THE EFFECTS OF PROTEIN NUTRITION ON LACTATIONAL PERFORMANCE

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ABSTRACT

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The effects of an inadequate diet, in terms of both protein quality and quantity, on maternal protein metabolism and on the lactational performance of the Wistar rat have been studied. The rats received diets with a high or low protein content of a good quality (milk-based) protein, or poor quality (cereal-based) protein. Reduced food intakes resulted on the latter diets. The experimental design therefore included rats pair-fed the control diet, in amounts equivalent in energy content to that eaten by the cereal-protein fed rats. In an attempt to increase energy intakes, rats fed the high cereal diet were given additional energy in the form of a sucrose solution.

Liver, muscle and mammary tissues were examined. Measurements carried out included: protein, RNA and DNA concentrations of the three tissues, and also serum albumin concentrations; protein synthesis in the three tissues; lactose synthetase and galactosyl transferase activities; milk volume and composition and serum insulin and corticosterone concentrations. The effects of exogenous prolactin on lactational performance were also examined.

The results suggested that, in liver, reduction in dietary protein quality and quantity reduced the protein synthesising capacity but had no significant effect on the fractional synthetic rate (FSR). Serum albumin secretion was decreased by reduced dietary energy intake but not by the quality or quantity of the dietary protein.

The responses of muscle and mammary gland to dietary protein insufficiency were similar in many respects. In muscle, cellular protein concentration, protein synthesising capacity and FSR were reduced by poor dietary protein quality. Maternal muscle mass, as represented by tibialis weight, was affected more by quality than quantity of dietary protein.

In the mammary gland, the protein synthesising capacity decreased with dietary protein quantity, though the effects were not as pronounced as in liver. Reduced dietary protein quality significantly decreased FSR and had a greater effect on lactose synthetase activity, milk volume and total milk protein content than reduced dietary protein quantity. The reduction in lactose synthetase activity may be due to a specific decrease in mammary α-lactalbumin content. Reduced energy intakes decreased lactose content, whereas differences in protein quality did not.

Serum insulin and corticosterone concentrations were reduced by inadequate dietary protein quality and quantity. Insulin was reduced to a greater extent than corticosterone.

It could be concluded that, in general, when a diet inadequate in protein is fed to lactating rats, maternal metabolic adaptations take place in order to bring about mobilisation of nutrients, so as to supplement the nutrient requirements of the mammary gland, and thus improve lactation. However, the severe dietary restrictions imposed in this study rendered these adaptations ineffective, due to the high nutrient demands of mammary tissue in small animals like the rat.

TO

YEMBEH I, KANKU, ZAINAB, MAMA AND DEE

"Because I know
The customs of our people
When the baby cries
Let him suck
From the breast.
There is no fixed time
For breastfeeding.
When the baby cries
It may be he is ill;
The first medicine for a child
Is the breast.
Give him milk
And he will stop crying."

From "Song of Lawino" by Okot p'Bitek.

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GENERAL INTRODUCTION

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There are reported to be four-thousand two-hundred and thirty-seven species of mammal in existence. The essential characteristic distinguishing them from other animals is the possession by the female of mammary glands which, by secreting milk, provide a source of nutrients for the newborn. Female mammals are thus able to lactate. Other features usually associated with mammals, such as viviparity and possession of a hair coat, are not shown by all species.

The World Health Organisation Technical Report (1965) succintly emphasises the importance of lactation in the survival of mammals and the modifications it can impose on the metabolism and reproductive activity of the mother in the following statement : "Lactation is the final phase of the complete reproductive cycle in mammals. It is usually differentiated into three stages, namely: mammogenesis or mammary growth; lactogenesis or the initiation of milk secretion; and galactopoiesis or the maintenance of established milk secretion. In almost all species, the newborn are dependent on maternal milk during the neonatal period; in most, the young are dependent for a considerable length of time. Adequate lactation is therefore essential for reproduction and the survival of the species. Biologically, the failure to lactate can be just as much a cause of reproductive failure as a failure to mate or ovulate. In view of the necessity for lactation, it is not surprising that the lactating mother will produce, if necessary, milk at the expense of her own body tissues and that suckling young, like foetuses, can 'metabolically cannibalize' the maternal organism. Correspondingly, the reproductive cycle is usually in abeyance during at least the early stages of lactation."

Lactation could also be considered as a continuation of gestation. In both processes, maternal diet plays an active role in the provision of nutrients; maternal nutritional stores and endocrine adaptations serve to buffer short-term variations in the

maternal nutritional intake; blood flow plays an overriding role in the transfer of nutrients to the foetus and the newborn. The nutrient demands of the young animal are higher than at any other stage of development.

In other words, pregnancy and lactation both make demands on the metabolism of the female. In many species, lactation exerts very exacting demands. Table 1 illustrates this.

In rats, mice, cats, rabbits, sheep and pigs, the demands of the young on the mother, for nutrition after birth, are far greater than those for nutrition of the foetus before birth. Conversely, guinea-pigs, which have a long gestation period and are born large and mature, make considerably more demands on the mother before birth than after; their young depend on the mother's milk for only a short time and begin to nibble on other food soon after birth.

For the human species, pregnancy and lactation would appear to make equal demands on the mother (Table 1). This, however, is not so for a number of reasons. First, the time-span for lactation must be taken into consideration. The human baby takes twice as long to grow 3.5 kg before birth as it does to double that weight after birth. Moreover, the provision of milk for growth of the young after birth is a less efficient process than supplying the nutritional requirements of the foetus in the uterus. Neither the production of milk in the mammary glands, nor the absorption of nutrients in milk by the young is 100% efficient. Moreover, the cost of lactation to the mother includes not only the nutrients in the milk and their energy value but also the energy required to synthesise milk constituents, i.e. lactose, caseins, α-lactalbumin, fat, etc. (Gopalan & Belavady, 1961).

A more important reason why nutrition after birth is less efficient than nutrition before birth is that much of the energy in milk must inevitably be used for the maintenance of the young animal in an environment that would usually be cooler than in the uterus. Table 2 shows that, on average, the slower a young animal grows, the larger the proportion of energy used for

Table 1. Weight of litter young as a percentage of mother's weight.

Species	Weight		
	At birth	At end of full suckling	Gain during suckling
Mouse	28	160	132
Rat	28	120	92
Cat	12	100	88
Rabbit	15	50	35
Sheep	7	40	33
Pig	7	40	33
Man	6	12	6
Guinea-pig	50	-	-

From Spray (1950).

Table 2. Percentage of energy intake used by infants for maintenance and growth (in kJ/kg/day).

Age (months)	Intake	Expenditure	
		Maintenance	Growth[a]
0-2	527	389 (74%)	138 (26%)
2-4	464	389 (84%)	75 (16%)
4-6	418	389 (93%)	29 (7%)

(a) increment in protein and fat.

From Widdowson (1976).

maintenance and the smaller the proportion used for deposition of new tissues. All these facts go to emphasise that lactation is a highly energy and nutrient requiring process, much more so than is pregnancy. The increased nutrient and energy requirements are met firstly from within, i.e. from the contribution of the mother's tissues, and, secondly, from without, i.e. from the diet.

In any attempt, therefore, to study the association between maternal nutrition and lactational performance, it is important to distinguish between the maternal nutritional status and the nutritional adequacy of the mother's current diet. Habicht *et al.* (1973) have asserted that the mother's nutrition during her own childhood and her pre-pregnancy nutritional status are factors which must be considered in evaluating the effects of nutrition on lactational performance. This problem is very relevant in those populations where the potential effects of maternal diet on lactation are of most concern, i.e. in areas where mothers have often been malnourished since childhood. Thus, the differences in the quality and quantity of breast-milk found in populations where dietary intakes are inadequate may be due to the mothers' current situation, their prior nutrition or some combination of both. It could be reasonably expected that the effects of acute nutritional deprivation would be less marked in women with adequate stores of body fat.

1.1 Meeting the cost of lactation from within

An important physiological change in the mother's body during pregnancy is the deposition of fat. Table 3 shows the weights and protein and fat contents of the bodies of rats, immediately after the birth of their first litters and 15 days later (Spray, 1950). The values are compared to littermate virgins and show that the rats gained weight during pregnancy and lost most, but not all, of the extra weight during lactation. Well-nourished rats, like women, deposit fat in their bodies during pregnancy

Table 3. Composition of the bodies of mother rats and their young and of unmated control animals (g).

	After birth			At end of full lactation		
	Control	Mother	Young (9) at birth	Control	Mother	Young (7)
Weight	230	305	52.7	236	248	193
Total water	130	174.2	45.4	137	166.8	147
Total protein	40.4	49.1	5.7	41.5	43.4	30.7
Total fat	51.0	72.3	0.6	48.4	29.2	9.2

From Spray (1950).

(Spray, 1950; Naismith, 1969; Naismith *et al.*, 1982). The stored fat is subsequently lost during lactation, together with further amounts from maternal tissues (Spray, 1950; Naismith, 1982). The energy supplied from the fat presumably subsidises the high energy cost of lactation and provides substrate for milk components. Of course, as already stated above, the amount of fat stored during pregnancy will depend on the mother's current nutritional status.

Economies in energy expenditure occur in the lactating animal. Trayhurn *et al.* (1982) have reported that thermogenic activity of brown adipose tissue (BAT) is suppressed in mice during the physiological hyperphagia of lactation and suggested that this probably results in a reduction in the energy requirement for maintenance of the lactating animal.

There are also some hormonal adaptations during lactation that would maximise the use of the mother's available nutrients to support lactation. For example, Jones *et al.* (1984) have reported an increase in the mammary gland insulin-sensitivity in lactation, which would lead to a co-ordinated redirection of substances towards the mammary gland after each meal.

Studies in rats have provided conflicting evidence on whether protein is stored in the maternal body at the end of pregnancy. The rats in Spray's study (Spray, 1950) deposited protein in their bodies during pregnancy, as did those of Poo *et al.* (1940) and Kanto *et al.* (1980), but not those of Mayel-Afshar and Grimble (1982) and Naismith (1969), who explained that, in the conditions of their experiments, nitrogen was stored in the maternal body but much of this had been lost at term. Furthermore, the efficiency of protein utilisation during pregnancy and lactation is greatly enhanced by suppression of the activity of hepatic enzymes that control amino acid oxidation and the synthesis of urea (Naismith *et al.*, 1982). These alterations in the metabolism of protein in the maternal body are induced by hormones secreted by the feto-placental unit and result in protection of the foetus from shortcomings in the maternal food supply (Naismith, 1980).

From the evidence of various studies, it would seem unlikely that significant amounts of protein are deposited in the maternal tissues for use during lactation. In those studies where protein deposition has been reported, the amounts are insignificant compared with the need for milk protein production. At maximum lactation, the rat dam produces about 4-5 g protein per day (Godbole *et al.*, 1981). The size of the protein stores which have been reported are within the range of 2-10 g (Kanto *et al.*, 1980; Spray, 1950).

Although some requirements for milk production can be met by the mother's own body, there must be an increase in the mother's food intake in order to lactate successfully. Most of the cost of lactation, therefore, must be met from without.

1.2 Meeting the cost of lactation from without

Increased food intake is the main mechanism for supporting the demands of lactation in a small animal, like the rat, where the total litter weight, at the end of lactation, approximately equals the dam's weight (Widdowson, 1976), and in which the daily output of milk energy is a large proportion of the dam's total body energy. In contrast, larger animals, such as the human, where the lactational stress is not as intense as in the rat, the mother may derive a greater proportion of the energy for lactation from body stores (Hanwell & Peaker, 1977).

Kennedy (1952-1953) and Anderson and Turner (1963) found that rats, in late lactation, ate more than twice as much as non-pregnant, non-lactating females. During lactation, the digestive tract of the dam enlarges in response to this increased food intake. The stomach increases in weight by 60%, the small intestine by over 100% and the caecum by 65% (Fell *et al.*, 1963). The surface area of the gastric mucosa increases and there is hyperphagia in the parietal cells (Crean & Rumsey, 1971). There is alsohypertrophy of the mucosa and muscle of the small intestine and caecum (Fell *et al.*, 1963). The villi of the intestinal mucosa are lengthened and proliferation is stimulated (Cairnie & Bentley, 1967). Rolls and Porter (1973) found a trebling of the

intestinal dipeptidase activity in the small intestine of the rat, at the second week of lactation, and suggested that the increase in Enzymatic activity was a response to the increased intake of protein. The liver has also been reported to increase significantly during lactation. Kennedy (1958) showed that liver weight and food intake increase in proportion to the number of pups. He also showed that liver protein nearly doubled during lactation. The DNA content also increased but not so markedly. Therefore, liver growth was due to increase in both the size and number of cells.

1.3 The influence of malnutrition on lactation

The above discussion has tried to show that lactation makes great demands on the dam and that these are satisfied from both within and without by adjustments in maternal structure and physiology.

The nutritional status of the mother to a large extent determines its ability to lactate adequately. Sampson and Jansen (1983) fed three groups of rats a diet containing 23% casein, 23% wheat gluten and 23% casein, pair-fed to the amounts taken by the wheat gluten group, respectively. They found that the litter weights on day 15 of lactation for these groups were 217, 155 and 171 g, respectively.

Many studies have indicated that malnutrition affects lactation, mainly by causing a reduction in the milk volume and, to a lesser extent, by altering its composition in a large number of species (Blaxter *et al.*, 1964; Rook *et al.*, 1968; Jelliffe & Jelliffe, 1978a). The mechanisms behind these effects will be examined later in this thesis.

The significance and usefulness of any conclusions reached in the present study will depend on how they can be used to improve lactation in humans. Studies which have been done on the effects of environmental factors on human milk and on breast-feeding will now be reviewed.

In 1847, Anthony Trollope remarked: "How is it that poor men's wives, who have no cold fowl or port wine on which to be coshered up, nurse their children without difficulty, whereas the wives of rich

men, who eat and drink everything that is good, cannot do so?" He was implying that malnourished mothers and mothers living in low socio-economic sectors of society are capable of producing milk that is adequate for their offspring in terms of its energy content (fat and lactose), protein content, minerals and vitamins, as has been more recently reported by Lonnerdal *et al.* (1976). This can only be possible if the maternal tissues are used to complement the insufficient dietary supplies that such mothers are known to take. This adaptation does not always occur. Karmarker *et al.* (1960) reported reduced milk protein concentrations from malnourished mothers and Lindblad *et al.* (1974) reported that, while milk of women from a low socio-economic group contained a normal concentration of total nitrogen, fat and lactose, the amino acid profile of the total protein fraction suggested a reduction of the whey protein, α-lactalbumin. There was a comparative decrease in the two essential amino acids, lysine and methionine, thereby lowering the nutritional quality of the milk protein. Earlier studies by Lutz and Platt (1958) had also shown that the concentration of total milk protein was reduced in malnourished women and that there was a relatively greater reduction in the whey than curd proteins. More recent studies by Jensen *et al.* (1978) and Crawford *et al.* (1975) have also shown that women living under unfavourable socio-economic conditions have reduced total milk lipid and that supplementing the diets of these women increased the milk fat (Infull *et al.*, 1959; Belavady, 1978) and the milk protein (Edozien *et al.*, 1976). The milk lactose concentration has been reported to be generally unaffected by malnutrition (Morrison, 1952; Kon *et al.*, 1950; Lonnerdal *et al.*, 1976). Some workers, however, have reported low milk lactose concentrations from malnourished women in the New Hebrides (Peters, 1953).

