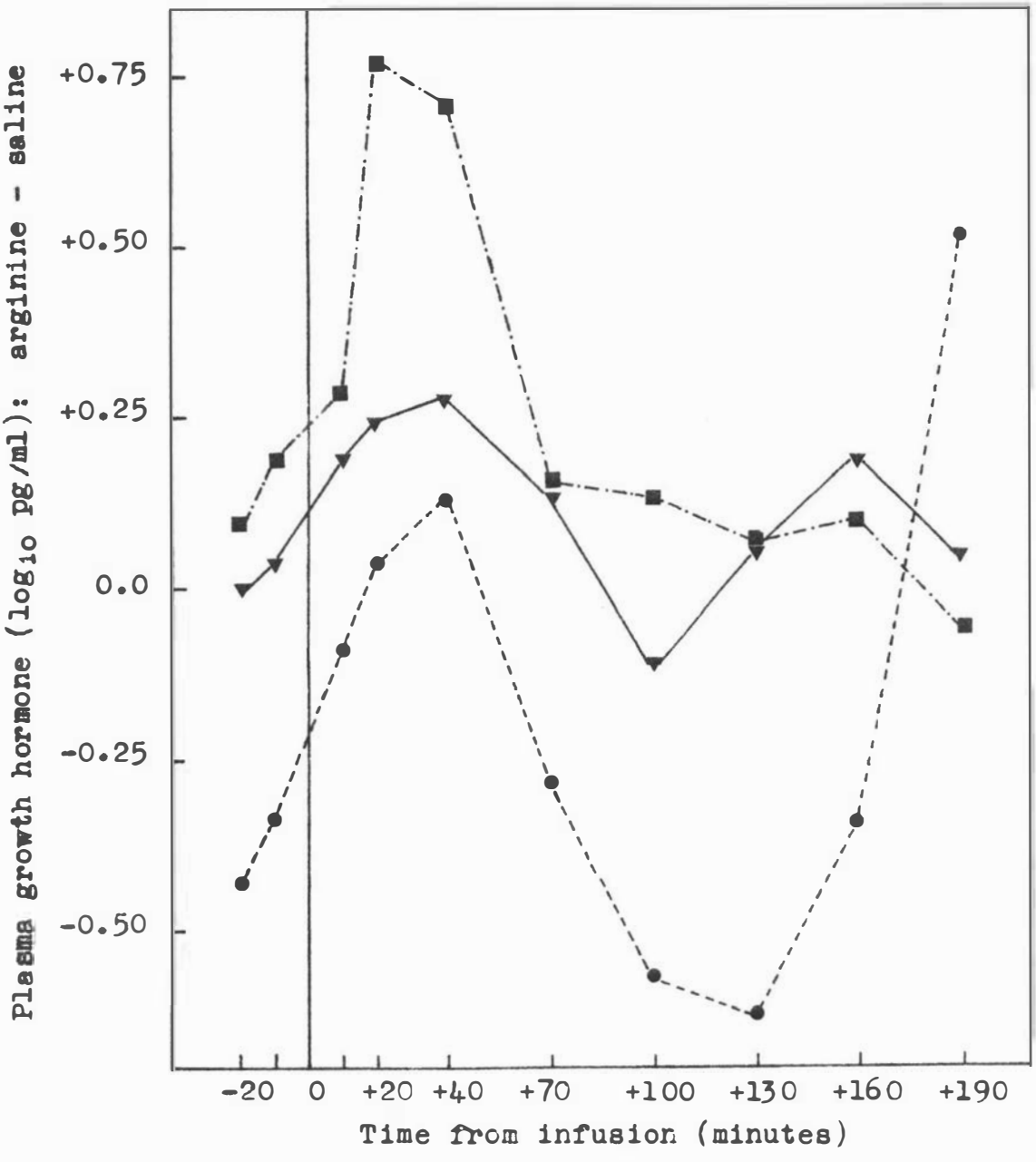

Figure 33 Mean GH levels after arginine - mean GH levels after saline.

——— Group M1
- - - - - - - Group M5
—·—·—·— Group R5

GH levels were highly variable, with large differences between calves and also peaks occurring apparently at 'random', within individual calves. The response to arginine was therefore rather difficult to detect, and plasma levels did not differ from those of controls.

Following the administration of 1 ml/kg body weight (bw) of 1.72 M intravenous saline, a decrease was noted in both GH and prolactin levels. Although it was not significant for prolactin, it indicated a possible inhibitory effect of the osmotic stimulus on pituitary function. Because the arginine infusion was likely to be bringing about the same osmotic effects, a more realistic indication of the effects of arginine per se may be given by the difference between the effect of saline treatment and of arginine treatment (Figure 33).

3.3.3 Discussion

Glucose levels in untreated calves were high in group M1 and M5 and significantly lower in group R5 (Figure 25 and Table XXVIII). The lower glucose levels appear to be associated with the transition to ruminant digestion, but it was clear from pre-treatment levels shown in Figure 29, that glucose levels were rather variable and that this relationship does not always hold. To some extent, therefore, the data support the conclusion of Preston (1963) and Huber (1968), that blood glucose levels show a decrease with age during the first six weeks of life of the young calf, but that the decrease

is not necessarily associated with the change to solid feed and ruminant status.

Insulin levels on the control day, followed a similar general pattern to glucose levels, being highest in group M1. Table XXVIII indicates that group M5 had lower levels than group R5, but again the pre-treatment levels shown in Figure 30 suggest that this was an age-related rather than a diet-related drop in insulin levels.

The glucose and insulin responses to arginine were not a result of the infusion itself, or of the change in osmolality, because no change was observed in response to saline infusion (Figures 29 and 30). The timing of the glucose and insulin responses indicated that the increase in both was a primary response to the arginine, and that the subsequent hypoglycaemia was a secondary effect probably attributable to the high insulin levels. The size of both peaks was significantly higher in group M5 than in either of the other two groups (Table XXIX and XXX).

The new-born ruminant is usually considered to be functionally a monogastric animal. By maintaining one group of calves on a diet consisting solely of teat-administered milk, it was expected that a monogastric mode of digestion would be maintained in group M5, which could then be compared with ruminant calves of the same age. A decrease in glucose utilisation rate, and an increased resistance to insulin, are developments which have been observed to correlate with rumen development

(Stern et al, 1971). What was observed here was contrary to what was expected in that there was virtually no difference between the week-old calves and the five-week ruminant calves, and the change to solid feed diet appeared to bring about no change in the responsiveness either of the insulin secretion, or of the subsequent hypoglycaemia.

The increased responsiveness of Group M5 is likewise hard to explain, because that group had one factor (either age or diet) in common with each of the other groups. It can only be explained after a brief consideration of the reason why arginine should elicit peaks of glucose and insulin. There are two endocrinological mechanisms by which a glucose increase could have occurred.

1. A glucose increase as a result of an epinephrine response.

There is very little evidence to suggest that the arginine infusion brought about an epinephrine response. There were no signs of adverse side-effects in the calves, and no glucose change following equimolar saline infusion (Figure 29 and Table XXIX). However, such a response should not be ruled out, since the arginine dose administered was very much higher than the arginine levels which would naturally be reached in the circulation, and it may have caused a severe physiological imbalance.

The five-week-old milk-fed calves were observed

to be very much more responsive to human contact (in terms of their physical activity and movements) than either of the other two groups, and their decreased body weight gain relative to group R5 (despite the fact that both groups were fed a ration calculated to produce the same growth rate) suggests that they were possibly in a state of continuous irritability as a result of being deprived of solid feed. If group M5 calves were in such a state, an increased epinephrine output in response to a minor adverse stimulus such as the arginine infusion, could well have been the result.

2. Glucose increase as a result of glucagon secretion.

The fact that glucagon has been found to be released in response to arginine infusion in isolated perfused rat pancreas (Gerich et al, 1974: Pagliara et al, 1974) makes a glucagon-mediated glucose response seem likely. Moreover, glucagon and insulin have been observed to be mutually stimulatory (page 6) and an insulin response of the magnitude of that observed in Figure 30 would be unlikely to occur in the absence of a glucagon response.

Glucagon levels have been relatively little measured in ruminants (Bassett, 1975), and so one can only speculate on possible reasons why group M5 should have higher glucose and insulin peaks after arginine infusion, than either of the other groups. Either it was because the glucagon : insulin ratio momentarily became higher in group M5 than in the other groups, resulting in a more intense stimulus for glucose release; or because

the same amount of glucagon may have been secreted in all calves, but group M5 calves were more immediately responsive to glucagon. The relatively shallow hypoglycaemia reached by calves of group R5, could also be a consequence of a more sluggish response to glucagon than that of group M5.

Basal levels of GH and prolactin were significantly higher in the week-old calves than in older calves. High neonatal GH levels have consistently been observed in ruminants (Reyneart et al, 1976: Hertelendy et al, 1969) and in non-ruminants (Tsushima et al, 1971). Prolactin levels, on the other hand, are lower in the human foetus (Friesen and Hwang, 1973) and neonatal mice (Sinha et al, 1972 b), and no change was observed with age in very young bulls (Tucker et al, 1974).

Following the arginine stimulus, a peak in GH levels was apparent only when account was taken of the decrease in GH levels after a saline infusion at the same volume and osmolality as the arginine infusion. Prolactin levels increased consistently following arginine treatment, although the change was less significant in week-old calves. The pattern of the prolactin response between different groups, follows that of insulin and glucose to some extent, with group M5 calves being significantly more responsive than either of the other two groups, which were not very different from each other.

The prolactin and insulin responses to the arginine treatment were consistent with those found by other workers (Davis, 1972), but the calves in the present

study appear to be unusual in not producing a similar and obvious increase in GH levels (Davis, 1972: Hertelendy et al, 1970: Stern et al, 1971: Reyneart et al, 1975). GH has also been observed to be released in response to glucagon stimulation in man (Cain et al, 1970) so the GH response usually observed could be either direct or an indirect response to the arginine infusion.

The reason for the discrepancy regarding a GH response, may be that the dose of arginine used was smaller than that used in other studies, most of which used 0.5 g/kg bw, as compared with 0.3 g/kg bw used in the present study. There have been observations (Hertelendy et al, 1970) that the GH response was less sensitive than the insulin response, and was very much smaller at the lower arginine dosage of 0.25 g/kg bw. In addition, the GH levels were highly variable between calves, and also individual calves showed very large increases in GH levels, which seemed to occur at random throughout the sampling. Despite the use of a logarithmic transformation, it was still apparent from these data that the mean GH level was often disproportionately influenced by the presence of just one abnormally high result. So a GH response, already small because of the low arginine dose, could easily have been masked by the random fluctuations which also occurred.

The presence of 'random' peaks in GH levels does not give any reason for the GH assay to be suspect, because episodic GH release is typical for most species (page 18 - 19). Moreover, the same GH assay system was

used in Experiment 5, and results of that experiment showed that sheep and lambs produced a repeatable GH peak in response to an arginine infusion.

It is of interest that group M5 GH levels fell within a greater range (Table XXXI) and had larger random peaks than in the other groups of calves. It seems that calves of group M5 were more responsive in all aspects of all hormones measured, than other calves. Since GH release has many times been associated with noxious stimuli and adverse environmental conditions in cattle and in primates (page 20), the general over-responsiveness of group M5 could be related to the abnormality of depriving calves of five weeks of age, of solid feed, in that a milk-only diet caused a severe degree of deprivation or hunger (page 159).

The significant decrease in GH following the osmotic stimulus of the saline treatment, could indicate that increased osmolality of the blood had an inhibitory effect on pituitary function, with a possible involvement of pituitary hormones in the maintenance of constant osmotic pressure in the circulation. The decrease in prolactin levels at that time was not significant, but it did occur in 24 out of 28 saline treatments, and therefore lends support to the observation with GH. Other workers have suggested a similar involvement of pituitary hormones in maintenance of osmotic balance, but results obtained have shown either no relationship (Mattheij, 1977) or a stimulatory effect of increased osmotic pressure on prolactin secretion (Buckman and Peake, 1973),

a situation which was the reverse of the results of this study.

3.3.4 Conclusions

1. Plasma levels of glucose and of the three hormones measured, all decreased with age during the first 6 weeks of life of the calves, but it was not clear whether or not the decrease was related to the change to the ruminant mode of digestion.

2. Plasma glucose and insulin levels both rose immediately following a single intravenous bolus of 0.3 g/kg body weight of arginine. Plasma glucose levels then fell to produce a deep hypoglycaemia, while insulin returned to basal levels more slowly.

3. The glucose and insulin responses were rather similar between the week-old calves and the five-week-old ruminant calves. However, the five-week-old milk-fed calves were significantly more responsive than the other two groups, both in the size and extent of the insulin and glucose peaks, and also in the extent of the hypoglycaemia.

4. There was no obvious GH response to the arginine infusion. However, if intravenous infusion of saline which was equimolar to the arginine dosage, was given, it brought about an immediate decrease in GH

levels. When the response to saline was taken into account, it appeared that the arginine was preventing the inhibition caused by saline, and therefore that the arginine *per se* was possibly having a stimulatory effect on GH secretion.

5. Plasma prolactin levels increased in response to the arginine infusion. The increase was smallest in the week-old calves, whose prolactin levels were already higher than in the other groups. The prolactin increase was significantly larger in the five-week-old milk-fed calves than in either of the other two groups, a situation which also applied in the case of the responses of insulin and glucose levels to arginine.

6. The five-week-old milk-fed calves showed larger oscillations in the episodic periods of GH secretion than the other calves. This observation, and the greater responsiveness of the same group of calves in the other respects studied, was discussed in relation to their possible adrenergic status and in relation to the role of glucagon.

3.4 EXPERIMENT 4

3.4.1 Experimental design

The aim of Experiment 4 was to investigate the relationships between energy substrate levels and insulin and GH levels in more detail, Experiment 2 having been only a pilot experiment. It was also of interest to conduct a further investigation into the different hormonal characteristics of the three groups of calves treated in the same way as in Experiment 3.

Two points were considered:

(1) Would the treatment effects (effects of age and diet) on hormone and glucose levels shown in Experiment 3, be repeatable in another experiment?

(2) Because the different response to arginine in the three groups involved differences in insulin and glucose, the different responses to arginine seemed to be a reflection of a different type of energy substrate metabolism in the three groups. Further light would be shed on the phenomenon by direct investigation of the way in which insulin and glucose levels inter-relate in the three groups.

The experimental conditions of the groups of calves were identical to those followed in Experiment 3, except that there were consistently only four calves in each group, and calves were fed sufficient for a body weight gain of 0.5 kg per day, rather than 0.3. The calves were all female Friesian-Jersey cross-bred.

Table XXXIII Experimental procedure for each six-day sampling period.

