TRYPTOPHAN DEFICIENCY AND FOOD INTAKE DEPRESSION IN PIGS

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ABSTRACT

Two experiments are described, in which the effects of feeding pigs on a tryptophan deficient diet or supplemented diet were investigated.

The feeding patterns of 10 cross-bred pigs were measured by continuous recordings of feed-bin weights, in a double reversal design experiment. The pigs, fed ad libitum, ate an average of 9 "meals" per day (range 5 - 16) with an average meal size of 170 g. There was a distinct diurnal pattern of food intake; most meals were eaten in the light phase of the day with peaks in the early morning and at midday and a large peak mid afternoon. Pigs fed the deficient diet showed some depression in food intake on the first day and the depression had reached maximal levels by the third day. On the deficient diet pigs ate 17 - 20% less than on the supplemented diet and most of the depression in intake was accounted for by reduced meal size.

In the second experiment 4 pigs were trained to eat their daily ration in a 2 h period (0900 - 1100 h) and catheters were placed in the jugular veins. A double reversal experimental design was employed, with 3 periods of 5 days, and blood samples were taken over the feeding time on the second and fifth day in each period. The levels of plasma Glucose, Urea, Amino acids, Cortisol, Insulin, and Growth Hormone were measured. There were no significant differences between diets in levels of Growth Hormone or Cortisol. On day 2, Urea levels were higher in pigs fed the supplemented diet, while on day 5 there were no significant differences between diets. The lowered food intake

on the deficient diet meant that both protein quality and protein intake were altered, which may explain the differences in Urea levels.

The most consistent differences in plasma Amino acids levels occurred with tryptophan, the limiting amino acid, for which the levels were lower in pigs fed the deficient diet, although the differences were not statistically significant. Glucose rose higher in pigs fed the deficient diet and the differences could not be attributed to an altered Insulin response to feeding the deficient diet.

It was concluded that the early changes in glucose and tryptophan may be associated with the food intake depression on the deficient diet, but further studies would be required before the relative importance of either relationship could be established.

PREFACE

The relationships between growth and the quantity and quality of dietary protein have been extensively studied and a depression of growth on diets of poor protein quality is well documented. However, the relationships between food intake depression and protein quality are not well understood and much of the work has been carried out with a single species, the laboratory rat. In studies at the Pig Research Centre, Massey University, comparing the nutritional quality of opaque-2 and normal maize varieties, Stables and Carr (in press) observed increased feed refusals on some diets, which they attributed to amino acid imbalance.

With the demonstration of food intake depression on diets based on maize grain and a commercial protein source, it became of interest to study the relationship between protein quality and food intake in pigs. Experiments were designed to study:

(i) the depression of food intake on a diet of low protein quality,

(ii) the pattern of intake in ad libitum fed pigs including any changes in the pattern related to protein quality,

(iii) the effects of the diet on the responses of some metabolites and hormones related to protein and carbohydrate metabolism, to throw some light on the underlying causes of the depression in intake.

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CHAPTER ONE

REVIEW OF LITERATURE:

In their review, Finger and Mook (1971) wrote; "The literature on food and water intake continues to gain weight rapidly", and in 1976 there are still no signs of any reduction in growth rate. The present review deals with limited areas of the control of food intake, with special reference to the relationship between food intake and protein quality. It is intended to provide a background to the research outlined in later chapters and not as a general discussion.

The review is divided into three sections. The first deals with energy balance and the control of food intake. Emphasis is placed on mechanisms capable of sensing the state of energy balance. The second section deals with the relationship between food intake and the amino acid compositions of diets and the final section introduces the present study and outlines the objectives of the experiments undertaken.

1.1 ENERGY BALANCE AND THE CONTROL OF FOOD INTAKE:

For the maintenance of life animals require a continuous flow of energy, which must be met from the external environment or stores of energy within the body. Small errors in energy balance may lead to large changes in energy stores over long time periods (Hervey, 1969) and the control of food intake represents an important mechanism for balancing energy expenditure and energy intake.

Recent reviews on the subject include those of Epstein (1971), Finger and Mook (1971), Grossman (1975), Hoebel (1971), Le Magnen (1971), Liebelt, Bordelon and Liebelt (1973), Morgane and Jacobs (1969), Panksepp (1974), and Teitelbaum (1971). In addition, many aspects are covered in the Handbook of Physiology volume on Control of Food and Water Intake (1967) and the New York Academy of Science symposium entitled Regulation of Food and Water Intake (1969).

1.1.1 Brain Structures and Food Intake Control:

From early clinical observations and work with experimentally induced brain lesions, the hypothesis was developed that food intake is controlled by two areas in the hypothalamus; one area in the lateral hypothalamus designated the "feeding centre" and a second area in the ventromedial hypothalamus designated the "satiety centre". The most elaborate version of the hypothesis was discussed by Stellar (1954) when he proposed a general model for the control of several motivated behaviours including food intake. He proposed that feeding behaviour was controlled by activity in the "feeding centre" while the "satiety centre" influenced behaviour by direct inhibition of activity

in the "feeding centre".

The hypothesis gained wide acceptance and much subsequent work has lent support to it. However, over recent years, evidence has accumulated that the hypothesis is not adequate. Panksepp (1974) has reviewed the literature and proposed a new model, where the lateral hypothalamus, in association with other brain areas mediates "short term satiety", while ventromedial hypothalamic activity reflects depletion and repletion of body nutrient stores.

The revised hypothesis still represents a simplification. In an excellent review, Morgane and Jacobs (1969) discussed the complex interrelationships between brain areas and the control of feeding behaviour. Teitelbaum (1971) discussed parallels between the recovery of animals with bilateral lesions in the lateral hypothalamus and the development of food intake control. He suggested that the hypothalamic "centres", if in fact they exist, usually act as an integral part of an "highly encephalised system". Grossman (1975), reviewing evidence for the role of extra-hypothalamic structures in the control of food intake, concluded that neural pathways passing through the hypothalamus may have more significance for the control of food intake than had previously been accepted.

Thus it appears that the central mechanisms involved in the integration of feeding behaviour are not well understood and future explanations must take account of extra-hypothalamic structures and the role of non-specific arousal systems.

1.1.2 Sensory Signals and Energy Balance:

The second major area of research concerns the afferent limb of the food intake controlling mechanism. What areas of energy metabolism, including energy stores, signal the state of energy balance?

Several mechanisms have been postulated to explain the control of food intake, including shifts in body water between body compartments, the presence of food in the digestive tract, the availability and metabolism of glucose at various sites in the body, the specific dynamic action of ingested food, and unspecified metabolites or signals from adipose tissue (Brobeck, 1955). An additional mechanism involving blood amino acid levels has also been postulated (Harper, Benevenga and Wohlhueter, 1970; Rogers and Leung, 1973).

1.1.2 (i) Energy Stores and Food Intake:

The major energy store within the body is adipose tissue and in adult animals body weight, and by inference, energy stored in adipose tissue, remains remarkably constant over long periods of time. On the other hand food intake occurs in discrete "meals" (Duncan, Horne, Hughes and Wood-Gush, 1970; Hirsch, 1973; Kanarek, 1975; Le Magnen, 1969; Wiepkema, 1968) and energy expenditure can fluctuate over short time periods.

Several authors have addressed the question of the time relationship between energy expenditure and energy intake. Durnin (1961) summarised and extended observations on the relationship between expenditure and intake in humans. The daily relationship was very poor,

but 59 per cent of subjects achieved energy balance when whole-week data was considered. The lack of a daily relationship could not be explained by a delay of one or two days in balancing past energy expenditure. Mayer (1955), summarising early work of Gasnier and A. Mayer on rabbits, concluded that short term and long term components of energy balance regulation can be distinguished.

Direct evidence for an effect of adipose tissue stores comes from experiments where fat stores were manipulated experimentally. Electrolytic lesions placed in the ventromedial hypothalamus caused rats to eat large amounts of food and rapidly become obese (Kennedy, 1953). The hyperphagia was not maintained indefinitely. Instead, as obesity developed food intake declined and a stable body weight was achieved. Furthermore, animals would again become hyperphagic and rapidly gain weight if body weight had been reduced by dietary restriction. Powley and Keesey (1970) noted that rats with lesions in the lateral hypothalamus appear to regulate body weight at a lower level than control animals.

Other dietary manipulations suggest that animals defend a given level of body weight. Elevation of body weight by force feeding (Cohn and Joseph, 1962) resulted in a depression of food intake and a return to normal body weight at the end of the forced-feeding period. Injections of insulin caused a significant increase in food intake in the rat (Mayer and Bates, 1952). The resultant increased body weight was again lost when insulin injections were discontinued (Hoebel and Teitelbaum, 1966).

The ability of animals to defend some "set"

body weight led Kennedy (1953) to postulate a "lipostatic" hypothesis for the control of food intake. However, no mechanism capable of detecting the level of adipose tissue in the body has been clearly identified. Hervey (1959) postulated a humoral factor influencing food intake, after experiments involving ventromedial lesions in parabiotic rats. Further work led to the hypothesis that a steroid or similar fat soluble "tracer" could monitor fat stores, provided certain conditions were met (Hervey, 1969).

Van Itallie and Hashim (1960) suggested that plasma non-esterified fatty acids may play a role in signalling hunger and satiety. Walker and Remley (1970) concluded that although there was an interaction between fat content, energy expenditure, and food deprivation in determining circulating free fatty acid levels, the levels may have been important in controlling food intake. However, Hales and Kennedy (1964) pointed out that circulating free fatty acid levels increase in both obesity and starvation and Booth and Campbell (1975) showed that an infusion of a palmitic acid-albumin complex had no effect on subsequent food intake.

Thus evidence for a role of adipose tissue in the control of food intake is largely circumstantial and several conflicting reports are present in the literature. Ingle (1949) described a method of inducing obesity in normal rats, by simply restricting exercise and allowing free access to a palatable diet. Free access to a high fat diet reliably produced obesity in rats with no known genetic tendency towards obesity (Mickelsen, Takahashi and Craig, 1955). Corbit and Stellar (1964) showed that the weights of control animals could be manipulated by feeding diets

of differing palatability.

Liebelt and colleagues (reviewed by Liebelt, et al. 1973) have conducted a comprehensive investigation of adipose tissue metabolism in mice. They provided evidence that adipose tissue functions as an integrated organ and several lines of investigation suggest a relationship between adipose tissue and food intake control. However, no single experiment constitutes strong evidence. Two studies using parabiotic rats and measuring food intake directly, for the individual animals (Fleming, 1969; Han, Mu and Lepkovsky, 1963) do not support the conclusions of Hervey (1959).

Despite considerable circumstantial evidence, and a strong presumption in the literature of an active control of body weight and/or adipose tissue stores, there is very little direct evidence for any long term regulatory mechanism. The two other postulated mechanisms concerned with the state of energy balance and food intake (the "thermostatic" theory and the "glucostatic" theory) involve variables that alter over short periods of time. It will be of interest to determine whether alterations in metabolism and short term control of food intake are sufficient to maintain the long term stability of body weight in adult animals.

1.1.2 (ii) Food Intake as a Mechanism of Temperature Regulation:

In homeotherms there is a negative relationship between environmental temperature and food intake (Hamilton, 1967). As air temperature increases food intake

declines and in several species ceases altogether at rectal temperatures of about 40°C (Brobeck, 1960; Hamilton, 1967). Observations on the relationship between temperature and food intake led Brobeck (1948) to propose that food intake is controlled as a mechanism of body temperature regulation, "... animals eat to keep warm and stop eating to prevent hyperthermia."

Additional support for the hypothesis comes from experiments involving diets of differing compositions, fed over a range of environmental temperatures and experiments involving local manipulations of temperature in the hypothalamus. Hamilton (1967) reviewed the effects of changing dietary composition on food intake and concluded that although feeding diets with a low "specific dynamic effect" (S.D.E.) resulted in higher food intakes, the data were confounded by changes in palatability. Furthermore, at low environmental temperatures, the increased metabolic rate probably obscures any differences in S.D.E. between diets.

Hamilton (1967) also reviewed evidence for effects of direct heating and cooling in the hypothalamus, or hypothalamic lesions on food intake. Several lines of investigation suggest that the anterior hypothalamus and preoptic area may be important in the relationship between temperature and food intake, but Grossman (1968) concluded that the feeding system is not primarily affected by brain temperature changes. On the present evidence it appears unlikely that the primary control of food intake is achieved through mechanisms related to body temperature.

1.1.2 (iii) Carbohydrate Metabolism and Food Intake:

Carbohydrate metabolism occupies a central role in energy metabolism; the central nervous system requires a constant supply of glucose and stores of carbohydrates are relatively small. Mayer (see Mayer 1953 and 1955) proposed a "glucostatic" mechanism for the control of food intake, which postulates the existence of glucoreceptors sensitive to blood glucose ".... in the measure that they can utilise it." The receptors, possibly in the hypothalamus and also in the periphery, would provide information on the rate of glucose metabolism to central structures controlling food intake.

Mayer and his colleagues (Mayer and Bates, 1952; Mayer, 1953; Mayer, 1955) have presented and discussed evidence for changes in food intake, under conditions of altered glucose utilisation. Conditions leading to increased glucose availability depressed food intake, while decreases in blood glucose within physiological limits caused increases in food intake, although the changes were small. More dramatic changes in food intake were noted in hypophysectomised, diabetic rats unable to regulate blood glucose. Decreased liver glycogen and increased carbohydrate metabolism seen in cold-acclimatised animals (Baker and Sellers, 1953, abstract only) is interpreted as evidence that high food intakes in altered physiological states may be the result of altered carbohydrate metabolism.

Clearly, if glucoreceptors exist they must be sensitive to intracellular glucose utilisation, rather than levels of blood glucose. Patients with diabetes mellitus show increased food intake despite elevated levels of blood

glucose. Studies in humans indicate that food intake and sensations of "hunger" are closely associated with arterio-venous differences in blood glucose, if the effect of administered substances on blood flow is taken into account.

Evidence relating blood glucose and food intake can only be considered suggestive. No reduction in food intake following intraperitoneal or intravenous infusions of glucose was observed in either the rat (Janowitz and Grossman, 1948), dog (Bellinger, Trietley, and Bernardis, 1976; Janowitz, Hanson and Grossman, 1949), pig (Stephens and Baldwin, 1974), or rabbit (Vanderweele, Novin, Rezek and Sanderson, 1974).

In another approach to the problem, Schupf, Quartermain and Van Itallie (1976) studied food intake in rats fed triundecanoin. Feeding triundecanoin (a fatty acid with a carbon skeleton of 11 carbon atoms) as the major fat source resulted in considerable enrichment of rat fat depots with fatty acids containing odd numbered carbon skeletons. The propionate residue resulting from β-oxidation of odd numbered fatty acids can be used for gluconeogenesis and animals fed triundecanoin can maintain high levels of blood glucose (Anderson and Boggs, 1975) and liver glycogen (Van Itallie and Khachadurian, 1969) during fasting. Schupf et al. (1976) reported that fasted rats previously fed triundecanoin were just as motivated to eat as were control animals, but required less food before terminating feeding behaviour.

More direct evidence for a role of glucose metabolism in food intake control has been provided from experiments utilising glucose antimetabolites, such as 2-deoxy-D-glucose (2.D.G.), 3 O-methyl glucose, and phlorizin.

Rezek and Kroeger (1976) recently reviewed the literature on cellular glucoprivation and food intake. In all species so far studied, except perhaps the cat, intravenous infusions of 2.D.G. caused a large and sustained increase in food intake. However, an overdose of 2.D.G. can lead to drowsiness, stupor, and ataxia which will prevent the increase.

Intense debate of the "glucostatic" theory has centered around the site of the proposed glucoreceptors. Mayer (1953, 1955) postulated that the hypothalamus may be the site of central glucose receptors and changes in electrical activity in the hypothalamus appear to be closely related to the arterio-venous differences in blood glucose levels (Anand, 1967). If central glucoreceptors are important in controlling food intake, it has been argued that they must be responsive to insulin (unlike other neural tissue). A series of studies, on the susceptability of mice to injections of gold thioglucose (G.T.G.), by Debons and colleagues (Debons, Krimsky, Likuski, From and Cloutier, 1968; Debons, Krimsky, From and Cloutier, 1969; Debons, Krimsky and From, 1970) suggest that glucoreceptors may be present in the ventromedial hypothalamus and that the glucoreceptors may be sensitive to insulin. However, the unique role of G.T.G., in producing ventromedial lesions in mice, has been questioned by Rutman, Lewis and Bloomer (1966).

The role of peripheral glucoreceptors has been reviewed by Russek (1971). He concluded that glucoreceptors in the liver may be an important component of the glucoreceptor system. In particular, studies of Niijima (1969) on the discharge from the hepatic branch of the vagus, following glucose perfusion of the isolated guinea pig liver,

provide strong evidence for the existence of hepatic glucoreceptors. More recently, however, Bellinger et al. (1976) observed no decrease in food intake following intraportal injections of glucose in dogs and apparent total denervation of the liver was without effect on subsequent food intake. Evidence from glucose antimetabolite studies summarised by Rezek and Kroeger (1976) suggests that both peripheral and central glucoreceptors may be present and each can exert dominant effects on food intake, depending on experimental conditions. Epstein and Teitelbaum (1967) studied rats with lesions in the lateral hypothalamus and presented evidence for a specific loss of the ability to respond to glucoprivation, despite apparent regulation of food intake (although see Stricker, 1976).

Therefore, considerable evidence exists for a role of carbohydrate metabolism in the control of food intake. Nevertheless, there are many conflicting reports and under some circumstances food intake appears to function adequately in the absence of any "glucostatic" control.

1.1.2 (iv) Summary:

Food intake is influenced by many factors and no single postulated mechanism provides an adequate explanation for the control of food intake. Many reports of conflicting evidence have been mentioned. Rather than challenging the validity of published literature, they point more to the complexity of the mechanisms involved. The possible role of anticipatory reflexes in influencing respiratory quotient and blood glucose further confuses the issue (Morgane and Jacobs, 1969). Furthermore, carbohydrate

metabolism, fat metabolism, and body temperature are themselves subject to feedback control at many levels and all are interrelated. Stevenson (1969) demonstrated a very high correlation between blood glucose levels and rectal temperatures in rats following exercise and/or insulin treatment. In his review, Kennedy (1966) wrote "The metabolism of glucose and lipids is so interrelated that either one may decrease the oxidation of the other, even in the short term ..... Not only would it be difficult to imagine a feedback to the hypothalamus of information about one that is not affected by the other, but this applies in the short intervals between meals as well as to longer intervals."

1.2 DIETARY AMINO ACID BALANCE AND FOOD INTAKE:

Changes in the proportion of balanced protein in a diet, or altered amino acid balance, result in changes in food intake and growth which range from mild tempory effects to severe and persistent disturbances culminating in death. The effects of altered proportions of dietary amino acids including imbalances, toxicities, antagonisms, and deficiencies have been extensively reviewed (Harper et al., 1970).

In their review, the authors discussed operational definitions for altered amino acid balance. An imbalance occurs when amino acids, other than the growth limiting amino acid, are added to a low protein diet. Addition of one amino acid, especially the one second most limiting for growth, can cause an imbalance, but the most consistent effects have been obtained from the addition of a mixture of essential amino acids lacking the growth limiting amino acid. An amino acid deficient diet contains a lower level of the growth limiting amino acid.

Harper et al. (1970) made a clear distinction between deficient and imbalanced diets. "Investigations of deficiencies are concerned with responses of animals or subjects to an inadequate amount of an amino acid. Investigations of imbalances are concerned with the effects of surpluses of indispensable amino acids other than the one that is limiting for growth or maintenance." The distinction is important in interpreting experimental results, but a consideration of the definitions reveals an important point. The distinction does not relate to the experimental diet (which may be identical in both cases), but to the control

diet used for comparison and especially to the relative levels of amino acids in diets fed prior to the experimental period.

1.2.1 Amino Acid Balance, Food Intake and Growth:

Rats fed amino acid deficient or imbalanced diets have shown a depression in food intake and growth (Harper et al., 1970; Sanahuja, Rio and Lede, 1965). After a few days animals adapted to the imbalanced diet and began to increase food intake and growth (Harper et al., 1970; Leung and Rogers, 1970). It is not clear whether food intakes return to the level of intakes of animals fed the "basal" diet, or stabilise at some other value. Leung, Rogers and Harper (1968a) demonstrated that food intakes and growth rates approached levels seen on the "basal" diet, but in the experiment, there was no difference in the food intakes of animals fed either the "basal" or "corrected" diet, despite differences in growth rates. The food intakes of rats fed an isoleucine imbalanced diet returned to the levels of food intake of animals fed the "basal" diet, although the pattern of meals was not the same (Rogers and Leung, 1973). Stable food intakes for animals on the "corrected" diet were not reported.

The severity of the depression in food intake and growth depends on the protein content of the diet, the type of dietary carbohydrate and the environmental conditions of the experiment (Harper et al., 1970). Initial observations on amino acid imbalances were complicated by vitamin deficiencies and the food intake depression was considered a secondary phenomenon. More detailed studies emphasised the rapid onset of lowered food intake and suggested

that the depression in food intake may be an early response to the imbalanced diet. When the food intakes of rats fed an imbalanced diet were increased by force-feeding (Leung, Rogers and Harper, 1968a), insulin injection (Kumta and Harper, 1961) or cold exposure (Harper and Rogers, 1966) the animals gained weight as fast as controls and appeared healthy. Harper et al. (1970) concluded that the basic effect of the imbalance was on food intake regulation.

When offered a choice between two or more diets under normal conditions, rats reliably select against diets with imbalanced amino acid patterns (Harper et al., 1970). Given a choice rats ate a protein free diet which would not support growth rather than an imbalanced diet which would allow a modest growth rate. Robinson (1975) studied the food preferences of pigs offered a choice between an imbalanced diet and a protein free diet. The pigs responded in a similar manner to rats, rejecting the imbalanced diet in favour of the protein free diet. The response of rats offered a choice of diets has been altered by prior feeding of a high protein diet or injections of cortisol, but in the case of cold exposure, which increased the intake of an imbalanced diet, rats still chose the protein free diet and lost weight (Harper et al., 1970).

1.2.2 Plasma Amino Acid Patterns and Food Intake Control:

Amino acid imbalances and deficiencies cause a consistent alteration in plasma amino acid pattern in rats (Harper et al., 1970; Rogers, 1976). The concentration of the limiting amino acid falls to low levels while

the concentrations of the other essential amino acids rise. Similar alterations in plasma amino acid patterns have been reported in pigs fed amino acid imbalanced diets (Robinson, 1975). Harper et al. (1970) concluded that the alteration in plasma amino acid pattern coincides closely and possibly precedes the depression in food intake. The possible involvement of the altered plasma amino acid pattern in the food intake response led Leung and Rogers (1969) to a more direct study of the problem. In an important experiment, they compared the effect of the site of infusion of the limiting amino acid in rats fed threonine and isoleucine imbalanced diets. A small amount of the limiting amino acid infused into the carotid artery prevented the food intake depression observed in animals infused with saline. Infusion of the limiting amino acid into the jugular vein did not prevent the food intake depression. The results were interpreted as evidence that the plasma level of the limiting amino acid was capable of influencing a central food intake regulatory mechanism. However, animals with cannulae in the carotid artery consistently ate 10 - 20 percent less than animals with jugular vein cannulae. It was suggested that the difference in food intake may have been due to a restriction in the blood supply to the brain, but the possibility that the infused amino acid may have influenced brain blood supply was not discussed and infusions of other amino acids were not attempted.

When rats with lesions in the ventromedial hypothalamus were fed diets with imbalanced amino acid patterns (Krauss and Mayer, 1965; Leung and Rogers, 1970; Nasset, Ridley and Schenk, 1967; Scharrer, Baile and Mayer,

1970) they did not decrease their food intakes on mildly imbalanced diets, but more severely imbalanced diets caused a reduction in food intake. Animals with lesions in the lateral hypothalamus also reduced their food intakes in response to amino acid imbalance (Scharrer et al., 1970), suggesting that the "central food intake regulatory mechanism" sensitive to plasma amino acid pattern must reside outside of the areas of the brain traditionally accorded a role in food intake control. In a series of lesion experiments Rogers and Leung (1973) implicated the medial amygdala and anterior prepyriform cortex as brain areas involved in the response of animals to amino acid imbalanced and amino acid deficient diets.

1.2.3 Dietary Amino Acid Balance and Altered Carbohydrate Metabolism:

While most studies on amino acid imbalances or deficiencies have concentrated on the effects on protein metabolism, other alterations in metabolism have been reported. Animals fed amino acid imbalanced diets for two weeks had increased liver weights. The protein and glycogen content in the liver was increased, but the lipid content decreased (Sanahuja, et al., 1965). The most striking feature was the increase in liver glycogen which reached almost 10 percent of the liver weight.

Following earlier studies, which demonstrated that young rats force-fed diets devoid of a single amino acid develop pathological lesions including increased liver glycogen, Sidransky, Wagle, Bongiorno and Verney (1969) studied glucose tolerance and glycogen metabolism in

similar force-feeding experiments. After force-feeding animals for 1, 2 or 3 days, on a diet devoid of threonine, blood glucose levels were similar to levels in control animals 16 to 18 h after the final feeding. However, glucose tolerance was impaired on all 3 days, especially on the third day. Measurements of hepatic glycogen indicated that the levels were similar up to 6 h but at 16 h the animals force-fed the threonine devoid diet had higher glycogen levels. Breakdown of liver glycogen, tested using epinephrine, glycine, insulin, or glucagon, appeared to be similar in the two groups. Clark and Barron (1972) have also observed increased hepatic glycogen in animals force-fed diets devoid of a single amino acid, although the changes depend on the age of the animal and the diet fed. Data on label incorporation indicated that the increased glycogen levels resulted from increased synthesis of glycogen. When animals were force-fed a threonine deficient diet, rather than a diet devoid of threonine, significant changes in liver glycogen were observed after 3 days but not when animals had been fed for longer periods (Sidransky and Verney, 1968). The situation is complex, since no change in liver glycogen was observed in animals force-fed a diet devoid of threonine, after a prolonged period of force-feeding either the complete or deficient diet.

Peret, Chanez, Cota and Macaire (1975) recently reported an elegant study of the relationship between dietary protein and the activities of certain enzymes of carbohydrate metabolism in rat liver. As the percentage of dietary protein increased, the activity of phosphoenolpyruvate carboxykinase increased while the activity of pyruvate

kinase decreased. The equilibrium between gluconeogenesis and glycolysis was altered by proteins of different quality and the cross-over point in enzyme activity coincided closely with the maximum growth rates achieved for each protein. Peret et al. (1975) concluded that the results support their hypothesis that, "for a given protein, maximum growth depends not only on its ability to meet quantitative and qualitative needs for amino acids, but also, on the maximum energy available in the form of ATP as shown by the metabolic equilibrium between glycolysis and gluconeogenesis."

1.2.4 Summary:

In summary one of the early effects of feeding amino acid deficient or imbalanced diets is a depression in food intake. Despite considerable circumstantial evidence that altered amino acid pattern may play a role in the reduced food intake, the more direct evidence is not compelling. In view of the multiple effects of lesions in other brain areas (Grossman, 1975; Hoebel, 1971; Hustvedt and Løvø, 1972; Stricker, 1976), further studies of animals with lesions in the prepyriform cortex would be justified before concluding that it is the site of receptors sensitive to the level of limiting amino acids in blood plasma. The role of altered carbohydrate metabolism in the food intake response to imbalanced or deficient diets also awaits further elucidation.

1.3 AIMS AND OBJECTIVES OF THE PRESENT EXPERIMENTS:

The depression of food intake and growth observed on feeding diets with disproportionate levels of amino acids has been mainly studied in rats. However, Robinson (1975) observed that pigs fed amino acid imbalanced diets exhibit depressed food intake, altered plasma amino acid patterns, and diet choice patterns similar to results reported for rats (Harper et al., 1970). Pigs fed diets based on maize, with meat and bone meal as a protein supplement, showed depressed growth rates and increased feed refusals, especially when the diets were supplemented with lysine and methionine (Stables and Carr, in press).

For a study of the rapid alterations in metabolism that may occur when animals are fed diets with deficient or imbalanced amino acid patterns, the pig has several advantages over the rat. The greater blood volume and the ability to maintain indwelling catheters for long periods of time (Harris, 1974; Shearer and Neal, 1972; Wingfield, Tumbleson, Hicklin and Mather, 1974) allows for serial blood samples to be taken. The size of samples can be sufficient to measure the concentrations of a broad spectrum of metabolites and hormones. The previous sections dealt with the control of food intake and observations on amino acid imbalance and food intake. The discussion stressed the complex nature of the interacting metabolic systems, leading to difficulties in the interpretation of results from many of the experiments.

Therefore, it was decided to use the pig as a model for food intake responses on an amino acid deficient diet and in the first instance measure the changes in some

blood metabolites and hormones in response to the diet. In addition to plasma amino acid patterns, the metabolites or hormones to be measured were decided upon, on the basis of a central role in intermediary metabolism and/or a postulated role in the control of food intake.