The commonly held view that the most significant effect of malnutrition on lactation is the reduction in milk volume is more difficult to examine critically. If true, it might mean that the baby is more likely to become malnourished through a reduction in total milk supplies than by any alterations in milk composition.

Data on human milk volume are limited; many are out of date and difficult to compare because of the different methods used in their measurement. The psychosomatic aspect of lactation has a profound effect on milk volume (Jelliffe & Jelliffe, 1978b) and statements about amounts collected must be suitably qualified in respect to the stage of lactation, time and method of collection and the sensitivity of the mother during the test period. Furthermore, it should be remembered that, physiologically, the milk secreted depends to a great extent on the demand, i.e. the intensity of sucking by the infant. It is obvious that these difficulties have a severe impact on the ability to calculate the effects on maternal nutrition on lactational performance, as well as the nutritional cost of lactation to the mother.

In well-nourished mothers, production of milk gradually increases from 600 ml per day or less in the first month to 700-750 ml per day in the third month and 750-800 ml per day in the sixth month (Wallgren, 1944; Lonnerdal *et al.*, 1976). A slightly higher volume of milk may be produced when male infants suckle, possibly because they have a higher birth-weight and consequently greater demands. After the first 6 months, a slow decline in the production of milk has been noted. Diurnal variations also exist. The maximum yield occurs in the morning and the lowest in the evening.

Studies in many parts of Africa, Asia, Central America and New Guinea show that generally the milk volume produced by poorly-nourished women is somewhat less than that produced by well-fed women. In the first six months of lactation, yields from the former tend to vary from 500-700 ml per day, in the second semester between 400-600 ml per day and in the second year between 300-500 ml per day. It is known that lactation will eventually cease in severely malnourished mothers or in conditions of prolonged famine (Jelliffe & Jelliffe, 1978b).

1.4 The advantages of breast-feeding over bottle-feeding in poor communities

The human is unique in using milk from another species to suckle its young. This practice can lead to severe nutrition and health problems in poor communities. It can create far greater malnutrition of infants than the changes in the volume and composition of human milk brought about by maternal malnutrition. Moreover, on teleological grounds alone, it is rational to suppose that the very different milks produced by each type of mammal would be species-specific, after millenia of selective adaptation to ensure optimal growth, development and survival of the species (Blaxter, 1961). In the case of human milk, one of the most significant of the many differences from cow's milk appears to be the abundant supply of nutrients most needed for the rapid growth and development of the central nervous system. Particularly high levels of lactose, cystine and cholesterol and specific polyenoic fatty acids are found in human milk. It has been found that the young human infant is able to absorb fats from human milk more efficiently than from cow's milk because human milk has higher amounts of acid-resistant lipases, and also because the human infant is able to absorb palmitic acid monoglycerides much more efficiently than stearic acid monoglycerides (Filer *et al.*, 1969). It has been demonstrated that in human milk fat the beta position mainly contains palmitic acid while in fat from cow's milk this position is occupied mostly by stearic acid (Freeman, 1965).

In terms of nitrogen, human milk is also significantly different from cow's milk. The non-protein nitrogen in mature human milk (made up of urea, creatinine, creatine, uric acid, small peptides and free amino acids) accounts for 25% of the total nitrogen. The caseins in cow's milk form by far the dominant group of proteins, whereas the whey proteins, i.e. α-lactalbumin, lactoferrin, secretory immunoglobulin A, lysozyme and serum albumin form 60-80% of the true protein content of human milk.

There is now some evidence for the superiority of human milk, as compared to milk of other origins for feeding the human newborn (Royer, 1978). It is based on the biological development related to the digestive, metabolic and excretory functions during foetal and postnatal life. Several metabolic functions, e.g. synthesis of cysteine from methionine, or tyrosine from phenylalamine and of urea from ammonia are still limited at birth. The capacity for the excretion of sodium and hydrogen ions is sub-optimal at this time.

The considerable protective effect afforded by breast-feeding is not related simply to the cleanliness and lack of opportunity for contamination but because of the presence in human milk of anti-infective agents like IgA, lysozyme, the bifidus factor (György, 1971), lactoferrin and other substances. The protective effect of human milk is evident especially in relation to intestinal infections, including diarrheal disease due to *E. coli*, other organisms and enteroviruses (Gerrard, 1974; McClelland *et al.*, 1978).

For the above reasons and for other reasons outlined below, mothers, especially in the Third World countries, where breast-feeding has been in the decline, must be advised to breast-feed their babies on a continuous and frequent basis. The reality of the situation stems from the fact that infant milk formula, to be properly prepared, needs sterile bottles, sterile water and correct measurements. For a poor village or slum-dweller, such conditions are difficult, costly and usually impossible to obtain. More often than not, formula is made with contaminated water and/or the formula is over-diluted to 'stretch' it because of its high cost. The result of this practice is infant malnutrition and disease, especially diarrhoea, which often leads to dehydration and death.

Jelliffe says that human milk in fact should be recognised as a national resource in economic, agronomic and nutritional planning (Jelliffe, 1968). For example, the recorded decline in breast-feeding in Kenya in 1968 resulted in an estimated $11.5 million loss in the breast-milk, which was equivalent to two-thirds of the Health Budget (Cook, 1968).

1.5 Nutritional status and infection

In a discussion concerned with the effects of protein nutrition on the mother's ability to lactate, especially in the context of a developing country, where sanitation is still deficient, one must always bear in mind the role of infection on lactational performance.

It is well established that the nutritional status of the mother and of the child are usually as much the product of infection as of diet (Scrimshaw, 1968). Clinical malnutrition is often precipitated by infections like measles, other systemic infections or diarrhoea (Morley, 1969). Infections are usually accompanied by anorexia, vomiting and increased peristalsis, so that food is digested and absorbed for only a limited period in the intestinal tract. The systemic manifestations of infection often result in marked reduction in food intake, impaired digestion and malabsorption. Other alterations are loss of tissue, particularly epithelium, blood, muscle and liver, depending on the type of infection, its localisation and pathogenesis. In many infections, there is increased loss of nitrogen, amino acids, electrolytes and vitamins. Even subclinical, or silent infections, induce increased nitrogen excretion in the urine (Beisel *et al.*, 1967). Tomkins *et al.* (1983) reported that infection in young children caused a rise in protein breakdown which was larger than the concomitant rise in synthesis, leading to net loss of protein.

On the other hand, malnutrition affects the host capacity to respond to infection, an important issue when one considers that more than half of the world's population suffers from malnutrition to varying degrees, while at the same time they are exposed to far greater risks of infection than well nourished societies (Mata, 1979). It is apparent that the increased incidence of infection in malnourished individuals is due to an inability to rapidly initiate some defence mechanism which requires protein synthesis.

1.6 Effect of nutrient supplementation on lactational performance

As has already been pointed out, the nutritional benefits of human milk are well documented (Jelliffe & Jelliffe, 1978a,b) and are of special significance for the health of infants, especially in developing countries. It has already been mentioned that maternal nutrition is one of the factors that influence successful lactation.

"Feed the nursing mother and thereby the infant" (Sosa *et al.*, 1976) is a slogan which has attracted increasing attention in the Third World. The underlying idea is that a more adequate maternal energy and nutrient intake will lead to an improved lactational performance. This simple measure would mean that the introduction of contaminated and potentially hazardous weaning foods could be delayed until the child's immune system was able to cope (Roland *et al.*, 1978). However, studies in humans have reached conflicting conclusions, even about whether maternal dietary intake and nutritional status do (Edozien *et al.*, 1976; Forsum *et al.*, 1980) or do not (Gopalan & Belavady, 1961; Lindhlad *et al.*, 1974; Prentice *et al.*, 1980) affect lactational performance. It has been difficult to measure milk yield accurately in field settings, to choose a protocol for milk sample collection such that the results will not be confounded by within-feed and diurnal variations in milk composition, and to select subjects who differ from one another solely by nutritional status.

The work of Prentice and co-workers in The Gambia (1980) suggested that large differences in energy intake did not manifest themselves in substantial differences in lactational performance, especially in the first three months of lactation. During this period, milk outputs were generally the same irrespective of nutritional status. During the dry season, when maximal lactational performance occurred, intake was only about 64% of internationally recommended amounts of energy.

In a later study, Prentice and co-workers (1983a,b) gave food supplements to lactating Gambian mothers, such that their total energy, protein and vitamin intakes were in line with. or better than, internationally recommended values. However,

the supplement did not improve milk output, composition or infant growth rate significantly. The mothers gained weight, although the weight gain represented only about 7% of the extra energy available, and their general health was believed to be improved. The most likely explanation of these observations is that adaptations exist, especially during lactation, that improve the efficiency of energy and nutrient utilisation, particularly when energy intakes are low. Supplementation brings about a relaxation from a state of high metabolic efficiency so that much of the extra energy derived from the supplement was simply wasted through a decreased energetic efficiency in the women.

As has already been mentioned, a similar type of adaptation has been reported by Naismith and Fears (1971) for nitrogen utilisation in pregnant rats. Galler and Zartarian (1981) investigated the reproductive performance of rats with different histories of malnutrition. They measured reproductive competence by examining weight gain during pregnancy, duration of pregnancy and foetal outcome. The following conclusions were reached : (i) that although weight gain was reduced in rats exposed to malnutrition for several generations, the duration of pregnancy and the number of live births did not differ from well-fed controls, suggesting adaptation to long-term dietary deprivation; (ii) that the rats malnourished from birth through pregnancy (one generation of malnutrition) had fewer live births than did rats with chronic, inter-generational malnutrition or well-fed control rats.

Moreover, Warman and Rasmussen (1983), looking at the effects of malnutrition during the reproductive cycle on nutritional status and lactational performance of rats, came to the conclusion that milk yield was compromised in food-restricted rats, and that the acutely-malnourished rats were much more severely affected than those which were chronically underfed.

All the studies cited suggest that, as a result of long-term adaptive mechanisms, the low levels of dietary energy intake reported for many communities need not be associated with serious impairment of reproductive performance, particularly during lactation.

1.7 Lactation and postpartum amenorrhea

Prolactin is thought to be an important hormone for successful lactation. However, it also has an important effect on maternal metabolism in that it limits the fertility of the mother during the period when she is lactating. An important advantage of breast-feeding over formula-feeding, therefore, is postpartum amenorrhea, that is, lactation contraception. A number of studies have shown that the duration of lactation is positively associated with the duration of postpartum amenorrhea (Salber *et al.*, 1966; Berman *et al.*, 1972). It has also been reported that menstruation is absent practically in all women during the early months of lactation and that, as lactation proceeds, the proportion of women menstruating increases (McKeown & Gibson, 1954). This indicates that there is an inhibitory influence of lactation on ovulatory functions which progressively loses power as the duration of lactation increases. Tietze (1961) suggested that lactation could delay pregnancy,even after ovulation has been resumed, by interfering with the process of implantation.

The length of postpartum amenorrhea is associated not only with the duration of lactation but also with whether or not an infant is receiving supplementary foods (Pérez *et al.*, 1972). It was therefore postulated that the nutritional supplements may discourage lactation through a 'substitution' effect, which leads to diminished suckling and thereby shortens the period of postpartum amenorrhea (Chen, 1973).

There is now considerable evidence to indicate that the pituitary hormone prolactin plays a central role in controlling this reproductive mechanism operating during lactation. Prolonged postpartum amenorrhea occurs only in women who breast-feed their babies, and the duration of the period of infertility appears to be closely related to the plasma concentration of this hormone (Duchen & McNeilly, 1980). Although the exact nature of the interaction of prolactin with the reproductive tissues at the molecular level remains to be fully elucidated, there is little

doubt of the overall effect. Hyperprolactinemia resulting, for example, from a prolactin secreting pituitary microadenoma, is in women invariably associated with amenorrhea, but when these elevated values are suppressed by bromocriptine treatment, a resumption of normal reproductive cycles is allowed (Robyn, 1980).

Marked differences in plasma prolactin concentrations have been found between women in developing countries and those in the more affluent western societies (Duchen & McNeilly, 1980; Lunn *et al.*, 1980). In general, it has been assumed that this variation occurred as a consequence of the considerable differences in feeding patterns between the two populations (Delvoye *et al.*, 1977; Konner *et al.*, 1980). In particular, the much higher frequency of breast-feeding episodes practised by Third World mothers, by operation of the suckling reflex mechanism, would be expected to stimulate a greater amount of pituitary prolactin and thus lead to higher plasma levels (McNeilly, 1977).

Moreover, Lunn *et al.* (1980) have shown that significant changes in plasma prolactin values occurred in conjunction with variation in maternal food consumption in lactating Gambian women. In particular, the introduction of a high energy supplement to the maternal diet was associated with a reduction in plasma prolactin concentrations through the whole period of lactation. This in turn had allowed an earlier resumption of the menstrual and ovulatory activity postpartum. Lunn *et al.* (1984) went further and showed that if food supplementation is practised throughout pregnancy as well as during lactation, a further decrease in the plasma prolactin and an even earlier resumption of menstrual and ovulatory activities were observed.

From the above discussion, therefore, it is obvious that breast-milk is regarded as the ideal early food for normal, healthy infants in terms of the balance of the nutrients supplied, their bio-availability and their immunological and protective advantages. Current controversy centres around the time of supplementation of the infant diet and, in this context, energy is conventionally regarded as the limiting factor.

Calculations have shown that milk from British mothers contains a mean energy content of 2890 kJ/ℓ (DHSS, 1977). This value matches others obtained from well-nourished subjects (Macy & Kelly, 1961). The intakes of breast-milk needed to fulfil the energy requirements at different stages of lactation are shown in Table 4 (Roland *et al.*, 1981). A value below 800 ml for the output at 3 months represents a realistic target falling well within the range of values documented in different surveys. The 6-month value in excess of 900 ml, by contrast, exceeds the volumes reported in almost all studies. The general conclusion is that the onset of weaning (i.e. supplementation) on conventional nutritional grounds must be advocated before 6 months. In fact, the recommendation in the UK (DHSS, 1974) that weaning should be avoided before 4 months appears to be a reasonable guide for industrialised countries. In Third World communities, such as Sierra Leone, however, sub-optimal maternal health and nutrition may result in lower or less well-sustained milk outputs. This, coupled with the required flexibility in milk energy to permit catch-up growth in small birth-size babies and infants with frequent infections, adds up to a frequent need for earlier supplementation of the infant's diet. Waterlow and Thompson (1979) concluded that the supplementation should realistically occur at 2-3 months. Chavalittampong *et al.* (1981), working with Thai children, came to the same conclusion.

At the same time, however, just as different individuals are able to utilise dietary energy and nutrients for anabolic purposes with different efficiencies (James & Trayhurn, 1981), there should be no reason why these differences should not also exist between babies. Thus, a seemingly inadequate amount of milk produced by a given mother may be perfectly satisfactory, if her baby happens to be metabolically efficient. It is not impossible that natural selection in conditions of food shortage could have encouraged the survival of the most metabolically-efficient individuals. It would, of course, be dangerous to assume that this selection has already taken place without further detailed investigation, but it is equally illogical

Table 4. Calculated breast-milk volumes which alone would satisfy lowest current estimated energy requirements for young healthy infants.