Period	Day	Treatment	Dosage	Volume ml/kg bw
1	1	(saline	0.9% NaCl	0.625
	2	(insulin	0.75 U/kg bw	0.625
	3	(glucose	1.4 mM/kg bw	0.625
2	4	(saline	0.9% NaCl	0.625
	5	(insulin	0.75 U/kg bw	0.625
	6	(glucose	1.4 mM/kg bw	0.625

The experimental procedure is outlined in Table XXXIII. In the present experiment, no attempt was made to allow for the different osmolality of the infused solutions. Glucose (May and Baker Ltd., U.K.) was dissolved before sterilising the solution by autoclaving, and insulin was added in a sterile manner by syringe, to physiological saline which was pre-autoclaved. Insulin was given in the form of protamine-zinc insulin (Burroughs Wellcome Co. (NZ) Ltd.). All treatments were administered in a 30-second pulse through the catheter.

As in experiments 2 and 3, each calf acted as its own control within each of period 1 and period 2. In the present experiment, however, the sequence of the three treatments within each period, was chosen at random, in order to overcome the effects of external factors which could have been changing from day to day. The order was therefore different and unpredictable within each period for each individual calf. The blood-sampling regime was identical to that followed on the treatment days of Experiment 3.

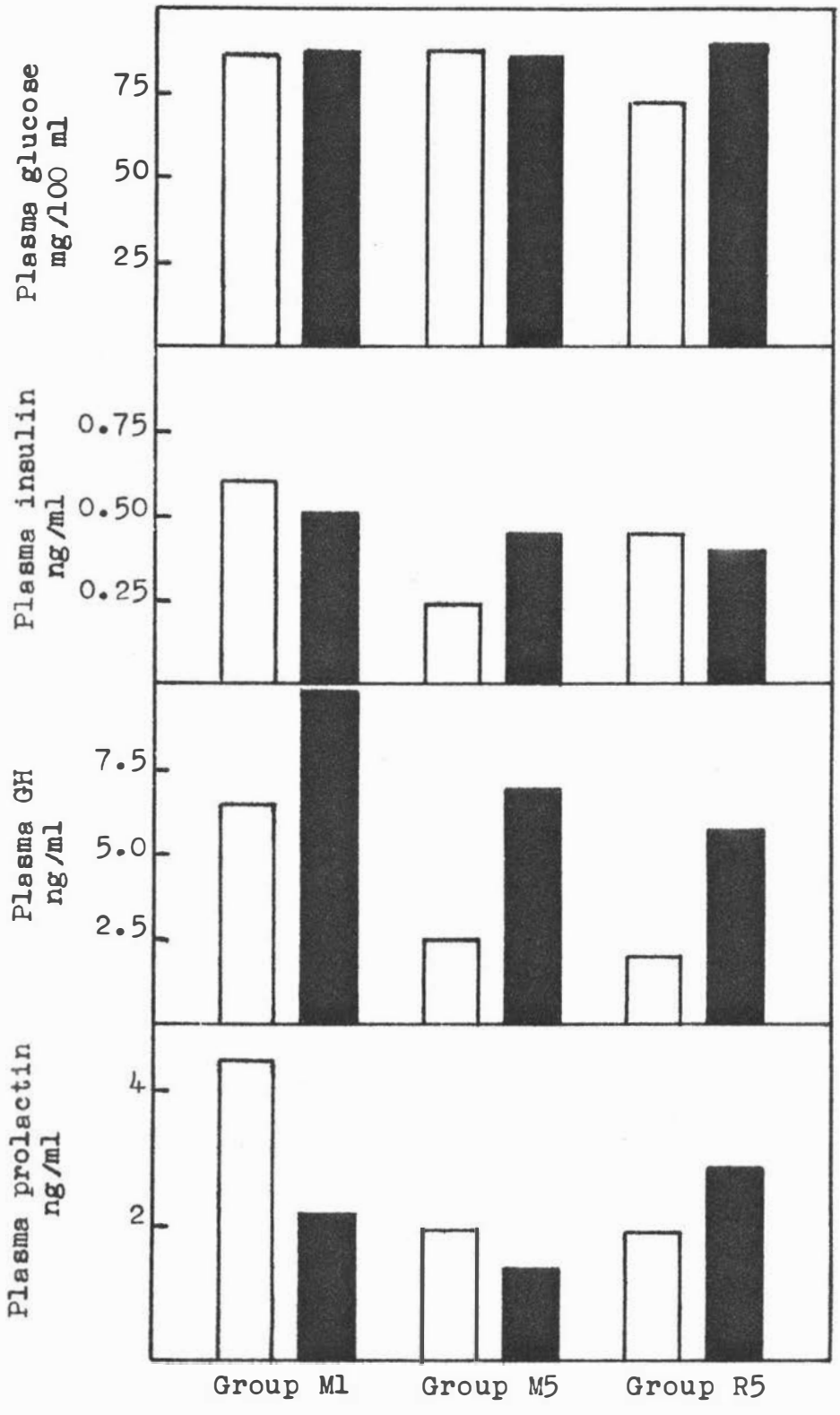

Figure 34 Average of medians for individual calves, of hormone levels in plasma samples taken on control day during Experiment 3 (white bars) and Experiment 4 (black bars).

Table XXXIV Average of medians and ranges for individual calves of samples taken on control day: untransformed data.
+ = $p<0.1$: ++ = $p<0.05$: +++ = $p<0.01$:
each group compared with other two groups combined by Mann-Whitney U test.

	Median			Range		
	M1	M5	R5	M1	M5	R5
Glucose mg/100 ml	87.5	85.9	89.9	13.5	15.9	13.0
Insulin ng/ml	0.51^{+}	0.45	0.40	0.81	0.47	0.58
GH ng/ml	9.83	6.93	5.76	12.27^{+}	23.38^{++}	16.55
Prolactin	2.18	1.39^{+++}	2.89^{+++}	3.07	3.12^{+}	2.37

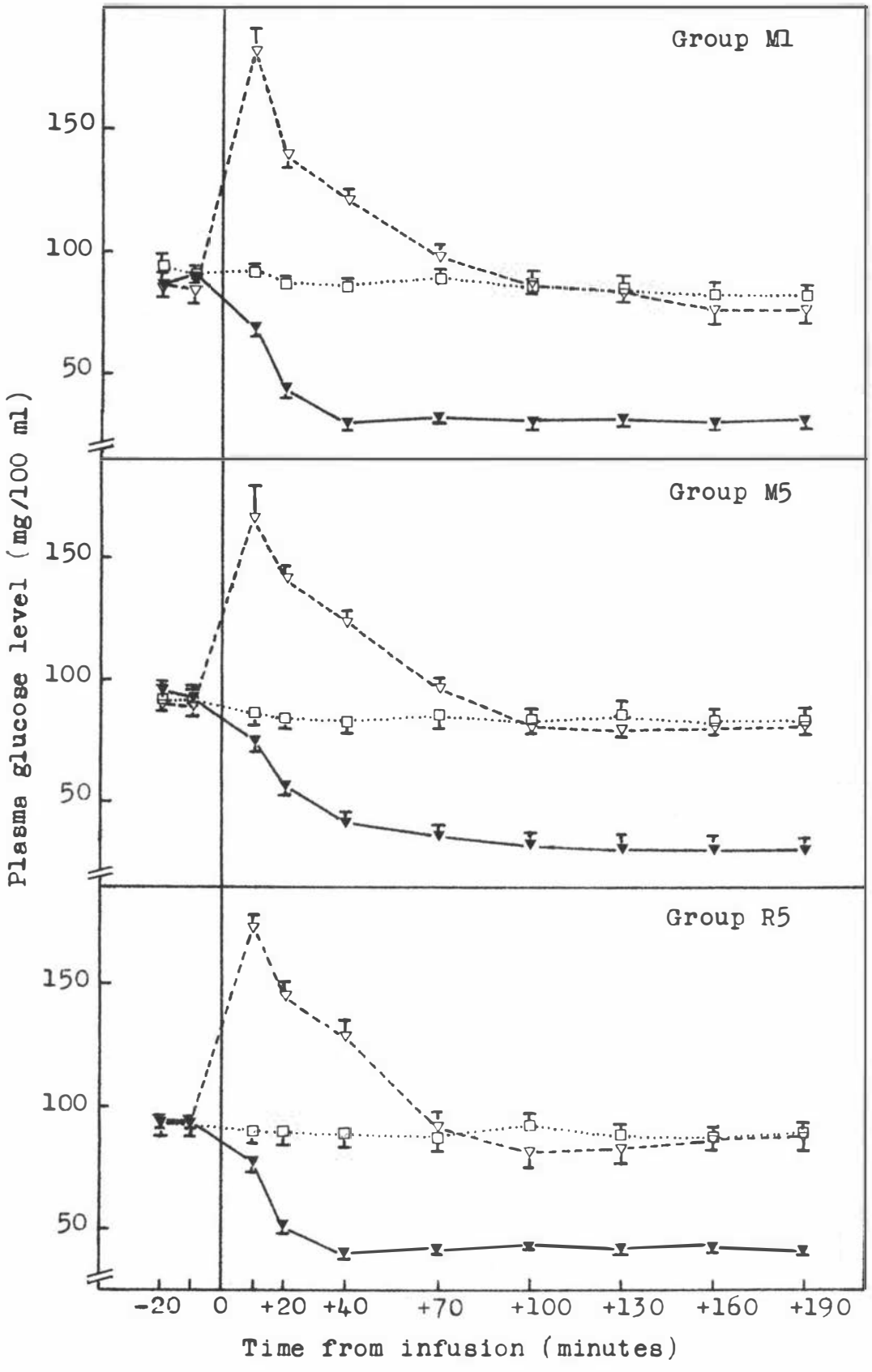

Figure 35 Plasma glucose levels after treatment with insulin (solid lines), glucose (broken lines) or physiological saline (dotted lines). Mean for values obtained on day of each treatment, with vertical bars representing SEM based on variance between calves within different sampling days.

Table XXXVa Plasma glucose levels: mean values for different sample times on days of three different treatments.

NS = not significant: + = $p<0.1$: ++ = $p<0.05$: +++ = $p<0.01$: difference between post-treatment samples adjusted by covariance for differences in pre-treatment levels.

Treatments: S=saline, G=glucose. I=insulin.

Group	Samples	Treatment			Significance		
		S	G	I	G vs I	S vs I	S vs G
M1	1-2	90.9	85.2	89.1			
	3-5	88.3	147.4	47.6	+++	+++	+++
M5	1-2	91.1	87.1	93.9			
	3-5	84.6	141.1	58.2	+++	+++	+++
R5	1-2	92.7	90.4	94.3			
	3-5	89.6	145.7	55.5	+++	+++	+++

Table XXXVb Plasma glucose levels: significance of differences between groups of calves at particular times after treatment.

	G, sample 5	I, sample 4-5	I, sample 7-10
M1 vs M5	NS	+++	NS
M1 vs R5	NS	+	+++
M5 vs R5	NS	NS	+++

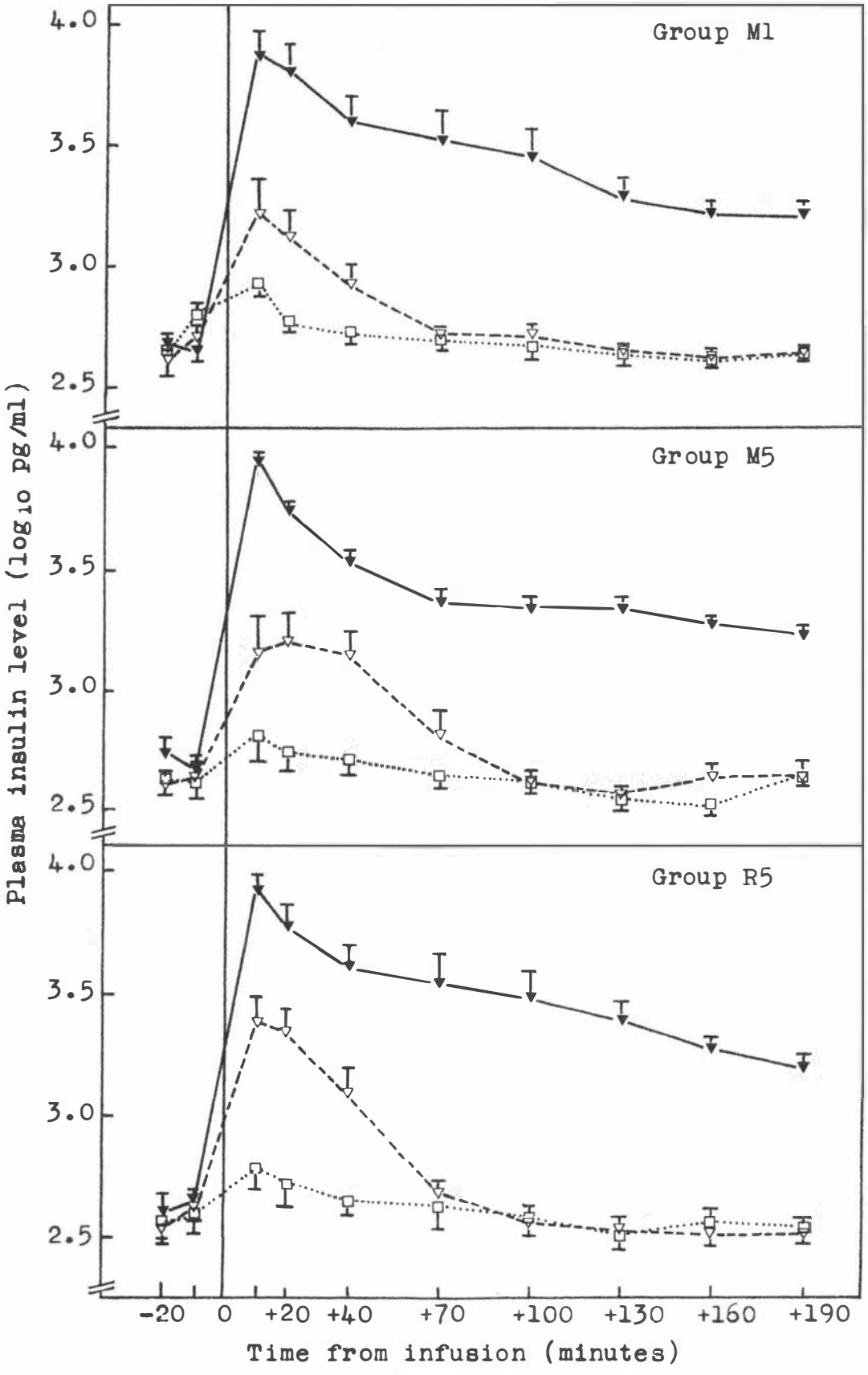

Figure 36 Plasma insulin levels after treatment with insulin (solid lines), glucose (broken lines) or physiological saline (dotted lines). Mean for values obtained on days of each treatment, with vertical bars representing SEM based on variance between calves within different sampling days.

Table XXXVIa Plasma insulin levels: mean values for different sample times on days of three different treatments. Data presented as $\log_{10}$ pg/ml with untransformed data (ng/ml) in parentheses.

NS = not significant: + = $p<0.1$: ++ = $p<0.05$: +++ = $p<0.01$: difference between post-treatment samples adjusted by covariance for differences in pre-treatment levels.

Treatments: S=saline, G=glucose, I=insulin.