The "glucostatic" hypothesis (Mayer, 1955) is perhaps the most studied hypothesis concerning the control of food intake. Of special interest is the role of the pancreatic hormone, insulin. Insulin is important in protein metabolism (Cahill, Aoki, Brennan and Müller, 1972; Manchester, 1970a; Manchester, 1970b; Wool, Wetlenhall, Klein-Bremhaar and Abayang, 1972), carbohydrate metabolism (Ashmore and Weber, 1968; Frohman, 1969; Narahara and Cori, 1968), and lipid metabolism (Frohman, 1969; Krahl, 1974). Lowered plasma insulin levels have been reported in animals fed a low protein diet (Young, Vilaire, Newberne, and Wilson, 1973). Altered insulin levels have been implicated in the obesity produced by lesions in the ventromedial hypothalamus (Hustvedt and Løvø, 1972) and it has been suggested that insulin (in conjunction with other factors) may play a role in the control of food intake (Kennedy, 1966; Woods, Decke and Vasselli, 1974).

Pituitary growth hormone (GH.) has multiple actions, including effects on protein synthesis and amino acid transport (Korner, 1968; Kostyo, 1968a; Kostyo, 1968b; Manchester, 1970b) and effects on carbohydrate and lipid metabolism (Altszuler, Rathgeb, Winkler, de Bodo and Steele, 1968; Batchelor and Stern, 1973; Goodman, 1968). Plasma GH. levels are elevated during starvation although not during protein depletion (Adibi and Drash, 1970) and Woods et al.,

(1974) have suggested a role for GH., in conjunction with insulin, in controlling body weight.

Glucocorticoids have important actions in the control of gluconeogenesis (Ashmore and Weber, 1968). Injection of cortisol prevented the depression in the food intake of animals fed imbalanced diets (Leung, Rogers, and Harper, 1968b; Sanahuja and Rio, 1971), and caused an increase in the plasma levels of essential amino acids including the limiting amino acid threonine (Leung et al., 1968b).

The metabolites, glucose and urea, are also of interest. The importance of intracellular glucose metabolism in the control of food intake and alterations in glucose metabolism observed in animals fed amino acid imbalanced or deficient diets has been discussed. Prior, Milner and Visek (1975) demonstrated increased blood urea and increased urea excretion rates on amino acid deficient diets. Since urea excretion is not concentration dependant and the rate of urea excretion varies directly with plasma urea concentration (Pitts, 1968), plasma urea may indicate changes in amino acid catabolism.

Therefore, the programme of research discussed in the next two chapters was undertaken to fulfil the following objectives:

1. To study the depression of food intake in pigs fed a maize-meat and bone meal diet, relative to the same diet supplemented with tryptophan, and observe the rapidity with which the depression in intake is manifest.
2. To study the feeding pattern of pigs fed ad libitum and any changes in the feeding pattern associated with the deficient diet.

3. To develop radio-immunoassays capable of measuring the levels of insulin and growth hormone in porcine plasma samples.
4. To establish indwelling catheters in the blood vessels of pigs, and on the basis of the free feeding patterns, take plasma samples to study the relationships between the deficient and supplemented diets, and the plasma concentrations of glucose, urea, amino acids, insulin, growth hormone, and cortisol during meals.

CHAPTER TWO

EXPERIMENTAL SECTION:

Discussion of the experimental work is divided into three sections. The first describes the feeding patterns of pigs and changes in the feeding patterns associated with a tryptophan deficient diet. The second section describes analytical methods used to measure metabolites in plasma and discusses the development of radio-immunoassays for plasma hormones. The third section describes an experiment on the effects of feeding a tryptophan deficient diet on changes in plasma levels of metabolites and hormones over the daily 2 h feeding period.

2.1 OBSERVATIONS ON FREE FEEDING PATTERNS IN PIGS:

2.1.1 MATERIALS AND METHODS:

2.1.1 (i) Diets:

The compositions of the two diets used in all experiments are shown in Table I. The diets were based on crushed maize grain and a commercial meat and bone meal. A vitamin/mineral supplement including free niacin (12 mg/kg of feed) was added to both diets.

TABLE I: Compositions of the tryptophan supplemented and deficient diets (kg/100 kg).

Constituents	Supplemented diet	Deficient diet
Maize	89.4	89.5
Meat and bone meal	10.0	10.0
Salt	0.25	0.25
Vitamin/mineral mix[a]	0.25	0.25
L Tryptophan[b]	0.1	-

a Stated to provide /kg feed: Vit.A 6,000, Vit.D 1,000 ius; Vit.E 8, Vit.B_1 1, Vit.B_2 4, Vit.B_6 2, Niacin 12, Pantothenic acid 10, Choline 90, Fe 200, Co 1.5, Mn 40, Cu 180, Zn 80, I 1.5, Se 0.15 mg; Vit.B_{12} 8.5 µg.

b L Tryptophan. Daiichi Seiyaku Co. Ltd., Tokyo, Japan. Lot No. 46099.

Because meat and bone meals vary considerably depending on the proportions of animal by-products used as starting ingredients, one batch of meat and bone meal was purchased from the Co-operative Wholesale Society freezing works (Longburn) and used throughout the series of experiments. The meat and bone meal was stored at -12°C and mixed with freshly ground maize obtained from the Feed Processing Centre (Massey University) before each experiment. Analyses of the diets for dry matter, fibre, and protein are shown in Table II. The diet designated as deficient caused a rapid depression of food intake and growth which could be corrected by supplementation with L Tryptophan.

TABLE II: Dry matter content and chemical analyses (% dry matter) of the diets.

	Supplemented diet	Deficient diet
Dry matter	87.03	87.02
Crude fibre	6.4	6.2
Protein	13.99	13.69

2.1.1 (ii) Animals, Housing and Experimental Procedures:

Twelve Large White Landrace cross pigs, six barrows and six gilts (initial weight range 23 to 39 kg) were housed individually in metabolism crates in a controlled environment room. The ambient temperature was 18 ± 1°C and the light-dark cycle was 10 h light (0800 to 1800 h) and 14 h darkness. Water was freely available through nipple drinkers

attached to the sides of the crates. Following a preliminary period of 10 days to allow their adjustment to the experimental diets and individual housing, the animals were moved into metabolism crates. A further period of at least 3 days was allowed for the pigs to adjust to the crates and for taking some preliminary measurements. Restrictions of time and facilities dictated that the experiment be carried out on three independent groups of four animals.

Because food intake varies greatly between individual animals and increases with increasing age or weight, the experimental design employed was a double reversal design described by Brandt (1938). Reversal designs are useful for detecting small treatment differences because they take account of between animal variation and linear changes during the course of the experiment. One disadvantage is the possibility of carry-over effects from one experimental period to the next. However, one objective of the present study was to observe any changes in food intake pattern during adjustment to the diets, and food intake patterns were recorded from the first day following switches of diet.

The two experimental treatments imposed were sequences of dietary switching as shown in Table III. Each experimental period consisted of 5 days, because preliminary experiments had indicated that depression of food intake on the deficient diet occurred within 3 days. Pigs were allocated to the two treatments at random with the restriction that, within one experimental run, equal numbers of animals of each sex received each treatment. Continuous readings of food intake patterns and daily spillage measurements were obtained during the 15 days of the experiment.

TABLE III: Details of the "cross-over design" used in the experiment on free feeding patterns.

	Preliminary Period	Period 1	Period 2	Period 3
Days in each period	3 - 5	5	5	5
Treatment 1	Supplemented diet	Supplemented diet	Deficient diet	Supplemented diet
Treatment 2	Supplemented diet	Deficient diet	Supplemented diet	Deficient diet

During the third run of the experiment, one of the barrows developed a rectal prolapse, not related to the treatments, and was subsequently dropped from the experiment. As it was too late in the experiment to replace the animal with another and the design requires pairs of pigs on different treatment sequences, the results reported were obtained from 10 of the 12 animals.

2.1.1 (iii) Food Intake Recording:

Food intake patterns were measured by continuously recording the weights of individual feed bins. The feed bins, free to swing within the confines of a metal cage bolted to the front of the metabolism crates, were suspended from modified Salter No. 60M Mark II spring balances. The spindle axle of the spring balance was connected through a reduction gear to a 1.5 kΩ Relcon wire wound potentiometer (Reliance Controls Ltd., England). The potentiometer formed one arm of a Wheatstone bridge circuit, fed by a constant

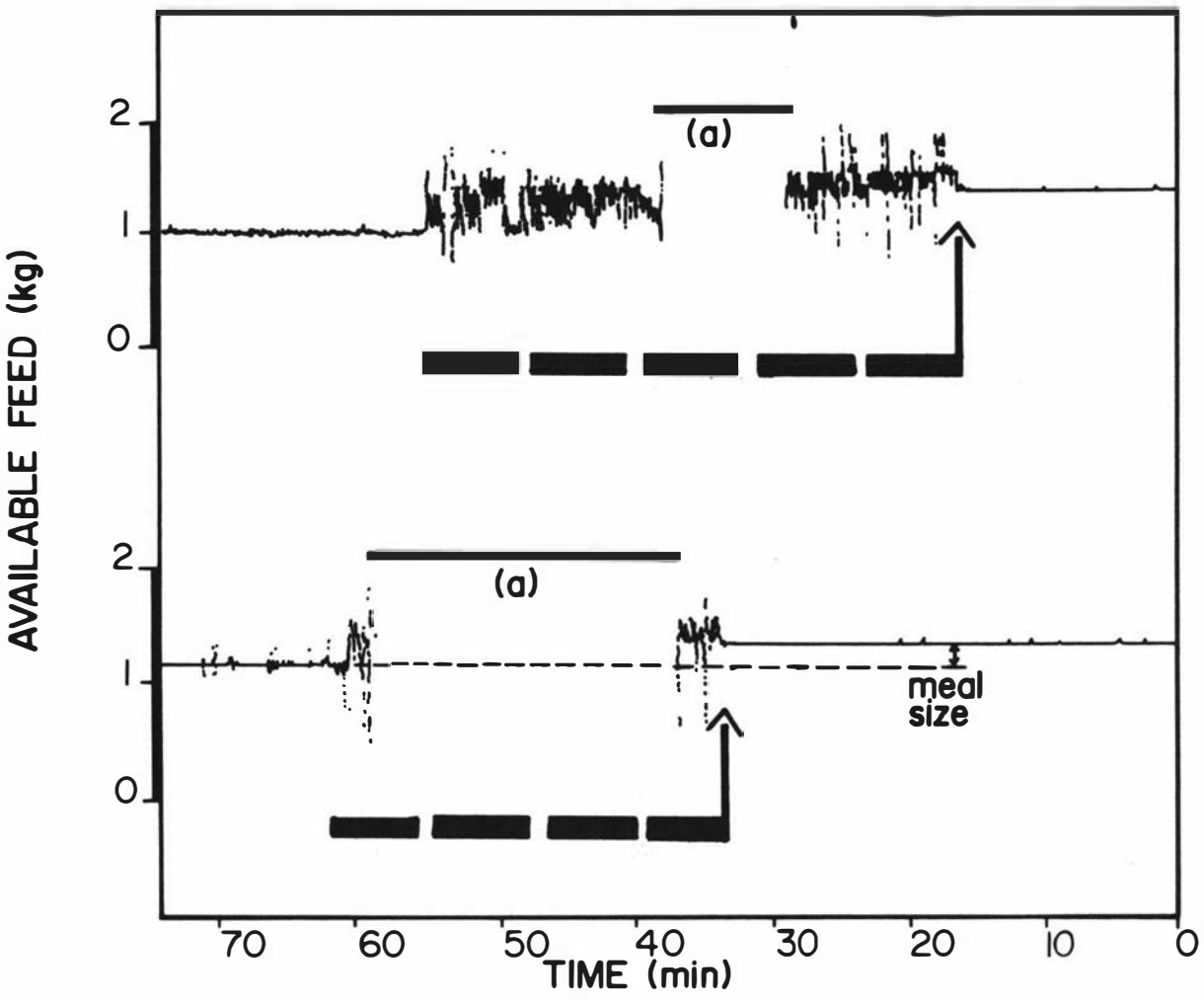

FIGURE 1 Two typical meals from the chart recordings. The broken bar indicates the meal duration and the arrow indicates the start of the meal. (a) indicates the pig standing in the feed bin during the meal.

voltage source, and output from the bridge was recorded on a Devices multichannel recorder (Devices Instruments Ltd., England).

Fig. 1 shows typical meal records from individual pigs. Feeding behaviour was apparent on the chart recording trace as rapid movements of the feed bin, although some animals preferred to stand in the feed bin during feeding, causing the chart recording to go off scale.

The vertical scale on the chart was calibrated from 0 to 2 kg at the beginning of each experiment and checked at the end of each period. A running check of calibration was also kept, since food in the bins was replenished twice daily at approximately 0830 and 1630 h, and all food added was weighed and recorded.

Estimates of food eaten at each meal were obtained from the resting position of the chart recording before and after the meal, the difference between resting positions giving the meal size. These estimates had some limitations, because the full scale deflection of the recorder was only 5 cm and meal sizes could only be measured to ± 25 g. More accurate estimates of food intake would have required more sophisticated and considerably more expensive equipment, which could not be justified at this stage. Despite the limitations in measuring meal size, the food intake measurements from the charts and from records of food added, corrected for the residual food in the bins at the end of each period, agreed to within 4% and this difference was not statistically significant ($P > 0.25$).

Estimates of meal duration and the intervals between meals could be obtained from the horizontal chart

distance during meals and between meals. These intervals could be measured to ± 0.5 min. In reading the charts a meal was defined as a bout of feeding activity where any pause did not exceed 10 min, and where total intake was greater than 25 g. Feeding activity separated by intervals longer than 10 min were considered different meals.

2.1.1 (iv) Food Spillage:

The food intake recording apparatus was unable to prevent pigs spilling food. All spilt food was collected in trays underneath the feed bin and weighed daily. However, water from the nipple drinkers caused some contamination, and the food spilt by individual pigs was bulked separately for dry matter analysis at the end of each period. Figures were corrected back to air dry weights of daily food spilt.

2.1.1 (v) Statistical Analysis:

Each meal has several associated variables: meal size, meal duration, and the intervals to the two adjacent meals. In future, the associated variables will be collectively referred to as meal pattern characteristics. Meal patterns were analysed in two stages. First, individual meal pattern characteristics were analysed for differences due to diets; second, the relationships among different meal pattern characteristics were examined.

Inspection of the raw data for individual pigs suggested that the meal pattern characteristics were not normally distributed. Frequency distributions for the two diets were calculated, taking account of the variation between pigs, by subtracting the means for the relevant

periods within pigs from each record. The distributions were skewed with large numbers of small meals and small intervals between meals.

To analyse the effects of diet on individual meal pattern characteristics, it was necessary to use daily means, because meals occurred at different times during the day and the daily number of meals varied. The central limit theorum states that means drawn from a non-normal frequency distribution tend towards normality as the number of observations in the mean increases (Snedecor and Cochran, 1967), so the use of average data reduced the problem of the non-normal distributions. Daily food intake and mean daily figures for meal size, meal duration, and intermeal interval were analysed by the method of analysis for reversal experiments described by Brandt (1938).

Frequency distributions were also analysed using a non-parametric or distribution-free test as an alternative approach. Although such tests do not depend on the assumptions of normality and additivity necessary for analysis of variance techniques, they are generally less efficient in detecting departures from the null hypothesis. The non-parametric test chosen was the Log Likelihood Ratio Test described by Sokal and Rohlf (1969).

The effect of diet on the number of meals per day (a discontinuous variate ranging from 5 to 16) was analysed by summing the number of meals over the 5 days in each period for each pig. A "one-way" analysis of variance between diets was carried out separately for each period on the individual pig totals after square root transformation.

The use of a reversal design complicated the

calculation of the mean dietary effects because pigs contributed two values to one diet mean and only one value to the other mean. The difference between diets was therefore calculated on a within pig basis. For each pig the difference between the diets (d) is given by:

$$d = \tfrac{1}{2}(\text{period } 1 + \text{period } 3 - 2 \times \text{period } 2)$$

The sign of d depends on the sequence of diets within each treatment, and the mean dietary difference (D) is given by:

$$D = \tfrac{1}{2}\left[\frac{\Sigma d_{t1}}{n} - \frac{\Sigma d_{t2}}{n}\right]$$

where t1 = treatment 1

t2 = treatment 2

n = number of pigs in each treatment.

The effect of the diets was calculated from the overall mean of the data (M).

$$\text{supplemented diet} = M + \tfrac{1}{2} D$$

$$\text{deficient diet} = M - \tfrac{1}{2} D$$

In the second part of the analysis the relationship between meal pattern characteristics was considered. A logarithmic transformation gave distributions more closely approximating a normal distribution, and further analysis used logarithmic transformations of meal size, duration, and the intervals preceding and following meals.

There was considerable diurnal variation in both number of meals and average meal size and the data were sub-divided into the light phase and dark phase before correlation analysis. The within day correlation coefficients for individual pigs were calculated for all combinations of the variables; time of day and meal size through to following

interval. Z transformations of the correlation coefficients (de Castro, 1975; Snedecor and Cochran, 1967) were analysed for the reversal design as earlier described. The analysis could only be carried out for meals in the light phase since the number of meals in the dark phase was often insufficient to allow calculation of the correlation coefficient.

It was assumed that data for individual days within periods within pigs could be pooled, and the correlation coefficients were calculated for periods within pigs, for both the light and dark phases. The correlation coefficients were then combined using the Z transformation (Snedecor and Cochran, 1967). Unless the differences between pigs were statistically significant ($P < 0.01$) a single estimate of the correlation coefficient was calculated for each diet. Differences between diets were tested and a single estimate calculated where appropriate. The range of correlation coefficients is reported for any significant differences between pigs.

2.1.1 (vi) Spectral Analysis of Daily Food Intake Patterns:

The food intake time series was submitted to spectral analysis to determine the dominant frequencies present before any averaging of the series to records of fixed length. Graphs calculated on a fixed record length show the presence of rhythms only where the period is harmonically related to the record length. The techniques and computer programs used in the spectral analysis have been described in detail elsewhere (Sarelius, 1971), and a brief description is included in Appendix 1.

Data on food intake, time spent eating, and

number of meals were allocated to respective half-hour intervals according to the time a meal started and the meal duration. Means and standard errors within each half-hour interval were calculated to present a composite "average" day.

2.1.2 RESULTS:

With unrestricted access to a dry, coarse ground diet, the pigs ate between 5 and 16 discrete meals per day. The means, standard errors of the means, and the ranges of observations for individual meal parameters are shown in Table IV. The average daily intake of the pigs comprised a little over nine meals, each of about 172 g.

TABLE IV: Mean daily *ad libitum* intakes of all experimental animals and the characteristics of their feeding behaviour.

Feeding pattern characteristics	Mean	Std. error	Range
Total food intake (g)	1589.3	34.81	725 - 2950
Number of meals	9.26	0.16	5 - 16
Meal size (g)	171.63	3.08	25 - 1050
Meal duration (min)	26.35	0.05	2 - 129
Meal interval (min)	129.23	3.23	10 - 947

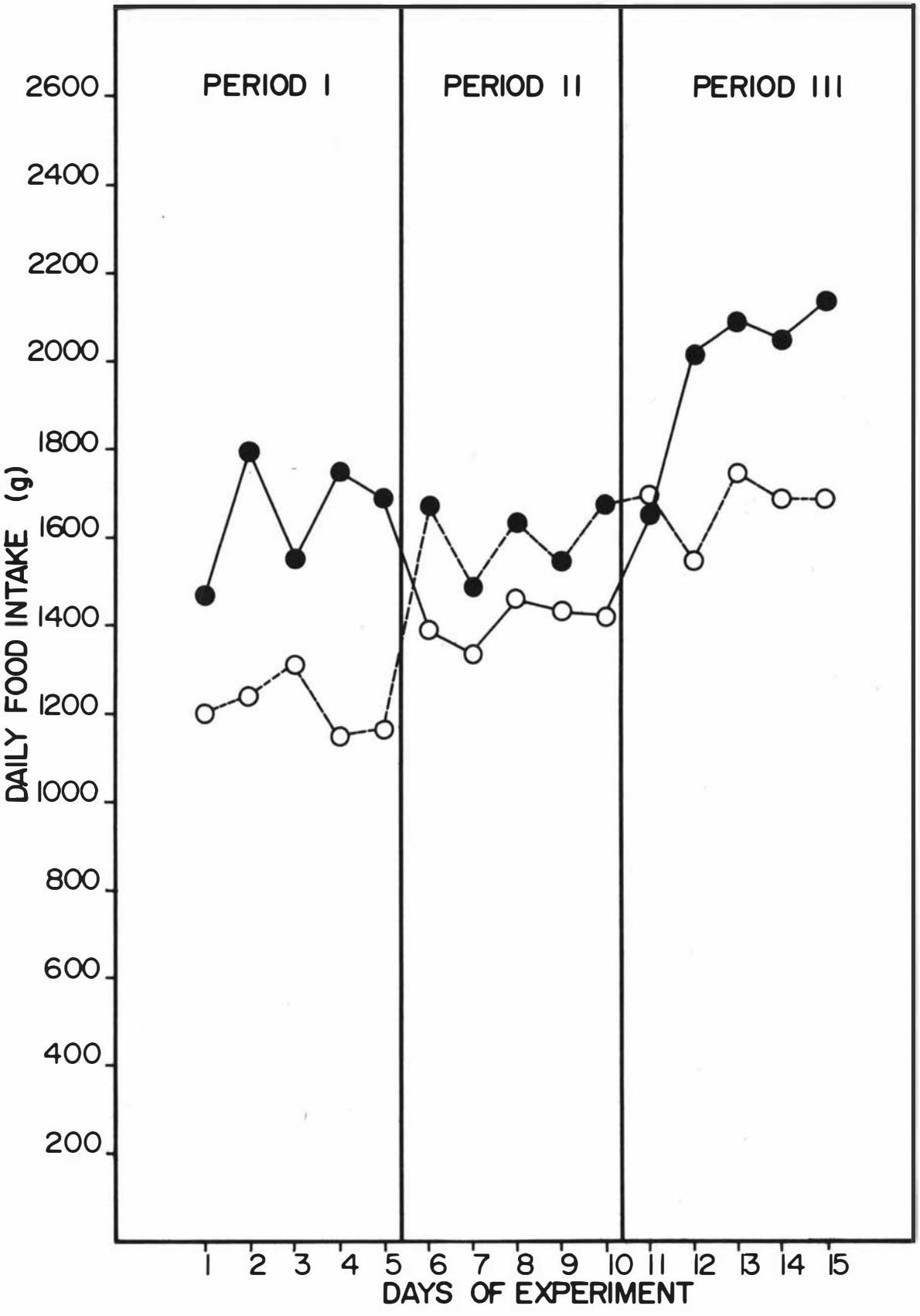

FIGURE 2 The effects of alternating a tryptophan deficient and supplemented diet on daily food intakes. Each point is the mean for 5 animals.

● Supplemented Diet

○ Deficient Diet

2.1.2 (i) Food Intake, Sex, and Diet:

To allow for the possibility of an interaction between sex and diet, both barrows and gilts were included in the experiment. In the last run the two barrows were dropped because one suffered a rectal prolapse, and a possible interaction between sex and diet was tested for by using data from the first 8 animals. The experimental model did not allow a direct test of the interaction between sex and diet. However, the algebraic sum of the total food intakes in each period, used in the analysis of the reversal design (Brandt, 1938), gave a measure of the effect of diets within pigs. The figures were submitted to a "one-way" analysis of variance between sex. The F ratio was not significant ($P > 0.1$), and it was concluded that there was no statistically significant interaction between sex and diet.

2.1.2 (ii) Effect of Diet on Meal Patterns:

Average daily food intakes for pigs on the two sequences of dietary switching (Fig. 2) showed a cross over of intakes during the second period. Intakes reversed again following the dietary switch in the third period, clearly demonstrating the effect of diet on food intake. It is evident that changes in intake began during the first 24 h following the diet switch and were almost complete within 48 h.

When the three periods were considered together, the effect of feeding either the supplemented or the deficient diet for 5 days could be calculated as described in the Methods Section (2.1.1 (v), p. 34). The results are

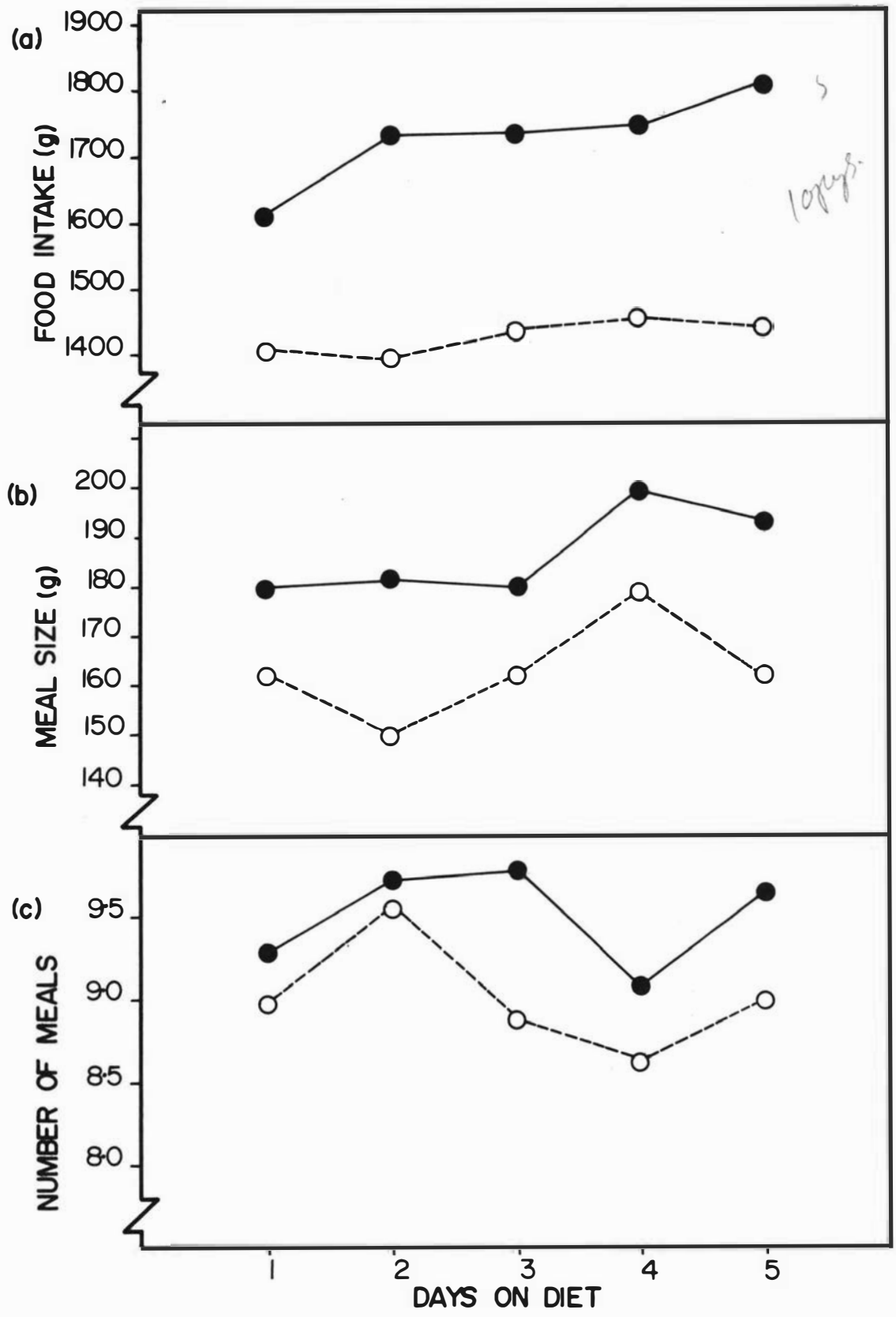

FIGURE 3 The effects of feeding a tryptophan deficient or supplemented diet on (a) food intake
(b) meal size
(c) number of meals per day.
Each point is the mean for 15 pig days.

●——● Supplemented Diet
○- - -○ Deficient Diet

presented in Table V and where the difference between diets was statistically significant it is indicated.

Daily intake appeared to be depressed by day 1; the difference became significant by day 2 ($P<0.05$) and was established and maintained for the remaining 3 days ($P < 0.01$). The widening difference between diets was due to a decrease in the intake of animals on the deficient diet and an increase in the intake of animals on the supplemented diet (Fig. 3a). During the second and third period animals switched to the supplemented diet showed an increase in intake over that in the period of feeding on the deficient diet (Fig. 2).

Daily food intake was made up of several meals of varying size. A consistent difference in the daily average meal size of pigs on the two diets (Fig. 3b) was almost significant on days 2 and 3 ($0.1 > P. > 0.05$) and was significant on days 4 and 5 ($P < 0.05$). The average difference in food intake of pigs on the two diets during days 2 - 5 was 329.31 g/day, or about 19 percent of the intake of pigs on the supplemented diet. The average difference in meal size during days 2 - 5 was 24.95 g or about 13 percent.

The daily number of meals for pigs on the supplemented diet was also consistently higher than for those on the deficient diet (Fig. 3c). The difference was never statistically significant, but, at 0.54 meals per day or about 6 percent, it accounted for the remainder of the difference in daily food intake.

A change in the daily number of meals should be reflected in longer intervals between meals for pigs on

TABLE V: The effects of feeding tryptophan deficient or supplemented diets on daily food intake and meal pattern characteristics of pigs.