Age (months)	Weight* (kg)	Energy require-ments (kJ/kg)	Energy require-ments (kJ)	Breast-milk intake** (ml)
0	3.25	481	1565	542
3	5.7	397	2268	786
6	7.4	364	2694	914

* Mean of sexes

** Based on 2887 kJ/ℓ

From Roland *et al.* (1981).

to assume that physiological findings obtained in western countries can necessarily be applied to the Third World without modification (Whitehead, 1983).

It therefore seems that the mother's nutritional status has a significant influence on her ability to lactate. The purpose of the research presented in this thesis, therefore, was to examine in greater detail just how the mother's diet affects her ability to lactate and how she responds to variations in dietary protein intakes.

1.8 Preliminary study

In a preliminary study, the supervisor of this work looked at the effects of dietary protein quality and quantity on the metabolism and milk composition of lactating rats, and drew the following conclusions from that study (Grimble, 1981) :

(i) When good quality protein was fed, reduction from 27% to 13.5% dietary energy as protein had little or no effect on milk volume or composition.

(ii) Milk volume was more severely reduced than milk composition by poor protein quality.

(iii) Milk total protein content was reduced by poor quality dietary protein.

(iv) Not all milk proteins are equally affected; a diet low in both quality and quantity caused a large reduction in α-lactalbumin content.

(v) Changes in maternal serum insulin, corticosterone, albumin and body weight suggested that maternal metabolism could be changing to provide nutrients for maintaining milk composition at the expense of yield.

(vi) The synthesis of milk proteins showed a similar sensitivity to dietary protein inadequacy as other proteins produced on membrane-bound polysomes, e.g. serum albumin.

The reduced α-lactalbumin reported in this study supports the findings of Lindblad and Rahimtoola (1974) where changes in the amino acid composition suggested a relative reduction in milk α-lactalbumin in the milk of malnourished women. It is therefore reasonable to expect that lactose synthetase, a two-protein enzyme of which α-lactalbumin is a component, would be influenced by diet and exert an effect on milk volume.

1.9 Present study

This project was therefore an attempt to examine the adaptations in the maternal body of the rat regarding protein metabolism, during normal and dietary protein restricted lactation, using the following lines of investigation :

(i) To study the effects of diets, sub-optimal with respect to energy and the quantity and quality of protein they supply, on the lactose synthetase, galactosyl transferase and α-lactalbumin content of rat mammary gland.

(ii) To investigate the response of protein metabolism and hormonal changes of the lactating rats to these diets.

(iii) To relate the changes in milk volume and composition caused by the proposed dietary situations to the findings of (i) and (ii) above.

(iv) To make a comparison of α-lactalbumin concentrations and volumes of milk of women experiencing successful and unsuccessful lactation.

However, this last aspect of the work could not be undertaken owing to time limitation on part of the investigator and is recommended as one of the ways in which this study could be pursued further in the future.

CHAPTER 2

EFFECTS OF PROTEIN QUALITY AND QUANTITY ON PROTEIN-RELATED CONSTITUENTS IN THE LIVER, MAMMARY GLAND, MUSCLE AND SERUM OF LACTATING RATS

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2.1 Introduction

During pregnancy and lactation, rapid changes in body weight and alterations in the contents of major chemical components of the body have been reported (Spray, 1950; Johnson, 1973). Table 5 illustrates the effects of lactation on dietary intake, cardiac output, organ weights and organ blood flow in rats. There is evidence that tissue hydration occurs in the late stages of pregnancy (Flanagan, 1964) and during lactation (Foot *et al.*, 1979), which could partly explain the weight increases. Kanto *et al.* (1980) reported a greater weight loss at the end of lactation than was gained during pregnancy. They also reported that more than 100% of the fat, protein and ash deposited during pregnancy was catabolised during lactation. Naismith *et al.* (1982) showed that rats on a high-protein diet gained weight during the course of lactation, whereas those on a low-protein diet lost a substantial amount of carcass protein and its associated water. Thus, the tissues of the rat dam could undergo adaptations during lactation. The direction and extent of these adaptations could be affected by the diet, in particular the quality and quantity of dietary protein. The details of a study to these effects are reported in this chapter.

The effects of a low protein and a poor quality protein diet on different aspects of protein metabolism in the lactating rat were examined. Three tissues were studied: skeletal muscle, liver and mammary gland.

2.1.1 Skeletal muscle

Muscle represents a major depot for the retention of free amino acids and is a major component of body protein, being 45% of the adult body weight (Munro, 1970). Malnutrition, due to a diet low in quality and quantity of protein, results in changes in gross chemical composition of body tissues (Cabak *et al.*,

Table 5. The effects of lactation on dietary intake, cardiac output, organ weights and organ blood flow in rats.

Measurement	Lactating		Non-lactating
	Day 1	Day 12	Day 12
Body weight (g)	252	315	285
Dietary intake (g/24 h)	18	50	16
Cardiac output (ml/min)	91	143	89
Mammary gland			
Wet weight (g)	13.2	24.2	5.7
Blood flow ($ml.100g^{-1}.min^{-1}$)	33	62	12
Liver			
Wet weight (g)	12.8	16.3	11.7
Blood flow ($ml.100\ g^{-1}.min^{-1}$)	38	74	50
Small intestine			
Wet weight (g)	12.2	21.5	10.7
Blood flow ($ml.100\ g^{-1}.min^{-1}$)	66	94	80

Non-lactating rats refers to rats which had pups removed at birth.

From Chatwyn *et al.* (1969).

1963). In total body protein metabolism, therefore, the contribution made by the skeletal muscle is important, not only because of its size, but also because this tissue shows marked changes in chemical composition during protein-energy depletion in experimental animals (Widdowson *et al.*, 1960) and man (Waterlow *et al.*, 1957). During prolonged protein-energy malnutrition, skeletal muscle loses a greater percentage of its initial protein content, and makes up a greater proportion of total body protein loss than from other tissues (Young, 1970). Studies with rats have shown that the *in vivo* rate of muscle protein synthesis is rapidly reduced by feeding a low-protein or protein-free diet (Waterlow & Stephen, 1966; 1968), and this can be related to a lowered protein synthetic activity of muscle ribosomes.

2.1.2 Liver

There are many factors which point to the liver as potentially the key organ in the regulation of amino acid supply to other tissues. The anatomical position of the liver gives it access to dietary amino acids ahead of other tissues. Most amino acid catabolism and urea synthesis take place in the liver. Liver proteins, together with plasma protein, synthesised by the liver, comprise a major part of labile protein reserves. Deposition or loss of protein is faster in the liver than in any other organ, except the pancreas and parts of the gastro-intestinal tract. Of great significance is the fact that catabolic hormones, such as glucocorticoids, which cause protein-depletion in most other tissues, cause protein-deposition in the liver. Conversely, under special circumstances, anabolic hormones cause protein-deposition in some tissues but protein-depletion in the liver (Munro, 1964).

2.1.3 The mammary gland

Milk is produced by the vast number of cells that make up the mammary gland. The secretory cells are formed into billions of pear-shaped, hollow structures called alveoli (Fig. 1). Each cell of an alveolus discharges its milk into

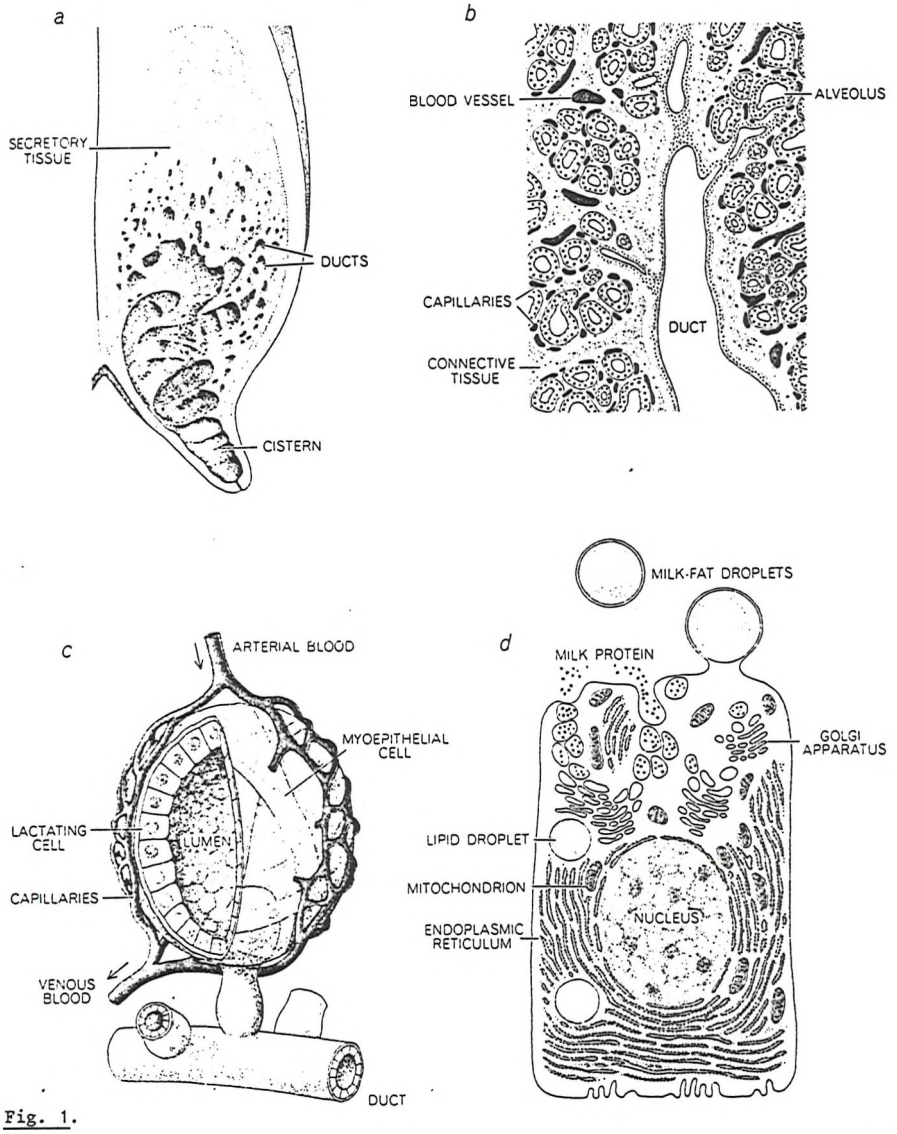

Fig. 1.

MILK-PRODUCING TISSUE of a cow is shown at progressively larger scale. At (*a*) is a longitudinal section of one of the four quarters of the mammary gland. The boxed area is reproduced at (*b*), where the arrangement of the alveoli and the duct system that drains them is apparent. A single alveolus (*c*) consists of an elliptical arrangement of lactating cells surrounding the lumen, which is linked to the duct system of the mammary gland. A lactating cell (*d*), similar to the one in the electron micrograph on page 114, is shown as it discharges a droplet of fat into the lumen. Part of the cell membrane apparently becomes the membranous covering of the fat droplet. Dark circular bodies in the vacuoles of Golgi apparatus are granules of protein, which are discharged into the lumen.

(From Patton, S. 1978).

the lumen, which is the hollow part of the structure. When an alveolus is full, its outer cells (the myoepithelial cells) contract under the influence of oxytocin, causing the alveolus to discharge milk into the duct system which connects eventually with the exterior through the nipple.

Satisfactory lactation can be expected only when the mammary glands have reached a proper state of development.

The various methods available for assessment of mammary growth include measurement of mammary volume, weight, area, DNA content (as a measure of cell number), DNA synthesis, qualitative and quantitative histological methods, and labelling indices (using autoradiography to visualise nuclei incorporating ^{3}H-thymidine into DNA). Two particular and related problems arise. The mammary gland consists of epithelial tissue (parenchyma) and connective tissue (stroma). The relative proportions of the two tissues vary enormously with the physiological state of the animal. The gross size of the mammary gland is a poor guide to development, especially in non-pregnant ruminants or women, in whom the udder or breast consists largely of stroma. In addition, growth of mammary parenchyma is not just a question of an increase of cell number but involves a pattern of formation, giving rise to well-defined structures in the normal gland.

From histological studies, the changes occurring in the mammary glands of unmated females have long been known (Folley, 1952). From birth to just before puberty, the gland is quiescent, consisting only of ducts which may either remain short or show moderate extention in proportion to body growth (Folley, 1952). A new phase of more rapid mammary growth occurs with the onset of ovarian activity. In some rodents and heifers, cyclic changes in mammary development in the oestrous cycle have been reported, while rather extensive mammary growth comparable to early pregnancy and involving lobulo-alveolar formation may accompany spontaneous or induced pseudo-pregnancy, as in the bitch and rabbit, respectively. Otherwise, the formation of true alveoli in the non-pregnant female is quite uncommon.

During pregnancy in mammals, the mammary gland develops and stroma is replaced with lobulo-alveolar tissue, the most striking changes often occurring about mid-pregnancy.

Measurement of DNA content indicates that mammary growth is essentially complete in sheep (Anderson, 1975), cow (Baldwin, 1966) and hamster at parturition, while a substantial proportion (20-40%) of the increase in mammary DNA occurs during early lactation in rats, mice (Munford, 1964) and rabbits (Denamur, 1963).

In pregnant women, extension and branching of the duct system occurs during the first trimester, alveoli form early in the second trimester with some secretory development by mid-pregnancy, continuing through the third trimester (Cowie & Tindall, 1971; Salazer & Tobon, 1974). Substantial quantities of both α-lactalbumin and lactose are present in human secretions at least 18 days prepartum (Kulski *et al.*, 1977).

In rats and mice, the biochemistry of mammary development has been extensively studied (Cowie & Tindall, 1971). Allometric duct growth (i.e. disproportionate in relation to the rest of the body) begins before the onset of oestrous cycles and continues until about 60-100 days of age. Bresciani (1971) has shown by autoradiography that the cells incorporating thymidine into DNA in the mammary glands of virgin female mice are located almost exclusively on the end buds and alveoli of the mammary tree and not on the ducts. By contrast, in pregnancy and early lactation, both alveolar and ductal cells show thymidine incorporation, and the percentage of cells labelled with thymidine greatly increases (Bresciani, 1971), leading to lobulo-alveolar development and an increased intricacy of the ducts.

Rat mammary glands undergo dramatic changes during the initiation of lactation (at about parturition). Parturition is accompanied by a moderate increase in the weight of the mammary glands. The functioning mammary gland, when empty, weighs 5-7% of the body weight of rats and mice in full lactation, and is therefore larger than the liver (Linzell, 1968).

Changes in the fat and water contents are considerably bigger than weight changes. Fat, which forms 47% of the weight of the gland at late pregnancy, falls to 22% within 22 h post-partum (Kuhn & Lowenstein, 1967). The percentage of water increases from 42% to 65% within the same period. The fat-free weight increases by about one-third from a level of 10% pre-partum. This change represents mainly an increase in the endoplasmic reticulum.