Group	Samples	Treatment			Significance		
		S	G	I	G vs I	S vs I	S vs G
M1	1-2	2.72 (0.52)	2.66 (0.46)	2.66 (0.46)			
	3-5	2.81 (0.64)	3.09 (1.24)	3.74 (5.48)	+++	+++	NS
M5	1-2	2.63 (0.43)	2.63 (0.42)	2.71 (0.51)			
	3-5	2.76 (0.58)	3.17 (1.48)	3.75 (5.61)	+++	+++	+++
R5	1-2	2.56 (0.37)	2.57 (0.38)	2.62 (0.42)			
	3-5	2.72 (0.53)	3.27 (1.87)	3.78 (5.98)	+++	+++	+++

Table XXXVI b Plasma insulin levels: significance of differences between groups of calves at particular times after treatment.

	G, 3-5	I, 6
M1 vs M5	NS	NS
M1 vs R5	NS	NS
M5 vs R5	NS	NS

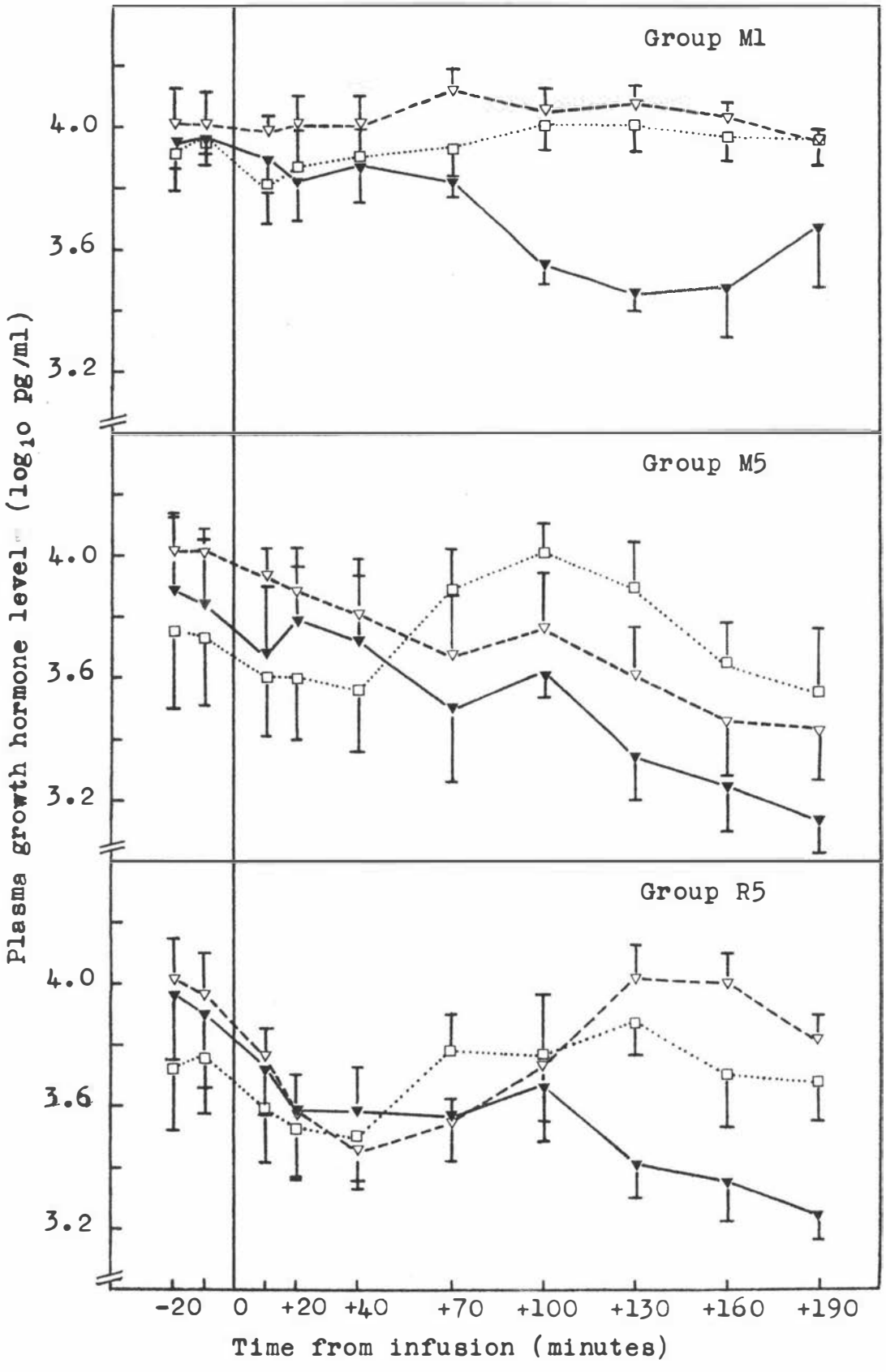

Figure 37 Plasma growth hormone levels after treatment with insulin (solid lines), glucose, (broken lines) or physiological saline (dotted lines). Mean for values obtained on days of each treatment, with vertical bars representing SEM based on variance between calves within different sampling days.

Table XXXVIIa Plasma GH levels: mean values for different sample times on days of three different treatments. Data presented as $\log_{10}$ pg/ml with untransformed data (ng/ml) in parentheses.

NS = not significant: + = $p<0.1$: ++ = $p<0.05$: +++ = $p<0.01$: difference between post-treatment samples adjusted by covariance for varying pretreatment levels.

Treatments: S=saline, G=glucose, I=insulin.

Group	Samples	Treatment			Significance		
		S	G	I	G vs I	S vs I	S vs G
M1	1-2	3.94 (8.74)	4.01 (10.2)	3.95 (8.96)			
	3-5	3.86 (7.18)	4.04 (11.0)	3.90 (8.04)	NS	NS	NS
	8-10	3.99 (9.77)	4.02 (10.4)	3.54 (3.44)	+++	+++	NS
M5	1-2	3.74 (5.60)	4.16 (14.6)	3.86 (7.30)			
	3-5	3.59 (3.89)	3.93 (8.45)	3.73 (5.34)	NS	NS	NS
	8-10	3.69 (4.92)	3.48 (3.00)	3.23 (1.70)	+	++	NS
R5	1-2	3.74 (5.50)	3.99 (9.71)	3.93 (8.61)			
	3-5	3.59 (3.43)	3.60 (3.96)	3.63 (4.27)	NS	NS	NS
	8-10	3.75 (5.66)	3.94 (8.80)	3.33 (2.16)	+++	+++	++

Table XXXVIIb Plasma GH levels: significance of differences between groups of calves at particular times after treatment.

Groups	Saline		Glucose		Insulin	
	3-5	8-10	3-5	8-10	3-5	8-10
M1 vs M5	NS	NS	NS	+++	NS	NS
M1 vs R5	NS	NS	+++	NS	NS	NS
M5 vs R5	NS	NS	++	+++	NS	NS

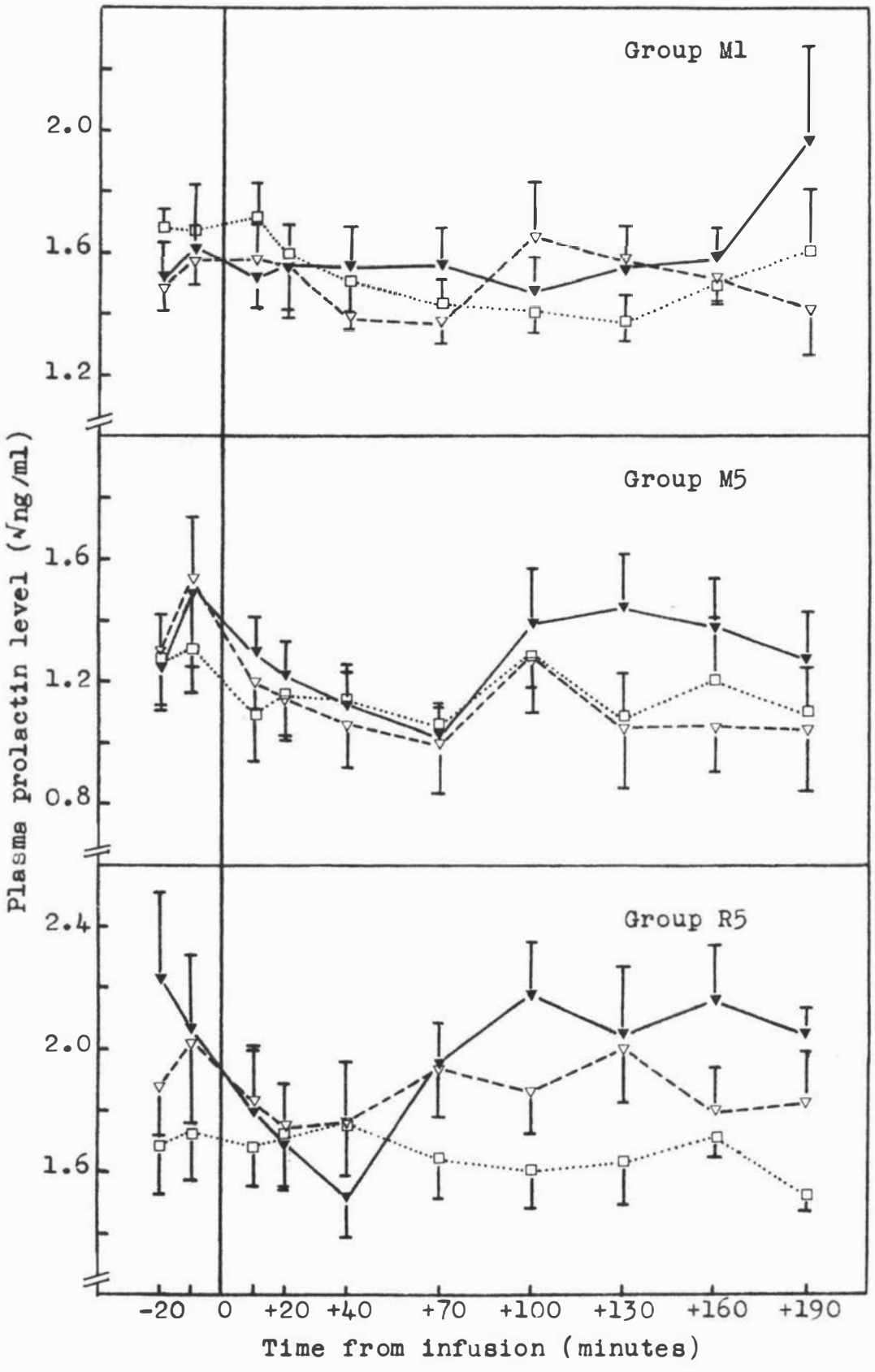

Figure 38 Plasma prolactin levels after treatment with insulin (solid lines), glucose (broken lines) or physiological saline (dotted lines). Mean for values obtained on days of each treatments, with vertical bars representing SEM based on variance between calves within different sampling days.

Table XXXVIIIa Plasma prolactin levels: mean values for different sample times on days of three different treatments. Data presented as √ng/ml with untransformed data (ng/ml) in parentheses.

NS = not significant: + = $p<0.1$: ++ = $p<0.05$: +++ = $p<0.01$: difference between post-treatment samples adjusted by covariance for varying pre-treatment levels.

Treatments: S=saline, G=glucose, I=insulin.

Group	Samples	Treatment S	Treatment G	Treatment I	Significance G vs I	Significance S vs I	Significance S vs G
M1	1-2	1.67 (2.80)	1.53 (2.33)	1.55 (2.41)			
	3-5	1.60 (2.57)	1.51 (2.29)	1.55 (2.39)	NS	NS	NS
	8-10	1.49 (2.23)	1.51 (2.27)	1.70 (2.89)	NS	NS	NS
M5	1-2	1.28 (1.64)	1.37 (1.88)	1.37 (1.87)			
	3-5	1.13 (1.28)	1.14 (1.30)	1.21 (1.47)	NS	NS	NS
	8-10	1.12 (1.27)	0.99 (0.97)	1.35 (1.83)	+++	NS	++
R5	1-2	1.71 (2.91)	2.02 (4.07)	2.14 (4.59)			
	3-5	1.71 (2.95)	1.77 (3.14)	1.67 (2.78)	NS	++	NS
	8-10	1.62 (2.63)	1.90 (3.60)	2.08 (4.33)	NS	NS	NS

Table XXXVIIIb Plasma prolactin levels: significance of differences between groups of calves at particular times after treatments.

Groups	Saline 3-5	Saline 8-10	Glucose 3-5	Glucose 8-10	Insulin 3-5	Insulin 8-10
M1 vs M5	+	NS	++	++	NS	NS
M1 vs R5	NS	NS	NS	NS	NS	NS
M5 vs R5	++	NS	+	+++	NS	NS

3.4.2 Results

The weekly body weights of the calves are recorded in Figure 49. All calves maintained excellent health, except Calf 20 (group M1), which developed severe blood scouring after the first sampling week was over. For the five-week-old sampling week, it was replaced by Calf 18, a calf of the same age, which had previously been fed a diet consisting only of liquids administered through a teat. Because of the lack of continuity brought about by using two different calves, however, the body weights of neither have been recorded in Figure 49. Calf 18 had also previously been shown by radiography that all of its diet was by-passing the rumen (Dr. I.M. Brookes[1], personal communication), a result which confirmed that teat-feeding was effective in stimulating oesophageal groove closure.

Table XXXIV shows the average within each group, of the median hormone level for each calf, of the samples taken on the days of saline treatment, and Figure 34 shows a comparison between median data, and that obtained on control days of Experiment 3. In the present experiment, insulin levels were significantly higher in week-old calves than in five-week-old calves of either group, and GH levels were also higher in young calves, although the difference was not significant. Prolactin levels were significantly higher in five-week-old ruminant calves.

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[1] Dr. I.M. Brookes, Department of Dairy Husbandry, Massey University, Palmerston North.

Following glucose infusion, there was a large insulin secretion in all groups of calves (Figure 36, broken line). The increase in insulin levels was of the same order in all groups, but was highly significantly different from the saline treatment in only the two groups of five-week-old calves (Table XXXVI). The insulin levels of week-old calves also returned to pre-treatment levels more rapidly than in the other groups. There were no significant differences between groups in the 'glucose tolerance', i.e. the time taken for glucose levels to return to basal levels (Figure 35). There were no significant GH or prolactin responses to glucose treatment (Figures 37 and 38).

After insulin treatment, glucose levels decreased dramatically (Figure 35), the response being more rapid in week-old calves than in either of the other two groups, as shown by the significant difference between glucose levels between groups during samples 4 - 5 after insulin (Table XXXV). The fall in glucose levels was highly significant in all groups (Table XXXV), lasting until after the termination of sampling. Table XXXV also illustrates that it was significantly deeper in the two groups of milk-fed calves, than in the ruminant calves.