		Day				
		1	2	3	4	5
Total food intake (g)	Supplemented diet	1613.95	1732.29	1737.62	1750.00	1815.21
	Deficient diet	1412.71	1391.04	1430.37	1455.00	1441.46
	Difference	201.24	341.25*	307.25**	295.00**	373.75**
Meal size (g)	Supplemented diet	180.48	181.74	180.39	199.47	193.19
	Deficient diet	162.13	150.49	162.47	179.43	162.61
	Difference	18.35	31.25+	17.92+	20.04*	30.58*
Number of meals per day	Supplemented diet	9.28	9.71	9.78	9.08	9.65
	Deficient diet	8.98	9.56	8.88	8.63	9.00
	Difference	0.30	0.15	0.90	0.45	0.65
Meal duration (min)	Supplemented diet	28.09	28.96	24.49	27.39	25.63
	Deficient diet	26.93	24.89	25.74	31.02	26.22
	Difference	1.16	4.07	-1.25	-3.63	-0.59
Interval preceding meals (min)	Supplemented diet	127.95	125.88	126.87	114.53	143.29
	Deficient diet	146.37	123.41	126.39	148.21	154.91
	Difference	-18.42	2.47	0.48	-33.68*	-11.62
Interval following meals (min)	Supplemented diet	131.65	127.71	123.23	136.51	134.79
	Deficient diet	134.17	135.80	125.67	148.11	147.85
	Difference	-2.52	-8.09	-2.44	-11.60	-13.06

+ $P < 0.1$, * $P < 0.05$, ** $P < 0.01$

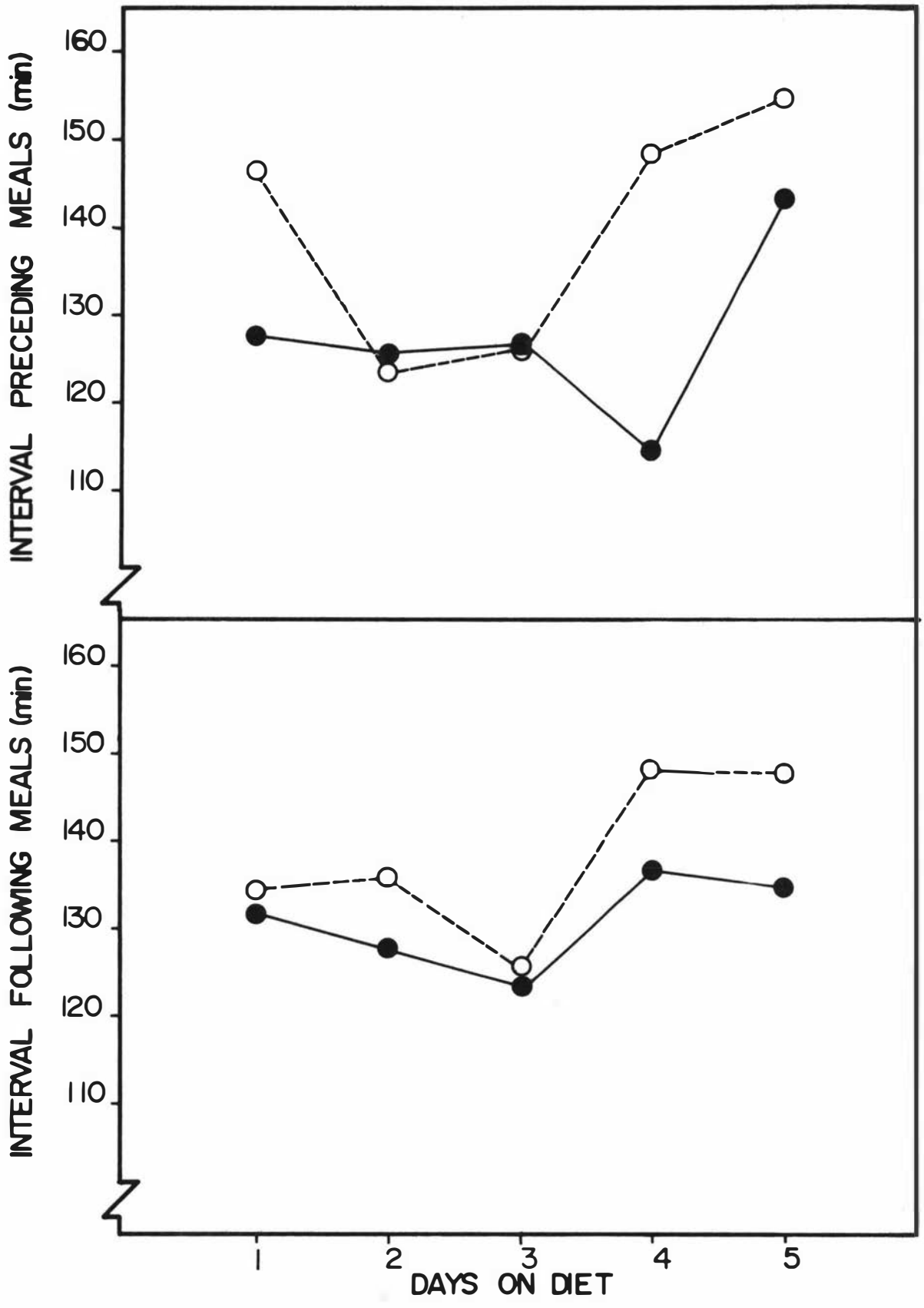

FIGURE 4 The effects of feeding a tryptophan deficient or supplemented diet on the intervals between meals. Each point is the mean for 15 pig days.

●——● Supplemented Diet

○- - - -○ Deficient Diet

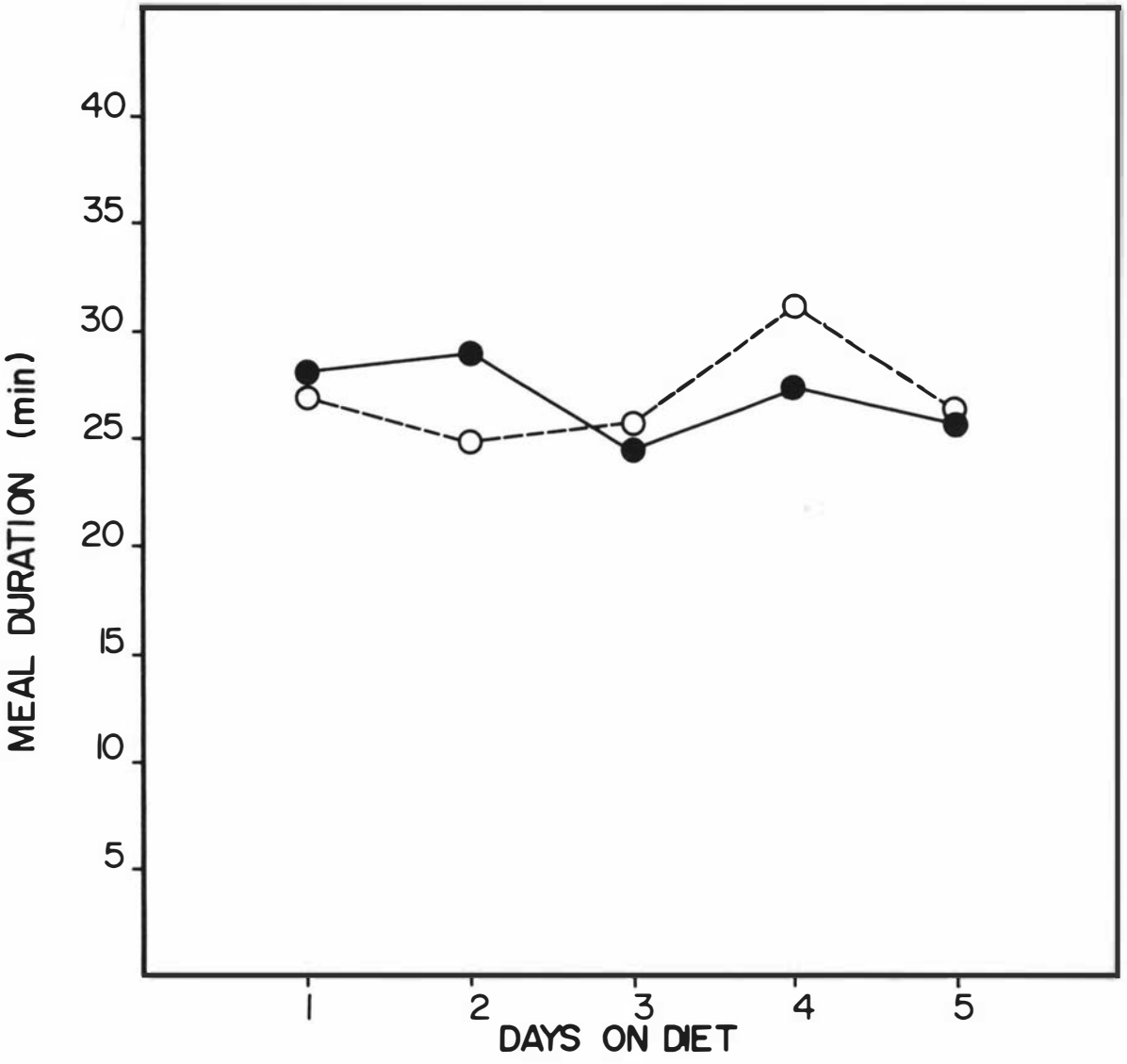

FIGURE 5 The effects of feeding a tryptophan deficient or supplemented diet on meal duration. Each point is the mean for 15 pig days.

●——● Supplemented Diet

○- - -○ Deficient Diet

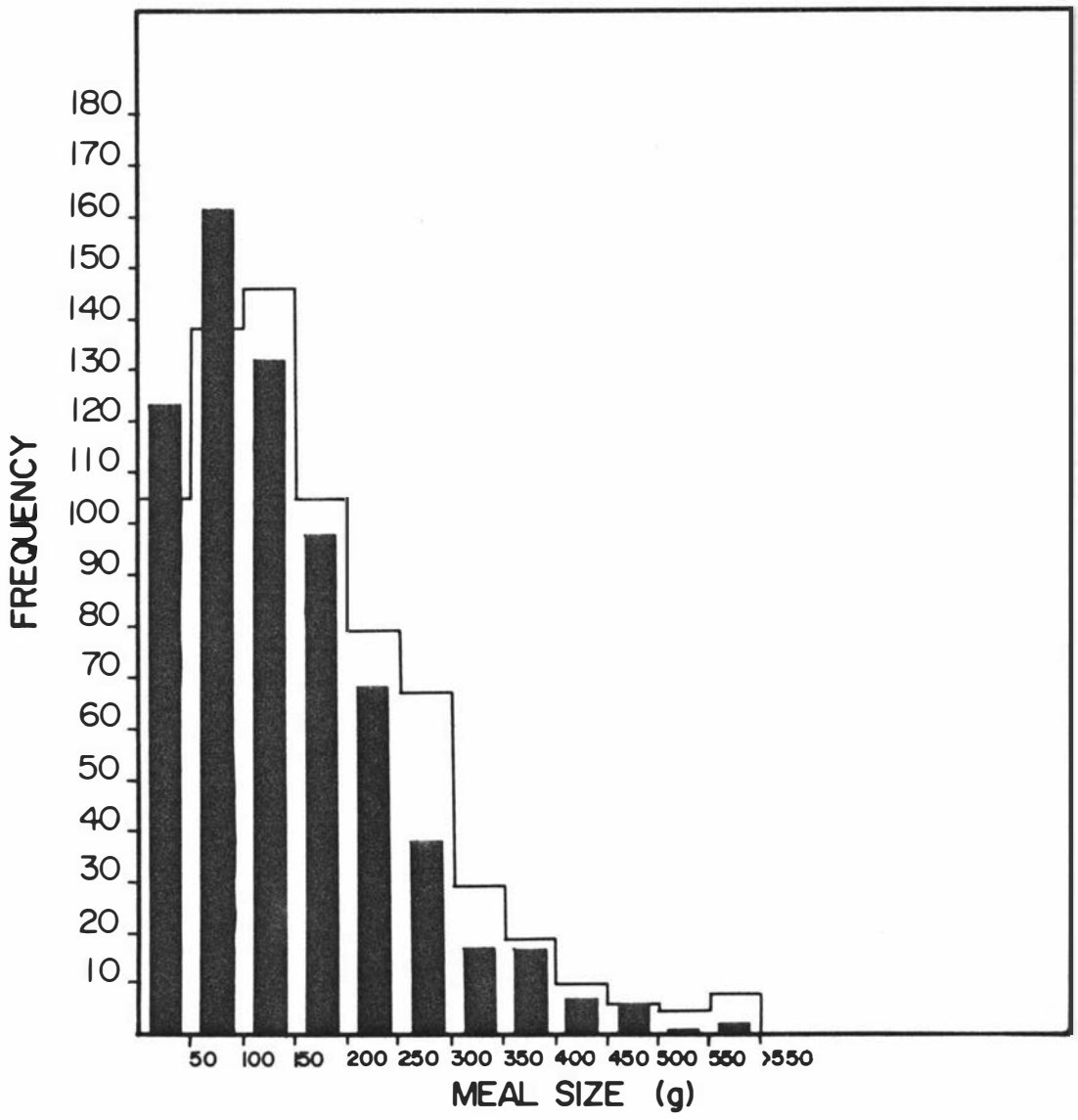

FIGURE 6 Frequency distributions of meal size for the supplemented diet (open columns) and the deficient diet (solid columns). The distributions differ significantly ($P < 0.025$).

the diet associated with fewer meals per day. Results for the average intervals between meals are shown in Fig. 4. Generally, the intervals were longer for animals on the deficient diet with the largest differences in the last 2 days. On day 4 the average interval preceding meals was significantly longer for animals on the deficient diet ($P < 0.05$), but this finding did not show up in the intervals following meals. A small change of little biological significance in the night meal pattern could have contributed to the changes in intervals preceding meals. The inconsistency in the dietary difference between intervals before and after meals suggests that the level of significance reached should be viewed with caution.

The results of feeding the two diets on average meal duration are shown in Fig. 5. No consistent pattern emerged despite significant differences in average meal size - a somewhat surprising result. The relationships between meal parameters will be discussed in greater detail in the following section.

An alternative approach to the analysis of individual meal parameters was the Log Likelihood Ratio Test (Sokal and Rohlf, 1969) for comparing distributions. The results confirmed the analysis of daily means. The only significant effect of diet was on the distribution of meal sizes shown in Fig. 6 ($P < 0.025$). On the deficient diet pigs ate more meals in the small class sizes and fewer meals in the large class sizes.

2.1.2 (iii) Relationships Between Meal Pattern Characteristics:

Product moment correlation coefficients for combinations of the meal pattern characteristics were calculated separately for both light and dark phases. The coefficients with their associated probabilities are shown in Tables VI and VII respectively.

In general the correlations are low and the probability levels are deceptive because mathematical significance does not necessarily confer biological significance on any relationship. The proportion of the variance of one parameter related to changes in the other parameter is given by r^2, the square of the correlation coefficient (Snedecor and Cochran, 1967). The meal size - meal duration correlations of about 0.6 are the highest in the two tables, but, for the only combined value given (0.63), less than half the variation in one variable($r^2 \Omega$ 0.4) is related to changes in the other variable. If the poor relationship shown between size and duration is considered it is not surprising that the deficient diet caused significant changes in meal size without affecting meal duration.

Where correlations could not be combined because of significant differences between animals, wide variation will be noted in the ranges reported. Correlation coefficients calculated from small samples show considerable sampling variation (Snedecor and Cochran, 1967), and wide variation could be expected between days for individual pigs. However, when data were combined for the 5 days within periods there was still considerable variation between animals. It is interesting that during the light phase pigs

TABLE VI: Correlations between different aspects of feeding behaviour during the light phase of the day. Coefficients were calculated for individual pigs and combined into estimates for each diet and a single estimate over all pigs and diets, unless the differences were statistically significant.

Variable pair	Supplemented diet	Deficient diet	Combined correlation or the significance of the difference between diets
Time[a]/meal size	0.13	0.05	0.09*
Time/meal duration	0.00	0.00	0.00
Time/preceding interval[b]	-0.34**	-0.42**	$P < 0.05$ some days
Time/following interval[c]	0.02	-0.05	-0.02
Meal size/meal duration	0.52** to 0.76**	0.63**	-
Meal size/preceding interval	0.06	0.12	0.09
Meal size/following interval	0.23	0.11	0.17**
Meal duration/preceding interval	0.25	0.17	0.21**
Meal duration/following interval	0.20	0.11	0.16**

a The clock time at the beginning of meals.
b The time interval elapsed since the previous meal.
c The time interval elapsed before the following meal.
* $P < 0.05$, ** $P < 0.01$. Probability levels for correlation coefficients significantly different from zero.

TABLE VII: Correlations between different aspects of feeding behaviour during the dark phase of the day. Coefficients were calculated for individual pigs and combined into estimates for each diet and a single estimate over all pigs and diets, unless the differences were statistically significant.

Variable pair	Supplemented diet	Deficient diet	Combined correlation or the significance of the difference between diets
Time[a]/meal size	-0.12	-0.12	-0.12*
Time/meal duration	-0.23 to 0.25	0.05	-
Time/preceding interval[b]	0.13	0.38**	$P < 0.01$
Time/following interval[c]	-0.84* to 0.10	-0.96** to 0.41**	-
Meal size/meal duration	-0.08 to 0.87**	-0.11 to 0.90**	-
Meal size/preceding interval	0.10	0.07	0.09
Meal size/following interval	0.21	0.09	0.16**
Meal duration/preceding interval	-0.76** to 0.65**	-0.48** to 0.67**	-
Meal duration/following interval	-0.50** to 0.62**	-0.66** to 0.64**	-

a The clock time at the beginning of meals.

b The time interval elapsed since the previous meal.

c The time interval elapsed before the following meal.

* $P < 0.05$, ** $P < 0.01$. Probability levels for correlation coefficients significantly different from zero.

differed significantly only in the meal size - meal duration relationship. In the dark phase, animals differed significantly in five relationships.

There were significant correlations between meal size and following interval and between meal duration and intervals both preceding and following meals. However, the proportion of variance in common is so low that little biological significance can be attached to the findings. Apart from the size-duration correlation the other relationship worth noting is the time - previous interval correlation. The proportion of variance in common was again low, but the interval between meals was more strongly related to the time of day than to meal size or meal duration.

2.1.2 (iv) Diets and the Relationships Between Meal Pattern Characteristics:

In the light phase daily correlation coefficients were analysed for an effect of diet. A significant difference between diets ($P < 0.05$) was noted for the relationship between time and previous interval (days 2 and 3) and for the relationship between meal size and duration (day 4 only). The relationship between the time of the day at which a meal occurred and the interval preceding that meal was closer for the deficient diet. Meal size and duration were more closely related on the supplemented diet.

During the dark phase there were too few meals to calculate correlation coefficients for individual days and the five days in each period were combined. There was again a significant effect of diets on the relationship between time and previous interval.

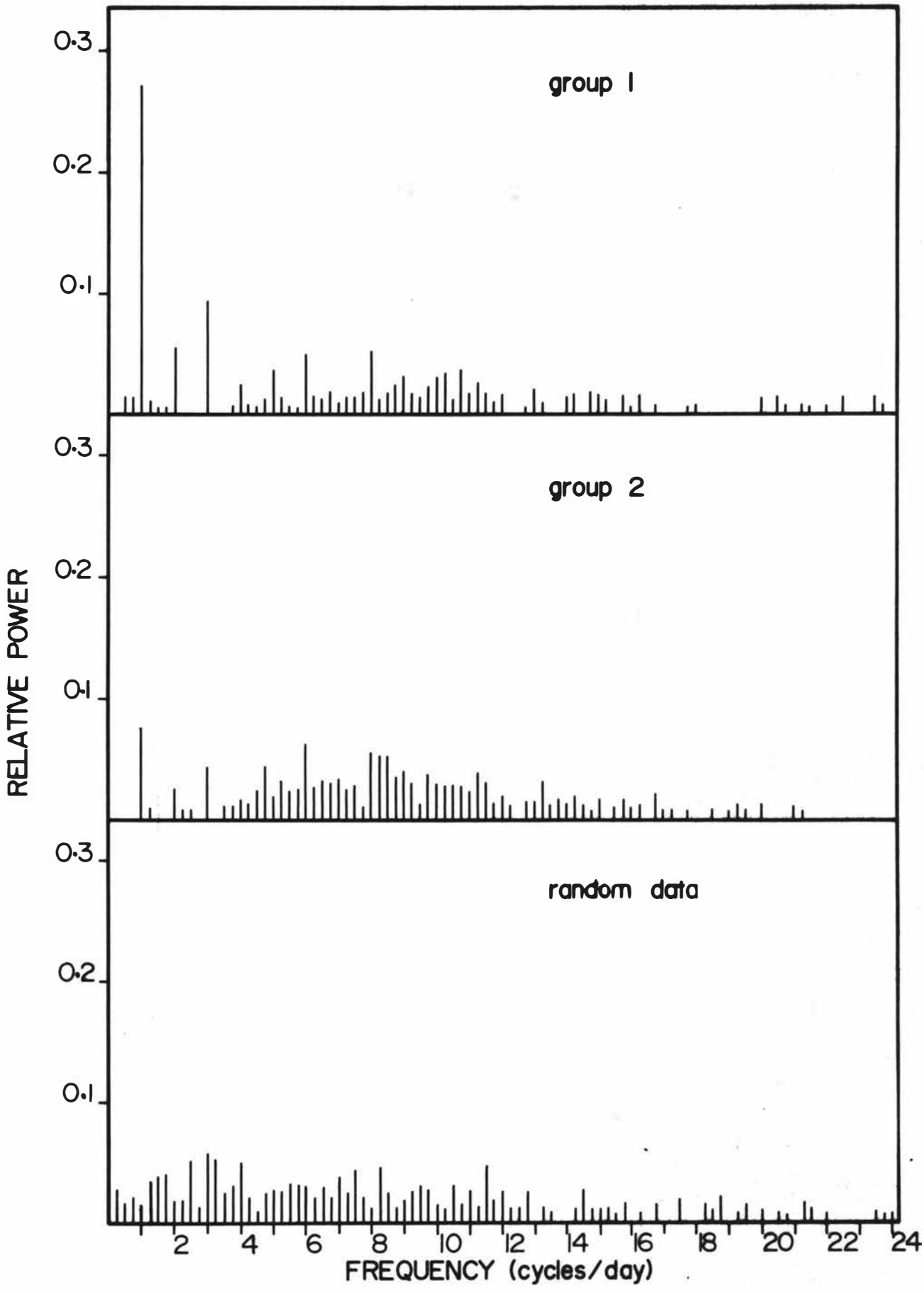

FIGURE 7 The dominant frequencies in continuous food intake records, from the 4 pigs in groups 1 and 2, compared with a set of random data. For further explanation see appendix 1.

Consideration of dietary differences must be qualified because of the low correlation coefficients discussed previously, but it is interesting that the relationship between time and previous interval was closer than other relationships and also differed significantly between diets in both the light and dark phases.

2.1.2 (v) Diurnal Patterns of Food Intake:

Spectral analysis was initially carried out on the five day records from each period. There appeared to be no effect of diet on the patterns of intake. Therefore, the 3 periods for each pig were combined and the 15 days of continuous records submitted to spectral analysis. Food intake patterns for individual animals showed some variation between pigs and that the variation was not random. Intake patterns were similar for all pigs in one experimental run, and the variability was largely between runs. The impression gained from inspection of the chart records was that the timing of meals was determined by one animal in each group consistently beginning meals a short time ahead of the other animals. Therefore, power spectra have been averaged for the four animals in each group, and the results for the first two groups plus an average spectrum for samples of random data are presented in Fig. 7. Results for the two pigs in the third group were quite similar to results for the second group and have not been presented.

In both samples of experimental data the major peaks occurred either at one cycle per day or at frequencies harmonically related to one cycle per day. Other peaks in the power spectra had little significance when compared to the random data sample. The periodic nature of the

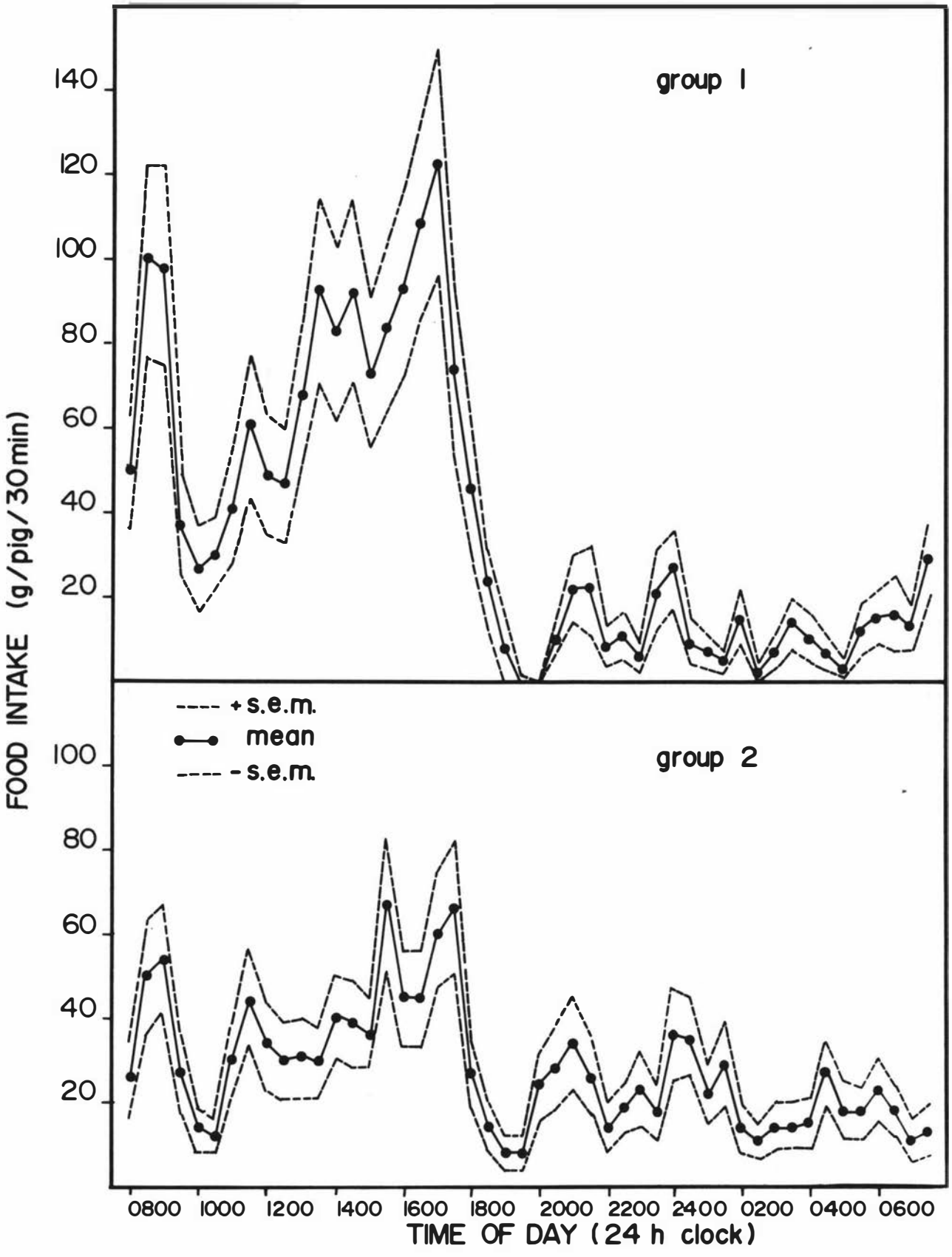

FIGURE 8 Diurnal patterns of food intake, calculated over both the deficient and supplemented diets, for two groups of 4 pigs fed ad libitum.
Mean ± standard error of the mean.
Lights were on from 0800 to 1800 hours.