At the same time, however, fatty acids synthesised in the mammary gland (for milk lipids) are considerably increased during lactation. Rosmos *et al.* (1978) showed that 60%, 40% and 0% of fatty acid synthesis takes place in the liver, adipose tissue and mammary gland of the virgin rat, respectively; 66%, 11% and 23% during pregnancy and 24%, 1% and 75% during lactation in the three tissues, respectively. Moreover, Agius *et al.* (1979) showed that the removal of pups for 24 h from rats at peak lactation decreased $^{3}H_2O$ incorporation into lipid *in vivo* in the mammary gland by 95%, whereas it was increased in the liver by 77% and in adipose tissue by 330%. These increases were prevented by the administration of prolactin. These observations imply that the suckling stimulation is essential to maintain the high level of lipid synthesis in the mammary gland during lactation.

During the first 48 h after parturition, there is a period of rapid cell proliferation (Munford, 1963), resulting in a two- to three-fold increase in DNA levels (Baldwin & Milligan, 1966). Griffith & Turner (1961), using DNA concentration in fat-free, dry mammary tissue of the rat as an index of growth, found no significant growth, compared with virgin controls, by the fifth day of pregnancy. However, a three-fold increase occurred during the remainder of pregnancy. Thus, some 60-70% of mammary gland development recorded on day 14 of lactation had occurred during pregnancy (Moon, 1962).

The beginning of the secretory phenomenon in the mammary gland corresponds with increases in cellular RNA concentrations (RNA/DNA) and RNA synthesis (Denamur, 1969). A

large increase in total quantities and cellular concentrations of RNA, corresponding to the stimulation of RNA synthesis has also been reported to take place from the beginning of lactation. Shortly after parturition, the RNA/DNA ratio is 1.9 but increases to 4.19 by day 16 of lactation (Denamur & Stoliaroff, 1966). These studies, and those of others, have drawn general conclusions in favour of a close relationship between mammary RNA and milk secretion levels. Moreover, it has also been shown that,in all species,the decreasing phase of lactation, e.g. during involution, corresponds to a rapid reduction of RNA content per cell (Cowie, 1969), in addition to the fact that there is also a slow disappearance of mammary cells, i.e. a fall in total DNA (Denamur, 1965).

Enzyme activities start increasing 12-24 h after the period of rapid cell proliferation. During the first three days of lactation, the activities of most enzymes increase 5-10-fold. For example, changes in the routes of carbohydrate metabolism occur in that more glucose is metabolised by the pentose phosphate pathway (McClean, 1958) and large increases in the activities of enzymes in the biosynthetic pathways for lactose (Malpress, 1961; Shatton *et al.*, 1965), protein (Bucovaz *et al.*, 1961) and fat (Abraham *et al.*, 1961) have been reported. These increases in the enzyme concentrations of the tissue reflect its increased metabolic status and all involve an increase in protein synthetic activity. A summary of some of the metabolic changes during lactation are shown in Table 6.

The quality and quantity of protein in the diet of the lactating dam will affect the amino acid pools of all the tissues which, in turn, will affect their protein synthesising activities. Millward (1970) measured the immediate effect of a protein-free diet or starvation on protein synthesis and breakdown in the liver and muscle. The results obtained demonstrated that, in muscle, protein synthesis was more sensitive than breakdown to starvation or the protein-free regimen. On the other hand, in liver, protein synthesis and breakdown were both sensitive to the two dietary regimens.

Table 6. Summary of metabolic changes during lactation in the rat.

Tissue	Process	Change
Mammary gland	Glucose utilization	Increased
	Lactose synthesis	Increased
	Lipogenesis	Increased
	Fatty acid esterification	Increased
	Triacylglycerol uptake	Increased
	Amino acid utilization	Increased
	Protein synthesis	Increased
Liver	Glucose utilization (glycolysis)	Increased
	Lipogenesis	Increased
	Fatty acid esterification	Increased
	Ketogenesis	Decreased
	Triacylglycerol secretion	Decreased
	Amino acid metabolism	decreased*
	Protein synthesis	?
White adipose tissue	Glucose utilization	Decreased
	Lipogenesis	Decreased
	Triacylglycerol uptake	Decreased
	Lipolysis	Increased
Brown adipose tissue	Glucose utilization	Decreased
	Lipogenesis	Decreased
	Fatty acid oxidation	?

? indicates where information is not available.

From Williamson (1980).

*Naismith et al. (1982)

The intensity of protein synthesis in a tissue is a function of the concentration of the translational apparatus, that is, the RNA content, and the efficiency with which translation takes place (i.e. the protein : RNA ratio). Millward and Garlick (1972) demonstrated that varying the diet, in terms of quantity, affects the RNA content. They showed that a protein-free diet resulted in a lower RNA content. In the present study, therefore, the DNA, RNA and protein contents were measured in rats fed diets varying in dietary protein quality and quantity, and the efficiency of the protein synthesising machinery was determined from these values.

2.2 Materials and methods

2.2.1 Animals and diets

Virgin female Wistar rats, from the Southampton University Medical School colony, weighing 210-230 g, were mated and fed a standard laboratory diet (PRD pellets, Christopher Hill Group, Poole, Dorset) throughout pregnancy. A white vaginal plug was taken as an indication of successful mating. All animals were kept in a light (12 h on, 12 h off) and temperature (23 ± 1°C) controlled experimental room. The pregnant rats were then housed in individual solid-bottom breeding cages with bedding and nesting material, about 6 days pre-partum (day 16 of pregnancy). Two days post-partum, the litter sizes of all dams were adjusted to 9 pups. The reason for this adjustment was to ensure that the dams had the same suckling stimulus and also because Wilde and Kuhn (1979) demonstrated that litter-size does affect mammary tissue volume.

The dams were randomly assigned to six dietary groups, which are shown below :

(i) 200 g/kg milk protein *ad libitum*;

(ii) 100 g/kg milk protein *ad libitum*;

(iii) 60 g/kg milk protein *ad libitum*;

(iv) 200 g/kg cereal protein *ad libitum*;

(v) 100 g/kg cereal protein *ad libitum*;

(vi) 200 g/kg milk protein pair-fed with group (iv).

Five groups received food *ad libitum* and a sixth group was pair-fed in a manner which will be explained later. Diets were fed until the animals were ready for sacrifice on day 15 of lactation. The diets were prepared in pellet form. A high and low quality protein source was used. A milk protein-based diet, supplemented with methionine, was used as the high-quality protein source. The low-quality protein diet contained whole-wheat flour, zein (BDH) and maize meal (Provender, Christopher Hill Group), previously analysed for protein content. Purified maize protein, zein, was added to the low-quality protein diets to bring the cereal protein concentrations to 200 and 100 g/kg diet. Zein is known to be deficient, especially in tryptophan, but also in lysine. As the maize meal and whole-wheat flour contain appreciable amounts of fibre, crude fibre contents were equalised by the addition of cellulose powder (Solkafloc, BW40, J.J.W. Greenock, USA) to the milk-based diets. Lipid contents were equalised to compensate for the lipid that would be present in the cereals. All diets provided 63 kJ metabolisable energy/g fibre, 13 kJ/g food and had 27, 13.5 or 8.1% dietary energy as protein. The rats also received tap water *ad libitum*. Full details of the composition of the diets are shown in Tables 7, 8, 9 and 10.

2.2.2 Experimental procedure

The weights of the dams and pups were measured two days after parturition and every third day until the mother was sacrificed. Day one of lactation was taken to be the morning on which the pups were seen. Pup growth was used as an index of lactational performance. Food intake was also measured during the experimental period.

The pups were removed from the dam on the evening of day 14 of lactation. The dam was anaesthetised with sodium pentabarbitone the following morning (Nembutal, May & Baker, Ltd.). Fur was removed from around the teats, which were washed with distilled water and thoroughly dried. Oxytocin (5 iu) was injected intraperitoneally to enhance milk secretion. Milk was manually expressed from all the teats and sucked into a collecting vial. As much milk as

Table 7. Casein diets.

	Protein (g/100 g)		
	20%	10%	6%
Casein (casumen)	127.2	63.6	38.16
Corn starch	300.0	400.0	493.07
Solkafloc	200.0	200.0	200.0
Dried skim milk (36.4% protein)	200.0	100.0	60.0
Sucrose	59.8	124.9	97.87
Mineral mixture (AIN)	40.0	40.0	40.0
Vitamin mixture (AIN)	20.0	20.0	20.0
Corn oil	50.0	50.0	50.0
DL-methionine	3.0	1.5	0.9
	1 kg	1 kg	1 kg
		13,426 kJ/kg	

Table 8. Cereal diets.

	Protein (g/100 g)	
	20%	10%
Maize (10.4% protein)	500.0	500.0
Whole wheat flour (13.2% protein)	250.0	250.0
Zein	115.0	15.0
Corn starch	-	100.0
Sucrose	75.0	75.0
Mineral mixture	40.0	40.0
Vitamin mixture	20.0	20.0
	1 kg	1 kg
		14,016 kJ/kg

Table 9. AIN-76 Mineral mixture (Am. Inst. Nutr., 1977).

Item	g/kg mixture
Dicalcium phosphate	500
Sodium chloride	74
Potassium chloride	42
Potassium sulphate	52
Magnesium hydroxide	35
Manganous carbonate	3.5
Ferric citrate	6.0
Zinc carbonate	1.6
Cupric carbonate	0.3
Potassium iodide	0.01
Sodium selenate	0.01
Chromic potassium sulphate	0.55
Sodium molybdate	0.01
Starch	285

Table 10. AIN-76 Vitamin mixture (Am. Inst. Nutr., 1977).

Item	mg/kg mixture
Thiamine	600
Riboflavin	600
Vitamin B_6	700
Nicotinic acid	3,000
Calcium pantothenate	1,600
Biotin	20
Folic acid	200
Vitamin B_{12}	1
Vitamin A (400,000 iu)	800
Vitamin D_3 (10,000 iu)	20
Vitamin E (5,000 iu)	20,000
Vitamin K (Menapthone)	5
Inositol	10,000
Choline	10,000
Starch supplement	952,454

possible was collected from each dam. Maternal blood was removed by cardiac puncture.

The tissues taken for analysis were the liver, inguinal mammary gland and mixed muscles from the thigh. These tissues were put in liquid nitrogen immediately and stored at -20°C until ready for use. The tibialis muscle was later removed and weighed. This muscle was sampled to see how maternal body muscle mass responded to the various diets.

The group on the 200 g milk protein/kg diet was taken to be the control group because many workers (McCoy, 1947;Nelson & Evans, 1958) have established that the optimal concentration of milk protein for growth, pregnancy and lactation in the rat is 18-24%, provided that diets are supplemented with methionine. The 10% milk protein group was the good quality, low quantity group. A 6% milk protein group was added to see the effect of further increasing the stress of protein deficiency on lactational performance. The 20% and 10% cereal groups represented the groups on poor quality protein fed at high and low quantities, respectively.

The pair-fed group of animals was included as the food intakes of animals on the cereal diets were significantly less than those on the control diet. This group received the 20% milk protein diet in amounts taken by the 20% cereal protein group (i.e. 24 g/day). A comparison of results obtained from the pair-fed milk protein group with those from the cereal protein groups would therefore show the effect due to protein quality, uncomplicated by differing energy intakes, as energy intakes would be similar.

Five non-pregnant non-lactating, virgin female rats, age-matched to the control group, were fed the control diet for 15 days. Blood serum was separated from all blood samples by centrifugation and stored at -20°C until analysis.

2.2.3 Experimental methods

An aqueous homogenate (about 20%) of the tissue was made, using an ultraturax homogeniser and 1.0 ml of the homogenate treated according to Fig. 2.

2.2.3.1 Protein

The method of Lowry *et al.* (1951) was used and standards were prepared from a solution of serum albumin (Sigma Chemicals Ltd., UK). 1.0 mg/ml in 0.1M NaOH stock solution was prepared. 1.0 ml aliquots were placed in small vials and stored at -20°C until needed. The strength of the Folin Ciocalteu stock solution was regularly checked and was adjusted to 1.0M with addition of distilled water. Aqueous 1.0% $CuSO_4.5H_2O$, 2% sodium tartrate and 2% Na_2CO_3 in 0.1M. NaOH were prepared and kept at 4°C. 0.5 ml of the sulphate and tartrate solutions were added just before use to 50 ml of the sodium carbonate solution. One ml of this solution was added to 0.1 ml of the unknown solution, containing 20-70 µg equivalents of bovine serum albumin, in a small glass test tube. The mixture was vortexed and kept for at least 10 min at room temperature. 0.1 ml of 1.0M Folin reagent was added and stirred immediately. After at least 30 min at room temperature, the blue colour formed was read at 750 nm using a Beckman Spectrophotometer. It was repeatedly found that the standard curve outside 20-70 µg protein range was not linear and therefore was not used outside that range.

2.2.3.2 RNA

The RNA content of tissue was estimated according to the method of Schneider (1957). Orcinol reagent was prepared by dissolving 1.0 g pure orcinol in 100 ml concentrated hydrochloric acid containing 0.1 g $FeCl_3$. A standard solution was prepared, using the sodium salt of yeast RNA, 250 mg/ml, in distilled water. To estimate RNA, 1.0 ml of the RNA extract (of muscle, liver or mammary tissue) was heated with 1.0 ml orcinol reagent for 30 min in a boiling water bath. The intensity of the green colour was read at 660 nm against a water blank. The water blank and standards were all made up to 1.0 ml and treated in the same way as the sample. To give a straight line standard curve, concentrations in the range of 10-70 mg were used.