There was no immediate GH change following the insulin treatment, but a significant decrease in plasma GH levels occurred consistently in all groups of calves, 2 - 3 hours after the infusion (Figure 37 and Table XXXVII). At the same time as the GH decrease, prolactin levels (also

initially un-responsive) were elevated, but not significantly in relation to levels after saline treatment (Figure 38, Table XXXVIII).

3.4.3 Discussion

(a) Saline treatment

For insulin and GH levels on the days of saline treatment, the results in Table XXXIV agree to some extent with Table XXVIII, Experiment 3, in that levels of both hormones were higher in week-old calves (Figure 34). However, the present experiment showed no age-related decrease in plasma glucose levels, and also the distribution of the prolactin data was very different from those observed in Experiment 3, with group R5 having significantly higher levels. The range of all hormone levels was generally much greater than in Experiment 3, especially in group M5.

One characteristic of the results obtained on saline treatment days, was an increase in insulin levels in samples 2 - 5. It is unlikely that the increased insulin levels were caused by insulin secretion in response to the infusion, because insulin levels began tc increase before Sample 2, before the infusion, and also no effect of saline was seen on insulin secretion in Experiments 2 or 3, or by other workers e.g. Ambo et al (1973), Horino et al (1968). The only feasible explanation for the phenomenon is that contamination was occurring between samples. The experimental design required that saline-treated calves

should be sampled within 1 minute of insulin treated calves, and all samples centrifuged immediately: the same syringe was used for sampling all four calves on any one day, and the same Pasteur pipette was used for each group of six samples being centrifuged. Although both syringes and pipettes were rinsed between samples, insulin adheres strongly to glassware (Yalow and Berson, 1960), and also the insulin solution being administered to the calves was 36×10^6 times as concentrated as the levels being measured in the plasma of control calves. Thus the degree of contamination which led to a doubling or trebling of basal levels, represents only a minute fraction of the amount being handled. The same problem was not encountered in the glucose levels, because the solution being administered was only 252 times plasma glucose levels.

(b) Glucose treatment

The insulin contamination could have been sufficient to render the insulin control data invalid in the present experiment. By the same process, the insulin levels following glucose treatment would also be expected to have the same degree of contamination with insulin: therefore a comparison of the levels measured after glucose treatment, with those measured after saline treatment (Figure 36) is not without merit for indicating that some insulin secretion was likely to have occurred as a result of glucose treatment. Thus the results appear to be in agreement with those of Experiment 2. To inspect

the data in sufficient detail to analyse the degree of responsiveness of individual calves, would be unwise under the circumstances, as would a comparison between the size of the insulin response to glucose treatment, between Experiments 2 and 4.

The uniformity of the time taken for glucose levels to return to normal after glucose infusion, confirms the results of Preston (1963) who observed no differences between glucose tolerance of calves on three different diets up to 6 weeks of age. Stern et al (1971) and Preston (1963) observed that glucose disappearance rates decreased with age in young ruminants exceeding 8 to 14 weeks of age.

The fact that GH and prolactin did not respond to the glucose treatment is also inagreement with Experiment 2, and the phenomena have been discussed on page 136.

(c) Insulin treatment

Following treatment with insulin, the hypoglycaemia was maintained for a very long time, because of the slow-releasing properties of protamine zinc insulin. The fact that week-old calves achieved the hypoglycaemia significantly more rapidly than older calves (Table XXXV, Figure 35) had an interesting parallel in Experiment 3, in that the hypoglycaemia as a secondary response to the arginine stimulus was also achieved more rapidly in group M1. Furthermore, the fact that the hypoglycaemia finally

reached was significantly less deep in group R5, was also the case for the hypoglycaemia after arginine. It seems likely, therefore, that the differences in the response of the different groups of calves to arginine, were a direct reflection of a basic difference in their responsiveness to insulin, whether the insulin was endogenous or administered. Calves appeared to become more resistant to insulin, both with increasing age, and also with advancing rumen development.

A similar phenomenon was observed by Stern et al (1971), in response to intravenous administration of insulin (2.0 U/$kg^{0.75}$), glucose levels decreased more rapidly and reached significantly lower levels in suckling lambs and kids, than in weanling or mature sheep and goats. It is interesting that in the present study, the increased resistance to insulin was detectable, while the decreased glucose disappearance rate with age noted by Preston (1963) and by Stern et al (1971), a phenomenon which is probably linked to the degree of insulin resistance, was not detectable until the young ruminants were more than 3 weeks older than the calves studied here. The early development of insulin resistance indicates that the changes involved in the maturation of the energy metabolism of the young ruminant begin at a very early age, and to some extent (e.g. in the speed of the hypoglycaemic response to insulin), the changes occur at 5 weeks of age even in animals which have not yet begun to use the rumen. The changes are to some extent constitutional for the young

ruminant, although the adoption of the ruminating habit speeds up the process of change.

The GH response to insulin-induced hypoglycaemia is well-documented in all other ruminants studied, as is the GH response to arginine (Wallace and Bassett, 1970: Stern et al, 1971: Hertelendy and Kipnis, 1973: Reyneart et al, 1975). The lack of responsiveness to insulin in the present study could be associated with the fact that protamine zinc insulin was used. In the case of man, it has been observed that the GH secretion occurs in response to the fall in levels of blood glucose (Roth et al, 1964) regardless of absolute levels of glucose. Possible slowing of the effects of insulin by the protamine zinc preparation could have rendered the blood glucose decrease too slow for the GH to respond. There are, however, two reasons why such a theory may not apply here:

(1) Blood glucose levels in the present experiment took the same length of time (30 - 40 minutes) to reach minimum levels after insulin, as they did in the study of Stern et al (1971).

(2) It has been postulated (Reyneart et al, 1975) that in ruminants it is the associated decrease in plasma FFAs which causes the GH response to insulin, rather than the hypoglycaemia. To answer the question of whether the protamine zinc preparation prevented the GH response for any reason, the experiment should be repeated with pure insulin as well as with protamine zinc insulin, with plasma FFA measurements made.

What appears to be more likely in view of the results of Experiments 2 - 4, is that in the calves used here, GH was not responsive to any of the stimuli which elicit its secretion in other young ruminants. It has already been noted in the Discussion of Experiment 2 (page 136) that it is not unusual for certain species of animals to appear to have an unresponsive GH secretory mechanism when the classic stimuli are used. Since it is now clear that many of the metabolic effects of GH take place via the secretion of somatomedins (pages 24 - 28), GH secretion and its relationship to metabolites may no longer be seen as a simple feedback situation. The classic stimuli for GH secretion may be less potent under conditions where somatomedin levels are relatively high. Without actually measuring plasma somatomedin, it is not possible to predict its levels, since McConaghey (1972) observed that a considerable time-lag existed in the liver production of somatomedins in response to changes in GH levels. In relation to the young calf, the importance of somatomedins in influencing the non-responsiveness of GH is open to some doubt in view of the fact that neonatal rats and rabbits had very low somatomedin levels for the first 11 days of life (Stuart et al, 1976: Charrier, 1978).

Another factor influencing the present results was the wide variation in plasma GH levels at all times. The large frequency of 'spontaneous' pulses of GH secretion would lead to a situation in which most calves would be under the metabolic influence of GH at some time within

the three hours preceeding the treatment. Under such circumstances it is possible that GH secretion is inhibited. Furthermore, the difficulty with which basal or peak GH levels could be detected, could lead to a situation in which an increase in GH secretion is masked. The use of analysis of covariance has overcome the problem to some extent, but it is still apparent that the GH levels of calves in the present study were behaving differently from young ruminants in other studies.

The phenomenon of significantly depressed GH levels after a period of 2 - 3 hours following the insulin infusion, has also not been noted in the literature. However, the present study is unique in measuring the effects of protamine zinc insulin on plasma GH levels, and the hypoglycaemia was very prolonged (Figure 36). Maintaining such low plasma glucose levels for a period of several hours, would have profound and possibly far-reaching effects on the calves' physiology. Therefore the lower GH levels, and also the higher prolactin levels at that time, could well be side effects of a hypothalamic response to long-term hypoglycaemia. Since GH and prolactin are thought to be under opposite types of adrenergic control (pages 22 and 31), and their responses to long-term hypoglycaemia were opposite to each other, a central adrenergic influence could well be the cause of both.

Alternatively, one very likely response to the long-term hypoglycaemia, would be a stimulation in glucagon secretion (Bassett, 1975). There is some evidence that glucagon also stimulates the secretion of GH (Cain

et al (1970). After a period of several hours of GH secretion, an exhaustion of the system - either of glucagon secretion or of GH secretion - could result in a dramatic decrease in GH levels. The fact that no initial increase in GH levels was detectable here, however, makes this explanation seem less feasible than the hypothalamic adrenergic response postulated above.

3.4.4 Conclusions

1. The age-related differences in glucose and hormone levels observed in untreated calves in Experiment 3, were not repeated in the present experiment. The differences were either not significant, or in the case of prolactin, the trend was reversed.

2. Plasma insulin levels increased following 1.4 mM/kg body weight glucose infusion, in a similar manner to the response to glucose in Experiment 2.

3. Neither GH nor prolactin responded to glucose infusion.

4. The infusion of 0.75 U/kg body weight of insulin, gave rise to a prolonged hypoglycaemia. There was evidence for increasing resistance to insulin, both with increasing age, and also with advancing rumen development. This evidence supported some observations made in Experiment 3.

5. There was no immediate response of either GH or prolactin levels, as a result of the insulin infusion.

6. After hypoglycaemia had been maintained for a period of 2 - 3 hours, there was a significant decrease in GH levels, and a non-significant increase in prolactin levels. These were thought to be an indirect consequence of the effects of maintaining low blood glucose levels for such a long period of time.

3.5 EXPERIMENT 5: WORK WITH LAMBS AND EWES

3.5.1 Experimental design

The aim of Experiment 5 was to investigate the relationship between hormone levels, energy substrate metabolism and the feeding or suckling stimulus in lambs and their respective ewes. Dr, J.M. Gooden[1] carried out the blood sampling and the glucose and free fatty acids (FFAs) assays, with the aim of relating the results to carcass characteristics of the ewes and lambs. The author of the present study carried out the hormone assays, with the aim of comparing the lambs' hormonal responses with those of the calves observed in Experiments 1 and 3.

Six Southdown ewes, each with a single male lamb, were used in the experiment. All animals were catheterised in both jugular veins when the lambs were three weeks of age, and then again when they were 12 weeks of age. The lambs were all weaned at eight weeks of age, so that catheterisation and blood sampling took place five weeks before weaning and four weeks after weaning.

Following each catheterisation, the blood sampling schedule was as follows. One day after catheterisation, ewes were starved for a period of 21 hours, and lambs for 17 hours. Blood samples were taken at 30-minute intervals for two hours, and then the starvation period was brought to an end by allowing them access to solid feed (lucerne chaff and crushed barley in a 4 : 1 ratio) and the suckling lambs were given access to their dams. Blood sampling continued at 30-minute intervals for

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[1] Applied Biochemistry Division, D.S.I.R., Palmerston North.

a further three hours, and then at hourly intervals for three more hours.

After a period of three days without blood sampling, ewes and lambs which had again been starved for 21 and 17 hours respectively were blood-sampled at 30-minute intervals for two hours. A 30-minute infusion of arginine was then given at the rate of 0.5 g/kg body weight, in a volume of 50 ml of water for ewes, and 25 ml for lambs. During the infusion, blood samples were taken from the opposite catheter, at five-minute intervals, and afterwards at 30-minute intervals until two hours after the start of the infusion.

Ewes and lambs were weighed on the day of each arginine treatment, and body weights are recorded in Table XLVIII.

3.5.2 Results

The effect of feeding on insulin and GH levels in the three-week-old lambs and their dams, is shown in Figure 39. Following feeding, a small, slow but significant increase in insulin levels occurred in ewes, and contrasted with a rapid, nearly ten-fold, increase which occurred in suckling lambs. GH levels were variable before feeding, but an increase which occurred during the two hours after feeding was nonetheless significant for both lambs and ewes (Table XL).

Table XXXIX Means of medians and ranges for individual lambs and ewes, of insulin and GH levels in plasma samples taken before feeding or arginine treatment: untransformed data (ng/ml).

+ = $p<0.1$: ++ = $p<0.05$: +++ = $p<0.01$: comparing each group with itself at a different age, by Mann-Whitney U test.

	Suckling lambs		Weaned lambs	Lactating ewes		Non-lactating ewes
Insulin						
Medians	0.82		0.73	1.29	+++	0.54
Ranges	1.12		0.83	1.37	+++	0.29
GH						
Medians	3.73	+	1.43	2.63	+++	0.76
Ranges	19.22	+	5.79	17.57	+++	4.91

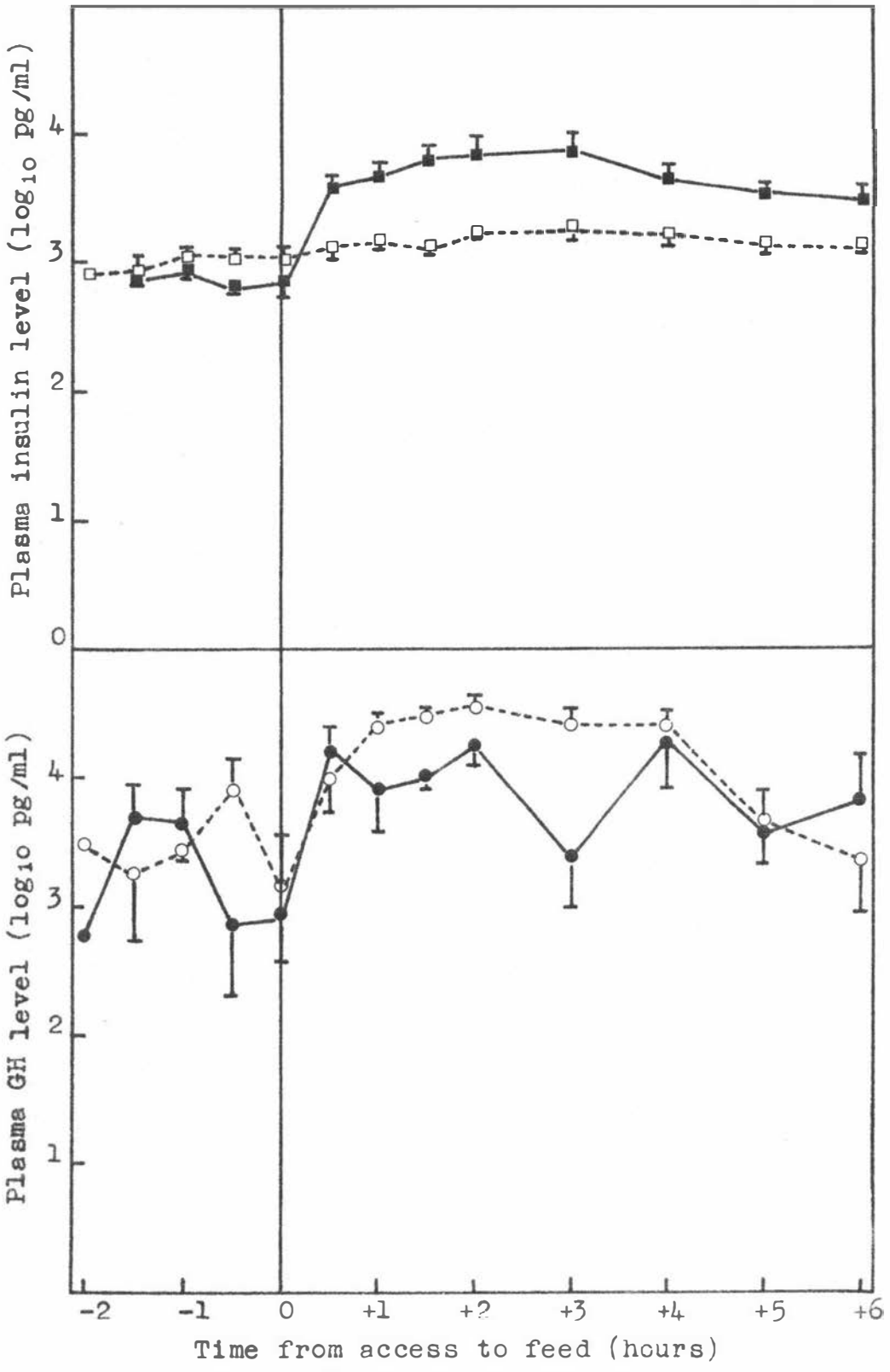

Figure 39 Plasma levels of insulin and growth hormone before and after feeding in suckling lambs (solid lines) and lactating ewes (broken lines).