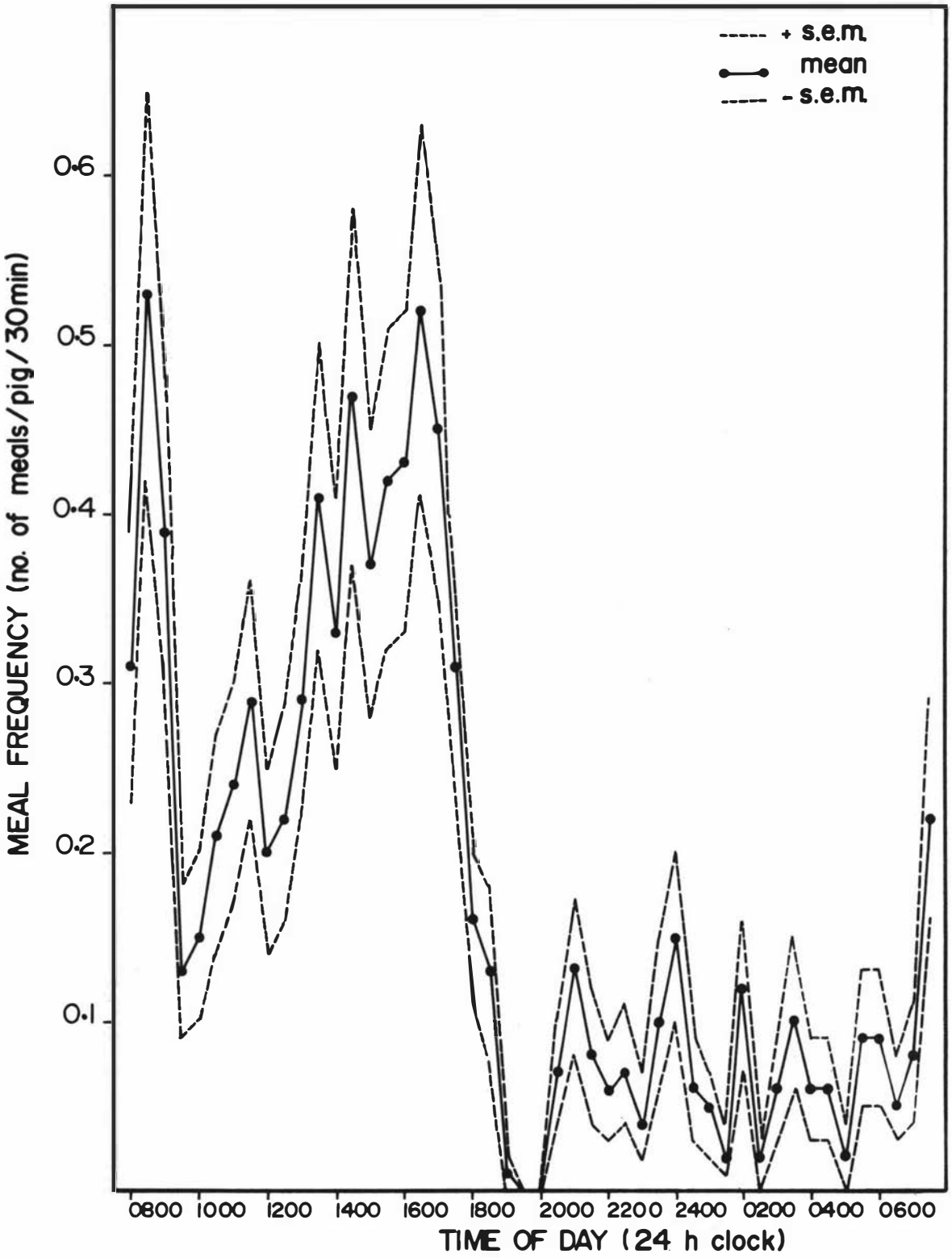

FIGURE 9 Diurnal pattern of meal frequency, calculated over both the deficient and supplemented diets, for the 4 pigs in group 1.
Mean ± standard error of the mean.
Lights were on from 0800 to 1800 hours.

feeding pattern appeared to be more consistent in the first group of pigs.

An average day has been calculated from the data, and the diurnal patterns in total food intake for the first and second groups are shown in Fig. 8. The intake pattern was similar in the two groups, although the standard errors were smaller for the first group. The pigs in the first group were heavier than any other animals, which explains the differences in intakes within any one half-hour period and may explain the more consistent intake pattern present in the first group.

About 70% of daily food intake was consumed during the 10 h light phase. A large peak in intake occurred 30 min after the lights went on. The peak coincided with the morning feeding time and probably related to the presentation of fresh feed. The afternoon peak in feeding activity was more sustained and less likely to be related to feeding time, since the afternoon feeding time was far less regular.

Total food intake in any half-hour period is the product of the number of meals and the average meal size. Patterns of results for all three groups were similar, and the pattern of the average number of meals for the first group is shown in Fig. 9. The diurnal pattern of the numbers of meals eaten was very similar to, and accounts for, most of the pattern of total intake. However, average meal size was consistently higher during the day, especially during the mid to late afternoon period, and does contribute to the diurnal pattern in total intake.

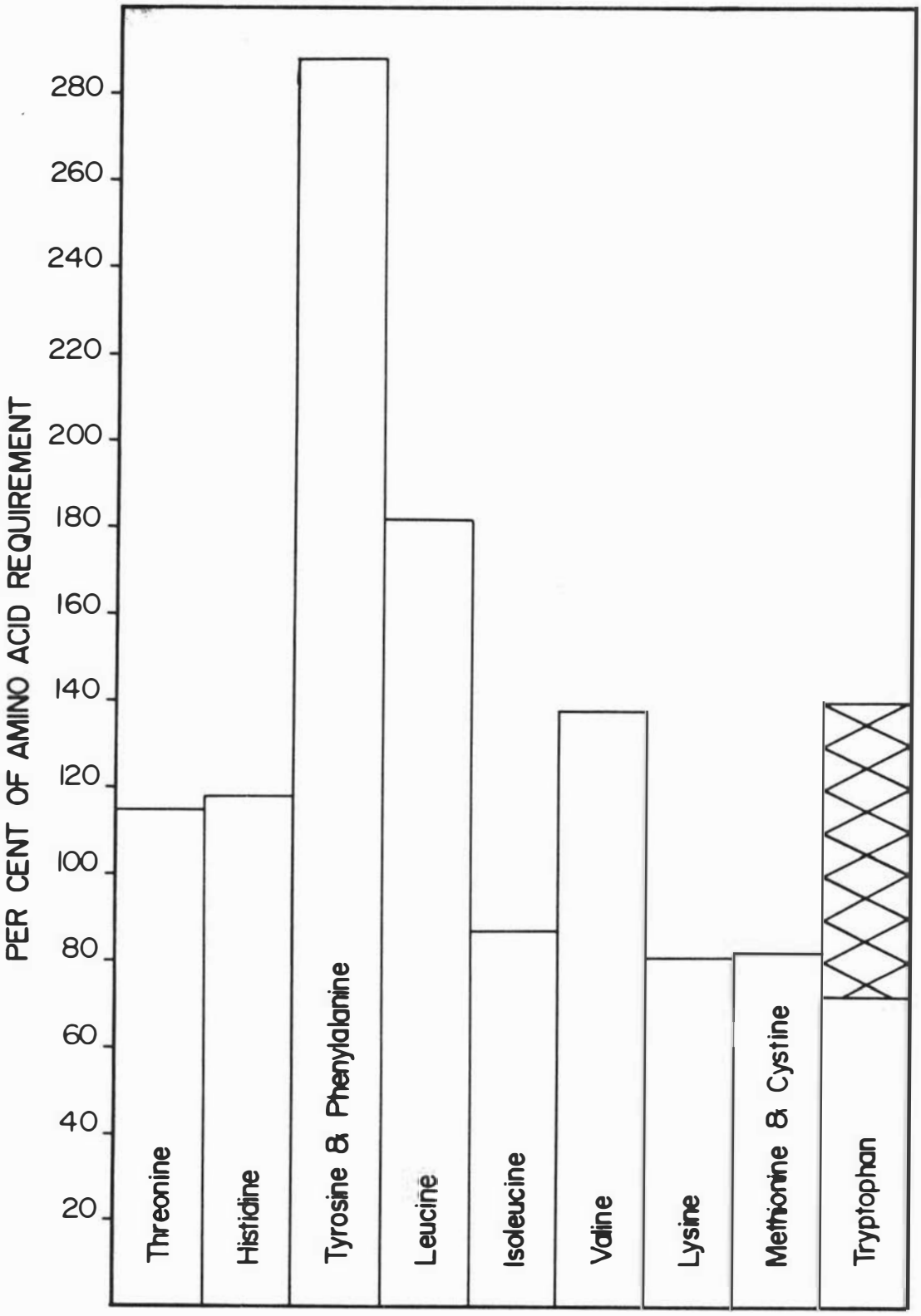

FIGURE 10 Percent of amino acid requirement supplied by the diets, calculated from amino acid requirements for the pig (Rerat and Lougnon, 1968) and the compositions of New Zealand feedstuffs (M. Adams, personal communication). The open column represents the deficient diet and the hatched plus open column the supplemented diet.

2.1.3 DISCUSSION:

2.1.3 (i) Diets:

Data on the amino acid compositions of the diets, apart from the added tryptophan, are not available. However, several investigators have reported on the poor performance of pigs fed diets containing meat and bone meal (MBM) as an additional protein source. Kennedy, Aherne, Kelleher and Caffrey (1974) attributed poor growth rates of pigs on diets containing MBM to poor protein quality of the feed. Batterham (1970), Stables and Carr (in press), and Stockland, Meade and Nordstrom (1971) concluded that tryptophan was the first and lysine the second limiting amino acid in diets based on maize grain with MBM as the only additional source of protein.

Batterham (1970) further concluded that niacin was the most limiting component of his basal diets. If this was correct a tryptophan response would also occur since tryptophan can be converted to niacin. However, in the present study the diets were supplemented with a vitamin-mineral mix which supplied 12 mg of niacin per kg of feed, the published Agricultural Research Council (1967) requirement for pigs of 20-90 kg.

The relative amino acid contents of the diets (Fig. 10) are based on the amino acid compositions of New Zealand feed stuffs calculated from a number of samples (M. Adams, personal communication[1]), and the amino acid requirements for the pig published by Rérat and Lougnon (1968). Amino acid compositions of New Zealand grown maize agree closely with figures published for North American maize

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[1] Dr. M. Adams, Ruakura Animal Research Station, Hamilton.

(National Academy of Sciences, 1971). New Zealand MBM has a low protein content and therefore has lower levels of the individual amino acids.

The amino acid requirements published by Rérat and Lougnon (1968) are in general agreement with values published by the Agricultural Research Council (1967), apart from a lower requirement for sulphur containing amino acids, which is more in line with values published by Braude and Esnaola (1973). Agricultural Research Council requirements refer to restricted feeding, so the ad libitum requirements of Rérat and Lougnon (1968) have been used.

Fig. 10 must be regarded as a simplification because of problems of amino acid availability and the variable composition of MBM. Nevertheless, it illustrates the point that the unsupplemented diet is best described as a deficient diet within the context of the definitions given in the review by Harper et al. (1970). The supplemented diet has higher levels of tryptophan but the same levels of other amino acids, and it therefore has a higher nutritional value.

2.1.3 (ii) Interpretation of Meal Patterns:

Calculations of meal patterns in the present experiments were based on an intermeal interval of 10 min. In studies in other laboratories and on other species the interval used to separate meals varied from 1 to 40 min (Kissileff, 1970; Le Magnen, 1969). Kissileff (1970), working with rats, used a minimum criterion of 1 min to separate bouts of feeding and then examined the effects of altering this criterion. His data show that in certain situations the criterion used to define meals may alter the interpre-

tation of the results. However, in a free feeding situation the criterion for defining meals did not alter the meal size distributions, and he concluded that 10-20 min after eating stops would be a natural dividing point for meals.

During the present experiment the pigs interrupted feeding to drink from the nipple drinkers and also if disturbed by noise or movement, so meals were not continuous bouts of feeding. The different diets did not radically alter the feeding pattern of the animals, and under these circumstances it did not appear that altering the criterion would alter interpretation of the data. Therefore, an interval of 10 min was chosen as the cut-off point between pauses within meals and intervals separating meals.

The chart recordings over-estimated meal size because spilt food was included on the recordings. An analysis of the effect of diet on the amount of food spilt revealed no significant difference ($P > 0.10$), and the average daily food spilt was 76.52 g or an average of 8.26 g per meal. The food spilt could not be apportioned to individual meals unless it was assumed that the amount of food spilt was proportional to meal size, an assumption not justified by observation of the animals. Therefore, raw data on meal size were not corrected for food spilt, and average meal size was over-estimated by some 4.8%. Considerable variation occurred between animals in the amount of food spilt, ranging from 1.27 percent to 9.92 percent of daily food intake. More accurate estimates of meal size would have to take account of spilt food and would require more sophisticated feeding apparatus, particularly when feeding dry meal.

2.1.3 (iii) Effect of Tryptophan Deficiency on Meal Patterns:

The effect of feeding amino acid imbalanced or deficient diets on free feeding patterns has received only limited attention. Rogers and Leung (1973) reported some limited observations on free feeding rats on diets with disproportionate levels of amino acids. The imbalanced diet caused a decrease in the number of meals on the first day after dietary switch. After the animal adapted to the diet, the daily number of meals was still lower, but food intake increased because of an increase in meal size. A diet devoid of one amino acid caused a depression in average meal size on the first day with no effect on meal number. After 14 days there was little increase in total intake, but the intake was spread over many very small meals, including several meals during the daylight hours.

Results of the present experiments are in general agreement with the above observations. The tryptophan deficient diet caused a depression in food intake that was apparent within 24 h and had reached about maximal levels by 48 h. The change in intake was largely due to a decrease in meal size of pigs on the deficient diet, and this difference in meal size was maintained throughout the 5 days on the diets. Although the decrease in the daily number of meals was not statistically significant, it accounted for a little less than one third of the difference in intake for days 2 to 5 and cannot be ignored. The short experimental periods prevent any direct comparison with the shift to many small meals observed in the rat. The level of food intake depression of about 20 percent attained within 48 h has

been maintained without adaptation for up to 15 days (unpublished observations), whereas pigs usually take less than 10 days to adapt to major changes in diet or feeding patterns. However, there was very little change in total intake of the rats on the amino acid deficient diet, and adjustment in meal patterns could occur without alteration in total intake. The longer meal intervals for pigs on the deficient diet on days 4 and 5 and the significant effect of the deficient diet on the preceding interval on day 4 indicate that longer term experiments might show a shift in meal patterns. Unlike the deficient diet used by Rogers and Leung (1973), the tryptophan deficient diet used in the present experiment was not devoid of tryptophan, would support a reduced rate of growth, and would have less severe effects on the animals.

2.1.3 (iv) Meal Pattern Correlations:

The relationships between meal pattern characteristics have been studied in many free feeding situations. Several authors, including de Castro (1975), de Castro and Balagura (1975), Le Magnen (1969), Le Magnen (1971), Snowdon and Wampler (1974), and Thomas and Mayer (1968), have reported a positive correlation between the size of a meal that a rat eats and the length of the following interval. This is cited as evidence that meal-to-meal regulation is brought about, in part, by a satiety mechanism related to the amount of food ingested at each meal (Le Magnen, 1969). However, the relationship is uncertain, and several studies have failed to replicate the findings (Kenney and Mook, 1974; Levitsky, 1974; Panksepp, 1973). The relationship has also

been observed in chickens (Duncan et al., 1970) but not in mice (Wiepkema, 1968), guinea pigs (Hirsch, 1973), or pigeons (Zeigler, Green and Lehrer, 1971). In the cat (Kanarek, 1975) there was no relationship between meal size and the interval following meals, but a positive relationship was shown between meal size and the interval preceding meals.

Studies of free feeding animals have used widely differing methods of data collection and analysis, and some of the confusion is undoubtedly related to methodological problems. Kissileff (1970) looked at the effects of changing the criteria for defining meals on the frequency distributions of meal sizes in both free and operant feeding situations. His data showed that the criteria for defining meals altered the frequency distributions of meal size and interval in the bar press situation. However, in the free feeding situation varying criteria had no effect on meal size distribution and a much smaller effect on intermeal interval distribution. Different laboratories have measured food intake by weighing feed bins, measuring meal duration, or using operant methods, and criteria for defining meals have included intermeal intervals from 1 to 40 min (Kissileff, 1970; Le Magnen, 1969). Despite a high positive correlation between meal size and meal duration in rats, the relationship between size and following interval is stronger than the relationship between duration and following interval (de Castro, 1975).

Differing methods of data collection are not the only problem. Panksepp (1973) has challenged the validity of some published correlations by pointing out

possible statistical bias due to pooling of data. One further source of statistical bias not discussed elsewhere relates to the shapes of frequency distributions of the meal parameters. The present results and published results for rats (Snowdon and Wampler, 1974) and chickens (Duncan et al., 1970) show frequency distributions that are not normal. This may give undue weight to the smaller numbers in the large class sizes, unless the data are transformed to give approximately normal distributions.

In the present study unordered data, log transformed to give approximately normal distributions, showed statistically significant correlations between several of the variables describing meal patterns. However, the correlation coefficients showed that the variables in question had such a small part of their variation in common that the relationship between intermeal intervals and meal size did not appear to be important in the free feeding pigs. In most of the studies with rats where significant correlations have been reported, there was considerable variation between animals and the variables generally had less than one quarter of their variance in common.

The timing of meals was not closely related to meal size and was clearly influenced by other factors including diurnal rhythms. In the present experiment the largest meals were often associated with the smallest intervals between meals, because of the time of day at which those meals occurred. If the diurnal pattern in intake was related to alterations in digestion, energy use, or energy storage, it may obscure a relationship between the energy content of a meal and the time that elapsed to the next meal.

2.1.3 (v) Diurnal Patterns of Food Intake:

There are few reports of feeding patterns in pigs fed ad libitum. Ingram and Legge (1974) reported a diurnal feeding pattern with higher food intakes in the afternoon although the data were confounded by alternating ambient temperatures. In an abstract published by Auffray, Bahy and Marcilloux (1974) it was reported that pigs consumed 10 - 14 meals per day with more meals consumed during the day than the night. Diurnal feeding patterns have also been reported in the chicken (Duncan et al., 1970), the pigeon (Zeigler et al., 1971), and the rat (Le Magnen, 1969; Levitsky, 1970; Siegel, 1961). In the chicken, the pigeon, and the pig, which are all active during the day, maximum intake occurred during the daylight hours. The rat, being nocturnal, showed maximum intake during the hours of darkness. The diurnal intake patterns observed in pigeons (Zeigler et al., 1971) and the rats (Siegel, 1961) showed remarkable similarity with the present data, when the phase shift in the rat is taken into account. The maximum intake of each animal occurred before the end of the active phase in the light-dark cycle.

At variance with the present results is the report of Le Magnen (1969) that the frequency of feeding in rats was similar in both the light and dark phases. The diurnal pattern in the intake of his rats was entirely due to changes in average meal size. Levitsky (1970) reported a diurnal pattern of meal frequency, but maximum intake occurred at the beginning of the dark phase. The diurnal pattern in intake in rats can be extinguished by maintaining the animals in constant, high intensity light (Siegel, 1961),

reversed by dietary manipulation (Panksepp and Krost, 1975), and affected by any patterns associated with routine feeding and cleaning. So differences in experimental or environmental conditions may account for the inconsistency of reported results.

If it is assumed that the results of Panksepp and Krost (1975) can be generalised to pigs, the diurnal pattern of intake does not represent an obligatory response to diurnal variation in any metabolic parameters. It is common practice in pig husbandry to feed a restricted amount of feed once per day, and pigs show satisfactory growth responses on this regime. However, meal feeding alters rhythms associated with hormone secretion and the metabolism of carbohydrates and lipids (Kaul and Berdanier, 1975; Leveille, 1970; Moberg, Bellinger and Mendel, 1975). Diurnal variation in glucose tolerance in humans has been reported (Melani, Verrillo, Maraso, Rivellese, Osorio and Bertolini, 1976). Ahmed, Gannon and Nuttall (1976) and Genuth (1973) studied the effects of feeding three isocaloric meals to humans. The maximal levels of glucose and insulin in plasma were similar after the three meals, but the fall in both insulin and glucose was delayed progressively from breakfast to dinner. If the same diurnal pattern in glucose tolerance was present in pigs it would raise interesting questions in relation to the diurnal pattern of food intake.

2.2 METHODS OF ANALYSIS FOR BLOOD METABOLITES AND RADIO-IMMUNOASSAY DEVELOPMENT:

2.2.1 Plasma Glucose Assay:

Plasma glucose concentrations were assayed by the glucose oxidase method described by Caraway (1970), using glucose oxidase (Fercozyme, Hughes & Hughes Ltd., England), horseradish peroxidase (Sigma Chemical Co., Type II), and o-dianisidine dihydrochloride as the oxygen acceptor. Four levels of a glucose standard (0, 50, 100 and 200 mg/100 ml, May & Baker Ltd.) were included in each assay and all standards and samples were analysed in triplicate. After full colour development the absorbance was read on a Unicam SP 500 spectrophotometer. There was a linear relationship between absorbance and glucose concentration up to 400 mg/100 ml, the highest concentration tested.

The glucose concentrations of the plasma samples were estimated in 14 assays. The mean optical density $\pm$ the standard error for the 100 mg/100 ml standard, from the 14 assays, was 0.120 $\pm$ 0.00093. The glucose concentrations of samples were calculated from the 100 mg/100 ml standard for each assay. Replicates within samples showed little variability and when all samples were considered, the variance due to replicates was less than 2% of the total.

2.2.2 Plasma Urea Assay:

Plasma urea was analysed on a Technicon Auto Analyser Mark I, using the manifold and reagents described

in the Technicon methodology publication N-16b. The method measured blood urea by a Diacetyl Monoxime colorometric reaction. A standard curve was included every 24 samples giving 8 values for calculating each standard point. The results as percent transmittance gave a curvilinear response with increasing urea concentration. When absorbance was calculated from percent transmittance (Absorbance = 2 - log [% Transmittance]), the relationship between absorbance and urea concentration was linear up to the highest urea standard included (30 mg/100 ml). A regression equation was fitted to the data for the standards and urea concentrations in the samples calculated using the regression equation.

2.2.3 Plasma Amino Acid Assay:

Amino acid analyses were carried out by Dr. G.G. Midwinter, Department of Biochemistry, Massey University, using a single column programme on a Beckman 120 C automatic amino acid analyser. The machine was calibrated for routine protein hydrolysate analysis, but no other analysis system for physiological samples was available. For each sample 0.6 ml of the supernatant from the ethanol protein precipitation step (0.2 ml of plasma equivalent) was evaporated to dryness in a rotary evaporator. The dried material was redissolved in 0.1 ml of 0.2 N citrate buffer, pH 2.2, and loaded into a Locarte automatic loading accessory on the amino acid analyser.

The analyser was regularly calibrated using a Beckman standard amino acid calibrant (0.05 µmoles of each amino acid). A tryptophan standard (0.05 µmoles and

0.01 μmoles) was run separately. Peaks on the chromatogram were integrated manually by the "half height by width" ($\frac{1}{2}$ H x W) method.

2.2.4 Plasma Cortisol Assay:

Plasma Cortisol (4-pregnen-11β, 17α, 21-triol-3, 20-dione) concentrations were analysed by P.C. Evans, Department of Biochemistry, Massey University, using a radio-immunoassay developed for measuring cortisol in human plasma samples. For each sample 0.1 ml of plasma was extracted into dichloromethane according to the method of Ruder, Guy and Lipsett (1972), and aliquots assayed directly. The method of assay was similar to the progesterone method of Fairclough, Hunter and Welch (1975). Antiserum, a gift from R.S. Fairclough, Ruakura Animal Research Station, was used at a dilution of 1 in 2000. 1, 2, 6, 7-^{3}H-Cortisol was purchased from the Radiochemical Centre, Amersham, and used at a dilution of 0.4 μCi/ml. Samples were assayed at random and, after separation, precipitates were counted in a Beckman LS 350 Liquid Scintillation Counter.

The specificity of the antiserum was determined from the cross-reaction with other steroids. The results (Table VIII) showed a 10 percent cross-reaction with cortisone but less than 1 percent cross-reaction for all other steroids tested. Dilution of plasma samples and recoveries of steroid added to plasma samples showed that over the central part of the standard curve the assay was reproduceable and samples were parallel to the standard curve. Therefore, all samples with levels greater than 100 ng/ml were diluted to lie within the range of 10-100 ng/ml.

TABLE VIII: Cross-reactions of steroids with the anti-serum used in the radio-immunoassay for plasma cortisol (courtesy of P. Evans, Department of Biochemistry, Massey University).

Compound	Cross-reaction[a] (%)
Cortisol	100
Cortisone	9.6
Deoxycortisone	0.9
Tetrahydrocortisol	<0.5
Tetrahydrocortisone	<0.1
17α OH - progesterone	<0.1
Progesterone	<0.1
Betamethasone	<0.1
Aldosterone	<0.1
Testosterone	<0.1
Androstenedione	<0.1
Estradiol - 17β	<0.1

[a] Cross-reaction calculated from (x/y) x 100
Where x is the mass of an unlabelled antigen required to displace 50% of bound labelled antigen;
and y is the mass of cross-reacting antigen required to displace 50% of bound labelled antigen.

Analysis of estimates of samples repeated in several assays showed no statistically significant difference between estimates ($P > 0.1$).

2.2.5 Plasma Insulin Assay:

A double antibody radio-immunoassay for measuring plasma insulin levels was developed jointly with G. Woolley, Dairy Husbandry Department, Massey University. Similar assays employing the same antiserum were used to measure plasma levels of both porcine and bovine insulin.

Antibodies against insulin were raised in guinea pigs immunised with either polymerised insulin or insulin conjugated to thyroglobulin. Porcine insulin (Schwarz/Mann, Lot No. 22-1123, potency 25.6 IU/mg) and bovine insulin (Sigma Chemical Co., Lot No. 121C-1350 26.4 IU/mg) were conjugated to thyroglobulin by the method of Frohman, Reichlin and Sokal (1970) and bovine insulin (Sigma Chemical Co., Lot No. 121C-1350 26.4 IU/mg) was polymerised with diethylpyrocarbonate according to the method of Wolf, Lesnaw, and Reichman (1970). 0.25 mg of hormone preparation, emulsified with complete Freunds adjuvant, was injected into guinea pigs at multiple subcutaneous sites. Injections were given at approximately monthly intervals for 4 to 6 months. Booster injections of the free hormone in sterile saline were then given and antiserum collected by heart puncture 7 to 10 days later. All animals produced antiserum which reacted with free insulin but one animal (Guinea pig 7) injected with the polymerised insulin produced antiserum of high titer which was used exclusively in the assay.

^{125}I-labelled insulin was prepared by the chloramine T method with the modification that bound and free radioactivity were separated on powdered cellulose columns (R.A. Donald, personal communication[2]). ^{125}I insulin was diluted and stored frozen until use.

Evidence from studies of the binding of standard insulin, in the absence of added protein, and with differing concentrations of bovine serum albumin, suggested that protein concentration was important in the relationship between the binding of standard insulin and insulin in plasma samples. The similarity of insulin structure between species precludes the use of untreated plasma from other species to equalise protein concentrations between standards and unknowns. Therefore, several methods of removal of insulin from plasma were tested. Molecular sieving through a Diaflow ultrafiltration Membrane PM 10 (Amicon Corporation, U.S.A.), high pH treatment, and charcoal stripping methods were employed, but plasma treated with a high pH or passed through an ultrafiltration membrane significantly inhibited the binding of the labelled hormone to the antiserum.

Therefore, a pool of heparinised porcine plasma was collected from the Longburn freezing works. The plasma was stirred for 1 h, at 4°C, with 10 percent by weight of decolourising charcoal (May & Baker Ltd.). After the charcoal was mixed with the plasma, many fine particles of charcoal passed through Watman No. 1

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2 Dr. R.A. Donald, Princess Margaret Hospital, Christchurch, New Zealand.

filter paper, and the plasma was spun at 13,000 r.p.m. for 45 min in an M.S.E. high-speed centrifuge. The supernatant was decanted and stored frozen. Before use in the assay, all diluent plasma was passed through a 6 μ filter (Ultrafiltration Membrane DP06, Amicon Corporation, U.S.A.).

The procedure used in the assay is shown in Table IX. The assay was carried out in 3 ml plastic LP 3 tubes (Luckham Co., England), and the first addition to all tubes was 0.15 ml of buffer containing 2% diluent plasma. All tubes were shaken before further additions to minimise any non-specific binding of protein to the assay tubes; a step which considerably reduced variation between duplicate estimates.

Throughout the development of both the porcine and bovine insulin assays, dilution of plasma samples yielded slopes differing from the slope of the standard curve. Small numbers of samples were used during development, and at the time of measuring the plasma samples it was thought that problems of non-parallel slopes between standards and unknowns had been overcome. Standards were made up in charcoal treated plasma and 0.05 ml of standard assayed in triplicate at each point. Two dilutions of each plasma sample (0.025 ml and 0.0125 ml) were assayed in duplicate, and charcoal treated plasma was added to all tubes to equalise the protein concentration. All the plasma samples from one animal were included in the same assay.

In the statistical analysis it was assumed that "dilutions" was a fixed variable. The results of analysis of the individual assays are shown in Table X.

TABLE IX: Reagents and procedures used in the radio-immunoassay for porcine plasma insulin.

Day		Reagent	Conditions
1	150 µl	P.B.S.[1] pH 7.4 0.01M EDTA[2] with 2% Diluent Plasma[3].	Room Temperature
	50 µl	Diluted Unknown Plasma or Standard[4] made up in diluent plasma.	
	100 µl	Guinea Pig γ Globulin 15 µg/ml and First Antibody (anti-insulin serum) at initial dilution of 1 in 20,000 made up in P.B.S. pH 7.4 0.01M EDTA with 2% diluent plasma.	
2	50 µl	^{125}I - Porcine Insulin[5] diluted in P.B.S. pH 7.4 0.01M EDTA with 2% diluent plasma.	Room Temperature
3	50 µl	Antibody against Guinea Pig γ Globulin diluted 1 in 2 with P.B.S. pH 7.4 0.01M EDTA.	Transferred to 4°C
6		All tubes spun at 2000 r.p.m. for 30 min at 4°C and supernatant aspirated. Precipitates counted in a Packard Auto γ Counter, Model 5285.	

1 Phosphate Buffered Saline.

2 Ethylenediaminetetra-acetate.

3 Charcoal stripped Porcine plasma. See text for description of method.

4 Insulin Standard. Porcine Insulin. Schwarz/Mann. Lot No. 22-1123 Potency 25.6 IU/mg.
34 mg were weighed into a siliconised glass vial (Cahn Electrobalance), dissolved in 0.5 ml of 0.003N HCl, and diluted with P.B.S. pH 7.4 10% diluent plasma containing 500 IU/ml Tracylol (Bayer Pharmaceutical Co.). Standards from 0-6400 pg/ml were included in each assay.

5 Porcine Insulin was iodinated by a chloramine T method.

6 Antiserum to Guinea Pig γ Globulin raised in sheep. See Radio-immunoassay for Growth Hormone for details.

TABLE X: Variance components from the analyses of individual insulin assays.