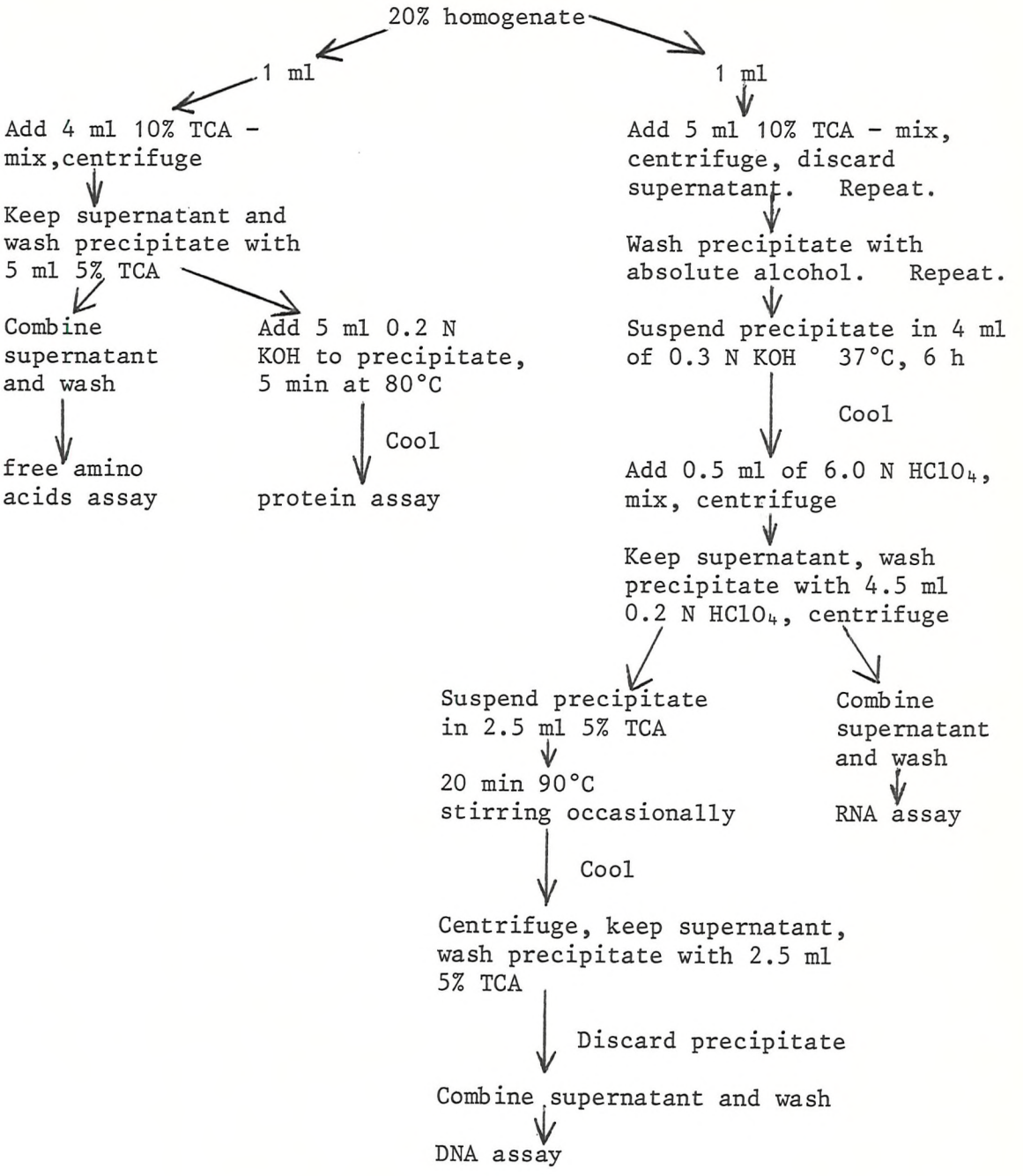

Fig. 2. Flow chart of separation of nitrogenous components of tissue homogenates.

2.2.3.3 DNA

The DNA content of tissue was estimated by the diphenylamine reaction as modified by Richards (1974). To 1.0 ml DNA extract was added 1.0 ml of 3.0 M perchloric acid ($HClO_4$), 2 ml of 4% diphenylamine in glacial acetic acid and 0.1 ml aqueous acetaldehyde solution (0.16%). The contents were thoroughly mixed and the blue colour allowed to develop at room temperature for 20 h. The blue colour was read at 600 nm. A stock solution of hydrolysed calf thymus DNA was used to make the standard curve. A solution containing 0.05 mg of hydrolysed DNA/ml 0.02 N $HClO_4$ was prepared by dissolving 12.5 mg DNA in 10 ml water in a 250 ml volumetric flask. The flask was immersed in a bath at 70°C and 1.7 ml of 3.0 N $HClO_4$ was added slowly with swirling. Heating was continued until all the DNA had dissolved (about 30 min), after which the flask was cooled, the contents diluted to volume with distilled water and stored at 4°C. The standard curve was prepared in the range 10-50 µg. All standards were made to a total volume of 1.0 ml and treated as for the samples above. All tubes were read against a water blank.

2.2.3.4 Serum albumin

Concentrations were determined by the bromocresol green dye-binding method of McPherson and Everard (1972). To 5 ml dye reagent was added 20 µl serum. The colour change was read immediately against a water blank at 635 nm: bovine serum albumin (1-6%) was used to obtain the standard curve. The colour reagent was prepared by adding to 800 ml distilled water 94.5 ml 1.0 N glycine, 5.5 N HCl and 3.0 ml of 0.02 M bromocresol green, and made up to 1.0 litre. The pH was adjusted to pH 4.0.

2.2.4 Statistical analysis

The data in the tables of this chapter and all subsequent chapters were analysed using the 't' test and all probability values -p- above 0.05 were considered non-significant. The control group was compared with every other group for any

significance. Then the group pair-fed with control diet was compared with the group on the 20% cereal protein diet. All results are given as values of the mean ± the standard error of mean (SEM).

2.3 Results and Discussion

2.3.1 Food intakes

The control group ate more than twice as much as the non-lactating group (Table 11). This is to be expected because, as has already been explained, lactation brings about very significant increases in food intake, in order to meet the high demands for milk production and maintenance (Moore & Brasel, 1984). The 6% milk protein group ate approximately half the amount eaten by the control group, as did those on the cereal diets. These diets might have had poor palatability. Low protein diets are known to reduce appetite. It has also been suggested that an imbalance in dietary amino acids can reduce food intake (Harper *et al.*, 1970). The cereal protein diets would have been considerably imbalanced in their amino acid composition due to deficiencies of lysine and tryptophan and excess of leucine (Table 12). It can be seen from the table that in consuming a sufficient quantity of tryptophan from the 20% cereal diet, the rat would receive $4\frac{1}{2}$ times its requirement of leucine.

It has also been reported that appetite regulation, which is normally controlled by the energy requirement of the rat, is influenced by the protein demand and supply under conditions of physiological stress, like pregnancy and lactation (Menaker & Navia, 1973). These workers showed that lactating rats, on an 8% casein diet, were not able to achieve normal levels of milk production or the associated increase in diet consumption, as a response to the demands of the suckling pups.

Even though decreased protein quantity and quality decreased appetite in the lactating animals, intakes were still above those of non-lactating animals. The increase in the food

Table 11. Food intakes and pup, dam, liver and tibialis weights (Mean ± SEM). Number of animals shown in parentheses

Diet	Food intake (g)	Utilisable protein intake (g)	Change in dam's weight (g)	Tibialis weight (mg)	Tibialis protein (mg)	Pup growth (g/day)	Liver weight (g)
20% milk protein (22)	692 ± 11	110.7	32	615 ± 15	86.7	1.81 ± 0.13	14.3 ± 0.35
10% milk protein (15)	561 ± 19	44.9	12	600 ± 32	81.6	1.36 ± 0.18(a)	14.9 ± 0.55
6% milk protein (18)	296 ± 22(a)	14.2	-49	500 ± 31(a)	64.0	0.51 ± 0.05(b)	12.0 ± 0.37(a)
20% cereal protein (10)	341 ± 19(a)	34.1	-44	410 ± 21(a)	58.6	0.56 ± 0.03(b)	9.2 ± 0.37(a)
10% cereal protein (10)	339 ± 21(a)	17.0	-47	390 ± 15(a)	51.9	0.57 ± 0.05(b)	10.0 ± 0.35(a)
20% milk protein (10) (pair-fed)	360(a)	57.6	-53	530 ± 20(a)	68.4	1.38 ± 0.04(a)	8.0 ± 0.2(a)
20% milk protein (10) (non-lactating)	270 ± 10(a)	43.2	19	550 ± 23	82.0	-	10.3 ± 0.44(a)

Significantly different from control group: a, $p < 0.05$; b, $p < 0.001$

Table 12. Essential amino acid content in the cereal diets and amino acid requirements of the rat (expressed as percentage of protein).

	Arg	His	Leu	Ileu	Lys	Met	Phe	Thr	Tryp	Tyr	Val
20% cereal diet	3.6	2.1	12.1	3.1	2.7	1.1	5.1	3.1	0.5	3.2	4.1
10% cereal diet	4.2	2.1	10.9	3.1	3.2	1.3	4.8	3.1	0.6	3.1	4.3
Amino acid requirement	5.0	2.5	8.0	5.0	6.0	4.5	5.0	4.0	1.5	4.0	5.5

intake ranged from 10% in the 6% milk protein group to 108% in the 10% milk protein group.

The pair-fed rats finished their rations about 3 h after the food was given in the morning. They developed a meal-feeding rather than the usual nibbling behaviour.

2.3.2 Pup growth

Milk production is seldom measured directly in small mammals. Therefore, indirect methods of evaluating lactational performance have commonly been used, for example, by weaning weights of the young, usually expressed as average weight per young or occasionally as total litter-weight. Another well used method is to measure average pup weight gained during the experimental period, which was done in the present study from day 2 of lactation to day 14. Any dam in which litter size was less than 7 pups was not used in the study.

Litters of dams on the good quality protein diet (with the exception of the 6% milk protein group) had satisfactory growth rates (Table 11). The 6% milk protein group did as poorly as the cereal groups because, as will be explained in Chapter 5, such a drastic decrease in the dietary protein content results in a severe reduction in milk volume. From the values of the pair-fed and 20% cereal protein groups, which took the same amounts of protein and energy in their diets, one could conclude that the quality of the dietary protein plays a crucial role in the lactational performance of the rat.

The decreased lactational performance of animals on the cereal diets was not merely due to the amount of utilisable protein in the diets. On the assumption that milk protein has an NPU of 80 and the cereal protein 50 in rats, it can be seen from Table 11 that, although pup growth was similar in rats of the 6% milk protein group and 20% cereal protein group, the rats on the former group consumed 14.2 g utilisable protein while those on the latter group consumed 34 g. Clearly, other factors, apart from utilisable protein consumed, were therefore limiting lactational performance on the cereal diets. One possible factor

is the amino acid imbalance, although the adequacy of energy intake of these three groups may have an effect.

2.3.3 Maternal weight changes

The results (Table 11) show that the non-lactating rats gained some weight, probably because they had not completely reached adult size. Older rats than the ones used in this study could not be used because the Animal House staff advised that they might be too old for breeding purposes. Indeed, it was observed in this study that using older rats resulted in mating difficulties. The control group had a good weight gain. They were therefore eating amounts of food that were adequate to provide for milk production needs, maintenance and even some gain in body weight. Weight increase in lactating rats is primarily brought about by increases in the liver and gut size (Wang, 1925; Resemer *et al.*, 1981) and, to a smaller extent, by nitrogen deposition (Spray, 1950), and also by tissue hydration (Foot *et al.*, 1979; Naismith *et al.*, 1982; Moore & Brasel, 1984). Moore and Brasel (1984) reported that the increased body weight in lactation was accounted for almost entirely by tissue hydration. It must be pointed out that the weight gain of the dams was highest around day 10 of lactation, but decreased towards the end of the experiment because the demands for milk production were greater at this time, as evidenced by the observation that the pups grew fastest during this period. Babicky *et al.* (1973) have shown that milk production is maximal around day 15 of lactation. At this time, the greatest stress will be put on the dam's homeostasis.

The cereal, 6% milk protein and pair-fed groups lost weight. All these animals had food intakes that were about half the control values. They were, therefore, under-nourished in terms of both energy and protein. Thus, tissues were catabolised to provide some of the substrate for milk production. Amongst these 4 groups, however, the pair-fed group had the best pup growth. An explanation for this

observation is that the main milk protein, casein, has a higher net protein utilisation than maize protein. The pair-fed group was thus able to retain more amino acids, per given amount of protein eaten, to meet their lactational needs in addition, of course, to the amino acids obtained from tissue protein catabolism. This is not an entirely satisfactory explanation of the differences in lactational performance, however, because when the 10% and 20% cereal groups were examined, it was observed that they did equally badly even though the second group were getting twice the utilisable protein of the first (Table 11). Moreover, the 6% milk protein group received a smaller amount of utilisable protein than the 20% cereal protein group. The poor lactational performance of the cereal groups therefore might be due to the amino acid profile of the dietary proteins, in particular to their amino acid imbalance.

Meal-feeding may have produced a more efficient utilisation of nutrients in the pair-fed group. This effect has been shown previously by Leveille and O'Hea (1967), who reported that meal-fed non-lactating rats, having access to food for a single daily two-hour period, utilised their food for weight gain more efficiently than *ad libitum*-fed (nibbling) rats. They suggested that the energy expenditure of the meal-fed animals was reduced as a result of a significant decrease in their activity (57% reduction in activity, especially during the evening hours). Cohn *et al.* (1958) observed a diminished thyroid activity, and thus reduced metabolic activity, in force-fed rats compared to rats feeding normally. These observations, of course, would imply that more nutrients would be available to the meal-fed rats for milk synthesis, and thus improve lactational performance. However, still it would seem as if the greater growth rate of the pups of the good-quality protein meal-fed dams, compared to dams on the cereal protein diet, would be due more to the superior quality of their dietary protein than to any decrease in activity. However, this parameter was not measured in the present study.

The cereal and 6% milk protein groups were unable to provide an adequate quantity of milk for their pups, despite losing similar amounts of weight as the pair-fed group.

The mammary tissues of the rats in the cereal and 6% milk protein groups, however, would have been exposed to a completely different pattern of nutrients and hormones than those of the pair-fed group. This may also have contributed to the differences in lactational performance. The results, therefore, suggest that a poor quality protein diet has a worse effect on lactational performance than reduced dietary protein content, and that the maternal tissues have only a limited ability to supplement nutrient supply to mammary tissue. This observation is supported by the results of measurements made on skeletal muscle of the dams.

2.3.4 Tibialis weight changes

The weights of the tibialis muscle of the dams were measured to examine the effects of the dietary variations on the dams' muscle mass. It was assumed, of course, that the effects of the diets on the tibialis weights were similar to dietary effects on the total muscle mass.

The results (Table 11) show that in the 20% and 10% milk protein groups the tibialis weights were almost identical but were somewhat reduced in the 6% milk protein group and very significantly reduced in the cereal groups. In the pair-fed group, the weight loss was considerably less. The results also show that lactation did not increase muscle mass despite the enormous differences in the body weights of the control and non-lactating groups. The weights of these animals were 327 g and 249 g, respectively. Resemer *et al.* (1981) and Poo *et al.* (1939) had also shown that, despite the increased body weight 10 days after parturition, striated muscle mass was not increased during the breeding cycle and so concluded that the increased body weight was due to increased carcass fat. However, Rosmos

et al. (1978) and Moore and Brasel (1984) have shown a decrease in carcass fat during lactation and the latter research workers have attributed the increases in body weight to increases in body water.

When the results from the pair-fed group are compared with those of the cereal groups, it can be concluded that the large loss in maternal muscle mass is not entirely due to lower energy intakes but also to poor protein quality. It could also be concluded that the large loss in maternal body weight in the pair-fed animals might have been due more to the loss of non-protein body reserves like lipids, glycogen and body water than to loss of protein.

2.3.5 Changes in liver composition

2.3.5.1 Liver weights

The liver weights for the control group were significantly heavier than those of the non-lactating group (Table 11). This is a normal adaptation during lactation (Beaton *et al.*, 1954; Schwenk *et al.*, 1961; Resemer *et al.*, 1981). The increase is not solely due to increased water content. Liver protein content increased in the present study.

The liver weights of the cereal protein groups were significantly lower than the controls. This finding is to be expected since undernutrition results in depletion of cell glycogen, protein and lipid. The liver weights of the pair-fed group were even lower than those of the cereal groups. This observation again suggests that the pair-fed group adjusted liver metabolism to a greater extent in order to improve their lactational performance by provision of substrate from liver tissue to synthesise milk components.