Table XL Plasma levels of insulin and GH before and after feeding in suckling lambs and lactating ewes. Data expressed as $\log_{10}$ pg/ml, with untransformed data (ng/ml) in parentheses.

NS = not significant: + = $p<0.1$: ++ = $p<0.05$: +++ = $p<0.01$: for analysis of variance comparing hormone levels before and after feeding, within each group of animals.

	Time	Insulin	Growth hormone
Suckling lambs	before	2.88 (0.76)	3.29 (1.93)
	after	3.88 +++ (7.53)	4.10 +++ (12.61)
Lactating ewes	before	3.05 (1.11)	3.43 (2.68)
	after	3.23 ++ (1.69)	4.37 ++ (23.66)

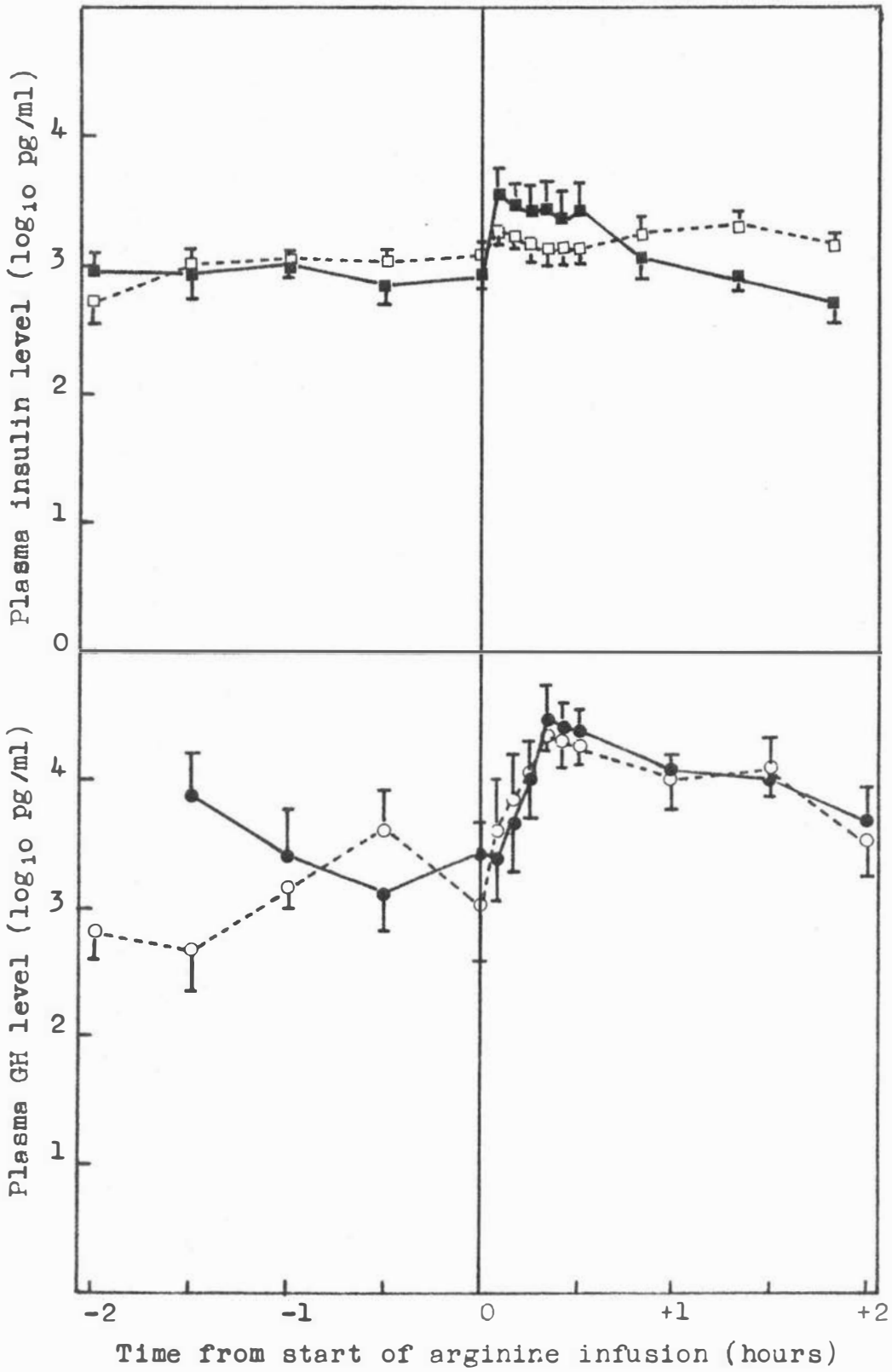

Figure 40 Plasma levels of insulin and GH before and after arginine treatment in suckling lambs (solid lines) and lactating ewes (broken lines).

Table XLI Plasma levels of insulin and GH before and after arginine treatment in suckling lambs and lactating ewes. Data expressed as $\log_{10}$ pg/ml, with untransformed data (ng/ml) in parentheses.

NS = not significant: + = $p<0.1$: ++ = $p<0.05$: +++ = $p<0.01$: for analysis of variance comparing hormone levels before and after arginine treatment, within each group of animals.

	Time	Insulin	Growth hormone
Suckling lambs	before	2.94 (0.87)	2.88 (0.77)
	after	3.52 +++ (3.34)	3.61 +++ (4.13)
Lactating ewes	before	3.06 (1.14)	3.11 (1.29)
	after	3.25 ++ (1.78)	4.24 ++ (17.50)

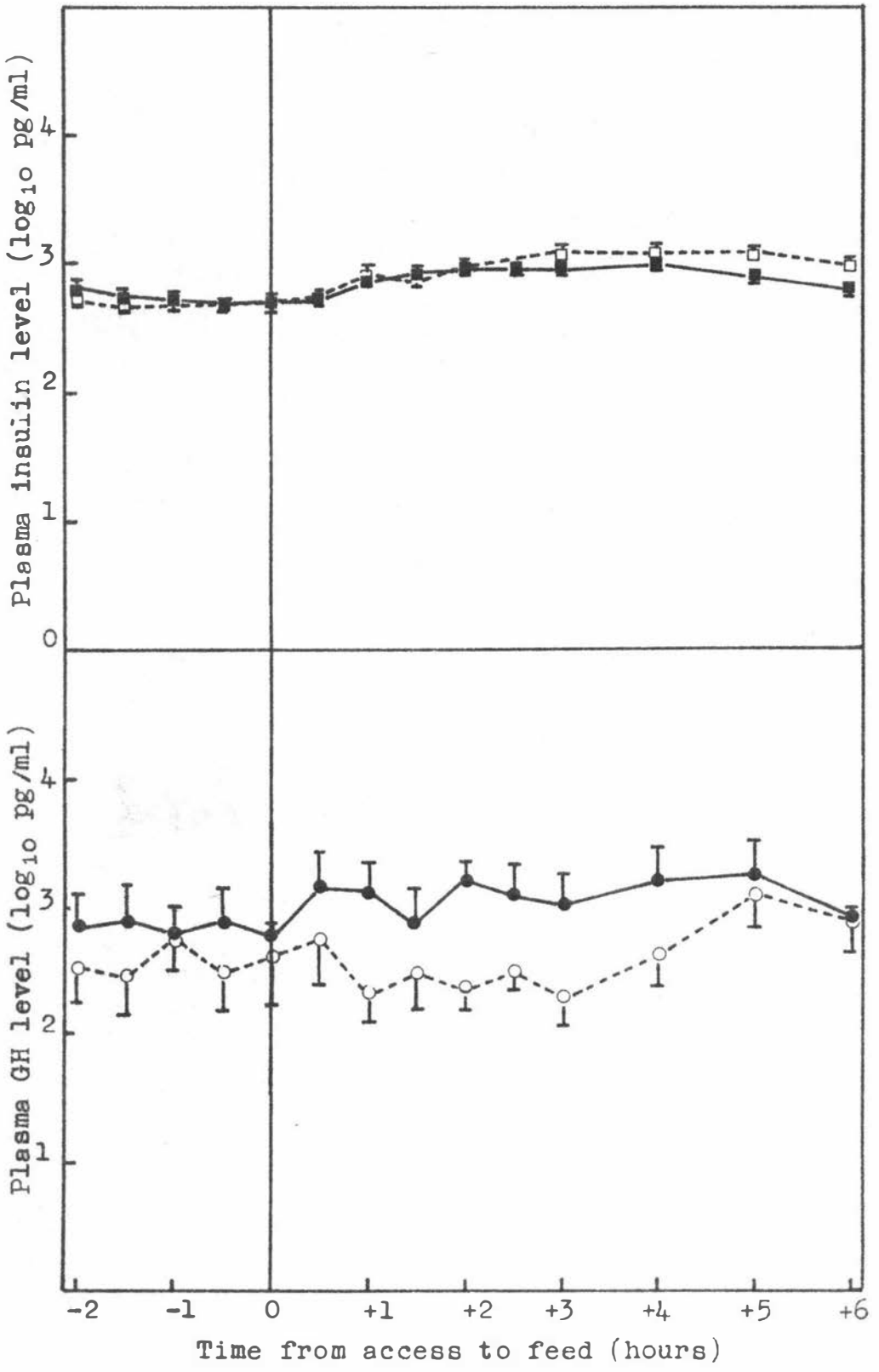

Figure 41 Plasma levels of insulin and GH before and after feeding in weaned lambs (solid lines) and non-lactating ewes (broken lines).

Table XLIIa Plasma levels of insulin and GH before and after feeding in weaned lambs and non-lactating ewes. Data expressed as $\log_{10}$ pg/ml, with untransformed data (ng/ml) in parentheses.

NS = not significant: + = $p<0.1$:
++ = $p<0.05$: +++ = $p<0.01$: for analysis of variance comparing hormone levels before and after feeding, within each group of animals.

	Time	Insulin	Growth hormone
Weaned lambs	before	2.74	2.62
		(0.55)	(0.42)
	after	2.98 NS	2.90 NS
		(0.95)	(0.80)
Non-lactating ewes	before	2.69	2.71
		(0.49)	(0.51)
	after	3.01 +++	2.75 NS
		(1.01)	(0.56)

Table XLII b Significance of the difference between feeding effects on suckling lambs versus weaned lambs, and on lactating ewes versus non-lactating ewes.

	Insulin	GH
Suckling vs weaned lambs	++	NS
Lactating vs non-lactating ewes	+++	++

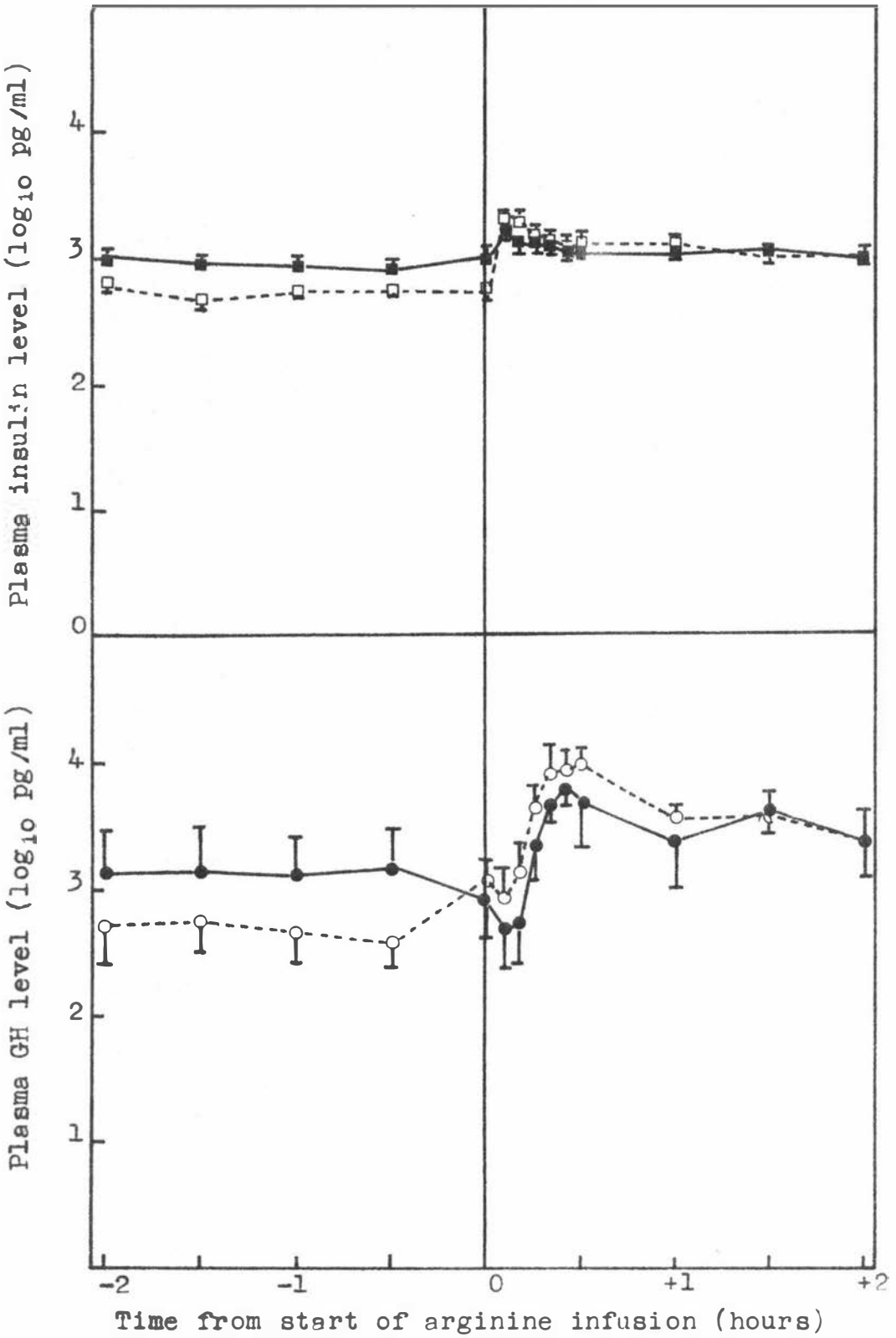

Figure 42 Plasma levels of insulin and GH before and after arginine infusion in weaned lambs (solid lines) and non-lactating ewes (broken lines).