	Assay 1	Assay 2	Repeat Assay 1
Between samples (s)	0.08366**	0.07714**	0.04767**
Between dilutions (d) within s	0.01295**	0.00088	0.00352**
Between duplicates within d & s	0.00762	0.00982	0.00663

** $P < 0.01$

The first assay with samples from pigs 3-26 and 3-27 showed a lack of parallelism that was statistically significant. The variance between dilutions within samples accounted for 12 percent of the total variance. The first assay was repeated using similar concentrations of all reagents, except that the proteinase inhibitor Tracylol (100 IU/ml, Bayer Leverkusen, Germany) was included in all tubes, to prevent label damage by the action of proteases.

The difference between dilutions was still significant ($P < 0.01$), but the proportion of variance involved was halved. The difference between dilutions in the second assay was not statistically significant. Although the variance of duplicates in the second assay was a little higher, the variance between dilutions within samples was one quarter of that in the repeat of assay 1.

The equilibrium situation, leading to the final estimate of bound counts in a radio-immunoassay, represents a complex sequence of chemical reactions. The

subject has been discussed in Radioimmunoassay Methods, edited by Kirkham and Hunter (1971). Many factors could contribute to the lack of parallelism but are ruled out because the effect was always in one direction. Undiluted samples gave higher estimates of hormone concentration than samples diluted with charcoal-stripped plasma.

Differences in the binding of the purified insulin or insulin present in plasma samples could have resulted in different slopes for the standards and unknowns. However, it appears unlikely that such differences contribute to the lack of parallelism since it is present with some samples but not others.

The presence of complement in some plasma samples may interfere with the reaction between the antibody and the precipitating sera, causing erroneously high estimates of hormone concentration. Addition of EDTA to all tubes is thought to overcome the problem of complement. The assay was carried out in the presence of 0.01M EDTA (Table IX), and higher concentrations of EDTA had no further effect in the assay.

Small amounts of charcoal in the diluent plasma could have removed labelled insulin and contributed to the lack of parallelism. However, all diluent plasma was passed through a 6μ filter before use in the assay, and if any charcoal was still present it should have affected all samples. Errors in pipetting appear unlikely because they should have increased the variance between duplicates, although systematic errors at the low dose cannot be ruled out entirely.

The effect of using the combined estimate

from both dilutions resulted in a difference of 32 pg at 500 pg/ml or 160 pg at 2500 pg/ml, a difference of 6 percent from those obtained by using the estimate at either end of the scale. Based on the variance between duplicates, within dilutions and samples, for the repeat of assay 1, the least-significant-difference that could be detected (at the 5% level of significance) between samples was 190 pg/ml at 500 pg/ml and 950 pg/ml at 2500 pg/ml.

Therefore, it was considered that the lack of parallelism, although statistically significant, was not a major source of error within the assays. In addition, all samples from the same pig were included in the same assay and the range of plasma insulin was very similar to values reported elsewhere (Anderson, 1973; Anderson, 1974; Machlin, Horino, Hertelendy and Kipinis, 1968). The mean recovery of insulin added to five samples was 105 percent with a range of 95-114 percent.

In each assay, three stock samples were included at three positions within the assay. The statistical analysis of the estimates obtained is shown in Table XI. The three samples included showed a significant difference between dilutions which has been discussed above.

The first order interaction between samples and assays was also statistically significant, although it represented only about 4 percent of the total variance. The means for each sample were similar in each assay (Table XII). Based on the variance of duplicates (within replicates, dilutions, samples, and assays) the least-significant-differences (5% level of significance) at 500, 1000, and 2500 pg/ml respectively were 283, 567, and 1417

TABLE XI: Variance components from the analysis of stock samples included at three positions in each insulin assay.

Source	Variance Component
Between assays (a)	0.00000
Between samples (s)	0.11336*
s x a	0.00548*
Between replicates (r) within s and a	0.00197
Between dilutions (d) within r, s and a	0.00883*
Between duplicates within d, r, s and a	0.01271

* $P < 0.05$

pg/ml. The differences between estimates in different assays for all 3 samples were less than the least-significant-difference. There were no significant differences between assays or the positions of samples within assays.

TABLE XII: Mean estimates of the hormone concentrations of three plasma pools in two different assays.

	Pool 1	Pool 2	Pool 3
Assay 1	964	2632	695
Assay 2	1068	2760	462

The cross-reactions of guinea pig 7 antiserum

with possible contaminants of the pure hormone preparation were tested by the addition of porcine glucagon or chemically denatured insulin to the assay. The disulphide bridges of porcine insulin (Schwarz/Mann Lot No. 22-1123) were reduced and acetylated by methods similar to those described by Fish, Mann and Tanford (1969) modified to include 50 mM Dithiothreitol and 2 mM EDTA in the reduction reagents. The reduction was carried out at 37°C for 3 h before acetylation. The treated hormone was dialysed against de-ionised water and lyophylised. Polyacrylamide gel electrophoresis showed that the reaction went to completion. Porcine glucagon (Lot No. TLM 599 A, Eli Lilley, Indianapolis, U.S.A.) and the chemically denatured insulin preparation were dissolved in buffer containing 10 percent of the charcoal-treated plasma and the proteinase inhibitor tracylol (10,000 IU/ml, Bayer Leverkusen, Germany), and included in the assay to a concentration of 640 ng/ml. Neither preparation showed any inhibition of the binding of ^{125}I-insulin with guinea pig 7 antiserum. In this context, although the word immunoreactive has not been included throughout the text, it is recognised that radio-immunoassays measure immunoreactive compounds present in plasma as distinct from biologically active compounds.

2.2.6 Plasma Growth Hormone Assay:

During the development of the radio-immunoassay for porcine growth hormone (pGH), considerable time was spent in resolving difficulties surrounding the poor affinity of antiserum for ^{125}Iodine-labelled pGH. As problems of this nature may be more widespread than one

might infer from reading the literature, they will be discussed in some detail.

pGH. used throughout was a gift from Prof. A.E. Wilhelmi, Department of Biochemistry, Emory University, Atlanta, Georgia. Two preparations were used: NIH GH P 526 B (porcine), with a specific activity of 2.0 IU/mg, was used for labelling and as a standard; NIH GH P 501 B (porcine), with a specific activity of 0.7 IU/mg, was used to raise antiserum against pGH. in guinea pigs.

Five guinea pigs were given monthly injections of either free pGH. (three animals), or pGH. conjugated to bovine serum albumin (Frohman et al., 1970). Both hormone preparations were emulsified with complete Freunds adjuvant and injected at multiple subcutaneous sites. Following a course of six injections at monthly intervals, a booster injection of free pGH. in normal saline was administered and serum collected by heart puncture 7-10 days later. Three of the antisera produced showed a reaction with pGH. in Ouchterlony and binding of labelled pGH. One antiserum in particular showed a strong reaction with ^{125}I-labelled pGH., which could not be repeated with any subsequent preparation of ^{125}I-pGH.

On arrival, fraction P 526 B was opened and iodinated products of high specific activity were obtained by the chloramine T method, used successfully with ovine and caprine growth hormone (Hart, Flux, Andrews and McNeilly, 1975). However, several months later when the same methods were used, there was almost no incorporation of radioactive iodine onto the hormone molecule. Between iodination attempts, the pGH. was stored at 4°C in a desiccator. With some fractions of ovine growth hormone problems of iodination

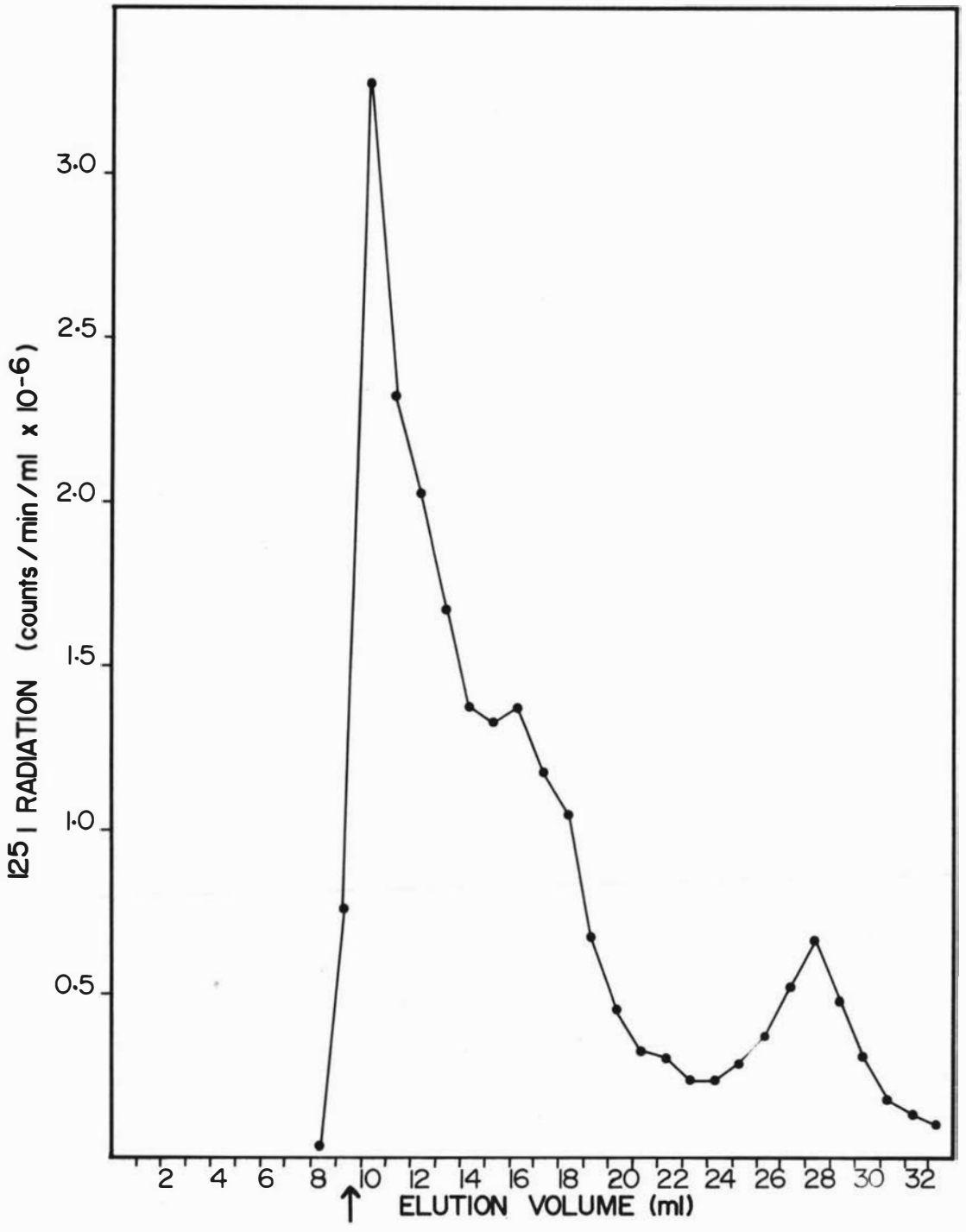

FIGURE 11 Gel filtration of ^{125}I-pGH (P 526B), after 42 days storage at -20°C, on Sephadex G-100 (equilibrated with 0·025 M borate buffer pH 8·4). The arrow indicates the void volume of the column.

were overcome by prior treatment in 2M urea (Hart et al., 1975), resulting in a stable, immunoreactive product. Urea treatment of pGH. prior to iodination gave a modest increase in the specific activities of the iodinated product. However, variable results in the reaction of the iodinated hormone with the antiserum raised against pGH. cast doubts on the stability of the iodinated product, and the gel filtration behaviour of the labelled hormone was examined.

A 50 cm Sephadex G-100 column (Pharmacia), equilibrated with 0.025M borate buffer (pH 8.4), was calibrated with proteins of known molecular weight (Andrews, 1965). A fraction of ^{125}I-labelled pGH. (P 526 B), stored at -20°C for 5 weeks, was applied to the top of the column and eluted. Fractions of 1 ml were collected and counted in a Philips γ counter, and the results are shown in Fig. 11. The major peak of radioactivity eluted at or near the void volume of the column, indicating that the protein with which the radioactivity was associated, had an apparent molecular weight greater than 100,000. The molecular weight of pGH. given by Frieden and Lipner (1971) is 41,600, and it is likely that the large molecular weight species represented aggregated pGH. and may not have been immunoreactive (Schwartz and Batt, 1973).

^{125}Iodine was obtained from the Radiochemical Centre, Amersham, England, and there was about 1 month's delay between placing an order and the receipt of fresh ^{125}I. While awaiting fresh ^{125}I, attempts were made to reverse the aggregation. Aliquots of the aggregated hormone were subjected to the following treatments: 4M urea for 8 h, 0.01M β-mercaptoethanol for 24 h, 4M urea and 2 x 10^{-4}M

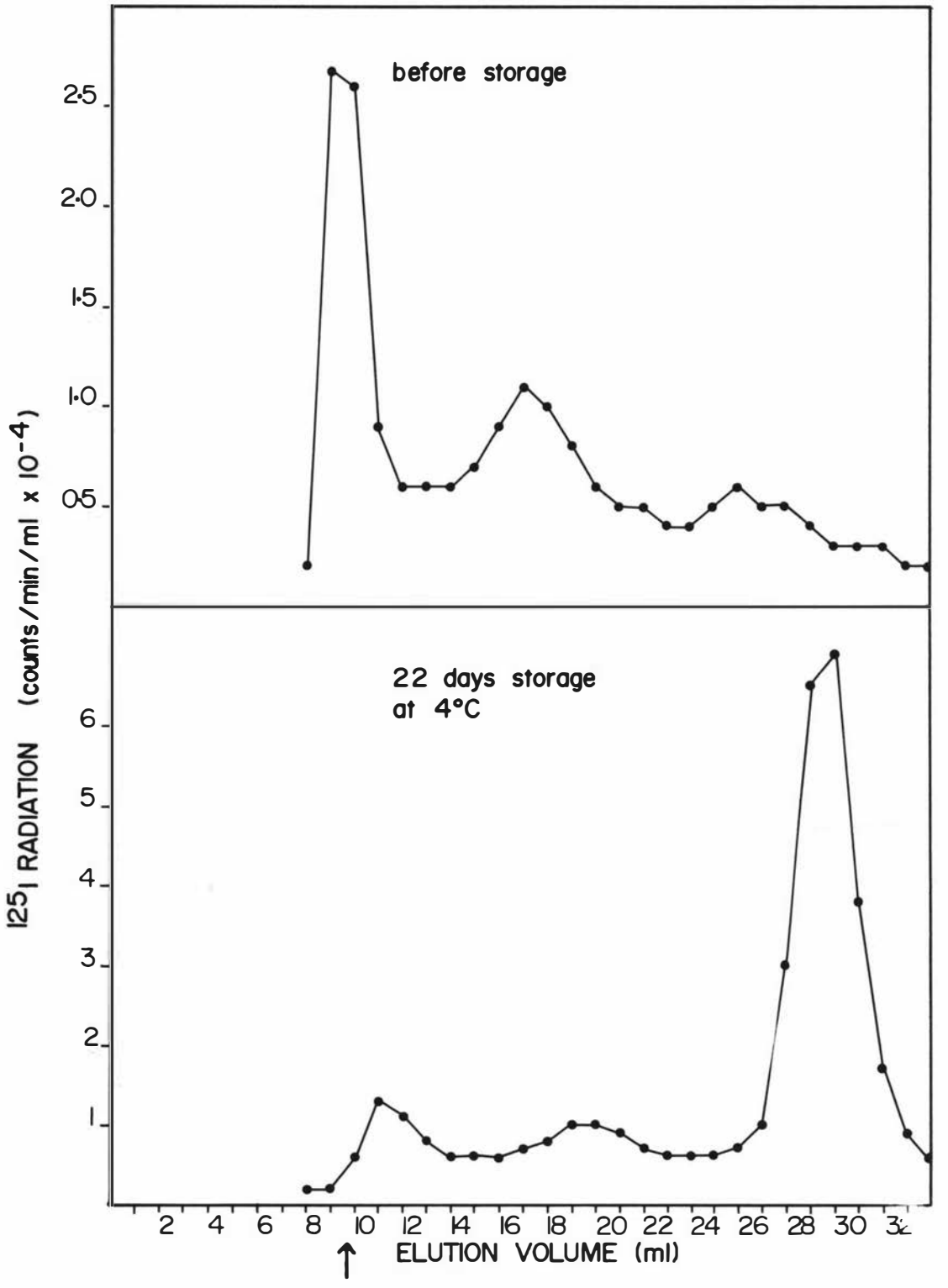

FIGURE 12 Gel filtration of ^{125}I-pGH (P 526B), before storage and after 22 days storage at 4°C, on Sephadex G-100 (equilibrated with 0·025 M borate buffer pH 8·4). The arrow indicates the void volume of the column.

β-mercaptoethanol for 24 h, and pH 11 for 45 min. After the various treatments the solutions were applied to the Sephadex G-100 column and eluted in borate buffer (0.025M pH 8.4). None of the treatments used had any detectable effect on the elution profile of the protein. It is possible that reassociation during the running of the column may have masked effects of the reagents, but it was concluded that for practical purposes the aggregation was irreversible.

A further aliquot of P 526 B pGH. was iodinated with a fresh batch of radioactive iodine. The reaction mixture was divided and the protein and salt fractions separated on two Sephadex G-50 columns, one column eluted in borate buffer (0.025M pH 8.4), and the other column eluted in borate buffer (0.025M pH 8.4) in the presence of 2×10^{-4}M β-mercaptoethanol. An aliquot from the first peak off each of the columns was further chromatographed on the calibrated Sephadex G-100 column. The remainder of both protein peaks was divided in two and stored at either 4° or -20°C.

Fig. 12 shows the elution profiles for the ^{125}I-pGH. eluted in borate buffer before and after storage at -20°C. The patterns were similar for both buffers and both storage temperatures. The four storage conditions had no significant effect on preserving the central peak, and it was clear that when urea was used as a solvent prior to iodination, the iodinated product from P 526 B showed considerable aggregation immediately after iodination. The central peak, probably corresponding to immunoreactive hormone, was destroyed after 3 weeks of storage.

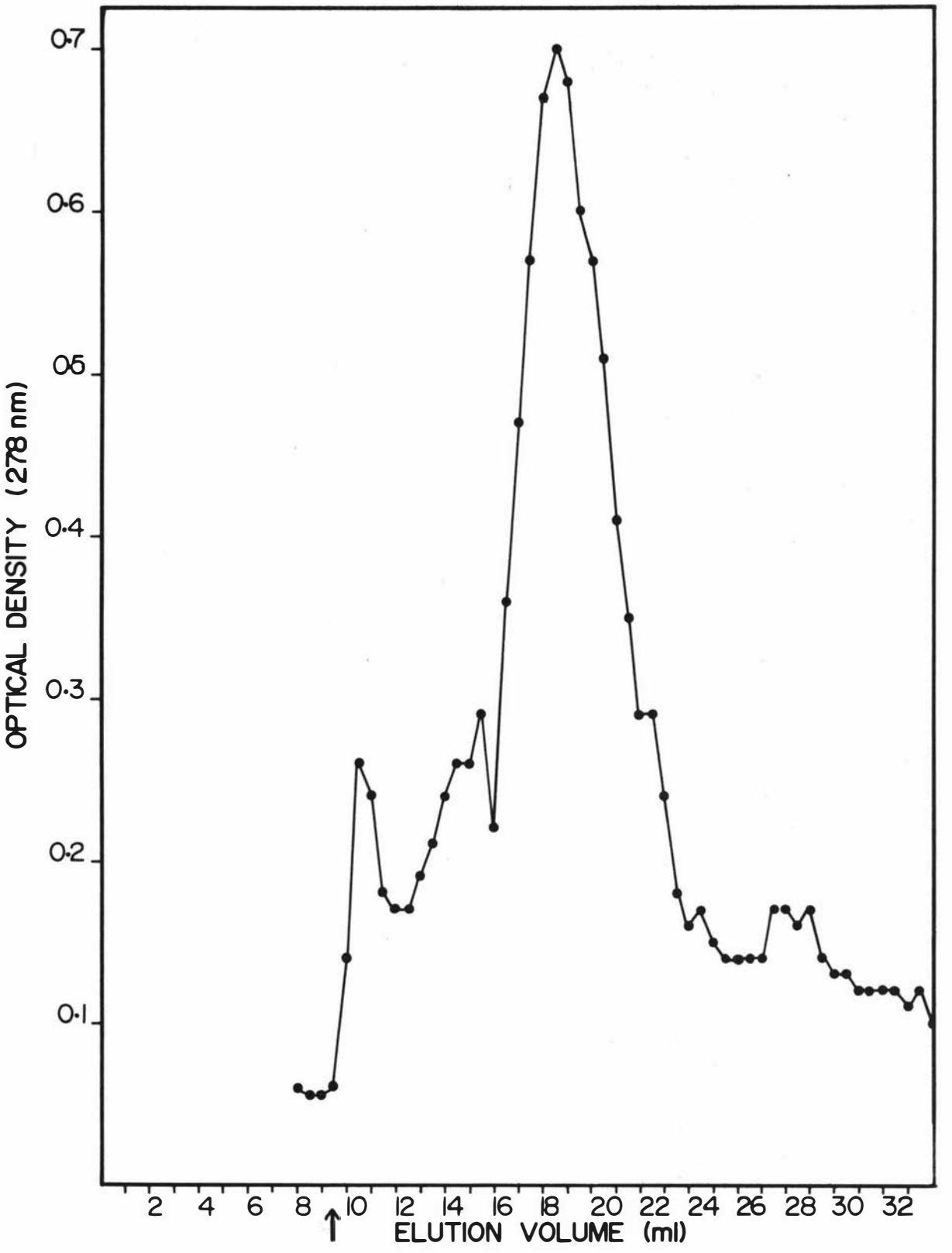

FIGURE 13 Gel filtration of 5mg of pGH (P 501 B) on Sephadex G-100 (equilibrated with 0·025 M borate buffer pH 8·4). The arrow indicates the void volume of the column.

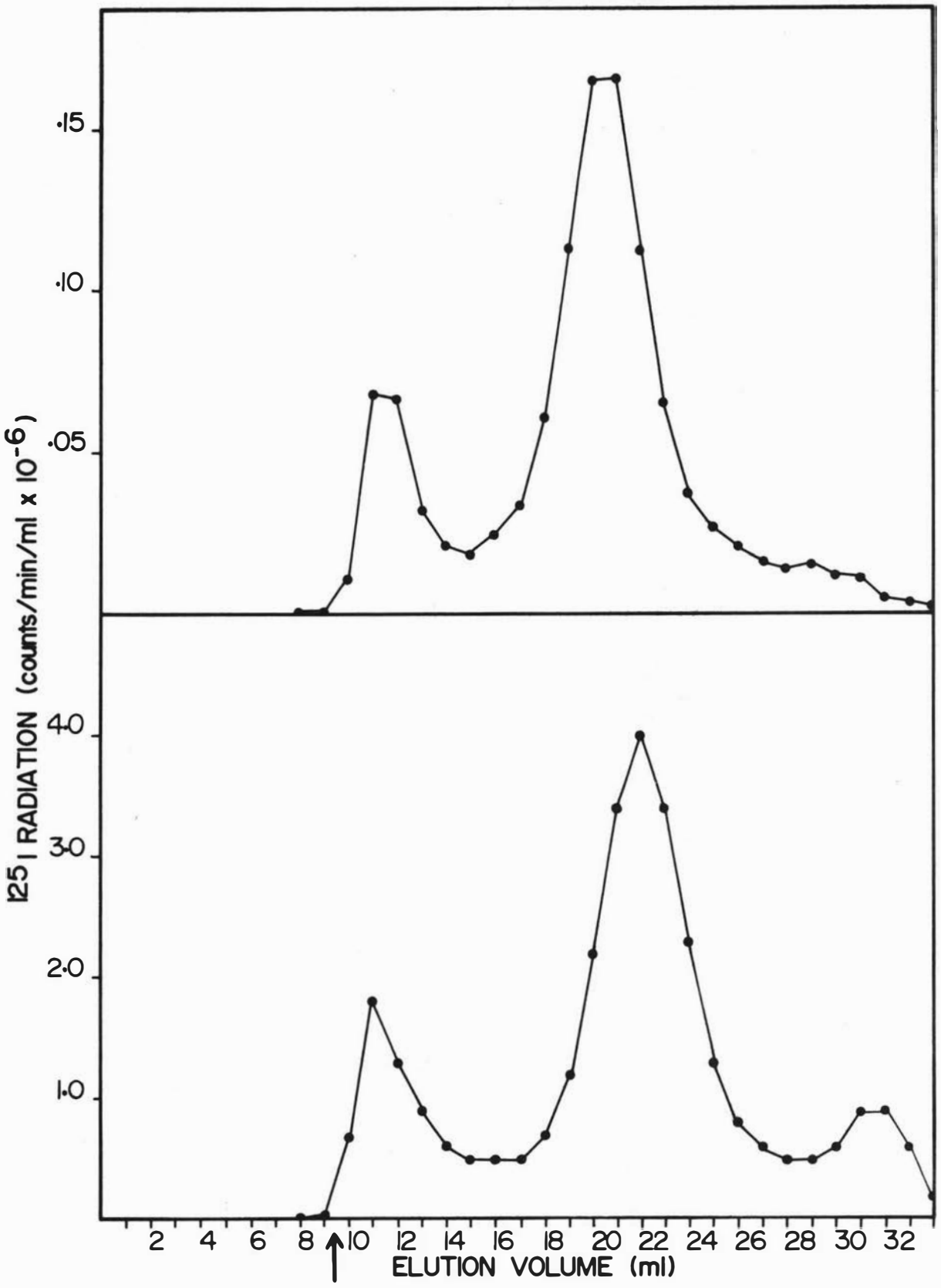

FIGURE 14 Gel filtration of ^{125}I-pGH (P 501B), before storage and after 39 days storage at -20°C, on Sephadex G-100 (equilibrated with 0·025 M borate buffer pH 8·4). The arrow indicates the void volume of the column.

The high degree of aggregation following iodination raised questions about the state of the freeze-dried hormone preparations. The presence of aggregated hormone in a sample of human growth hormone prepared by similar methods has caused difficulties with iodination (A.E. Wilhelmi, personal communication[3]). Column chromatography of P 526 B would have required the total sample available, and a 5 mg fraction of P 501 B was dissolved in borate buffer (0.025M pH 8.4) and chromatographed on Sephadex G-100. Fractions of 0.5 ml were collected and read at 278 nm on a Unicam SP 500 spectrophotometer. The elution profile is shown in Fig. 13. A small peak of aggregate was present at the void volume, but the majority of protein appeared in a single large peak with an apparent molecular weight of 40,000, which corresponds to the estimate of 41,600 given by Frieden and Lipner (1971). Fractions from the protein peak were pooled and dialysed first against de-ionised water and then against 0.01M ammonium bicarbonate before being freeze-dried.

An aliquot of the freeze-dried material was dissolved in 4M urea and iodinated using previously described methods. After separation of the protein and salt peaks, an aliquot of the protein peak was chromatographed on the G-100 Sephadex column. The elution pattern is shown in Fig. 14 with an elution pattern of another aliquot of the same iodinated material after storage at -20°C for 39 days. After storage a third peak of breakdown products appeared, but the major peak of radioactivity ran in the same position as the original hormone both before and after storage.

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3 Prof. A.E. Wilhelmi, Department of Biochemistry, Emory University, Atlanta, Georgia, U.S.A.

Subsequently, R.M. Gray (Dairy Husbandry Department, Massey University) studied the migration of P 526 B and another sample of pGH. (porcine somatotrophic hormone, Lot No. 400521, B grade, Calbiochem, California) in a polyacrylamide-gel-electrophoresis system. The majority of the protein from fraction P 526 B ran in a single well-defined peak (54%). One narrow peak of higher molecular weight (17%) and a broad band of lower molecular weight (19%) accounted for the remainder.

In contrast, the fraction of pGH. purchased from Calbiochem eluted as a broad band of protein, covering a large range of molecular weights, with only poorly defined peaks. Standards made from the material caused no inhibition of the binding of ^{125}I-pGH.-P 526 B with the antiserum to pGH. used in the assay.

One disturbing feature of the ^{125}I-labelled pGH. P 501 B fraction was a complete lack of any reaction with the antiserum raised in guinea pigs. Therefore, definite problems existed with the iodination of the more highly purified growth hormone preparation, and the antiserum produced was of unknown quality. In an attempt to resolve the issue of antiserum quality, Dr. E.D. Aberle of Purdue University was approached for advice and assistance. He kindly donated an aliquot of antiserum to pGH. (Marple and Aberle, 1972), which was used for the remainder of the project.