2.3.5.2 Tissue protein, RNA and DNA contents

Tissue analyses of the liver, muscle and mammary gland were carried out. The quantities of protein and

RNA are expressed as both their concentration, and in proportion to the DNA content of the tissue (Table 13). The ratios of protein and RNA to DNA give a more accurate impression of the cellular content of these components than when they are expressed on a wet weight basis. In addition, the RNA to DNA ratio indicates the amount of protein synthetic machinery available to the cell. The protein to RNA ratio indicates the efficiency with which it operates.

The total DNA content was measured to see whether cell number per given weight of tissue was affected by the various diets. The RNA content was measured to get an idea of the amount of protein synthetic machinery in cells. The RNA content would thus show how the protein synthesising machinery was affected by the various diets.

2.3.5.3 Relationships between RNA and protein synthesis in liver

The synthesis of peptide chains on ribosomes can be divided into three phases, namely :

(i) Initiation, in which ribosome subunits, mRNA, initiation factors and the initiator methionyl tRNA are required.

(ii) Peptide chain elongation, that is, the addition of successive amino acids to form the peptide chain. Some 60 aminoacyl-tRNA species, charged with amino acids, proceed to form complexes with elongation factors and GTP, and then attach to the ribosome for insertion of the correct amino acid indicated by the codons of the mRNA.

(iii) Peptide chain termination, in which the ribosome dissociates from the mRNA and dissociates into its subunits. The main changes observed in the rate of translation have been traced to effects on the rate of chain initiation and elongation.

The overall rate of protein synthesis in many types of cells is sensitive to amino acid supply (Munro, 1970). However, the regulatory effects of amino acid supply are not compatible solely with a mechanism involving simple substrate limitation. Protein synthesis occurs in polyribosomes (polysomes) which may have a number of ribosomes per mRNA molecule. It is well documented that the increase in supply of amino acids to many tissues and cell types results in an increase in the average polysome size (Sidransky. 1968) and must reflect an enhanced rate of initiation, relative to other steps in protein synthesis, since each ribosome on the mRNA strand will synthesise one protein molecule.

2.3.5.4 Liver protein

In vivo measurements of structural protein synthesis in livers of animals subjected to long-term variation in amino acids, as in the present study, suggest that the average rate of synthesis is not reduced and may even be increased (Waterlow & Stephen, 1968; Garlick, 1972). However, synthesis of albumin by the liver in protein-depleted animals is substantially depressed (Morgan & Peters, 1971). This apparently selective effect of protein malnutrition on the synthesis of exported proteins may be related to demonstrations of preferential disaggregation of membrane-bound, rather than free, polysomes.

The results of this work (Table 13) show that liver protein concentration is not significantly altered by lactation but the amount of protein per whole organ is significantly increased, mainly because of an increase in liver size. The total protein content was significantly decreased in all other dietary groups, suggesting that liver could be one of the organs which can be used by lactating animals as a source of reserve protein, when dietary protein quantity or quality or energy is inadequate. Liver protein metabolism thus behaves like that of muscle in this respect. These results

Table 13. Effects of dietary protein variation on liver protein, RNA and DNA concentrations and on the RNA/DNA, Protein/RNA and Protein/DNA ratios (Mean ± SEM).

Number of animals shown in parentheses

Diet	Protein (g/100 g tissue)	Total protein (g/liver)	RNA (mg/100 g tissue)	Total RNA (mg/liver)	DNA (mg/100 g tissue)	Total DNA (mg/liver)	RNA / DNA	Protein / RNA	Protein / DNA
20% milk protein (22)	19.13 ± 0.29	2.74 ± 0.07	349 ± 35	49.9	64 ± 3	9.15	5.51 ± 0.49	67.6 ± 4.7	314 ± 15
10% milk protein (15)	15.52 ± 0.37^{d}	2.31 ± 0.07^{d}	216 ± 37^{c}	32.2	76 ± 4c	11.3	2.80 ± 0.42^{c}	91.0 ± 5.4^{c}	208 ± 10^{d}
6% milk protein (9)	12.28 ± 0.57^{d}	1.54 ± 0.1^{d}	192 ± 29^{d}	23.0	100 ± 8^{d}	12.0	1.89 ± 0.17^{d}	74.0 ± 7.5^{a}	131 ± 4^{d}
20% cereal protein (10)	16.63 ± 0.6^{d,f}	1.53 ± 0.04^{d}	289 ± 26^{b}	26.6	125 ± 8^{d}	11.5	2.26 ± 0.16^{d,e}	61.1 ± 4.6	138 ± 7^{d}
10% cereal protein (10)	14.59 ± 0.56^{d}	1.51 ± 0.05^{d}	257 ± 24^{a}	25.7	111 ± 7^{d}	10.2	2.31 ± 0.19^{d}	61.3 ± 6.1	134 ± 4^{d}
20% milk protein (10) (pair-fed)	19.15 ± 0.31	1.53 ± 0.03^{d}	410 ± 38	32.8	128 ± 7^{d}	12.8	3.19 ± 0.37^{c}	51.6 ± 4.5^{b}	152 ± 7^{d}
20% milk protein (non-lactating) (10)	20.18 ± 0.46	2.09 ± 0.09^{d}	519 ± 57^{c}	53.5	133 ± 7^{d}	10.6	3.89 ± 0.37^{b}	44.0 ± 5.4^{d}	155 ± 7^{d}

Significantly different from 20% milk protein group:
a, $p < 0.05$; b, $p < 0.02$; c, $p < 0.01$; d, $p < 0.001$.

Significantly different from pair-fed group:
e, $p < 0.05$; f, $p < 0.01$.

could be interpreted as showing that during protein malnutrition in lactating animals protein synthesis in non-mammary tissues is decreased in order to provide amino acids for milk protein synthesis and so ensure the survival of the species. These observations were made in the present study (Table 17).

2.3.5.5 Liver RNA and DNA

There was a significant fall in RNA concentration when either dietary protein quality or quantity was reduced in lactating rats, with the exception of the pair-fed group (Table 13). This implies that the protein synthesising machinery (RNA concentration) is decreased when dietary protein quality or quantity was reduced. The pair-fed group had a higher value, probably because the liver was rapidly synthesising protein in response to meal feeding behaviour. It was also possible that the liver was devoid of most of its lipid and glycogen and so appeared to have a higher RNA concentration per given weight of tissue. Non-lactating rats had a higher RNA concentration than the lactating controls. This could be a dilution effect in the case of the controls, since the liver increases its content of fat, glycogen and water as a result of lactation.

Total DNA measurements showed that no hyperplasia occurred in lactation. This was in contrast to the 70% increase found in other studies (Kennedy *et al.*, 1958; Remeser *et al.*, 1981).

2.3.5.6 Liver protein/DNA ratio

The protein/DNA ratio was highest in the control group, indicating that lactation had caused a large degree of hypertrophy. The hypertrophy had been totally abolished in all experimental groups.

2.3.5.7 Liver RNA/DNA ratio

This ratio represents the cellular protein synthesising capacity. The value of this ratio is usually low in

situations when inadequate or poor quality protein diets are eaten. The results (Table 13) showed that compared with the control group the values for all other groups are significantly reduced. A comparison of the values of the lactating controls with the non-lactating animals suggests that lactation enhances the protein synthesising capacity of liver cells. The reduced values in the groups receiving a reduced quality and quantity of dietary protein indicate that protein and energy insufficiency not only suppresses the effect but reduces the protein synthetic capacity below normal. A comparison of values of the pair-fed and 20% cereal protein group, where dietary energy and total protein consumption were the same, show that the higher the protein quality, the larger the protein synthesising capacity.

2.3.5.8 Liver protein/RNA ratio

This ratio gives an indication of the efficiency of the protein synthesising machinery in the cell, i.e. the efficiency of the translational capacity of the ribosomes. The results (Table 13) show that lactation enhances efficiency in all dietary groups. However, it is only in the 20% milk protein group that this results in enhanced capacity, since reduced cellular concentrations of RNA occurred in all groups on inadequate dietary intakes. Thus, although the normal effect of lactation in cellular protein content was abolished by an inadequate diet, the effect of RNA efficiency was not. Other examples of the resistance of RNA efficiency to change by dietary inadequacy can be found in the literature (Garlick *et al.*, 1975). It can therefore be concluded that lactation enhances the protein synthesising activities in the liver by increasing both the protein synthesising capacity and the efficiency of protein synthesis. However, amongst lactating animals, diet seems to affect the protein synthesising activities by increasing capacity more than efficiency.

2.3.6 Serum albumin

In man, about 300 g protein are synthesised each day. The plasma proteins account for only 30 g, of which albumin represents about 12 g. Yet, a great part of our knowledge of adaptation to protein or amino acid deprivation is based on studies of albumin metabolism. This is not surprising when one contrasts the comparative ease of studies on albumin metabolism with the technical problems of isolation and measurements of metabolic changes in other proteins.

Even though the adaptive changes in albumin metabolism cannot be regarded as representative of all tissue proteins, nor even all plasma proteins, the effect of alterations in amino acid supply on albumin synthesis is of interest for two reasons. Firstly, because of the importance of this protein in maintaining plasma oncotic pressure and providing a transport mechanism for many substances; and, secondly, because the rate of its synthesis reflects one of the fundamental functions of the liver. Albumin represents at least a quarter of all protein synthesised by this organ. The liver plays a vital role in regulating total body amino acid metabolism through its modulation of the post-digestive surge of amino acids into the portal blood stream. Adaptive changes in the buffering action of the liver,perhaps reflected in the rate of plasma protein synthesis, could materially alter the amount of amino acids available for metabolic purposes elsewhere, such as the mammary gland.

In kwashiorkor or experimental dietary protein deprivation in man and animals, the plasma albumin concentration falls. This fall provides one of the most sensitive criteria for the diagnosis of protein malnutrition. Neither increased catabolism nor loss of albumin to extravascular sites can account for it. The rate of catabolism of albumin is in fact decreased and transfer of albumin has been demonstrated from extra- to intra-vascular compartments. Direct studies in the rat have shown quite clearly that the fall in plasma albumin concentration and mass after dietary protein deprivation results from reduced albumin synthesis.

The results of the present work for the effects of dietary protein variation on serum albumin concentration should be seen in this light and are presented in Table 14. They show that values are lower in lactating than non-lactating animals. All dietary treatments resulted in lower values. These results show that reduced protein or energy intakes could depress serum albumin concentrations in lactating animals as in non-lactating animals.

The results, taken as a whole, suggest that an adequate energy intake may be more important in maintaining serum albumin concentration than the amount or quality of dietary protein. The energy intakes of the 20% and 10% milk protein groups were almost the same but the latter group consumed about half the amount of protein. Serum albumin concentration was not affected. The energy intakes of all other lactating groups were again similar but lower than the former two groups. In all these groups, serum albumin concentrations were lower. However, the quantity or quality of protein in the diets had no influence on the degree to which serum albumin fell. This can be seen in a comparison of the values for the 6% milk protein and 20% cereal protein groups.

Indeed, most of the work on the effects of dietary protein on albumin concentration has been carried out on rats fed protein-free diets. It cannot therefore be concluded with certainty whether the reductions in the quality and quantity in the dietary proteins in the present study were severe enough to cause reductions in the serum albumin concentrations. Moreover, it has been shown in many studies that starvation produces an even more pronounced effect on serum albumin concentration than variation of the dietary protein. In the present study, although the animals in which reductions in serum albumin concentration was observed were not starving, their energy intakes were only about half that of the controls. It is therefore concluded that the reductions in serum concentrations in this study might have been due more to the effect of reduced energy intake and to a lesser extent to the effects of the reduced quality and quantity of the dietary proteins.

Table 14. Effect of dietary protein variation on the serum albumin concentration (Mean ± SEM).

Number of animals shown in parentheses

Diet	Serum albumin (g/100 ml)
20% milk protein (32)	4.20 ± 0.079
10% milk protein (20)	4.04 ± 0.119
6% milk protein (12)	3.06 ± 0.101[a]
20% cereal protein (12)	3.31 ± 0.09[a]
10% cereal protein (12)	3.31 ± 0.09[a]
20% milk protein (pair-fed) (11)	3.70 ± 0.06[a]
20% milk protein (non-lactating) (11)	5.29 ± 0.144[a]

Significantly different from 20% milk protein:
a, $p < 0.001$.

The results also show that the serum albumin concentration is reduced as a result of lactation. As has already been pointed out, hypo-albuminaemia is usually a result of inadequate protein or energy intake (Payne *et al.*, 1971). It is, however, recognised that other factors may sometimes be involved. In humans, Rothschild *et al.* (1972) suggested that these other factors could include elevated globulin levels, as seen in disease, severe burns or hormonal influences. Poo *et al.* (1939) have also reported a 10% decrease in plasma proteins during lactation in rats. Little (1974) observed that the serum albumin concentration of dairy cows was lower in cows 0-30 days after calving than non-lactating cows, although the concentration increased to higher levels in the latter stages of lactation. To explain the lower concentration in the lactating cows, he suggested the following :

(i) Reduced albumin synthesis resulting from a reduction in the availability of amino acids to the liver at a time when the secretion of milk is increasing.

(ii) Reduced albumin synthesis resulting from an increased globulin synthesis, as evidenced by the elevated globulin concentrations in blood.

(iii) An increase in plasma volume. Landesman and Miller (1963) showed that there was a post-partum plasma volume increase of approximately 15% in 50% of the women whom they examined. The percentage increase in plasma volume during lactation in rats would be even higher than that reported for humans, because the percentage increase in body weight of the rats in the present study is significantly higher than for humans.

(iv) Another cause of low serum albumin concentration may be due to leakage of albumin into other body fluids like milk and urine.

It could therefore be concluded that all these factors contribute, to different extents, to the reduced albumin concentration seen in the control group.

2.3.7 Compositional changes in skeletal muscle

In muscle, a protein-deficient diet results in a substantial reduction in the protein synthesis rate (Millward, 1970; Millward & Garlick, 1972). Analysis of *in vivo* data indicates that this is due to a rapid decrease in ribosomal activity, together with a progressive fall in the ribosomal content of the tissue (Millward *et al.*, 1973). The decrease in ribosomal activity is to some extent reflected in some disaggregation, i.e. loss of ribosomal efficiency. The muscle tissue was therefore analysed for protein, RNA and DNA to examine how these parameters were affected during lactation by variations of the dietary protein quality and quantity.

2.3.7.1 Muscle protein

The results (Table 15) show that the muscle protein concentration was not affected by lactation. This observation has been reported also by Remesar *et al.* (1981). It would therefore appear as if the increased body weight, resulting from lactation, would be due to increases in non-protein muscle components like lipids and glycogen. Water content would also increase as a result of the hydration of the tissues (Moore & Brasel, 1984; Remesar *et al.*, 1981).

The results of the present study also show that muscle protein concentration was significantly decreased in the rats fed the very low concentration of the good quality protein, i.e. the 6% milk protein diet, and in the pair-fed group. The reductions in protein concentrations in these two groups, plus the significant reduction of their muscle mass (as indicated by their tibialis weights) indicate that they responded appropriately to the conditions of dietary protein in sufficiency, by mobilising protein from the muscle. Muscle can be considered to be a less important tissue than, say, the liver for the survival of the dam, or than the mammary tissue for the survival of the pups. The mobilised amino acids would therefore be used by the mammary gland for milk protein synthesis. The muscle protein concentrations of the two groups on cereal protein diets

Table 15. Effects of dietary protein variation on muscle protein, RNA and DNA concentrations and on the RNA/DNA, Protein/RNA and Protein/DNA ratios (Mean ± SEM).