Table XLIIIa Plasma levels of insulin and GH before and after arginine treatment in weaned lambs and non-lactating ewes. Data expressed as $\log_{10}$ pg/ml, with untransformed data (ng/ml) in parentheses.

NS = not significant: + = $p<0.1$: ++ = $p<0.05$: +++ $p<0.01$: for analysis of variance comparing hormone levels before and after treatment, within each group of animals.

	Time	Insulin	Growth hormone
Weaned lambs	before	3.00 (1.01)	2.53 (0.34)
	after	3.22 ++ (1.66)	2.99 ++ (0.97)
Non-lactating ewes	before	2.73 (0.53)	2.78 (0.60)
	after	3.35 +++ (2.23)	3.90 +++ (8.11)

Table XLIIIb Significance of the difference between arginine effects on suckling lambs versus weaned lambs, and on lactating ewes versus non-lactating ewes.

	Insulin	GH
Suckling vs weaned lambs	NS	+++
Lactating vs non-lactating ewes	++	NS

Figure 39 may be compared with Figure 41, in which the effect of feeding was observed when the lambs had been weaned for four weeks. The insulin response to feeding was almost identical between weaned lambs and adult ewes. The weaned lambs and the non-lactating ewes had lower basal levels of GH than the suckling lambs and their dams (Table XXXIX), and feeding had no effect on their GH levels.

The effect of the arginine treatment is shown in Figure 40 and 42. In every case, the insulin levels underwent an immediate small, but significant increase following the start of the infusion. The GH levels increased ten-fold during the infusion, but the increase was delayed in the weaned lambs and lactating ewes, and was significantly smaller in the weaned lambs, than before they were weaned (Table XLIII).

3.5.3 Discussion

Following feeding, the insulin response of suckling lambs was comparable in magnitude to that of very young suckling calves, as shown in Figure 18. Elevated insulin levels were retained in lambs for three hours, and even after six hours, they had not returned to pre-suckling levels, whereas the return to pre-suckling levels was more rapid in calves (Figure 18). Without measurements of the amount of milk consumed, it is not possible to conclude that the difference was related to constitutional differences between lambs and calves.

The older lambs and non-lactating ewes, showed an insulin response which was typical for mature ruminants (Bassett, 1975) and was very slow as a result of the fact that production of energy substrates from the rumen is a much slower process than production from the abomasum.

GH levels were of the same order in three-week-old lambs before suckling or arginine treatment, as those of young calves (Figure 18, 31, 37) although the range was greater for lambs. The significant GH increase following suckling (Table XL) was in agreement with the findings of Wallace and Bassett (1970) and Stern et al (1971), who observed a GH increase in response to increased blood glucose levels in lambs. However, it is likely that the response is confined to lambs, among ruminants, because it was not observed in goats (Tindal et al, 1978), nor was a post-feeding GH increase observed in suckling calves in the present study (Figure 18).

The ewes showed a parallel increase in GH at the time of suckling. Since non-lactating ewes did not respond to feeding (Figure 41), it seems likely that the GH response was brought about as a result of being suckled. The observation of a maternal GH response to suckling is in agreement with findings of other workers with sheep and goats (Martal, 1975: Hart and Flux, 1973: Tindal et al, 1978), although the response has not been observed in cows (Koprowski and Tucker, 1973, and page 18 of review).

The observation that both ewes and lambs responded in parallel, is of interest in that it may be related to a hypothalamic or 'emotional' change rather than being mediated in lambs simply by increased plasma glucose levels. The importance of adrenergic influences acting at the level of the hypothalamus to control GH secretion is discussed on page 22. The endocrinology of maternal behaviour and of the responses of the young to the mother is an area which would be a promising field for further research (page 122).

GH levels in both lambs and ewes were very much reduced after weaning (Figure 41). The situation for lambs confirms the results of other workers (reviewed on page 15) who have observed that neonatal GH levels are higher than after weaning. For the ewes, the result serves to emphasize indirectly the importance of GH in maintenance of ruminant lactation (reviewed by Cowie and Tindal, 1971).

The insulin response to arginine was much smaller in lambs and ewes, than it was for all groups of calves (Figure 40 and 42, compared with Figure 30). It appeared to decrease with age in lambs (Table XLI and XLIII), a situation which was also not in agreement with the calf data in Figure 30. Since the arginine dose was larger in the sheep experiment than it was in Experiment 2, it appears that the different responsiveness could reflect a constitutional difference between lambs and calves, although it is not conclusive, because infusion time and also feeding regime were different between the two experiments. Feeding regime is known to influence the

number of insulin receptors per cell, and thus the insulin utilisation rate (Gavin et al, 1974): thus feeding regime could have an apparent influence on the size of the response.

The GH response to arginine was very much more repeatable and significant in ewes and lambs than it was in calves (Figure 40 compared with Figure 31). The data in Figure 40 lend support to the findings of all other workers testing the response to arginine infusion of GH levels in sheep (reviewed on page 9). The importance of the data to the present study, lies in the fact that they serve to verify that the GH assay was able to give results which were comparable to those of other workers, in some situations. Thus, in the young calf, where results were not in agreement with those of other workers, the discrepancy was likely to be due to a real difference between calves and other ruminants, rather than being due to a faulty assay. The 'parallelism problem' encountered in the GH assay (discussed on pages 62 - 93) occurred in 25% of all GH assays, and was more closely related to the use of certain second antibody preparations, rather than to the unknown plasma samples being of calf or of sheep origin. The assay results are thus fully comparable between the different species.

3.5.4 Conclusions

1. As a result of feeding following a period of starvation, suckling lambs showed an insulin response which was more similar to that of non-ruminants than ruminants, while that of weaned lambs and adult ewes was typical of ruminants.

2. The feeding and suckling stimulus caused GH levels to rise to the same extent in the suckling lambs, as they did in the dams: the rise did not take place in either after the lambs had been weaned, so the response was clearly caused by stimuli which are specific to suckling.

3. A 30-minute infusion of 0.5 g/kg body weight of arginine brought about an insulin response which was smaller than that observed in calves in Experiment 3, and a GH response which was larger and very much more consistent than that of the calves. The results indicate that calves' GH secretion may be under a different control mechanism from that of lambs, but until comparable arginine dosage and infusion time have been tested for both groups, firm conclusions may not be drawn.

3.6 GENERAL CONCLUSIONS

1. There is insufficient data for definite conclusions to be drawn concerning the basal plasma levels of glucose, insulin, GH or prolactin in relation to age and ruminant status. Experiments 3 and 4 indicated that glucose levels were higher in week-old than in five-week-old calves, but it was not clear whether the change was associated with rumen development. Experiments 3, 4 and 5 all indicate that GH was higher in the neonatal calf and lamb, with an age-related decrease, but the decrease was only statistically significant in Experiment 3.

2. Both GH and prolactin appeared to follow a pattern of 'episodic' increases in plasma in all animals studied. The secretory episodes were not able to be related conclusively to events, either external or internal, especially with respect to the GH levels of calves.

3. Young calves and lambs responded to suckling with a 2- to 5-fold plasma insulin increase, a response which is typical of the monogastric mode of digestion, and differed greatly from the small, sustained, insulin response to feeding in the ruminating lambs and ewes studied in Experiment 5.

4. Following suckling the lambs and their ewes showed a significant increase in plasma GH levels, a phenomenon which was not detected in suckling calves. The difference between lambs and calves could have been

related to the fact that calves were not GH responsive to hyperglycaemia, while the literature contains several reports of lambs responding to hyperglycaemia with an increase in GH levels.

5. Arginine infusion brought about some increase in plasma levels of all hormones in all animals studied. The insulin response was greater in calves than in lambs and ewes, while the GH response was greater in lambs and ewes, and almost non-existent in calves. The differences may have been due either to a constitutional difference between bovines and ovines, or it may have been due to different dosage, infusion time, and feeding regimes between the two experiments.

6. Five-week-old calves fed only on milk showed increased responsiveness to arginine treatment as compared with either week-old or five-week-old ruminant calves. They also showed a larger range of GH levels in both experiments 3 and 4. The increased responsiveness of this group cannot be brought about either by increasing age, or by the monogastric mode of digestion, since neither of the other groups was comparable. It must therefore have been related to the combination of age and diet, and draws attention to the fact that the experimental design may not have been appropriate for animals which, under natural conditions, would have eaten some solid feed from the time of birth.

7. Results of Experiments 3 and 4 both indicate an increasing insulin resistance in calves, both as a result of increasing age and increasing rumen development.

8. A sudden increase in plasma osmolality brought about a decrease in plasma levels of both pituitary hormones, GH and prolactin, in calves of both ages, although the prolactin decrease was not significant. It was not possible to tell whether this was a non-specific inhibition of pituitary function, or whether it was of functional significance for either hormone in relation to osmoregulation.

9. Despite the fact that the literature gives ample evidence to suggest that GH and prolactin are under opposing mechanisms of adrenergic control, the only instance in the present study, where they were observed to respond in opposite directions was when GH levels fell and prolactin levels rose after 2 - 3 hours of continuous insulin-induced hypoglycaemia. The response could have been associated with the prolonged insufficiency of glucose reaching the hypothalamus.

APPENDIX

Table XLIV a Results of insulin levels in twenty calf plasma samples, as calculated in three different ways, using data from two different plasma dilutions, from Assay 1 described on pages 62 - 70. Results expressed as pg/ml.

(1) Results obtained by reading from a graph plotting raw data (counts per minute) against hormone level known to be included in each standard tube.

(2) Results obtained by method (C), page 63, without using a weighting procedure.

(3) Results obtained by method (C), page 63, using the weighting method of Finney (1964).

(1)		(2)		(3)	
100 µl	50 µl x 2	100 µl	50 µl x 2	100 µl	50 µl x 2
331	300	356	330	349	309
609	476	595	540	602	521
525	464	528	519	530	500
368	327	385	352	380	331
392	401	405	444	400	423
406	290	380	305	374	284
6,871	6,123	4,225	5,017	4,802	5,527
2,044	1,991	1,983	1,956	2,154	2,037
965	919	935	933	972	930
630	570	610	613	617	596
561	503	557	567	561	548
368	314	386	352	380	332
1,595	1,660	2,049	1,999	1,771	1,656
865	740	927	762	962	751
613	570	598	631	604	614
585	540	577	602	582	584
710	700	665	742	678	729
610	635	597	714	604	700
480	540	487	571	487	553
1,105	1,130	1,109	1,106	1,164	1,113

Table XLIV b Results of insulin levels in twenty calf plasma samples, as calculated in three different ways, using data from two different plasma dilutions, from Assay 2 described on pages 62 - 70. Results expressed as pg/ml.

(1) Results obtained by reading from a graph plotting raw data (counts per minute) against hormone level known to be included in each standard tube.

(2) Results obtained by method (C), page 63, without using a weighting procedure.

(3) Results obtained by method (C), page 63, using the weighting method of Finney (1964).

(1)		(2)		(3)	
100 µl	50 µl x 2	100 µl	50 µl x 2	100 µl	50 µl x 2
395	310	572	632	420	416
3,580	2,640	1,985	2,624	1,829	2,241
1,077	860	1,165	1,206	974	894
552	486	746	868	575	605
508	295	700	612	533	400
455	255	637	520	477	332
532	415	728	790	559	542
489	330	677	656	513	434
504	253	698	522	532	333
520	293	714	596	546	388
485	340	675	682	512	455
550	348	748	685	577	454
450	265	632	545	472	349
513	384	695	735	529	497
410	250	592	505	438	319
1,565	1,083	1,440	1,469	1,251	1,129
1,810	1,210	1,521	1,605	1,336	1,254
1,735	1,155	1,505	1,572	1,318	1,224
3,150	2,245	1,915	2,388	1,754	2,006
3,300	2,180	1,950	2,334	1,792	1,953

Table XLV Average growth rate of each calf for the duration of Experiment 2.

Calf	7	28	29	30
Kg body weight gain/day	0.152	0.119	0.181	0.036

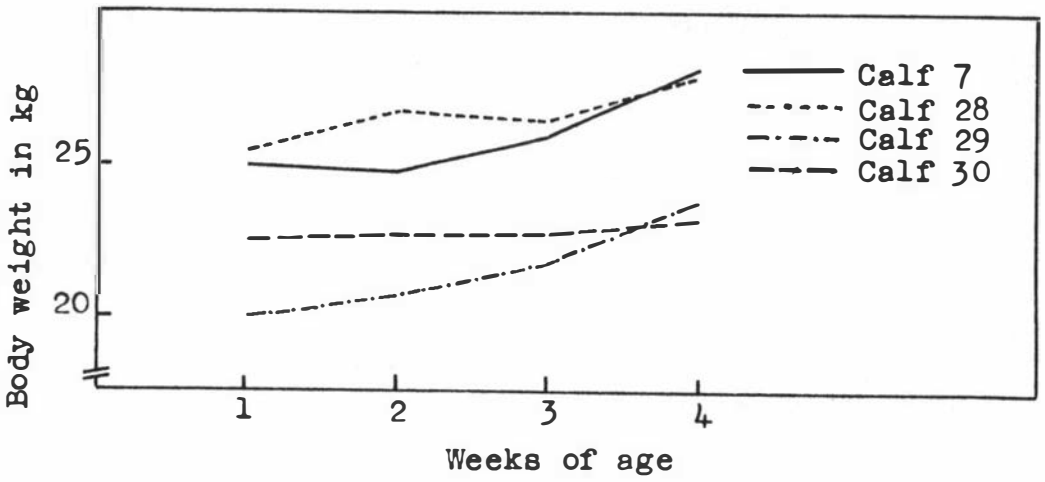

Figure 43. Body weights of each calf for the duration of Experiment 2.