Mills and Wilhelmi (1972) reported that pGH. may be more closely related to human growth hormone than to ruminant growth hormone. Therefore, the method of iodination of Greenwood, Hunter and Glover (1963), as modified for

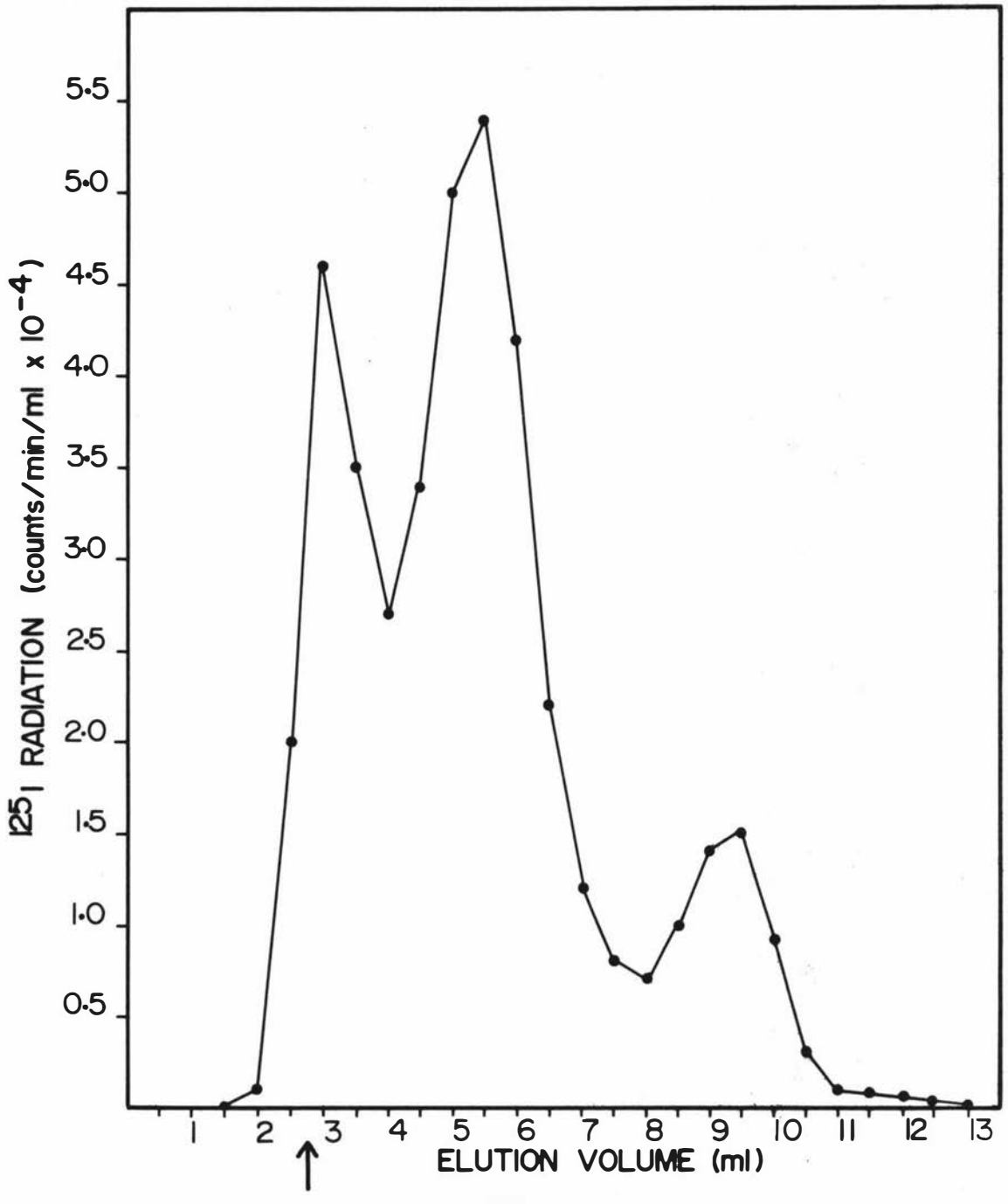

FIGURE 15 Gel filtration of ^{125}I-pGH (P526B; iodinated by the method used for human GH at Otago Hospital), after 5 days storage at -20°C, on Sephadex G-100 (equilibrated with 0·025M borate buffer pH 8·4). The arrow indicates the void volume of the column.

human GH at Otago Hospital (A. McCaw, personal communication[4]), was used to iodinate the pGH. The main differences between the Otago method and the previously described method were the use of 0.003 N HCl to dissolve the hormone before iodination and the use of phosphate rather than borate buffers throughout the procedure. The method yielded labelled pGH. with acceptable specific activities. After storage at -20°C for 5 days three peaks were present in the elution profile off Sephadex G-100 (Fig. 15): a peak at the void volume, a major central peak, and a peak of very small molecular weight which probably represents breakdown products of the hormone. Provided that the labelled hormone was chromatographed on Sephadex G-100 before use and only material from the central peak used in the assay, fraction NIH P 526 B pGH. labelled by the method for human GH proved quite satisfactory in the assay system.

The final assay routine for measuring all samples used the reagents and procedures described by Marple and Aberle (1972), with the following modifications. The assay was carried out in 3 ml plastic test tubes (Luckham Co., England), and the quantities of all reagents were halved. The pGH. was weighed on a Cahn electrobalance before each iodination, and ^{125}I from the Radiochemical Centre, Amersham, was used for the iodination. The reaction of first antibody with standards or plasma samples was carried out at room temperature, and the first antibody was used at an initial dilution of 1 in 128,000.

The assay routine is shown in Table XIII. A standard curve (0.5 - 64 ng/ml) was run with each assay.

4 A. McCaw, Otago Hospital, Dunedin, New Zealand.

TABLE XIII: The reagents and procedures used in the radio-immunoassay for porcine growth hormone.

Day	Reagent	Conditions
1	150 µl P.B.S.[1] 0.05M pH 7.4 1% B.S.A.[2]	Room Temperature
	100 µl sample or standard [3]	
	100 µl first antibody diluted to 1 in 128,000 in P.B.S. with 1 in 40 Normal Guinea Pig Serum	
2	50 µl ^{125}I-pGH.[4] diluted in P.B.S. with 1% B.S.A.	Room Temperature
3	50 µl antibody against Guinea Pig γ Globulin[5] diluted 1 in 2 with P.B.S.	Transferred to 4°C
6	All tubes spun at 2000 r.p.m. for 30 min at 4°C and supernatant aspirated. Precipitates counted in a Packard Auto γ Counter, model 5285.	

1 Phosphate Buffered Saline.

2 Bovine Serum Albumin (Sigma Chemical Co., Fraction V).

3 NIH P 526 B 59.3 µg weighed on a Cahn Electrobalance, dissolved in 0.5 ml 0.003 N HCl, and diluted to 12,800 ng/ml in P.B.S. containing 10% Expired Human Plasma. Aliquoted and stored at -20°C until use in the assay.

4 ^{125}I-pGH. was chromatographed on Sephadex G-100 just before use, and the peak corresponding to monomer-labelled hormone used in the assay.

5 Antibody against Guinea Pig γ Globulin was raised in sheep to a preparation of Guinea Pig Globulin prepared by standard salt precipitation methods. 4-5 fortnightly injections of 5 mg of Guinea Pig γ Globulin emulsified with complete Freunds adjuvant were given followed by a booster dose of free Guinea Pig γ Globulin prior to serum collection via jugular puncture.

All samples were run at two dilutions in duplicate, and three stock samples were included in each assay at three positions.

The specificity of the antiserum has been described previously (Marple and Aberle, 1972). The assay gave a reproduceable standard curve, sensitive to below 1 ng/ml. Above 2 ng/ml the dilution of plasma samples gave similar estimates for the pGH. concentrations of the plasma samples. The analyses for samples above 2 ng/ml in both assays is shown in Table XIV. There were no significant deviations from parallelism.

TABLE XIV: Variance components from the analyses of samples above 2 ng/ml in the individual pGH. assays.

	Assay 1	Assay 2
Between samples (s)	0.03840**	0.03097**
Between dilutions (d) within s	0.00009	0.00056
Between duplicates within d and s	0.00187	0.00127

** $P < 0.01$

Below 2 ng/ml, diluted samples gave higher estimates of the concentrations of pGH. in the plasma samples than did undiluted samples, and therefore only the estimates from the undiluted samples were used for samples with low pGH. concentration. The estimates appeared to be

reproduceable from sample to sample and assay to assay. The 3 stock plasma samples included in each assay were all below 2 ng/ml, and the analysis is shown in Table XV. The variance between samples was highly significant ($P < 0.01$), but there were no significant differences between samples measured in different assays or included at different positions within the same assay. Based on the estimate of duplicates within replicates, samples and assays, the least-significant-difference (5% level of significance) at 1, 10 and 20 ng/ml levels respectively was 0.1, 1 and 2.1 ng/ml.

TABLE XV: Variance components from the analysis of stock samples included at three positions within each pGH. assay.

Source	Variance Component
Between assays (a)	-
Between samples (s)	0.00260**
s x a	-
Between replicates (r) within s and a	-
Between duplicates within r, s and a	0.00062

** $P < 0.01$

2.3 OBSERVATIONS ON HORMONE AND METABOLITE LEVELS IN PIGS FED A TRYPTOPHAN DEFICIENT OR SUPPLEMENTED DIET:

2.3.1 MATERIALS AND METHODS:

2.3.1 (i) Animals and Experimental Procedures:

Four cross-bred barrows (average initial starting weight 29.5kg) were housed in individual metabolism crates under the same environmental conditions as those described for the experiment on feeding patterns (18 ± 1°C and 10 h light, 14 h darkness). Water was freely available at all times.

To follow the meal dependent changes in plasma hormone and metabolite levels, in ad libitum fed animals, a large number of plasma samples would be required. Analysis of the feeding patterns of pigs fed the tryptophan deficient diet indicated that a decrease in meal size accounted for most of the depression in food intake. Therefore, the pigs were trained to eat their daily intake in a two hour period between 0900 h and 1100 h. Blood samples were taken before, during, and after the meal in order to compare the effects of the tryptophan deficient or supplemented diets on changes in blood metabolite and hormone concentrations over the feeding period.

The pigs were moved into the metabolism crates and adapted to the 2 h feeding regime during the following 10 days. After 10 days, food intakes had stabilised and the pigs were catheterised prior to commencing the sampling experiment. The catheters were maintained for 17 days.

The pigs were fed the two diets, alternating each 5 days, in a reversal design similar to that used in the experiment on feeding patterns. Blood samples were collected on the second and fifth day in each period. The experimental design is summarised and presented in Table XVI.

2.3.1 (ii) Catheterisation:

Initial attempts at catheterisation, via the medial ear vein (Shearer and Neal, 1972), proved unsatisfactory for the weight range of pigs used in the experiments. Animal variation in the size of the medial ear vein made it difficult to introduce catheters into the vein in all cases. More importantly the catheters attached to the ear were a constant source of irritation to the animals, particularly while sampling. As a consequence, it was not possible to maintain catheters longer than a few days.

Therefore, the external jugular vein was catheterised under general anaesthesia using methods similar to those described by Harris (1974), and Wingfield *et al.* (1974). Anaesthesia was induced by direct inhalation of 8 - 10% Fluothane (I.C.I. Australia Ltd.) through a flexible rubber face mask, and maintained under the same system with 2 - 4% Fluothane. Excess rubber on the face mask was clamped off to provide a tight fit. After preparation for surgery, an incision was made and the external jugular vein located (Wingfield *et al.*, 1974). The jugular vein was temporarily occluded, and a medical grade Silastic tubing catheter (internal diameter 1.02mm and external diameter 2.16mm, Dow Corning Corporation), with two elastomer collars 8cm from the distal end, was inserted through a purse string

TABLE XVI Details of the "cross-over design", including blood sampling days, used for the experiment on the effects of a tryptophan deficient or supplemented diet on blood metabolites and hormones.

Day	Diets		Experimental Procedure
	Treatment 1	Treatment 2	
1	Supplemented	Supplemented	Catheterisation
2	"	"	-
3	Supplemented	Deficient	-
4	"	"	Blood sampling
5	"	"	-
6	"	"	-
7	"	"	Blood sampling
8	Deficient	Supplemented	-
9	"	"	Blood sampling
10	"	"	-
11	"	"	-
12	"	"	Blood sampling
13	Supplemented	Deficient	-
14	"	"	Blood sampling
15	"	"	-
16	"	"	-
17	"	"	Blood sampling

suture. The catheter was checked for patency and the purse string suture tied off between the elastomer collars. A trocar was passed subcutaneously around the neck from the point of incision to a point about 6cm out from the centre of the ear. A loop of catheter was tied off at the point of incision, to allow for movement of the neck, and the catheter passed up through the trocar and exteriorised. It was then led back over the shoulder blades and sutured to the skin. The neck incision was closed, the animals were allowed to recover and then returned to the metabolism crates.

During the remainder of the day of catheterisation the catheters were inspected, checked for patency, flushed with sterile saline and filled with sterile heparinised saline (250 IU/ml) every hour or so. After the first day, the catheters were checked twice daily. In the morning the catheters were filled with heparinised saline and in the evening they were filled with a solution of Triplopen (Glaxo Laboratories (N.Z.) Ltd.) in sterile heparinised saline, to prevent any occlusion of the catheter caused by infection.

2.3.1 (iii) Blood Sampling:

The catheters were checked each morning at 0815 h. On sampling days, two baseline samples were taken, at 0815 h and at 0845 h. The pigs were allowed access to the feed bins at 0900 h and samples taken at half-hourly intervals until the end of feeding at 1100 h. Two further samples were taken, one hour after the end of the feeding period at 1200 h, and three hours after the end of the

feeding period at 1400 h.

10 ml samples were collected into heparinised containers, mixed and immediately centrifuged. The plasma was separated, two aliquots taken for protein precipitation and the remainder immediately frozen for later hormone analyses. Protein in a 0.1 ml sample of plasma was precipitated by the Barium Hydroxide-Zinc sulphate method described by Caraway (1970), for later glucose analysis, while 1 ml of plasma was shaken with 2 ml of 95% ethanol, to denature and precipitate plasma proteins, for later amino acid analysis (Saini, 1971). Both supernatants were immediately frozen and all samples stored at -20°C until assay.

2.3.1 (iv) Data Processing and Statistical Analysis:

The concentrations of plasma hormones were calculated from the standard curve data, using a computer program developed from the system of analysis described by Burger, Lee and Rennie (1972). The program was implemented on an IBM 1620 by Prof. R.E. Munford, Department of Physiology and Anatomy, Massey University.

Peak areas of amino acid chromatograms were estimated by the "half height times width" ($\frac{1}{2}$ H x W) method and converted to concentrations using appropriate colour-yield factors. Plasma urea concentrations were calculated from the regression of absorbance on standard urea concentration and plasma glucose levels were calculated from the 100mg/100 ml standard.

The concentrations of all metabolites and logarithmic transformations of hormone concentrations in samples collected at the same clock time, on the same day

within each period, were analysed for differences between the two diets according to the method of Brandt (1938). The mean levels of hormones and metabolites, for each diet, were calculated using the method described in a previous section (Sect. 2.1.1 (v), p. 34).

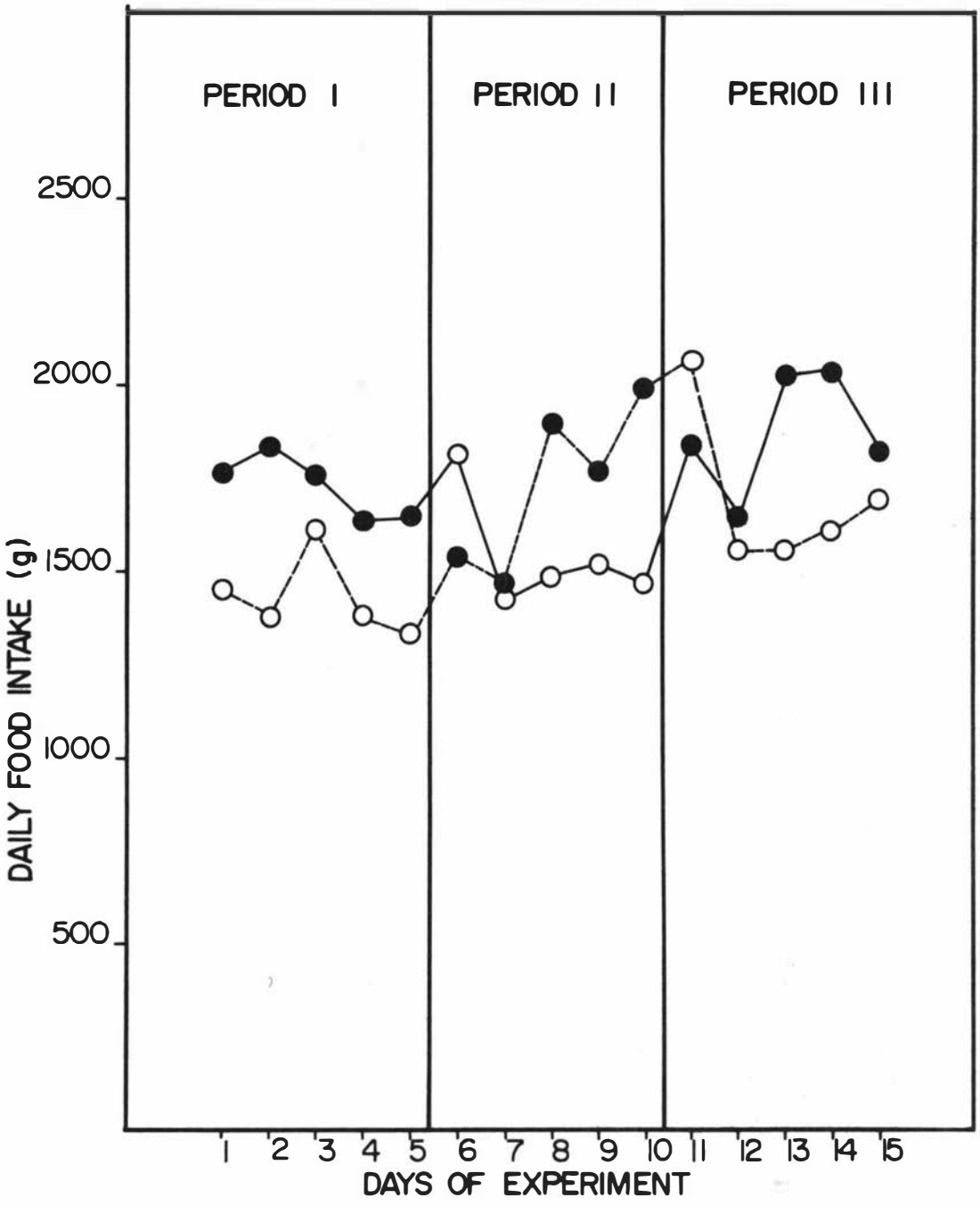

FIGURE 16 The effects of alternating a tryptophan deficient and supplemented diet on food intakes of pigs fed for two hours each day. Each point is the mean for 2 pigs.

● Supplemented Diet

○ Deficient Diet

2.3.2 RESULTS:

The pigs recovered rapidly after the catheterisation procedure and food intakes on the day after the operation were in the normal range for all animals. The experiment commenced on the third day after catheterisation.

The catheters remained patent in all animals until the last day of the experiment, when the catheter of one animal became blocked, preventing the collection of the last five samples (pig 3 - 29). All other samples were collected. Results from pig 3 - 29 were included in the analysis of results for day 5, by using the algebraic sum of values recorded in the first two periods, for the five missing samples.

2.3.2 (i) Food Intake:

Food intakes recorded during the preliminary period indicated that the pigs adapted rapidly to the schedule of a single 2 h feeding period each day. Intakes were low for the first two days but had reached stable levels by day 4.

Fig. 16 shows the mean food intakes of the two groups of two pigs given each sequence of alternating diets. The intakes showed a very similar pattern to the corresponding figure (Fig. 2) for the pigs fed *ad libitum*, but the day to day variation was considerably higher. In addition, the food intakes of animals fed the supplemented diet appeared to be depressed on the days that blood samples were taken, especially in the third period.

Considering the three periods together, the effects of feeding either the tryptophan deficient or

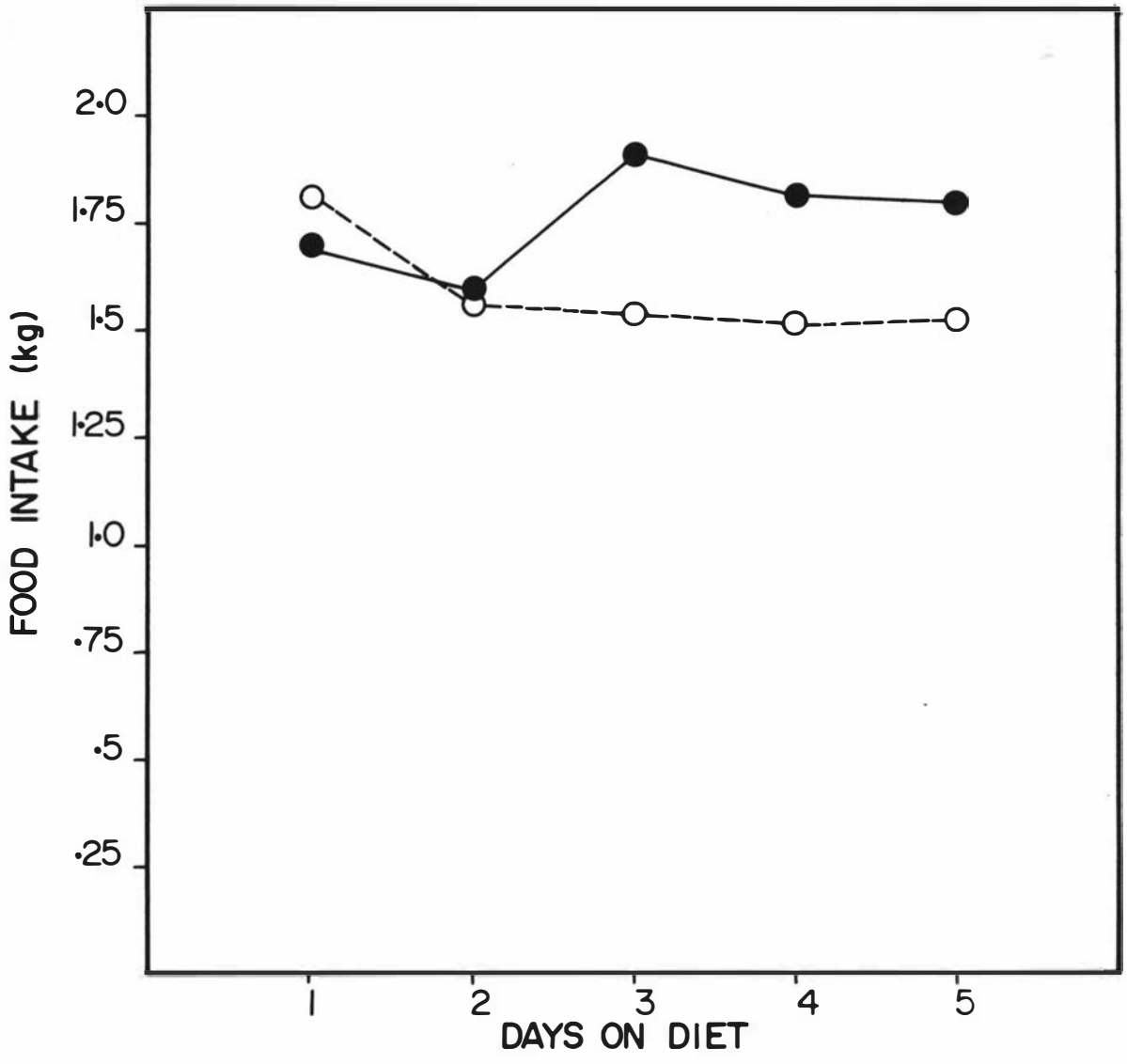

FIGURE 17 The effects of feeding a tryptophan deficient or supplemented diet on the mean food intake of pigs when the feeding period was restricted to two hours per day. Each point is the mean for 6 pig days.

●——● Supplemented Diet

○- - -○ Deficient Diet

supplemented diet for 5 days are shown in Fig. 17. The intakes of animals fed the deficient diet were depressed by day 2 and remained low for days 3 to 5. The intakes of animals fed the supplemented diet remained low on days 1 and 2 and a difference in food intakes between the two diets was not evident until day 3. The depression in the food intakes of pigs fed the deficient diet (21% on day 3 and 17% on day 5) was similar to that observed in animals fed ad libitum, but it was not statistically significant.

2.3.2 (ii) Plasma Amino Acid Levels:

Mean concentrations of amino acids in selected plasma samples are shown in Table XVII for day 2 samples and Table XVIII for day 5 samples. Restrictions on facilities made it unpractical to analyse the amino acid concentrations in all samples. Three samples on each day were analysed for all four pigs, before, during, and after the meal (0845, 1030 and 1400 h).

On day 2, the basal levels of most essential amino acids were higher in the plasma of pigs fed the supplemented diet. The levels of most amino acids rose during the meal. There was little difference between diets in the concentrations of non-essential amino acids, although proline concentrations were consistently higher in pigs fed the supplemented diet. This difference was statistically significant ($P < 0.01$) in the 1030 sample.

The levels of all essential amino acids rose higher during the meal in the plasma of pigs fed the supplemented diet. The difference for tyrosine and total essential amino acids reached statistical significance

TABLE XVII: Plasma amino acid concentrations (μmoles/100 ml) in pigs fed either a tryptophan deficient or supplemented diet for two days.

Amino Acid	Sample Time					
	0845		1030		1400	
Essential amino acids	Supplemented	Deficient	Supplemented	Deficient	Supplemented	Deficient
Lysine	1.8	2.1	5.6	5.1	2.1	2.9*
Histidine	2.9	2.4	5.1	2.8	1.6	2.1
Threonine	44.6	36.0	47.8	36.1	45.6	37.7
Methionine	1.9	1.5	1.7	1.6	2.8	1.6
Valine	16.9	15.2	21.9	19.8	20.8	20.0
Isoleucine	6.9	5.6	8.4	7.4	7.3	6.9
Leucine	16.9	12.8	20.6	19.3	22.9	22.4
Phenylalanine	6.7	5.6	8.8	8.2	9.1	8.3
Tyrosine	5.1	3.5	7.6*	5.7	7.4	6.5
Tryptophan	2.2	2.2	5.9	2.4	3.0	1.0
Total essential amino acids	106.0	87.1	133.2*	108.3	122.6	109.5
Non-essential amino acids						
Aspartic acid	15.5	14.7	17.9	17.2	21.3	19.0
Serine + Glutamic acid	32.4	39.1	37.7	38.2	37.1	41.7
Proline	26.5	17.5	42.9**	37.3	55.2	47.6
Glycine	84.6	89.3	87.6	93.9	124.6	116.1
Alanine	30.4	32.6	50.5	48.2	50.8	48.2
Total non-essential amino acids	189.4	193.1	236.6	234.8	288.9	272.6

* $P < 0.05$ ** $P < 0.01$

TABLE XVIII: Plasma amino acid concentrations (μmoles/100 ml) in pigs fed either a tryptophan deficient or supplemented diet for five days.

Amino Acid	Sample Time 0845		1030		1400	
Essential amino acids	Supplemented	Deficient	Supplemented	Deficient	Supplemented	Deficient
Lysine	1.6	1.4	5.7	6.4	3.0	3.0
Histidine	2.3	2.3	3.5	6.1	3.6	3.0
Threonine	30.5	36.2	33.3	54.8	41.1	42.9
Methionine	1.6	1.3	1.3	2.3	2.6	2.5
Valine	15.3	13.8	17.9	21.6	20.1	19.2
Isoleucine	6.7	5.4	6.8	8.1	6.7	5.7
Leucine	12.7	11.6	17.1	20.9	23.4	20.9
Phenylalanine	6.6*	4.7	8.3	8.5	9.4**	7.4
Tyrosine	4.3	3.5	7.1	7.1	7.8	6.3
Tryptophan	2.3	1.5	6.7	3.3	5.0	0.9
Total essential amino acids	84.0	81.7	107.7	147.2	122.6	111.8
Non-essential amino acids						
Aspartic acid	15.2	14.2	16.1	23.1	21.5	17.9
Serine + Glutamic acid	27.5	27.8	31.0	42.6	37.2	35.1
Proline	23.8	19.4	39.5	48.0	68.9	58.5
Glycine	92.8	81.7	88.6	103.0	139.2	104.9
Alanine	30.7	31.5	45.0	58.1	59.7	53.2
Total non-essential amino acids	190.1	174.5	220.2	275.0	326.4	269.6

* $P < 0.05$ ** $P < 0.01$

($P < 0.05$). The concentration of tryptophan more than doubled in the plasma of pigs fed the supplemented diet, but there was little change in pigs fed the deficient diet.

The concentrations of most essential amino acids were still high 3 h after the meal. However, the concentrations of histidine and lysine had decreased to about pre-feeding levels and differed between diets. The concentrations were higher in the plasma of pigs fed the deficient diet and the difference in plasma lysine was statistically significant ($P < 0.05$). The concentration of tryptophan, in pigs fed the deficient diet, had declined to half the pre-feeding level and there was a three fold difference between diets.

On day 5, the pattern of essential amino acids prior to feeding was similar to the pattern on day 2. In most cases, higher concentrations were present in pigs fed the supplemented diet. At the 1030 sample time the situation had reversed. The concentrations of essential amino acids, except tyrosine and tryptophan, were higher in pigs fed the deficient diet. Three hours after the meal, pigs fed the supplemented diet had higher concentrations of all essential amino acids except threonine and lysine. The difference in phenylalanine concentrations was statistically significant ($P < 0.05$).

Plasma tryptophan concentrations were lower, at all sampling times, in pigs fed the deficient diet. The concentration in pigs fed the supplemented diet was five times that of pigs fed the deficient diet at the 1400 h sampling time.