Number of animals shown in parentheses

Diet	Protein (g/100 g tissue)	RNA (mg/100 g tissue)	DNA (mg/100 g tissue)	RNA/DNA	Protein/RNA	Protein/DNA	Tibialis weight (mg)
20% milk protein(27)	14.1 ± 0.33	138 ± 14	26 ± 6	5.97 ± 1.05	100 ± 13	644 ± 99	615 ± 15
10% milk protein(15)	13.6 ± 0.71	117 ± 18	25 ± 3	4.65 ± 0.24	127 ± 16	589 ± 82	600 ± 32
6% milk protein(8)	12.8 ± 0.52[a]	121 ± 15	20 ± 2	5.4 ± 0.54	118 ± 21	661 ± 89	500 ± 31[a]
20% cereal protein(10)	14.3 ± 0.36[e]	107 ± 10[e]	26 ± 2	4.17 ± 0.33[f]	136 ± 16[g]	564 ± 47	410 ± 21[a]
10% cereal protein(10)	13.3 ± 0.41	97 ± 5[a]	22 ± 2	4.52 ± 0.19	139 ± 8[b]	621 ± 44	390 ± 15[a]
20% milk protein(10) (pair-fed)	12.9 ± 0.53[a]	188 ± 23	32 ± 5	5.6 ± 0.22	76 ± 13	446 ± 87	530 ± 20[a]
20% milk protein (10) (non-lactating)	14.9 ± 0.35	110 ± 6[a]	19 ± 1	5.82 ± 0.26	137 ± 9[b]	788 ± 51	550 ± 23

Significantly different from the 20% milk protein group:
a, $p < 0.05$; b, $p < 0.025$; c, $p < 0.01$; d, $p < 0.001$.

Significantly different from the pair-fed group:
e, $p < 0.05$; f, $p < 0.01$; g, $p < 0.005$.

were not significantly decreased. However, these two groups adapted mainly by reducing their muscle mass. There was a significant difference between the pair-fed group and the group fed the 20% cereal protein diet, showing that the differences in response were due to protein quality rather than the reduced energy intakes. Moreover, a calculation of the amount of protein in the tibialis (Table 11) indicated that muscle protein may decrease with protein quantity. This effect is seen in both cereal and milk groups. A comparison of the protein contents of tibialis muscles from the 20% cereal protein and pair-fed 20% milk protein groups shows that reduced protein quality also caused depletion of muscle protein (Table 11).

2.3.7.2 Muscle RNA and DNA concentrations

The results for the RNA concentrations show that lactation resulted in an increase in the RNA concentrations and that low concentrations of the good quality protein diets (10% and 6% milk protein) and both poor quality protein diets resulted in a decrease. The values were, however, only significantly lower in the 10% cereal protein group and nearly so in the 20% cereal protein group. This finding agrees with observations made by other workers with non-lactating rats under similar dietary conditions (Young & Alexis, 1968).

The muscle DNA concentration was not affected by lactation or quality and quantity of dietary protein. These findings were in contrast with those of Remesar *et al.* (1981), who reported that lactation resulted in a decrease in the DNA concentration of striated muscle. The decreased concentration could have resulted from an increase in water content following utilisation of glycogen and fat. It is therefore possible that the dilution effect resulting from the increase in muscle water content, reported by Remesar, did not take place in the present study.

2.3.7.3 Muscle RNA/DNA ratios

Lactation did not affect this ratio (Table 15). However, the values of all experimental groups were lower than that of the control, although no significant decrease was recorded, probably because the value for the control group had a large standard deviation.

2.3.7.4 Muscle protein/RNA ratios

The results (Table 15) show a significant decrease as a result of lactation. A significant increase occurred in the cereal groups (compared with the control). Both observations are difficult to explain and are, in fact, in contradiction to what is generally known, at least for non-lactating rats, i.e. that the efficiency of protein synthesis is reduced under conditions of poor-quality dietary protein. The observation of a decrease in the efficiency of muscle protein synthesis during lactation may be of benefit to this state. The decrease in muscle protein synthetic activity would result in reduced utilisation of amino acids by this tissue and greater availability of amino acids for protein synthesis in mammary gland.

2.3.7.5 Muscle protein/DNA ratios

Lactation results in decreased cellular protein concentration, though not significantly so (Table 15). Pair-feeding dramatically reduced cellular protein concentration. This is a reasonable observation, since muscle protein could be mobilised during lactation to complement dietary protein intake. Thus, substrate would be provided for mammary protein synthesis. The mobilisation is even further enhanced in the pair-fed group.

2.3.8 Compositional changes in mammary gland

The key to an understanding of the alterations in whole body metabolism during lactation is knowledge of the substrate requirements of the mammary gland for the production of the main components of milk: lactose, lipid, caseins and α-lactalbumin. On a normal laboratory diet (high in carbohydrate but low in fat), all the lactose and casein and about 50% of

lipid are synthesised within the gland. It can be estimated (Williamson & Robinson, 1977) that at peak lactation the glucose uptake by the rat mammary gland is about 30 mmol or 5.4 g per 24 h, of which about 23% is used for lactose synthesis (Carrick & Kuhn, 1978), and the rest for lipogenesis.

The lactating mammary gland is a very active tissue. It is, for example, the most active site of lipogenesis *in vivo* during lactation. The rate of lipogenesis on a gram tissue basis is five-times higher than that of the liver (Robinson *et al.*, 1978). The remainder of milk lipid is derived from triacylglycerols and non-esterified fatty acids taken up from the blood (Hawkins & Williamson, 1972).

Mammary amino acid utilisation and protein synthesis are also significantly increased in the lactating mammary gland (Table 6). It is also known that the absorptive capacity of the secretory cells is most marked for certain amino acids; for example, of the methionine reaching the glands in the arterial blood, more than 70% may be absorbed (Mepham, 1976). This large requirement of the gland for glucose, triacylglycerols and amino acids implies that the rate of production or entry of those substrates is increased and/or their rate of utilisation by other tissues is decreased. The results shown earlier indicate that the requirement of muscle for amino acids may be reduced.

Part of the increased substrate requirement can be met by the higher dietary intake and by increased blood flow to the mammary gland. However, part must be met by changes in the metabolism of other tissues.

2.3.8.1 Mammary protein concentration

Mammary protein concentration was not significantly affected by dietary protein, with the exception of the group fed the 6% milk protein diet. This group showed a significant decrease (Table 16). The pair-fed group showed a significant increase, possibly because these meal-fed animals were given food on the morning that they were killed. Mammary tissue would probably be rapidly sythesising protein at this time. Millward and Garlick (1972) showed that, by meal-

Table 16. Effects of dietary protein variation on rat mammary protein, RNA and DNA concentrations and on the RNA/DNA, Protein/RNA and Protein/DNA ratios (Mean ± SEM).

Number of animals shown in parentheses

Diet	Protein (g/100 g tissue)	RNA (mg/100 g tissue)	DNA (mg/100 g tissue)	$\frac{\text{RNA}}{\text{DNA}}$	$\frac{\text{Protein}}{\text{RNA}}$	$\frac{\text{Protein}}{\text{DNA}}$
20% milk protein(28)	11.03 ± 0.23	177 ± 22	68 ± 4	2.57 ± 0.25	87 ± 7	185 ± 11
10% milk protein(15)	10.55 ± 0.63	143 ± 23	81 ± 9	2.13 ± 0.14	88 ± 8	156 ± 15
6% milk protein(8)	8.97 ± 0.22[d]	148 ± 6	70 ± 4	2.21 ± 0.11	61 ± 2[d]	132 ± 10[c]
20% cereal protein(10)	10.32 ± 0.75[f]	181 ± 31[e]	105 ± 14[c]	1.74 ± 0.11[b,f]	68 ± 8[a]	110 ± 13[d,e]
10% cereal protein(10)	10.51 ± 0.54	166 ± 23	104 ± 8[d]	1.69 ± 0.19[b]	73 ± 9	105 ± 6[d]
20% milk protein(10) (pair-fed)	13.87 ± 0.27[d]	267 ± 28[c]	78 ± 7	3.39 ± 0.17[c]	58 ± 7[d]	192 ± 18

Significantly different from the control group:
a, $p < 0.05$; b, $p < 0.02$; c, $p < 0.01$; d, $p < 0.001$.

Significantly different from the pair-fed group:
e, $p < 0.01$; f, $p < 0.001$.

feeding rats for four hours at the beginning of each 24 h period, muscle protein synthesis varied throughout each 24 h cycle. The rate reached a peak between 12 and 18 hours after the feed. Since the absorptive capacity of the mammary tissue is far greater than that of the muscle, and mammary tissue has higher turnover rates, the peak period could be reached at an earlier time. This would account for the higher RNA and protein concentrations observed.

A problem that must be taken into account in mammary cellular protein measurement is that both structural protein (which stays) and milk proteins (which leave the tissue) are involved. Therefore, greater suckling could change this value.

2.3.8.2 Mammary nucleic acid content

Mammary nucleic acid content, as well as being affected by the factors outlined above for liver and muscle, is also determined by the suckling intensities of the pups. Single litters of 8 pups per 12 mammary glands have been shown to provide sufficient suckling stimulation to maintain DNA content (a measure of cell number) to 24 but not 28 days of lactation (Tucker & Reece, 1963). It has also been shown that suckling by foster litters, at 12-16 days of age, when nursing intensity was greatest, failed to prevent declines in DNA, RNA and RNA/DNA, and cumulative litter-weight gain between days 20 and 36 of lactation (Thatcher & Tucker, 1968).These workers showed a high correlation between litter-weight gain and mammary RNA content. They also demonstrated that losses in RNA during extended lactation were much greater than losses in DNA, and suggested that factors controlling protein synthesis limited milk synthesis more than factors influencing cell numbers. Tucker (1964) had also shown that mammary DNA and RNA content and the RNA/DNA ratio were increased as the number of suckling pups per teat increased.

Carrick & Kuhn (1978) had also demonstrated that lactose synthesis in the rat showed a diurnal variation. The

minimum occurred at 1800 h. Food withdrawal for 6 hours did not affect lactose synthesis in the early morning but greatly decreased it in the afternoon or evening. However, Sampson and Jansen (1983) observed no cicardian variation in the fractional synthetic rate (FSR) in the mammary, liver or muscle tissues of lactating rats, but observed that a poor quality protein diet (wheat gluten) did significantly decrease the FSR in all three tissues. These observations would imply that protein synthetic efficiency and capacity (RNA content and RNA/DNA ratio), or the cell numbers (DNA content) would be affected by the dietary protein (Sampson & Jansen, 1983), or by the time of day (Carrick & Kuhn, 1978).

The results of the present work (Table 16) show that there are no statistically significant differences in the mammary RNA concentrations resulting from the dietary protein variations, with the exception of the pair-fed group which had significantly higher values than in the controls. The results of the present study, therefore, are at variance with those of Sampson and Jansen (1983). However, results from the pair-fed group partly support those of Carrick and Kuhn (1978), since part of the diurnal variation can be explained by the intermittent intakes of food. The sudden appearance of an abundant supply of amino acids following meal-feeding may have stimulated RNA synthesis to high concentrations.

Although the RNA concentration tended to decrease with reductions in dietary protein quality and quantity, these differences were not significant. A possible explanation for this could be that the composition of milk within the gland had a diluting effect on the RNA content observed. As will be shown in Chapter 4, higher milk volume and lactose concentrations were found in milk from the control group. Milk fat was not measured in this study but milk protein and lactose alone would have made the milk solids about 45% higher in the control group than in the 20% cereal protein group.

The results for the DNA concentration show, as was the case for the liver, that as dietary protein quality or quantity are decreased, the DNA concentration is increased (Table 16). The possible explanation for this observation is the same as the one given for mammary RNA above, i.e. a dilution effect. It is very probable that, if these analyses were carried out on the fat-free tissues, at least the RNA concentration would have been significantly higher for the control group. However, since the rats on the 20% and 10% milk protein diets had higher mammary tissue weights, they would therefore have larger RNA and DNA contents per organ.

2.3.8.3 Mammary protein synthetic capacity (RNA/DNA)

The protein synthetic capacity was significantly reduced in the cereal groups (Table 16) and significantly increased in the pair-fed groups, for the same reasons as already given for the liver.

2.3.8.4 Mammary protein synthetic efficiency (protein/RNA) and cellular protein concentration (protein/DNA)

As was the case for liver, reduced dietary protein quantity (6% milk protein) decreased the protein synthetic efficiency (Table 16). A similar finding occurred in the cereal groups.

The results suggest that the mammary protein synthetic activity is affected during lactation, partly by quantity, but especially by the quality of dietary protein. They also suggest that the cellular protein concentration (protein/DNA) is depressed if either dietary protein quality or quantity is reduced. Moreover, a comparison of the values of the pair-fed and 20% cereal protein groups indicates that protein quality affects cellular protein concentration more than protein quantity. This effect may be because, in poor lactation, the mammary glands may be emptied to a greater extent by the pups.

CHAPTER 3

EFFECTS OF PROTEIN NUTRITION ON PROTEIN TURNOVER

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3.1 Introduction

It is well established that the protein mass and concentration of animal tissues are maintained by a continuous balancing of the processes of protein synthesis and breakdown. The collective name for these two processes is protein turnover (Millward & Garlick, 1972; Waterlow & Stephen, 1968). Turnover, then, is a general term used to describe the process of renewal or replacement of a given substance, either by synthesis and breakdown or by exchange with the same material in another tissue compartment. The expression 'turnover rate' is therefore appropriate when the arguments apply equally well to both synthesis and breakdown, even when these processes are not equal in magnitude. These two processes are equal only in a 'steady state' situation. In an anabolic state, such as growth or lactation, the rate of synthesis is greater than that of breakdown (Conde & Scornik, 1977), whereas in a catabolic state, such as starvation or infection, the opposite holds true (Beisel, 1966). Measurements of rates of synthesis and breakdown thus give a better idea of how tissue protein mass is changing than do nitrogen balance techniques.

3.1.1 Why do proteins turnover?

It would be natural to speculate why proteins turn over at all. Initially, it was believed that there was no evidence that they were degraded because of 'age'. Indeed, protein degradation was considered to be first order process, i.e. breakdown was random and 'newer' proteins were just as subject to degradation as 'older' ones. However, more recent evidence (Millward *et al.*, 1975) has suggested that, in myofibrillar proteins at least (these proteins comprise two-thirds of muscle proteins), the assumption that muscle protein breakdown is random may not be correct, as will be explained later.

A number of suggestions have been proposed as to why proteins turnover, suggestions which have not been validated. These suggestions are :

(i) Dinman (1972) suggested that protein molecules, particularly those located in the plasma membrane, may be susceptible to a process of irreversible degradation caused by the binding of metal ions coming from the environment of the cell or of the organism. A continuous replacement of those molecules, through a process of turnover, would increase the stability of the proteins, taken as a whole, and of the membrane. A further conjecture is that for cells or organisms, which have to respond to environmental changes through changes in enzymatic patterns, a system of constant protein turnover is the optimal one.