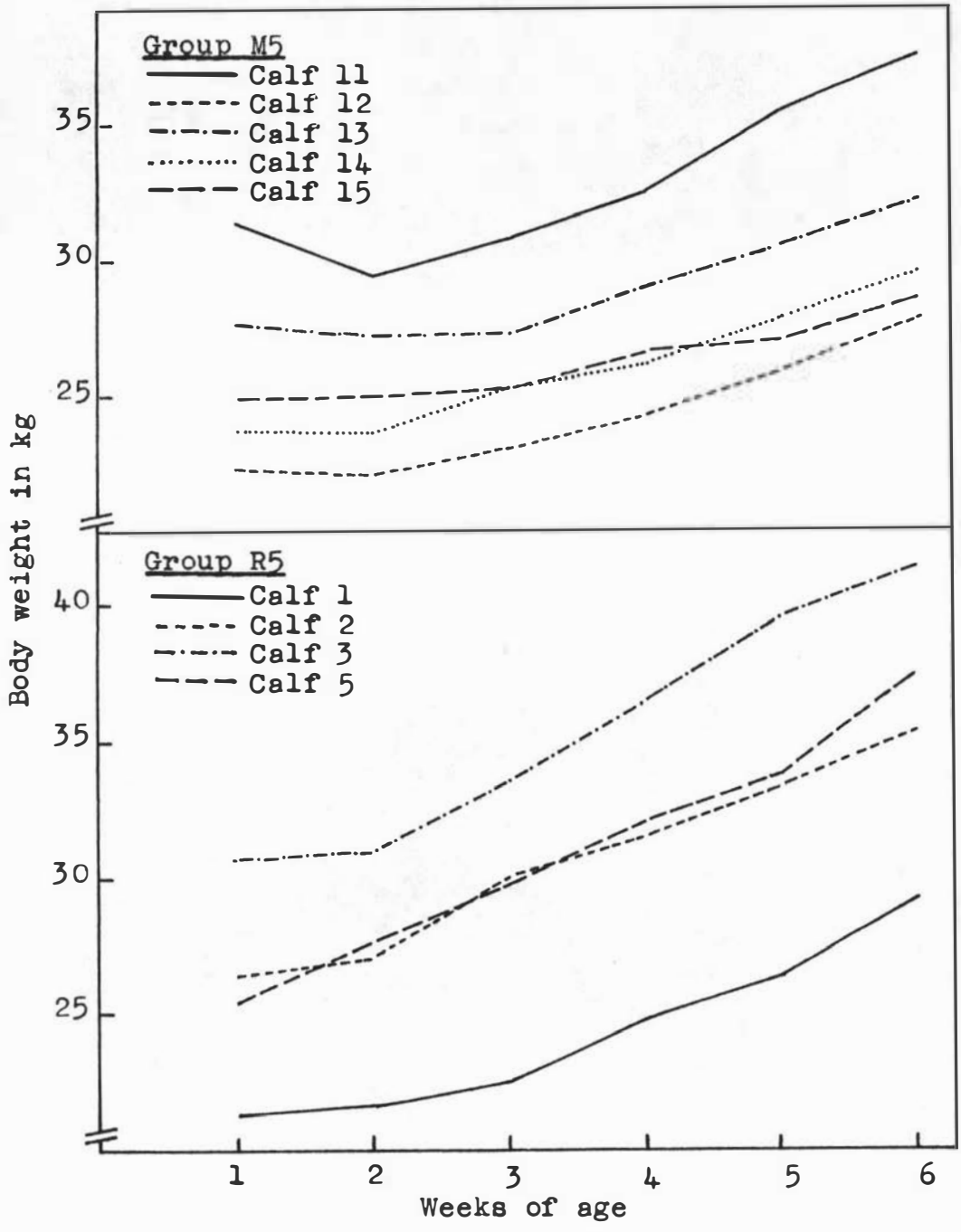

Figure 44 Body weights of each calf for the duration of Experiment 3.

Table XLVI Average growth rate of each calf for the duration of Experiment 3.

Group M	Calf 11	Calf 12	Calf 13	Calf 14	Calf 15
Kg bw gain per day	0.178	0.166	0.137	0.171	0.114
Group R	**Calf 1**	**Calf 2**	**Calf 3**	**Calf 5**	
Kg bw gain per day	0.227	0.258	0.309	0.350	

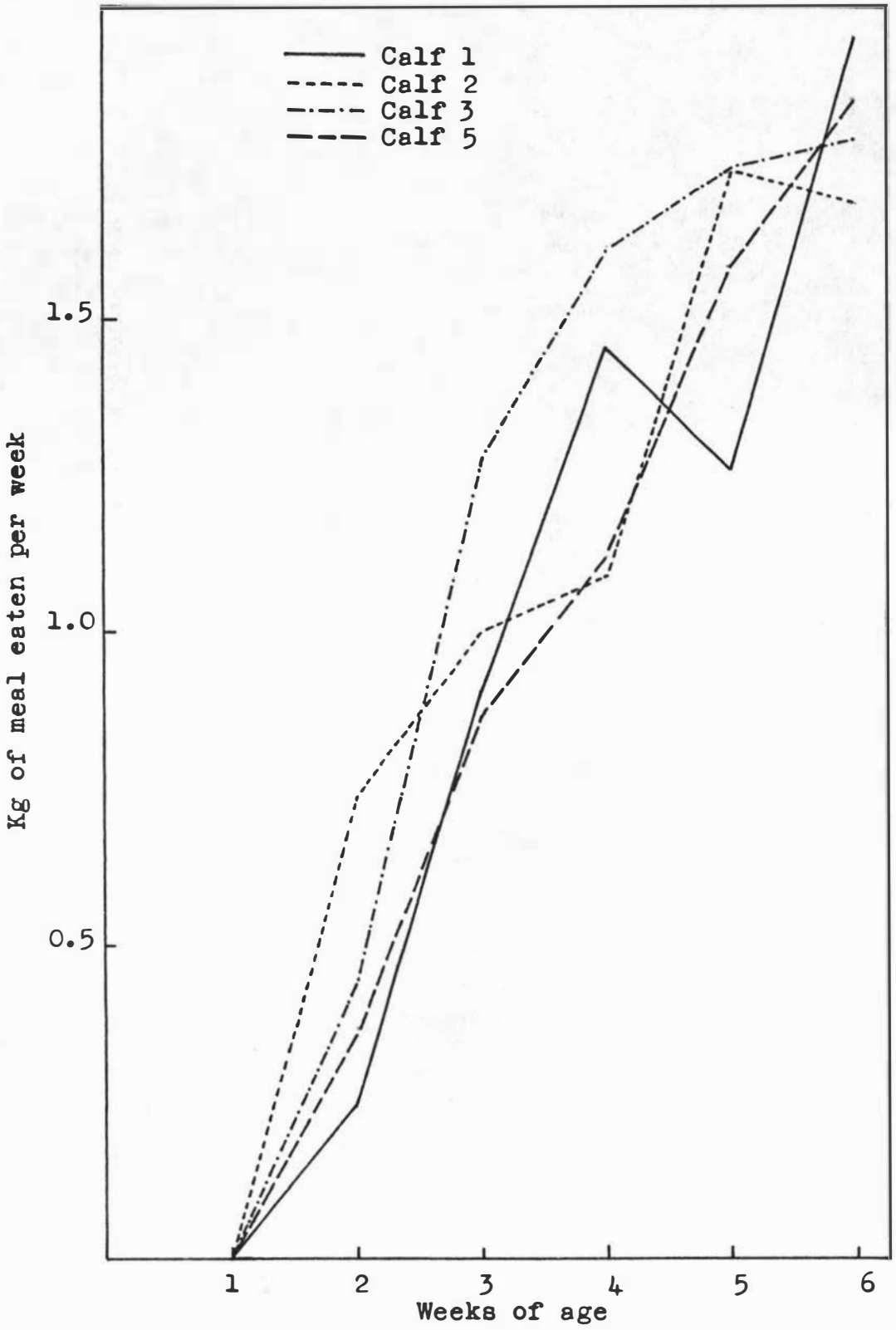

Figure 45 Amount of meal eaten during each week of Experiment 3, by individual calves of Group R.

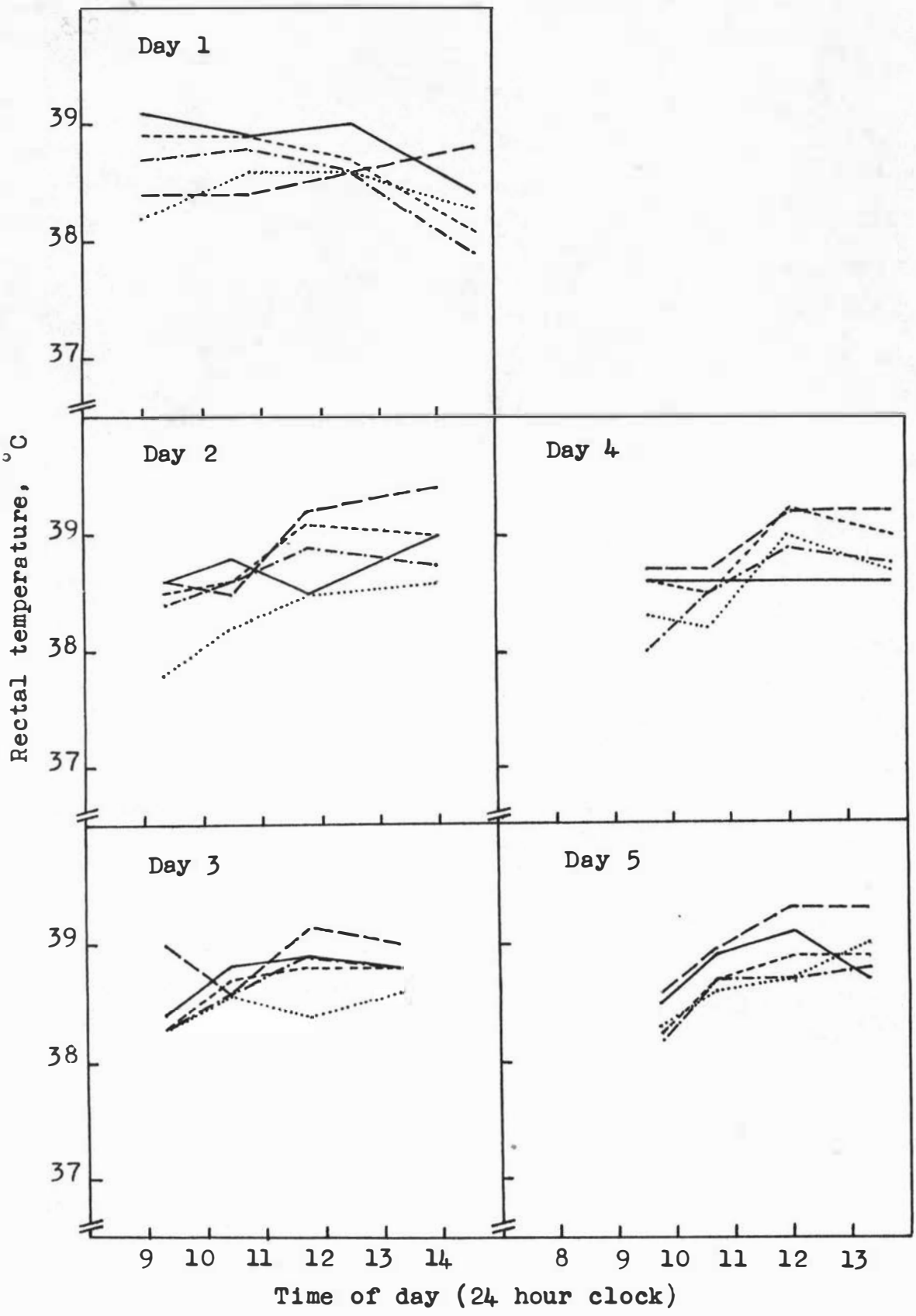

Figure 46 Rectal temperatures during sampling days of Experiment 3.

Group M1 ——— Calf 11 ····· Calf 14
- - - - - - - Calf 12 — — — Calf 15
—·—·— Calf 13

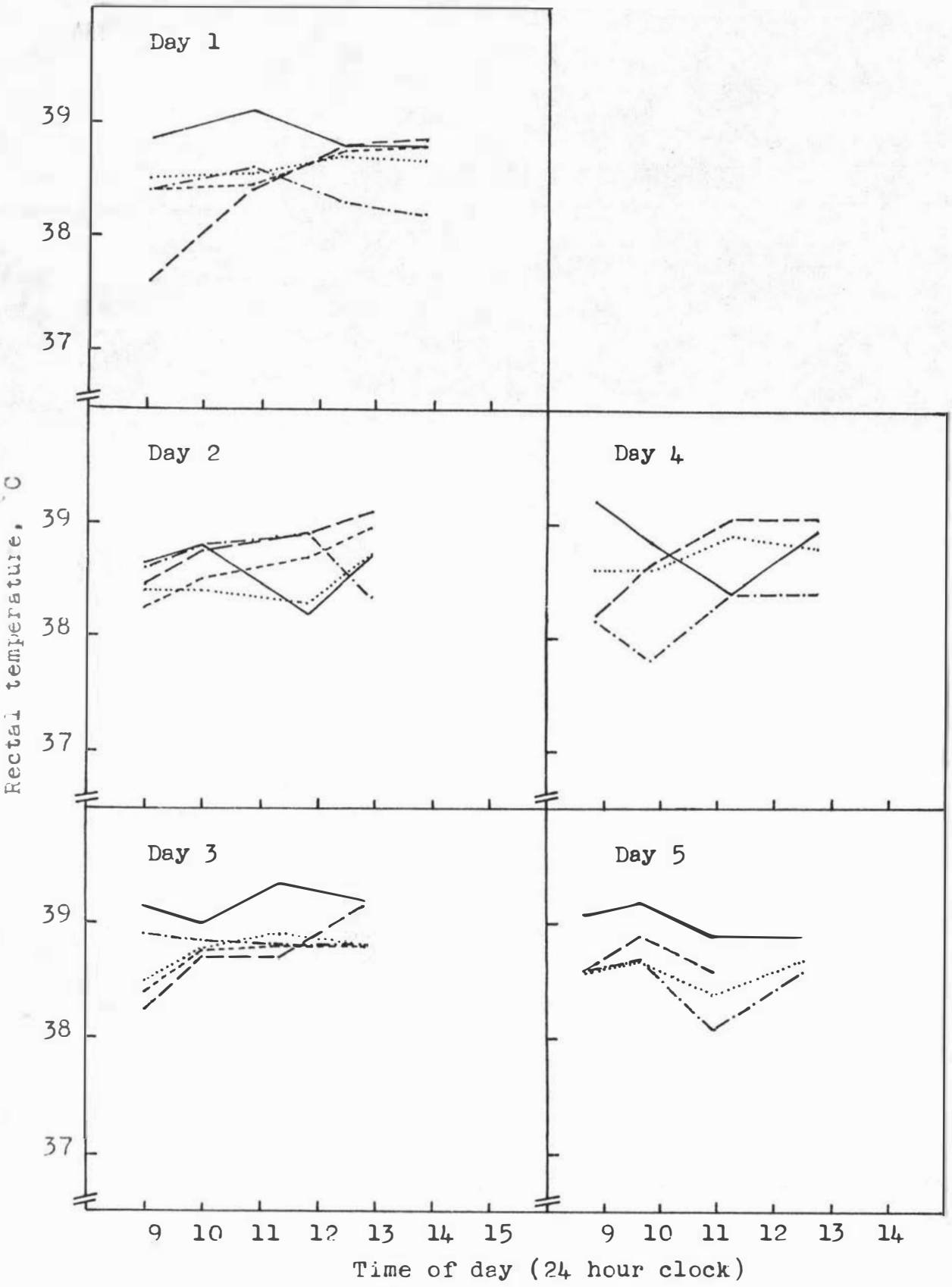

Figure 47 Rectal temperatures during sampling days of Experiment 3.