The change in non-essential amino acids

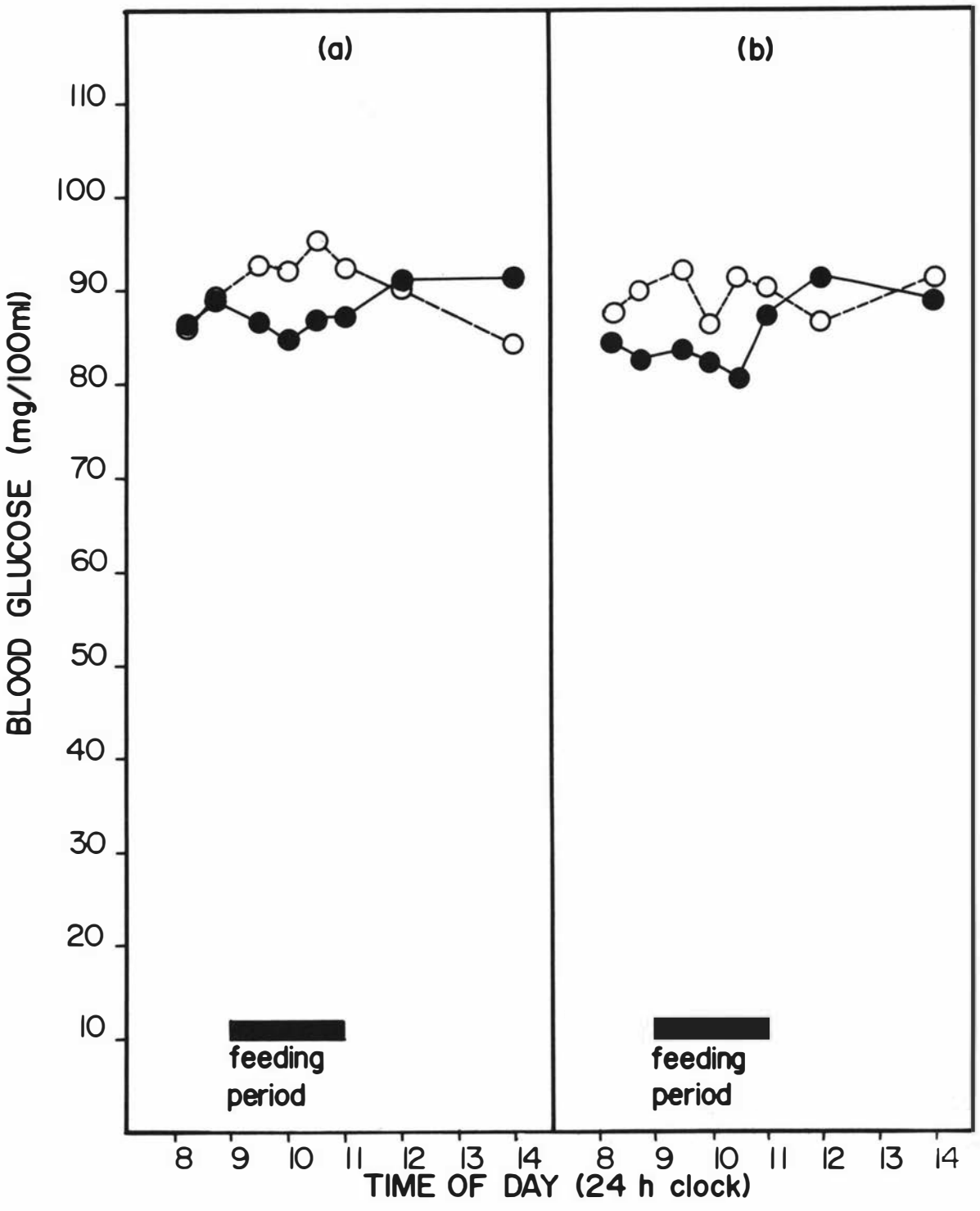

FIGURE 18 Changes in the plasma concentrations of Glucose during and following the daily two hour feeding period in pigs fed a tryptophan deficient or supplemented diet for (a) two days
(b) five days.

Each point is the mean for 6 pig days.

●——● Supplemented Diet

○------○ Deficient Diet

followed similar patterns to the changes in essential amino acids.

2.3.2 (iii) Plasma Glucose Levels:

The effects of feeding the tryptophan deficient or supplemented diet on plasma metabolite and hormone concentrations are given in Table XIX for day 2 samples and Table XX for samples collected on day 5. The plasma glucose values are shown in Fig. 18.

One striking feature of the plasma glucose results was the small response in peripheral plasma glucose during the meal. One animal, on one sampling day, showed a rise in plasma glucose from 100 - 140 mg/100 ml but the rise seldom exceeded 10 mg/100 ml and in many cases the levels declined.

On both sampling days the animals fed the supplemented diet showed a decline in plasma glucose concentrations during the meal, with a rise toward the end. At both sampling times after the end of feeding the concentrations were higher than the pre-feeding values.

On day 2, the glucose concentration in the plasma of pigs fed the deficient diet rose and by the 1030 sampling time the difference between diets had reached statistical significance ($P < 0.05$). Glucose values then declined and three hours after the meal, relative levels had reversed.

On day 5, the basal glucose level of pigs fed the deficient diet was higher than that of pigs fed the supplemented diet. The difference at the 0845 sampling time was statistically significant ($P < 0.05$). The difference

TABLE XIX The concentrations of metabolites and hormones, measured in plasma samples collected from pigs fed tryptophan deficient or supplemented diets for two days. The pigs were fed from 0900 h to 1100 h.

		Sample Time							
		0815	0845	0930	1000	1030	1100	1200	1400
Urea concentration (mg Urea N/100 ml)	Supplemented	14.62	14.50	14.15	14.46	14.81	14.64	14.42	15.14
	Deficient	13.05	13.06	12.55	12.92	13.31	13.44	13.66	13.32
	Difference	1.57*	1.44	1.60	1.54	1.50	1.20	0.76	1.82
Glucose concentration (mg/100 ml)	Supplemented	86.38	88.99	86.77	84.82	87.01	87.13	91.26	91.27
	Deficient	86.14	89.11	92.89	92.28	95.51	92.37	90.20	84.37
	Difference	0.24	-0.12	-6.12	-7.46	-8.50*+	-5.24	1.06	6.90
Insulin concentration (pg/ml)	Supplemented	895	832	1275	1426	1601	1137	1343	1466
	Deficient	956	662	1994	2301	2411	2080	1598	1171
	Difference	-61	170*+	-719	-875	-810	-943	-255	295
Growth Hormone concentration (ng/ml)	Supplemented	2.0	1.3	1.7	2.2	2.0	1.7	2.9	0.9
	Deficient	1.7	5.7	2.5	2.5	2.6	2.1	3.4	5.6
	Difference	0.3	-4.4	-0.8	-0.3	-0.6	-0.4	-0.5	-4.7
Cortisol concentration (ng/ml)	Supplemented	52	63	42	46	52	42	49	42
	Deficient	56	67	37	48	48	41	48	39
	Difference	-4	-4	5	-2	4	1	1	3

* $P < 0.05$ *+ $P < 0.025$

TABLE XX The concentrations of metabolites and hormones, measured in plasma samples collected from pigs fed tryptophan deficient or supplemented diets for five days. The pigs were fed from 0900 h to 1100 h.

		Sample Time							
		0815	0845	0930	1000	1030	1100	1200	1400
Urea concentration (mg Urea N/100 ml)	Supplemented	14.10	13.72	13.39	13.67	13.97	14.25	14.63	14.10
	Deficient	13.34	13.24	12.93	13.13	13.49	13.75	13.93	14.32
	Difference	0.76	0.48	0.46	0.54	0.48	0.50	0.70	-0.22
Glucose concentration (mg/100 ml)	Supplemented	84.47	82.81	83.86	82.22	80.87	87.30	91.63	89.08
	Deficient	87.83	90.01	92.16	86.38	91.45	90.14	86.79	91.44
	Difference	-3.36	-7.20*	-8.30	-4.16	-10.58	-2.84	4.84*	-2.36
Insulin concentration (pg/ml)	Supplemented	761	695	1705	2180	1944	1951	1358	1171
	Deficient	761	679	1892	1730	1479	1429	1171	1070
	Difference	0	16	-187	450	465	522	187	101
Growth Hormone concentration (ng/ml)	Supplemented	3.4	4.0	2.0	1.6	1.9	2.8	2.7	1.9
	Deficient	1.6	2.4	1.6	2.5	2.9	2.3	3.2	1.6
	Difference	1.8	1.6	0.4	-0.9	-1.0	0.5	-0.5	0.3
Cortisol concentration (ng/ml)	Supplemented	53	64	33	64	42	35	38	41
	Deficient	64	66	31	68	46	40	42	40
	Difference	-11	-2	2	-4	-4	-5	-4	-1

* $P < 0.05$

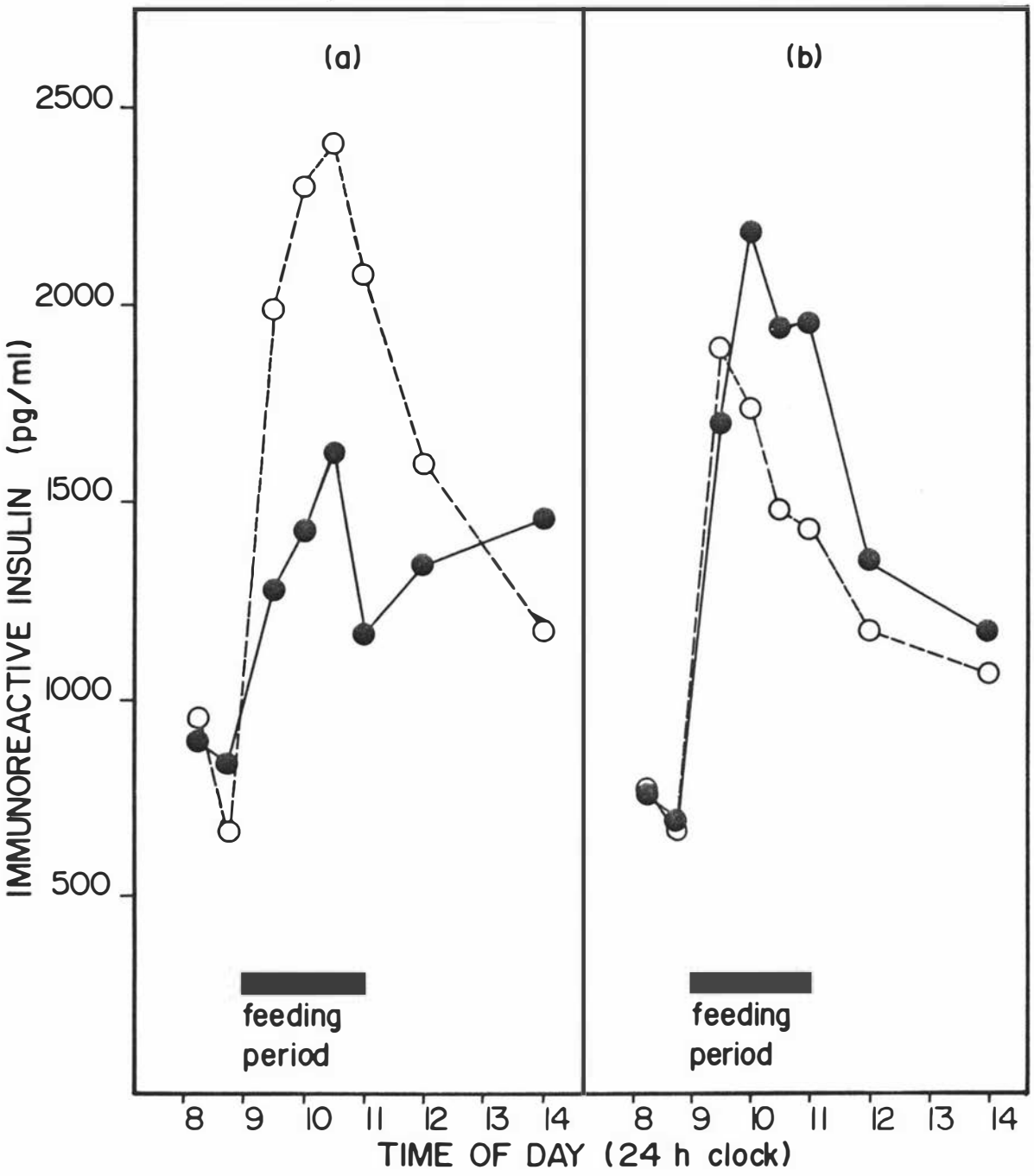

FIGURE 19

Changes in the plasma concentrations of Immunoreactive Insulin during and following the daily two hour feeding period in pigs fed a tryptophan deficient or supplemented diet for (a) two days (b) five days.

Each point is the mean for 6 pig days.

●——● Supplemented Diet

○------○ Deficient Diet

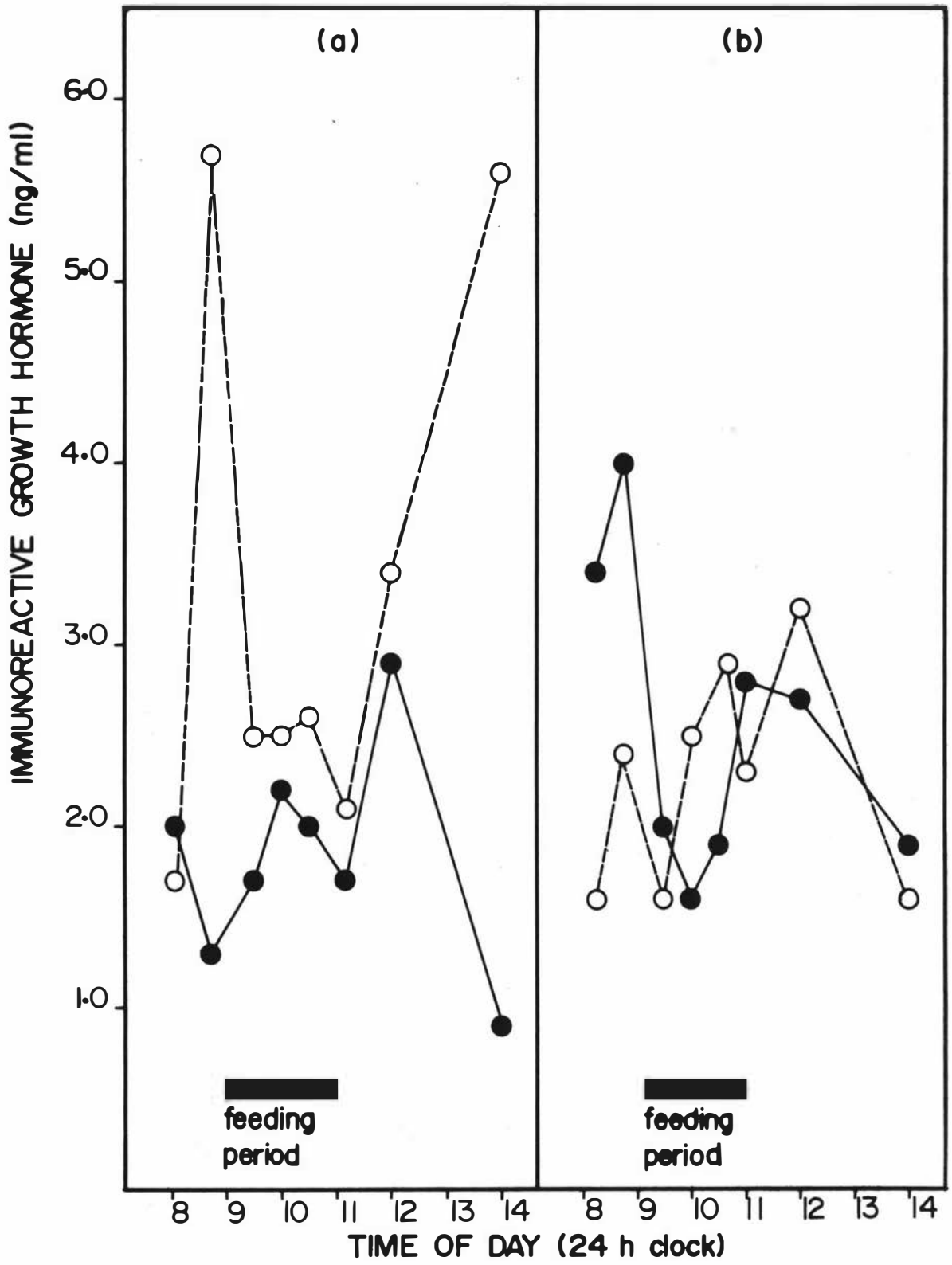

FIGURE 20 Changes in the plasma concentrations of Immuno-reactive Growth Hormone during and following the daily two hour feeding period in pigs fed a tryptophan deficient or supplemented diet for (a) two days (b) five days.
Each point is the mean for 6 pig days.

●——● Supplemented Diet
○——○ Deficient Diet

was maintained throughout the meal, although it was not statistically significant. Toward the end of the meal the concentration of glucose in the plasma of pigs fed the supplemented diet rose and the difference between the diets was reversed and statistically significant ($P < 0.05$) 1 hour after the meal.

2.3.2 (iv) Plasma Insulin Levels:

The results for plasma immunoreactive insulin are shown in Fig. 19. There was a rapid rise in plasma insulin level following the onset of feeding, which reached a maximum 30 to 90 min after the beginning of the meal. Insulin levels then declined, but remained elevated 3 h after the end of the feeding period.

Animals fed the deficient diet for 1 day prior to sampling had significantly lower insulin levels immediately before the meal ($P < 0.05$). During the meal, the situation reversed and although the difference was not statistically significant, the animals fed the deficient diet showed a greater increase in plasma insulin in response to the meal.

On day 5, animals fed both diets showed similar insulin responses during feeding. The maximum levels reached were intermediate between the levels reached by the two groups on day 2.

2.3.2 (v) Plasma Growth Hormone Levels:

The results for plasma immunoreactive growth hormone are shown in Fig. 20. Growth hormone levels were low in most samples, although a little under 10% of the

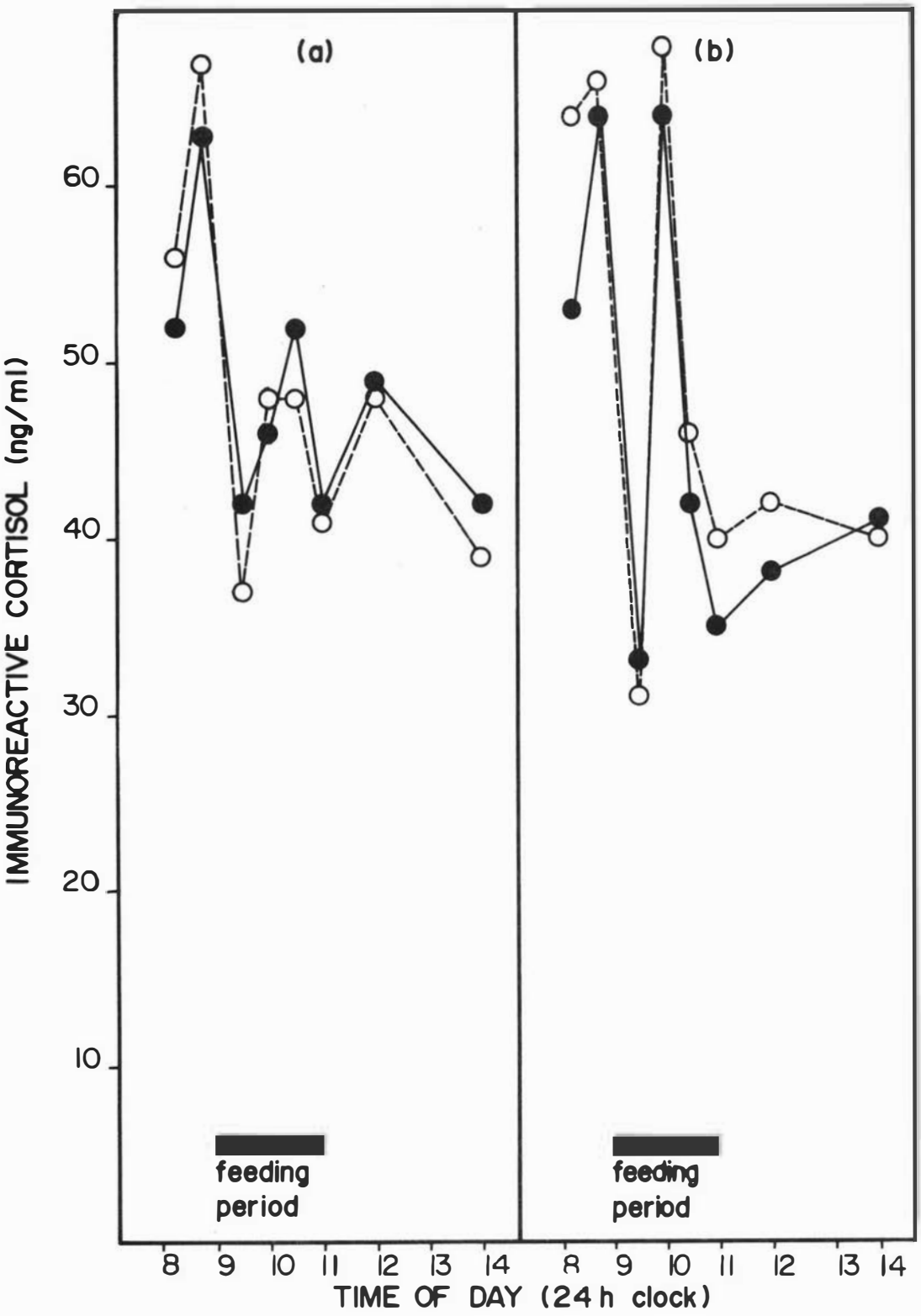

FIGURE 21 Changes in the plasma concentrations of Immunoreactive Cortisol during and following the daily two hour feeding period in pigs fed a tryptophan deficient or supplemented diet for (a) two days (b) five days.

Each point is the mean for 6 pig days.

●——● Supplemented Diet

○- - - -○ Deficient Diet

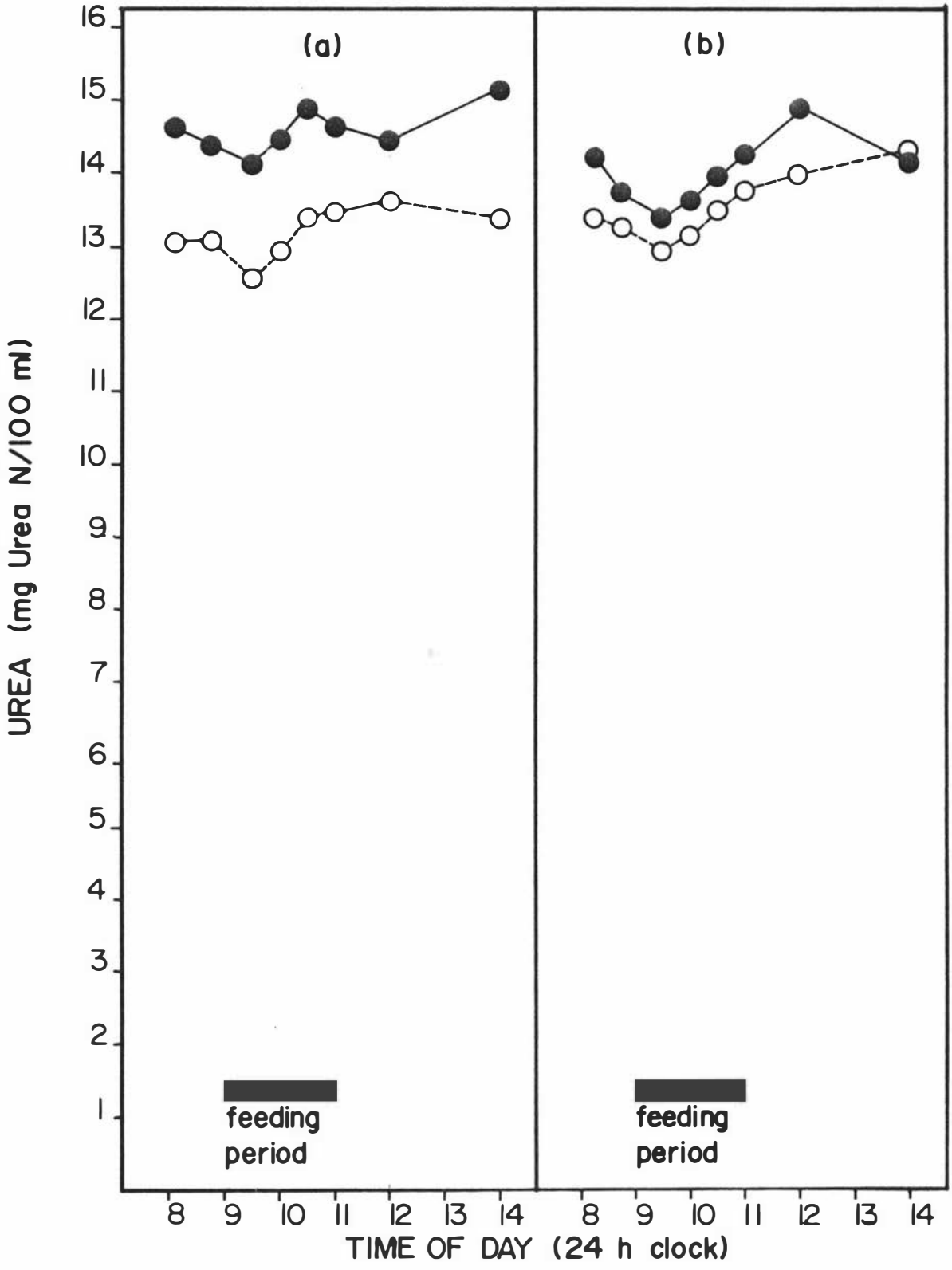

FIGURE 22 Changes in the plasma concentrations of Urea during and following the daily two hour feeding period in pigs fed a tryptophan deficient or supplemented diet for (a) two days
(b) five days.

Each point is the mean for 6 pig days.

●——● Supplemented Diet

○- - - -○ Deficient Diet.

samples exceeded 5ng/ml. There was no statistically significant difference between diets and peaks greater than 5ng/ml occurred on both diets. The highest recorded peaks (20ng/ml) occurred in pigs fed the deficient diet, contributing to the differences between diets on day 2. Only one peak greater than 5ng/ml was recorded during feeding (7ng/ml), although the mean levels during feeding were similar in most cases.

2.3.2 (vi) Plasma Cortisol Levels:

The concentrations of plasma immunoreactive cortisol are shown in Fig. 21. There was little difference between the levels in animals fed either diet and the differences were not statistically significant. On both sampling days the levels were elevated prior to the meal. After the beginning of feeding the levels fell, and apart from the fourth sample on day 5, they remained low for the rest of the sampling period.

2.3.2 (vii) Plasma Urea Levels:

The concentrations of plasma urea are shown in Fig. 22. The levels showed a drop in the first sample after the beginning of the meal. Levels rose during the remainder of the meal and were still elevated in the last two samples.

The basal levels of urea in pigs fed the supplemented diet were significantly higher on day 2 ($P < 0.05$). The urea levels in pigs fed the deficient diet rose during the meal and the difference between levels progressively decreased with each sampling time. By the end of the meal there was little difference between the

two groups. On day 5, there were no significant differences between diets.

2.3.3 DISCUSSION:

2.3.3 (i) Food Intake:

The pigs were allowed to eat as much as they wanted for 2 h each day (0900 to 1100 h). When corrected for differences in body weight, mean daily food intakes of pigs fed the supplemented diet were similar to the intakes of pigs fed ad libitum and also similar to the intakes of pigs of the same breed restricted to a 2 h feeding period (Ingram and Legge, 1974).

On the restricted feeding regime, the response of pigs to the two diets was similar to the response of pigs fed ad libitum, although animals appeared to respond more slowly to changes in diet. The intakes of animals fed the deficient diet were not depressed until day 2, and the intakes of animals fed the supplemented diet did not recover until day 3.

The slow recovery of food intake in pigs fed the supplemented diet may have been due to an interaction with the blood sampling procedure. Pigs fed the supplemented diet had low food intakes on both sampling days in the third period. During sampling it was necessary to close off the feed bins and although all the animals were quiet, pigs fed the supplemented diet made it clear that they resented the intrusion into their feeding time. Conversely, when on the deficient diet, the same pigs appeared content while samples were taken and were slower to recommence feeding after sampling finished. Longer catheters would have obviated the need to close off the feed bins and overcome the problem.

Differences in food intake between the two diets were not statistically significant. Inspection of the individual records revealed that 3 of the 4 pigs showed a consistent depression in intake when fed the deficient diet, while the fourth pig showed little response. The food intake of the pig that did not respond to the diet was higher than the intakes of the other three pigs. It is therefore unlikely that the lack of response was due to a general depression in food intake resulting from the catheterisation procedure.

The difference in intake between the two diets was similar in magnitude to the response of pigs fed ad libitum. The lack of statistical significance was probably due to the pig that did not respond to the deficient diet, although disturbances related to the blood sampling procedure could have been a contributing factor. The experiment on ad libitum feeding patterns was conducted with three groups. When the two groups of 4 animals were analysed separately the depression in intake on the deficient diet was statistically significant ($P < 0.05$) in one group, but not in the other. When all 10 pigs were included in the analysis, the differences in intake between the two diets were statistically significant ($P < 0.01$).