Group M5 ——— Calf 11 ····· Calf 14
------ Calf 12 — — — Calf 15
—·—·— Calf 13

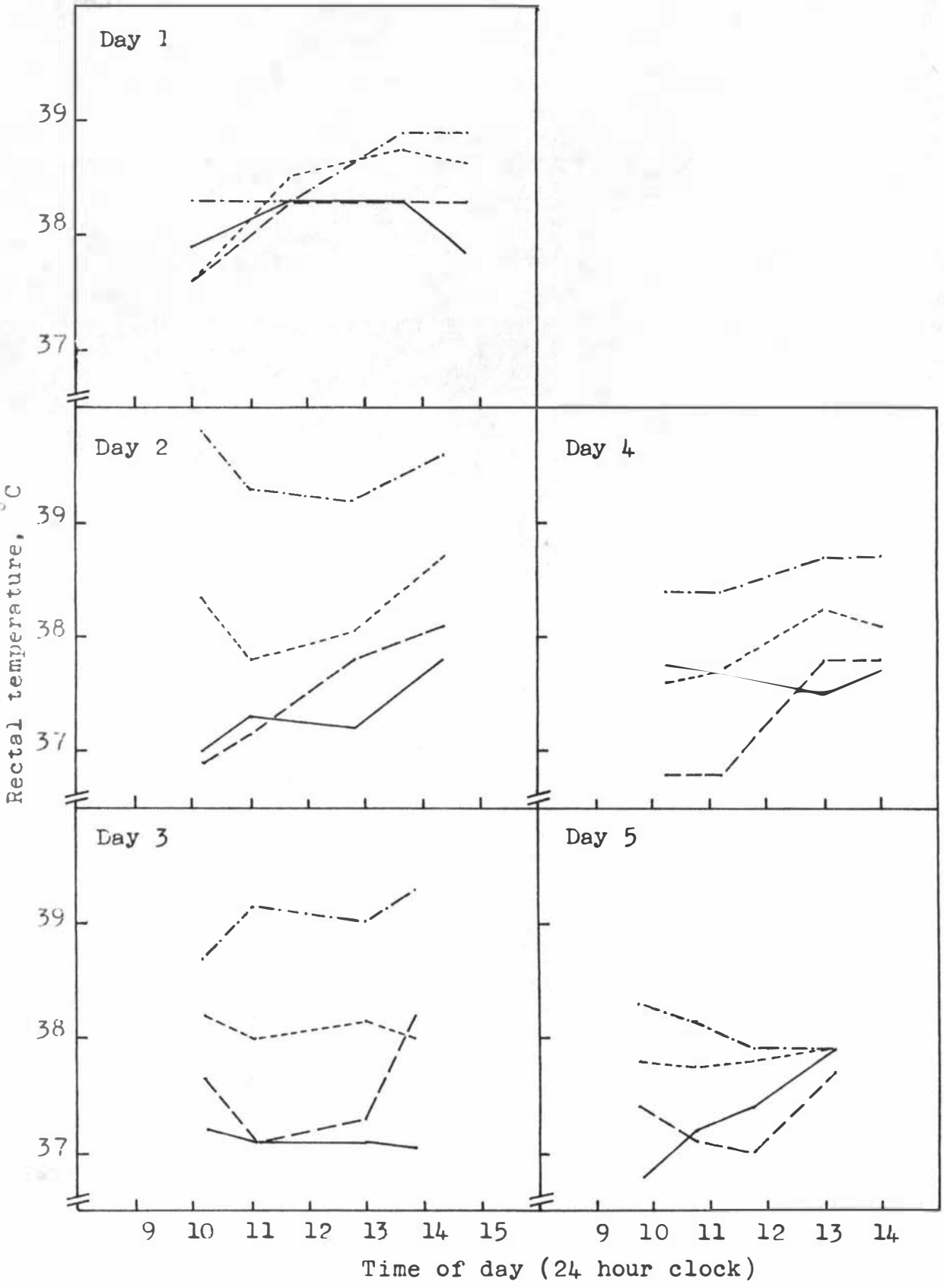

Figure 48 Rectal temperatures during sampling days of Experiment 3.

Group R5 ——— Calf 1 —·—·— Calf 3
------ Calf 2 — — — Calf 5

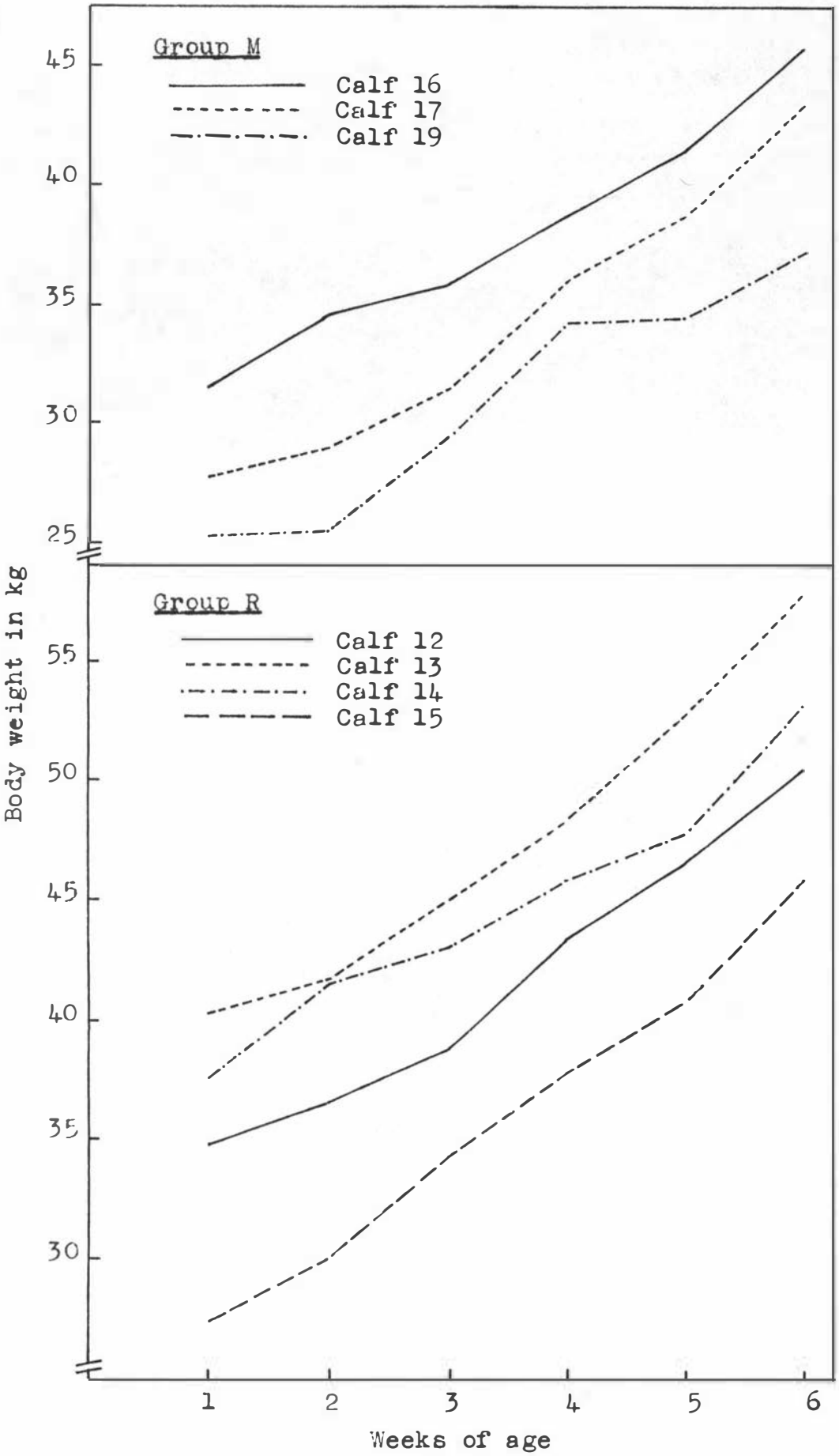

Figure 49 Body weights of each calf for the duration of Experiment 4.

Table XLVII Average growth rate of each calf for the duration of Experiment 4.

Group M	Calf 16	Calf 17	Calf 19	Calf 20 died
Kg bw gain per day	0.406	0.448	0.343	
Group R	Calf 12	Calf 13	Calf 14	Calf 15
Kg bw gain per day	0.448	0.503	0.448	0.528

Table XLVIII Body weights of lambs and ewes in Experiment 5.

No.	Lambs		Ewes	
	3.5 weeks old	12.5 weeks old	Lactating	Non-lactating
1	9.08	24.95	51.26	45.82
2	8.17	24.95	50.81	47.17
3	9.08	27.45	40.83	48.09
4	8.17	30.40	48.09	45.82
5	8.62	30.62	49.45	50.35
6	8.62	29.49	50.35	58.06

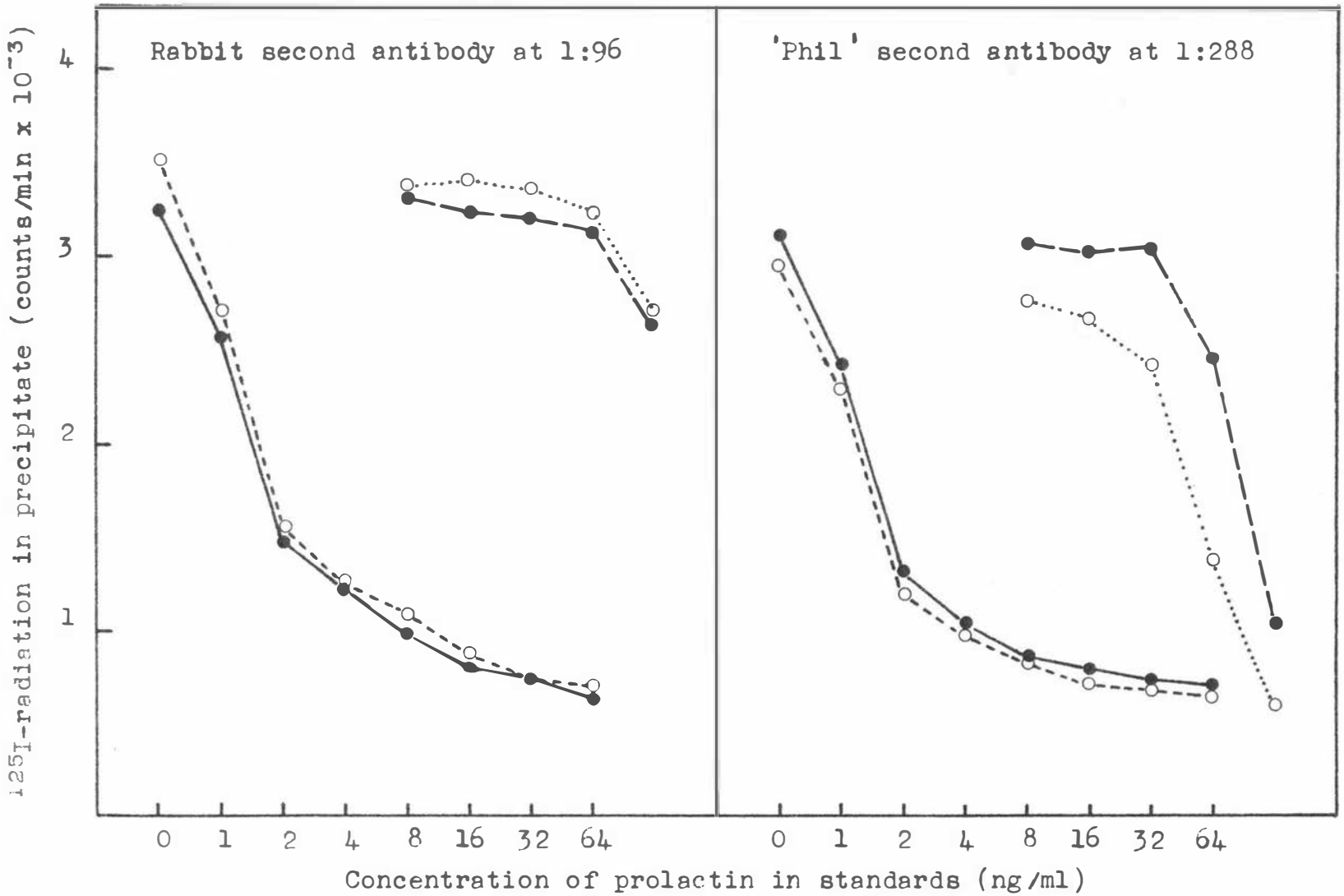

Figure 50 Standard curves and serial dilutions of calf plasma, with different second antibodies and with varying time period between second antibody addition and centrifugation of tubes.
----- Standard curve with 24 hours and ——— with 72 hours incubation.
..... Plasma dilutions with 24 hours and — — — with 72 hours incubation.

POST SCRIPT

Since the completion of the typing of the thesis, a more complete investigation was carried out into the efficiency of the precipitation reaction in prolactin assays, using a more recent bleeding of 'Phil' second antibodies than that described on page 78 (December 1975, as compared with November 1973). Different incubation times between the addition of 'Phil' and centrifugation of tubes, were also tested. The results of the investigation are shown in Figure 50 together with a similar investigation with rabbit second antibodies, included for comparison.

The main observation was that precipitation by 'Phil' was very rapid in the presence of diluent human plasma, while in the presence of unknown calf plasma samples it was very slow. Such a difference did not apply to precipitation by rabbit anti-GPγ. It was also found that the optimum concentration of 'Phil' for maximum precipitation was 1:288, whereas it had been observed (page 78) that the earlier bleedings of 'Phil' required a much lower dilution (1:16) for optimum binding.

These results are of great relevance to the assay development described on pages 58 to 93 because they indicate that insufficient incubation time with 'Phil' may have been the cause for some assays to show deviation from parallelism. This seems especially likely in view of the fact that, provided the concentrations of first antibodies and labelled hormone remained unchanged, the use of rabbit second antibodies always resulted in near

perfect parallelism, whereas the 44% of insulin, and 25% of GH assays which showed poor parallelism even after all other factors had been standardised, were those involving the use of 'Phil'. The reason why the problem was not detected earlier, was that in the 1973 bleedings of 'Phil' which had a very low titre, a large amount of 'Phil' plasma was required in each assay tube. Under these conditions of excess 'Phil' plasma, the discrepancy between time taken to reach maximum binding in diluent and in unknown plasma, was very little, and was not detected. Only after the titre of 'Phil' had increased sufficiently to require the addition of Phil at a final dilution of 1:288 instead of 1:16, was the difference detected. In between, however, many assays had been set up with 'Phil' at 1:32 and 1:64, parallelism had been variable and the cause remained undetected.

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