In one study of the effects of amino acid imbalance, rats trained to eat their daily ration in a two-hour period did not show depressions in growth or food intake until the threonine imbalance was severe (Leung et al., 1968a). The tryptophan deficient diet used in the present experiment was not a severe deprivation since it supported a modest rate of growth in pigs fed ad libitum. However,

Leung et al. (1968a) did not report results for rats fed a "corrected" diet and no direct comparison can be made.

2.3.3 (ii) Plasma Amino Acid Levels:

The concentrations of plasma amino acids in pigs fed the supplemented diet were similar to levels reported by Davey, Phelps and Thomas (1973), but lower than the levels reported by Grimble and Whitehead (1970), Robinson, Holmes and Bayley (1974), and Robinson (1975). Many factors influence the levels of plasma amino acids, including the content and quality of protein in the diet, the age and metabolic status of the animal, and the time of sampling in relation to previous feeding (Davey et al., 1973; Eggum, 1976; Pion, 1976). Amino acid levels reported by Robinson et al. (1974) and Robinson (1975) were obtained from Duroc pigs fed synthetic diets based on casein, sucrose, and corn oil, while control animals in the experiments of Grimble and Whitehead (1970) were fed diets containing 25% and 21.5% protein. The levels reported by Davey et al. (1973) are more directly comparable with the present results, because the pigs were fed diets based on maize with a similar total protein content.

The effects of feeding the deficient or supplemented diets on plasma essential amino acids, other than tryptophan, were not great. In most cases the levels in pigs fed the supplemented diet were a little higher and the difference reached statistical significance at times. However, the differences were not large and in some cases they were reversed. Tryptophan levels were higher in pigs fed the supplemented diet, in all samples except the pre-

feeding sample on day 2.

Low plasma levels of the first limiting amino acid have been reported on amino acid deficient diets. For example, lowered levels of isoleucine have been demonstrated in pigs fed diets low in isoleucine (Oestemer, Hanson and Meade, 1973). Plasma lysine concentrations were altered by feeding graded levels of dietary lysine, although they could not be used to estimate the lysine requirements of pigs (Braude, Fulford, Mitchell, Myres and Porter, 1974). Pigs fed an amino acid imbalanced diet showed a drop in the plasma levels of threonine, the limiting amino acid (Robinson, 1975). The concentrations of the other essential amino acids were elevated in pigs on the imbalanced diet.

The changes in the levels of tryptophan were not statistically significant, but they were similar to the results of other studies. The lower levels in pigs fed the deficient diet suggest that tryptophan was the first limiting amino acid in this diet, in agreement with other reports (Batterham, 1970; Stables and Carr, in press; Stockland et al., 1971) and the conclusions reached in section 2.1.3 (i), p. 56.

2.3.3 (iii) Plasma Glucose and Insulin Levels:

Basal plasma glucose concentrations measured in growing pigs have ranged from 85 - 120 mg/100 ml (Anderson 1974; Freeman, Noakes and Annison, 1970; Riis and Grummer, 1969; Topel, Weiss, Siers, and Magilton, 1973). The higher basal levels reported by Anderson (1974) and Freeman et al. (1970) are probably due to the sampling of right atrial

and arterial blood, rather than jugular venous blood. Freeman et al. (1970) reported an arteriovenous difference of between 5 and 9 mg/100 ml across the heads of pigs and a difference in plasma glucose levels in fed and fasted pigs.

One interesting feature of the glucose results was the small change in plasma glucose that accompanied feeding. Blood glucose rose promptly after meals in rats (Steffens, 1969) and in humans (Ahmed et al., 1976). Anderson (1974) observed a rapid rise in the plasma glucose concentrations of pigs fed a single meal. The levels reached a peak of about 125 mg/100 ml, 1 to 2 h after the meal.

The lack of response in the present experiment was not directly related to plasma insulin levels. The range of plasma insulin was similar to values reported elsewhere for the pig (Anderson, 1973; Machlin et al., 1968) and the changes in plasma insulin levels were similar to values reported by Anderson (1974). Aumaitre, Rérat, Vaissade and Vaugelade (1973) studied levels of blood glucose following single meals of glucose or maize starch and showed that blood glucose rose more rapidly following a meal of glucose than one of maize starch. The pigs showed a limited response to 800g of maize starch but a rapid rise in blood glucose following a meal of 1200g. The amount of maize starch eaten in one meal, in the present experiment, would lie between 800 and 1200g. Keys and De Barthe (1974), working with pigs, found no differences in the rate of passage of the diets studied. However, the amount of amylose digested before reaching a duodenal cannula was lower in pigs fed barley diets. No comparison can be made

between the present diets and those of Anderson (1974) because the source of nutrients was not described by that author.

Aumaitre, Rérat, Vaissade and Vaugelade (1975) described two patterns of peripheral glucose changes in pigs. In one instance, the basal level of blood glucose was low and a meal of 400 to 800g of starch, in addition to vitamins, minerals and protein, caused a large and persistent rise in portal vein glucose concentrations but little change in peripheral glucose concentrations. In the second case, where the basal glucose level was high, the meal caused a rise in both portal and peripheral glucose concentrations. It is possible that the different patterns observed in the present experiment and those of Anderson (1974) may fit the patterns described above. The basal glucose concentrations observed by Anderson (1974) were higher than those observed in the present experiment. Glucose levels observed by Topel et al. (1973) were similar to those in the present experiment and there was little difference between samples taken 1 h after the morning meal or 1 h before the evening meal. On the other hand, the so-called "high basal level" reported by Aumaitre et al. (1975) was similar to blood glucose levels reported by Topel et al. (1973) and basal levels observed in the present experiment.

The levels of food intake in the present study and that of Anderson (1974) appear to be quite different. The level of feeding used by Anderson (1974) is not stated, but it can be inferred from the remarks on protein feeding that the pigs were adapted to a very low level of intake.

The differences in plasma glucose response between pigs fed the deficient or supplemented diet suggest that early alterations in carbohydrate metabolism accompany the tryptophan deficiency. Sanahuja et al. (1965) reported an increase in liver glycogen in rats fed an amino acid imbalanced diet, although no change occurred in animals fed a deficient diet. Force-feeding rats for 1 to 3 days on diets devoid of, or deficient in, a single amino acid resulted in elevated levels of liver glycogen (Clark and Barron, 1972; Sidransky and Verney, 1968; Sidransky et al. 1969). The protein content and quality of diets has been shown to alter the balance between glycolysis and gluconeogenesis in rat liver (Peret et al., 1975). On low protein diets the predominant pathway is glycolysis. If amino acid balance further limits protein synthesis, surplus energy may be stored in the form of lipid, unless flux through the pathway is reduced.

Impaired glucose tolerance has been reported in rats force-fed diets devoid of threonine (Sidransky et al., 1969) and in pigs and dogs suffering from chronic protein-calorie deficiency (Heard, 1966; Heard and Henry, 1969). In dogs, the changes in glucose tolerance with age and protein status appeared to be independant of changes in plasma insulin (Heard and Henry, 1969). In the present experiment, the increase in insulin during feeding was similar for both diets and perhaps greater in pigs fed the deficient diet for 2 days. The relative roles of liver, muscle, or adipose tissue in contributing to the altered blood glucose curves cannot be ascertained without further studies.

The reason for the lowered pre-feeding insulin level in pigs fed the deficient diet for one day is not clear. The level of statistical significance achieved may be a chance phenomenon since the situation in the previous basal sample was reversed. Levels of hormones and metabolites often showed greater variability in the first sample of the morning, and it is considered that the second sample was more representative of basal levels. Adibi and Drash (1970) reported a small decrease in basal insulin levels in humans fed a protein-free diet and Young et al. (1973) reported lower basal levels and a smaller insulin response to force feeding in rats fed very low protein diets. It is unlikely that the lower basal insulin was due to impaired insulin production, since the rise in insulin during the meal was as great, or greater than, the rise recorded at other times.

The differences between diets, in the insulin response to feeding, were opposite to the changes in total essential amino acid levels, although the differences were not statistically significant. Infusion of arginine elicited a rise in insulin in pigs, but the response was slight when compared to changes observed in ruminants (Hertelendy, Takahashi, Machlin and Kipnis, 1970). Feeding a small amount of casein had little effect on plasma insulin in pigs (Anderson, 1974), suggesting that protein is not an important stimulus for insulin release in pigs.

2.3.3 (iv) Plasma Growth Hormone Levels:

Growth hormone (pGH) levels were similar to values reported elsewhere (Bidner, Merkel, Hafs, Swanson and Miller, 1973; Chappel and Dunkin, 1975; Siers and Hazel, 1970). Although higher, basal levels reported by Machlin et al. (1968) and Marple and Aberle (1972) were at the lower end of the range of values recorded in the present experiment. Levels were quite variable with occasional samples reaching 20ng/ml. Chappel and Dunkin (1975), who obtained plasma samples by venepuncture, reported similar variability. Although stress has been shown to increase pGH (Machlin et al., 1968), it was considered that the stress of bleeding was not closely related to the high pGH values obtained in some animals (Chappel and Dunkin, 1975). In the present experiment, samples were taken through indwelling catheters and it is unlikely that a stress effect is responsible for the occasional high values. Glick, Roth, Yalow and Berson (1965) reported spontaneous GH peaks in humans and Tannenbaum and Martin (1976) described a pulsatile type of GH secretion in the rat.

There were no clear-cut differences in pGH levels between the two diets, although the presence of occasional peaks makes it difficult to interpret results from the small numbers of animals in the study. Poor protein nutrition of dams has been reported to depress the synthesis of GH in rat pups (Shrader and Zeman, 1973) and humans fed low protein diets based on maize showed a reduced hGH response to arginine infusion (Merimee and Fineberg, 1972). However, the reduced response to arginine may not be related to protein quality, since the opaque-2 maize

variety, which permits faster growth rates in pigs (Stables and Carr, in press), also lowered the hGH response to arginine infusion (Merimee and Fineberg, 1973). Feeding a protein-free diet to humans had no effect on basal hGH levels (Adibi and Drash, 1970).

2.3.3 (v) Plasma Cortisol Levels:

Cortisol is the main glucocorticoid secreted by the adrenal gland of the pig (Heap, Holzbauer and Newport, 1966). Cortisol levels in the present study were similar to levels reported by Donald, Salisbury Murphy and Nabarro (1968) and Topel *et al.* (1973), although higher than values reported by Whipp, Wood and Lyon (1970). The latter authors used specific-pathogen-free pigs which may account for the difference.

Basal cortisol levels were higher than subsequent samples collected during feeding. Two studies of the diurnal pattern of cortisol secretion in pigs recorded the highest levels at 0800 h, in good agreement with the present results. Moberg *et al.* (1975) demonstrated that the diurnal rhythm in corticosterone levels in rats could be altered by feeding time and a similar effect may occur in pigs. An effect of feeding on the levels of cortisol in the studies of Topel *et al.* (1973) and Whipp *et al.* (1970) may be inferred when the sampling and feeding times are compared.

There was little difference in plasma cortisol levels between the two diets. Force-feeding diets devoid of single amino acids lowered corticosterone levels in rats (Clark and Barron, 1972). These authors suggested

that the decrease may have been due to poor synthesis of glucocorticoids, due to the involvement of a rapidly synthesised adrenal protein in ACTH-stimulated glucocorticoid production. The deficient diet used in the present experiment was not severely deficient and permitted a reduced rate of growth.

2.3.3 (vi) Plasma Urea Levels:

Plasma urea levels were similar to values reported by Braude et al. (1974) and Brown and Cline (1974), but lower than values reported for one pig by Eggum (1973). The rise in urea concentration from the beginning of the meal agrees with the results of Braude et al. (1974) and Eggum (1973) for pigs and Kumta and Harper (1961) for rats. In the above studies, urea levels reached a maximum 3 to 5 h after feeding.

Eggum (1973) discussed the role of plasma urea in determining protein quality. Plasma urea levels could be influenced by the quality and content of protein in the diet and time after feeding. By standardising protein content and time after feeding, plasma urea levels should give an index of protein quality. In the present results, there was no difference in urea concentrations between the two diets on day 5. The level of urea in pigs fed the supplemented diet was significantly elevated before feeding on day 2 and the difference declined in progressive samples taken after feeding.

The apparent discrepancy in the results can be resolved when food intake is considered. Although the protein content of the diets was similar, the protein intakes of the two groups of animals were different. To

overcome differences in food intake, Brown and Cline (1974) expressed urea values as mg/100 ml/kg of food eaten. On day 5, 3 h after feeding, the urea concentrations of pigs fed the deficient or supplemented diet were 9.42 and 7.83 mg urea N/100 ml/kg of food eaten respectively, and therefore the results are in agreement with the conclusion that urea concentration decreases with increasing protein quality (Braude et al., 1974; Brown and Cline, 1974; Eggum, 1973).

The increased basal urea concentrations, in pigs fed the supplemented diet for 1 day, may be related to adaptive changes in enzyme levels. The daily output of urea adapts only slowly to changes in diet (Elwyn, 1970). The activity of urea cycle enzymes has been shown to adapt to changes in the protein content of diets in rats (Schimke, 1962a, 1962b) and pigs (Krvavica, Pavić and Kućan, 1974). However, the enzymes appear to adapt slowly and changes were not complete until about 8 days after the changes of diet (Schimke, 1962a).

CHAPTER THREE

3.1 TRYPTOPHAN DEFICIENCY AND FOOD INTAKE DEPRESSION:

Feeding pigs on a diet with a relative tryptophan deficiency resulted in a rapid decrease in food intake. The depression in intake reached maximal levels within 3 days, whether the pigs were fed *ad libitum* or trained to eat their daily ration in one 2 h period. Experimental work reviewed earlier suggested that an altered pattern of plasma amino acids may be associated with food intake depression, but in view of the role of carbohydrate metabolism in mechanisms controlling food intake any changes in carbohydrate metabolism could also be important.

The hypothesis, that plasma amino acid pattern directly influences food intake, was developed from studies of amino acid imbalance (Harper *et al.*, 1970; Leung and Rogers, 1969; Rogers and Leung, 1973). The differences in plasma amino acid pattern between the two diets fed in the present experiment were in agreement with patterns observed in pigs fed an amino acid imbalanced diet (Robinson, 1975), although there was no consistent rise in essential amino acids other than tryptophan.

From the studies of Leung and Rogers (1969), it appears likely that the important factor in the plasma amino acid pattern is the depressed levels of the limiting amino acid and at most sampling times plasma tryptophan levels were depressed relative to the values in pigs fed the supplemented diet (see Figs. 23 and 24; Results presented earlier have been graphed together to facilitate comparison). Therefore, the results are consistent with

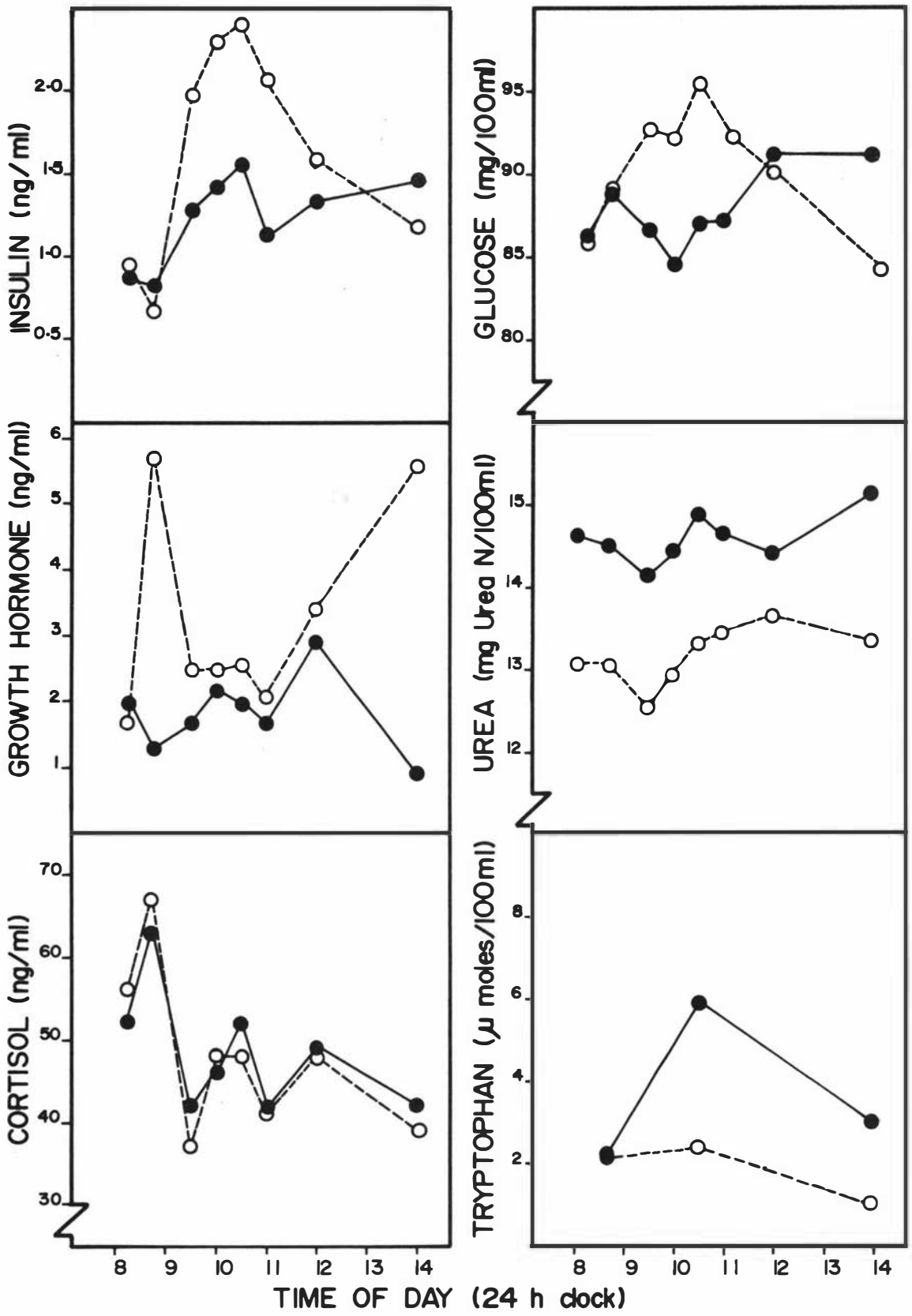

FIGURE 23 Changes in the plasma concentrations of hormones and metabolites, during and following the daily two hour feeding period, in pigs fed a tryptophan deficient or supplemented diet for 2 days.

●——● Supplemented Diet

○- - - -○ Deficient Diet

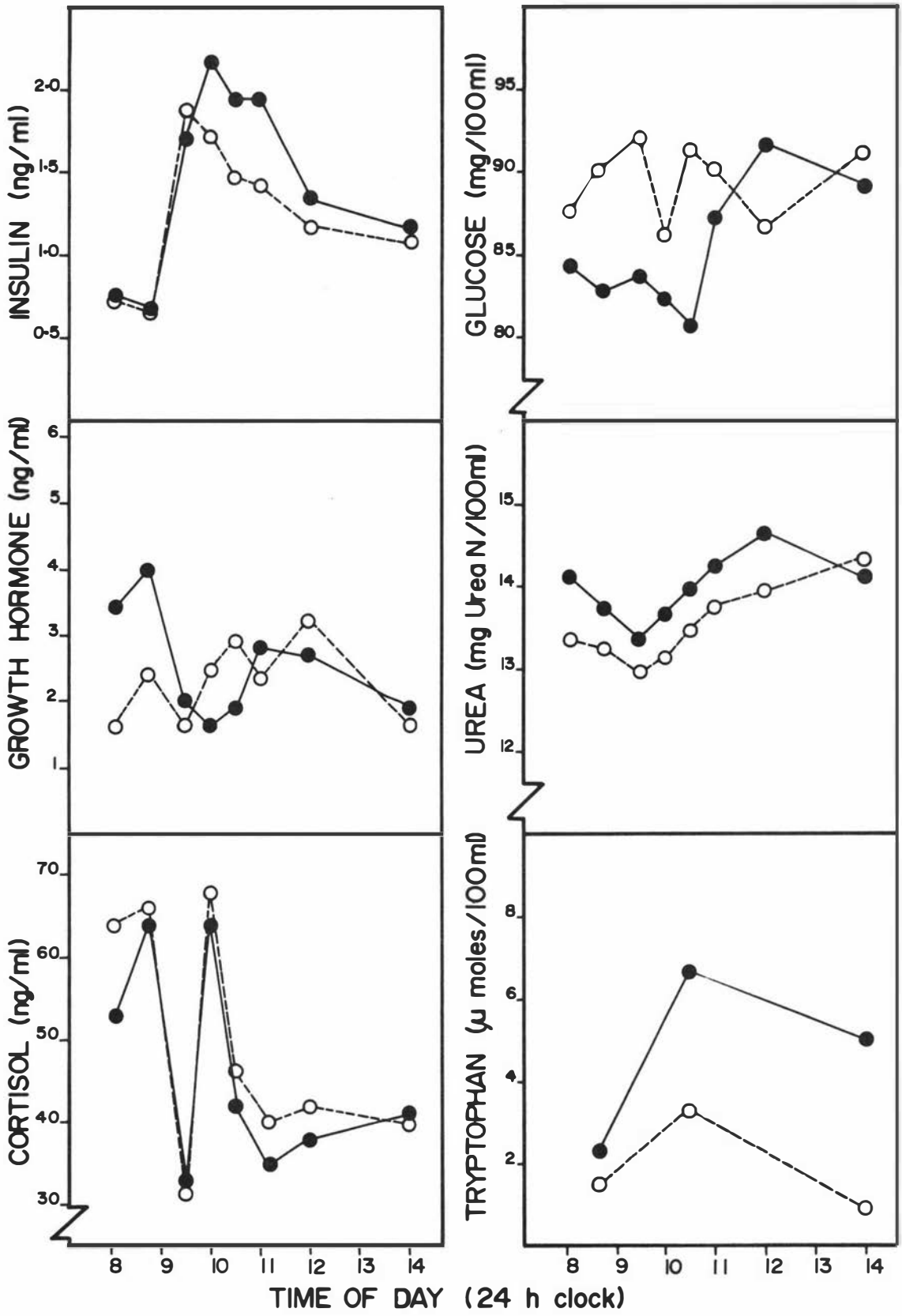

FIGURE 24 Changes in the plasma concentrations of hormones and metabolites, during and following the daily two hour feeding period, in pigs fed a tryptophan deficient or supplemented diet for 5 days.

●——● Supplemented Diet

○- - - -○ Deficient Diet

a direct role of plasma amino acids in the food intake depression.

In addition to a role in protein synthesis, tryptophan is a precursor for brain indole-amines and changes in plasma tryptophan could directly influence food intake through the levels of brain indole-amines. Alterations in the dietary levels of tryptophan have been shown to affect the levels of 5-hydroxy-tryptamine in brain tissue (Fernstrom, Madras, Munro and Wurtman, 1974). Rats fed a maize diet for 48 h had lower brain levels of tryptophan, 5-hydroxy-tryptamine and 5-hydroxyindoleacetic acid (Zambotti, Carruba, Vicentini and Mantegazza, 1975). However, if the deficient diet did lower brain indole-amine levels in the pigs, it is unlikely that this would be responsible for the depression in food intake, as it has been reported recently that depletion of 5-hydroxy-tryptamine caused hyperphagia and obesity in rats (Breisch, Zemlan and Hoebel, 1976; Saller and Stricker, 1976).

The altered plasma glucose response to feeding, in pigs fed the deficient diet, suggests that alterations in carbohydrate metabolism could also be responsible for the depression in food intake. Differences in blood glucose occurred on both sampling days. On day 2 plasma glucose rose significantly higher in pigs fed the deficient diet. On day 5, although the differences were not significant, plasma glucose levels were higher in pigs fed the deficient diet, despite the 17% lower food intake.

Although carbohydrate metabolism is important in the control of food intake (Anand, 1967; Rezek and Kroeger, 1976), the mechanisms involved are still not clear.

The higher plasma glucose levels in pigs fed the deficient diet could act on central glucose receptors and depress intake, although Stephens and Baldwin (1974) observed no change in the subsequent food intake of pigs given portal or jugular infusions of glucose and the differences in plasma glucose in the present experiment were not large. On the other hand, the pattern of plasma glucose changes would be consistent with altered liver carbohydrate metabolism and glycogen storage (Peret et al., 1975; Sanahuja et al., 1965; Sidransky et al., 1969). Glucose receptors in the liver have been described (Niijima, 1969) and their role in the control of food intake discussed elsewhere (Rezek and Kroeger, 1976; Russek, 1971).

In view of the small numbers of animals in the blood-sampling experiment, the results must be regarded as preliminary. The differences in food intake were not statistically significant, although it appears likely that the problems associated with the experiment may have obscured, rather than enhanced, the differences in blood metabolites and hormones. The alterations in carbohydrate metabolism associated with the amino acid deficiency need to be more clearly defined before the relationship between the changes observed and the depression in food intake can be evaluated. In particular, the importance of liver carbohydrate metabolism in determining the observed glucose patterns appears worthy of investigation.

Further studies should take account of the differences in food intake between the two diets, problems of taking point samples for rapidly changing variables, and the presence of spontaneous growth hormone peaks. The

simultaneous alterations in protein quality and protein intake have been discussed in relation to the interpretation of the plasma urea results. However, the lowered food intake on the deficient diet suggests that the elevated plasma glucose in response to feeding is all the more significant.

The maximum peaks in plasma insulin response to feeding ranged from 30 - 90 min after the beginning of the meal and samples taken every 30 min may not have coincided with peaks in plasma insulin, contributing to the variability between animals and sampling days. The presence of spontaneous peaks of growth hormone and the differences between the two diets suggest that the responses of hormones and metabolites to standard tests may yield further information on the changes associated with the deficient diet.

On the present evidence, it is not possible to assess the relative roles of the altered plasma glucose and amino acid patterns. Alterations in both the glucose and tryptophan response to feeding were evident at the earliest times studied and the mechanisms involved in the control of food intake are complex. However, the role of carbohydrate metabolism in the control of food intake is well documented and the altered carbohydrate metabolism in pigs fed the deficient diet deserves further investigation.

3.2 CONCLUSIONS:

Feeding pigs on a diet with a relative tryptophan deficiency resulted in a rapid depression in food intake. In pigs fed *ad libitum*, the depression in intake was evident within 24 h and fully established within 3 days. The major effect of the deficient diet on meal patterns was a depression in the average meal size. The deficient diet did not alter the diurnal pattern of feeding observed, but the marked diurnal feeding pattern raises interesting questions in relation to diurnal patterns of carbohydrate tolerance and growth.

When pigs were trained to eat their daily ration in a 2 h period a similar depression in food intake occurred in pigs fed the deficient diet. The changes in insulin, growth hormone, and cortisol that accompanied feeding were similar for both diets. When differences in food intake were taken into account, plasma urea levels appeared elevated in pigs fed the deficient diet. Plasma glucose levels were elevated and tryptophan levels appeared to be depressed on the deficient diet. It is concluded that the early changes in glucose and tryptophan may be associated with the food intake depression, but further studies would be required before the relative importance of either relationship could be established.

APPENDIX I

SPECTRAL ANALYSIS OF TIME SERIES DATA:

In the experiment on feeding patterns, meal patterns were recorded continuously for 15 days. The choice of 24 h as a period for averaging the record represented an arbitrary division and any information on rhythms not harmonically related to 24 h would be lost. A more objective approach to time series analysis is spectral analysis.

Spectral analysis is an established objective technique for showing periodic fluctuations in data, against a background of random variation. The analysis was carried out in two stages. Firstly, an autocorrelation function was calculated from the time series. The average food eaten in each half hour interval was calculated from the meal start time, meal size, and meal duration and the autocorrelation function calculated by multiplying each point on the time series with itself and shifting the two identical time series step-wise, relative to each other. When all peaks and troughs coincided with each other (e.g. comparing duplicate time series before any shift in the time axis) the autocorrelation function takes the value of 1. If all peaks and troughs exactly cancelled each other, the value would be zero. Calculating the autocorrelation function has two advantages; it separates the sine components from random fluctuations or "noise" (which by definition cancel each other) and reduces the data to a manageable size for calculation of the power spectrum.

The second stage of the analysis was the calculation of the power spectrum. Using a cosine transformation the relative power (the square of the amplitude) of the

sine component can be plotted against the independant variable frequency. Peaks in the power spectrum indicate the relative power and frequency of the sine components present in the time series. A pure sine wave will show power at a single frequency, but in the case of non-sinusoidal rhythms, harmonics related to the major periodicities may also show as peaks.

The scope of the analysis is limited by sampling frequency, the length of the record, and drift in the measured parameters during the course of the experiment. It is important to choose sampling frequencies that are much higher than any expected frequencies in the data. Only frequencies greater than twice the sampling frequency can be uncovered, and interference phenomenon will occur if sampling takes place at a frequency of similar magnitude, but out of phase with the frequency component in the sampled function. In the present experiment the effective sampling frequency was set by the meal definition criteria. However, expected frequencies would be considerably longer than this.

The length of the record will affect the resolution of spectral peaks. Initial analysis was carried out on the 5 day records for each period, but there appeared to be no effects of the two diets on feeding pattern. Consequently, the 3 periods for each pig were combined and the analysis performed on the 15 days of continuous records.

One disadvantage of power spectral analysis is the loss of all phase information. Therefore, where appropriate the phase information is shown in "averaged" data of appropriate fixed record length.

Further discussion and detailed descriptions

of the computer programs used in the analysis are given by Sarelius (1971).

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