(ii) The necessity for protein turnover is related to the possibility of translational mistakes occurring, despite all the correction factors built into the protein synthesising system. Specific proteins therefore appear in the cell with errors, which make them unsuitable for optimal biological activity. The protein turnover mechanism will thus get rid of useless proteins.

(iii) Another possible role that has been suggested for protein turnover is to increase the efficiency of energy utilisation by the cell. This is achieved by increasing the efficiency of the usage of nucleic acids. The same molecule will be used many times over.

(iv) The presence of enzymes, such as deoxyribonucleases and ribonucleases, in the cell is to protect the genetic machinery of the cell from being subverted by foreign invasive nucleic acid molecules, such as from viruses. At the same time, these enzymes could also degrade the cell's own DNA and RNA molecules.

Thus, nucleic acid synthesis, followed by breakdown, would be a logical concomitant with protein turnover. In other words, in order to maintain the efficiency with which energy is used in nucleic acid synthesis, protein turnover would have to occur. It would be of no use having the information processing machinery of the cell being turned over without it being given the opportunity to synthesise many protein molecules. Thus, the turnover of the latter should exceed, or at least equal, that of the former.

Protein turnover is known to serve a number of purposes in the maintenance of homeostasis. In fasting or in dietary situations, such as in the present study, degradation of protein, particularly in skeletal muscle and liver, provides amino acids for gluconeogenesis and for synthesis of essential proteins.

When an individual tissue is considered, net release of amino acids can result from an increased rate of protein degradation, and/or a reduced rate of synthesis. However, growth involves inverse changes in rates of synthesis or degradation. It may involve increases in both synthesis and degradation rates but with the synthesis rate being proportionately higher. Factors regulating protein synthesis include the availability of hormones, such as insulin, growth hormone and adrenal steroids; the supply of amino acids and oxidisable substrates, and the metabolic activity of the tissue. In the latter case, an increased rate of protein synthesis is an important component of muscular hypertrophy in response to an increased work load.

Control of protein degradation is poorly understood but insulin,glucagon, glucocorticoids, thyroid hormones, the supply of amino acids and the size and conformation of the protein, affect the rate.

3.1.2 Nutrition and protein turnover

Waterlow and Stephen (1967) measured whole body protein turnover in the rat and showed that, under a wide range of nutritional states and dietary intakes, the synthetic rate did not

change by significant amounts. Millward (1970), however, showed that protein turnover in different tissues was affected by nutritional variations. Waterlow and Jackson (1981) have also suggested that the rates of protein synthesis are sensitive to dietary intakes and that changes in protein synthesis and breakdown represent an important part of the adaptations to varying levels of nutrition.

Garlick *et al.* (1982) have demonstrated that, although the protein mass of animal tissues is maintained by a continuous balancing of the processes of protein synthesis and breakdown, this should not, however, be taken to imply that the rates of synthesis and breakdown are always equal. Even in the normal animal, fluctuations in body and tissue protein content occur in response to the discontinuous intake of food. This is illustrated by the results from a study by these workers. A normal diet (70 g protein and 8.4 mJ energy) was fed hourly for 12 h, followed by a 12 h fast. The rate of whole body protein synthesis was 121 g during the feeding period but fell to 92 g during the fasting period. However, the breakdown rates responded in the opposite direction and rose from 77 g during the fed period to 112 g during fasting. The resultant effect of these changes was that, during feeding there was protein retention in the body tissues. Conversely, during fasting, a similar amount of protein to that retained during the feeding period was oxidised and lost from the body. Storage probably occurred by expansion of the protein content of one or more tissues, since the change in the concentration of free amino acids in the blood was insufficient to account for storage in this form.

Garlick *et al.* (1982) also showed that the response to feeding was different when a protein-free, low-energy diet was given for three weeks. The rates of protein synthesis and oxidation were much lower than the rates on a normal diet. The rate of breakdown, by contrast, was lower than that observed during fasting but similar to that during feeding on a normal diet. The differences between the fed and fasted states,

however, were completely eliminated by the protein-free diet, indicating the importance of dietary protein, in the regulation of protein synthesis and breakdown in the whole body.

Golden *et al.* (1977) studied two sets of children: one set was children who were malnourished, and the other children who had been successfully treated for malnutrition. They found that, in the malnourished state, protein synthesis was 4.0 g/kg/d compared with 6.3 g protein/kg/d after recovery. Since protein turnover is an energy-requiring process, adaptation of protein turnover may be one of the main mechanisms by which the whole animal economises on energy expenditure.

If the energy cost of protein synthesis, based on what is known of the biochemistry of the process, is taken as 3.6 kJ/g protein (Millward *et al.*, 1976), then protein synthesis will account for 10% of basal oxygen consumption. This would, in fact, be an underestimation because there are additional costs in the process of protein synthesis over and beyond the cost of peptide bond formation; for example, the energy cost of mRNA and rRNA synthesis.

The responses of the whole body to food intake are a net result of the different changes in the protein synthesis and breakdown in individual tissues and also of their size. In the well-fed rat, the rate of synthesis is characteristic of each tissue. For example, measurement of the fractional rate of protein synthesis (FSR, the percentage of tissue protein renewed each day) made in young rats by Garlick *et al.* (1982), gave values of 123, 90, 86, 64, 20 and 17 for jejunal mucosa, bone, liver, skin, heart and gastronemius muscle, respectively. The rates of protein breakdown of the jejunal mucosa, liver and gastronemius muscle were 117, 47 and 11, respectively. These rates are slower than the rates of synthesis because the animals were growing and had tissues, like the liver, which were synthesising proteins, which were catabolised elsewhere.

Starvation for two days caused a decrease in the rate of synthesis in all tissues, but to varying extents. Skeletal muscle was the most sensitive with a 66% fall in synthesis, whereas that in skin and liver were less so, falling about 20-25%. The changes observed in animals on a protein-free diet for 9 days were similar to those seen in starvation.

There is also much evidence from experiments on rats to suggest that the mechanisms that regulate protein synthesis and breakdown are very different in liver and muscle. The liver is linked to the gut via the portal vein and so is the first tissue to receive amino acids and other absorbed nutrients. The changes in amino acid patterns and concentrations in the liver are therefore usually considered to reflect the inflow of amino acids from the intestine. Concentrations of amino acids in the systemic blood fluctuate much less after a meal than the concentrations in the portal vein (Elwyn, 1970). Muscle tissues are exposed to more constant concentrations of free amino acid than liver, and so it might be expected that the synthesis of muscle protein may not respond to sudden dietary changes to the same extent as the liver. *In vitro* experiments, however, have shown that muscle protein synthesis is increased by insulin (Manchester, 1970). Changes in metabolism in muscle protein could occur therefore after a meal, as a result of changes in plasma insulin, despite only small alterations in the amino acid concentrations. It would be reasonable, therefore, to expect the liver to be very sensitive to dietary variations. Nevertheless, in experiments on the acute response to feeding, Garlick *et al.* (1973a) were not able to show any significant differences in the fractional synthesis rate of liver proteins. Moreover, Garlick *et al.* (1975) demonstrated in rats on low protein diets that the rate of protein synthesis in the liver was unchanged, while that of muscle fell dramatically. Some studies have even suggested an increase in liver protein fractional synthesis rate (FSR) in rats with reduced protein intake (Waterlow & Stephen, 1968). At the same time, experiments with perfused livers indicated that the rate of protein breakdown in liver, unlike that in muscle, was quite sensitive to

amino acid supply (Woodside & Mortimore, 1972). Whether these *in vitro* observations correspond to the actual changes *in vivo* cannot be claimed with absolute certainty. Scornik *et al.* (1978) found that, in the liver, regenerating after partial hepatectomy, the increase in protein mass resulted partly from a decrease in protein breakdown. What this implies is that, because tissue proteins turnover, growth or protein mass regulation can be achieved by alteration of either the rate of synthesis or breakdown, as long as the turnover rate is rapid compared with growth.

In the liver, the breakdown rate seems to be the key control point, since the rapid alterations in protein content, which occur in response to feeding and fasting (Millward *et al.*, 1974) occur with little or no change in the FSR and therefore must reflect changes in proteolysis (Millward & Garlick, 1972; Garlick *et al.*, 1973). Food absorption appears to cause a depression in breakdown in meal-fed rats (Garlick *et al.*, 1973). The protein component of food appears to be important in this effect, since refeeding of protein to protein-deprived mice caused an immediate and complete cessation of proteolysis. However, liver protein breakdown was increased as soon as a protein-free diet was given (Garlick *et al.*, 1975).

In contrast to the mode of control of liver protein content, that of muscle is affected by such conditions as starvation and dietary protein deprivation, mainly by suppression of protein synthesis. Muscle protein breakdown is believed to be hardly affected. The breakdown rate is elevated only under very severe nutritional conditions, like long-term starvation or consumption of a protein-free diet (Millward *et al.*, 1976; Garlick *et al.*, 1975). Thus, muscle protein mass is regulated primarily through alterations in protein synthesis, and breakdown only becomes important under severe conditions.

Protein breakdown rates have a paradoxical relationship to growth. It has been shown in muscle that growth regulation is complex, since alterations in the breakdown rate,

far from facilitating growth may well limit it. Millward *et al.* (1975) demonstrated that the breakdown rate was directly proportional to the growth rate, so that rapid growth on a good diet was accompanied by high rates of protein breakdown, necessitating even higher rates of synthesis. Millward and Waterlow (1978) also observed that, during rapid-induced growth, rates of muscle protein synthesis were always increased to a greater extent than was necessary to achieve the observed growth, and suggested that this meant that the breakdown rate must be increased in such a situation.

The decreases observed in muscle protein synthesis rates in protein-deprived rats have been ascribed to reductions in the protein synthesising machinery (the RNA content) and its efficiency (protein/unit of RNA).

From the results of many studies, such as the ones outlined above, it is generally believed that the mechanisms which regulate protein synthesis and breakdown are very different in liver and muscle. Moreover, from the discussion above, it will be correct also to assume that there is now general agreement that whole body protein turnover is depressed by malnutrition.

3.1.3 The effects of dietary protein and physiological stress of pregnancy and lactation on protein turnover

In recent work carried out in the Department of Nutrition, University of Southampton, Mayel-Afshar and Grimble (1982) looked at the effects of a low protein diet on protein turnover in liver, muscle and whole body of pregnant rats and on their foetuses and placentas. They observed that there was no effect on liver protein FSR, as a result of malnutrition, but that muscle protein FSR decreased to a much larger extent in malnourished than in well-nourished rats during the catabolic phase of pregnancy. There was also a bigger loss in muscle weight and protein content in malnourished animals. Such a response would, of course, be of advantage to the foetuses and placentas. These workers calculated, after making certain assumptions, that the amino acids that would

be made available, as a result of their observed decrease in muscle protein synthesis, would be equivalent to 50% of the needs for growth for all foetuses and placentas.

In lactation, a much greater requirement for energy and protein exists than in the pregnant state. It has been noted by Spray (1950) that the rat, during pregnancy, will produce a litter about 28% of its body weight but, by the end of lactation, litter weight will be about 120% of the dam's body weight.

There are many studies on the effects of malnutrition on protein turnover in skeletal muscle, liver, heart, brain, pancreas, whole body in animals in a wide range of metabolic states and exposed to a multitude of dietary situations (Waterlow *et al.*, 1978). There is, however, very little data on animals undergoing malnutrition during various stages of the reproductive cycle. The studies described in this thesis were to provide information on this neglected area of nutrition.

It is interesting to note that other workers were of a similar opinion and initiated similar studies at about the same time. The experimental design had similarities to the studies described in this thesis (Sampson & Jansen, 1983). In their study, lactating rats were fed diets containing :

(i) 23% casein protein fed *ad libitum*.
These animals acted as controls (C).

(ii) 23% wheat gluten protein fed *ad libitum* (WG).

(iii) A group fed the 23% casein protein diet but pair-fed in amounts consumed by the wheat gluten group (CP).

The diets were given from day one to day 15 of lactation. On day 15 of lactation, protein synthesis was measured *in vivo* in the mammary gland, liver and gastronemius muscle, using a large dose of ^{3}[H]-phenylalanine. The results, with various other parameters, are shown below :

	Control	Wheat gluten	Control pair-fed
Litter wt (g)	271	155	217
Mammary FSR (%/day)	116	77	64
Liver FSR (%/day)	78	67	74
Muscle FSR (%/day)	6.7	3.4	4.0
Absolute protein synthesis (mg/day) :			
Mammary gland	1743	584	633
Liver	970	469	702

These results suggest that dietary protein quality and energy affect protein synthesis in the lactating rat.

The study reported in this thesis was undertaken to examine how protein turnover is affected by dietary protein variation during lactation. The fractional synthetic rate of protein in the main protein pools of liver, mammary gland and muscle, and the specific radio-activity of the serum free amino acid were determined.

The study carried out by Sampson and Jansen (1983), and described above, was similar in some aspects to the study reported in this thesis. However, there were some differences in the outcome of the two studies, as will be explained later.

3.1.4 General principles for measurement of protein turnover

In the measurement of protein turnover, a mass of protein (either a single protein or a mixture of proteins) is considered. The mass of protein is assumed to be exchanging with a pool of free amino acids. The exchange of the 20 or so amino acids found in proteins is not, however, independent. The process of protein synthesis joins together all the amino acid residues, which remain unchanged within the protein molecule until they are all released together by the process of protein

degradation. Therefore, it is inferred that the passage of one amino acid molecule, into and out of protein, involves the synthesis and subsequent breakdown of one protein molecule. The consequence of this is that the rate of incorporation of each individual amino acid within a protein is proportional to its frequency or concentration in that protein. In other words, the fractional rate of turnover of one component amino acid within a protein is equal to the fractional rate of turnover of the protein as a whole. This would not be so if protein-bound amino acids were able to exchange with free amino acids independently of their neighbours in the same protein chain. It also follows that different amino acids will be incorporated into (and lost from) a particular protein at the same fractional rate, so that turnover rates obtained with different amino acids should be directly comparable.

The technique by which protein synthetic rates have been determined in tissues has usually been by the constant intravenous infusion of a labelled amino acid over several hours (Waterlow & Stephen, 1968). The principle behind the constant infusion of a [^{14}C]-labelled amino acid is that the specific radioactivity of that amino acid in the plasma and tissue amino acid pools should rise to a plateau. The value of this plateau is used to calculate rate of whole body and tissue protein synthetic rates (Waterlow *et al.*, 1978). The technique would therefore ensure that the specific activity of the precursor pool is unchanging during the experimental period, so that the precursor concentration would not be a variable in determining the protein synthetic rate.

The fractional synthetic rate (k_S) therefore can be calculated from the ratio of the specific radioactivities of the protein-bound (S_B) and free amino acid (S_A) by the formula

$$k_S = S_B/S_A \times \text{time (in days)}.$$

As demonstrated previously in a number of studies (Hider *et al.*, 1971), the free amino acid pool of the body cannot be considered as a single homogenous compartment.

Intracellular amino acid specific radioactivities during infusion are lower than those in the blood, as a result of the dilution effect of tissue protein breakdown (Waterlow & Stephen, 1968; Gan & Jaffay, 1967). Thus, the FSR of tissues will be underestimated if measurements are made using the plasma free amino acid pool as S_A. Therefore, the specific radioactivities of the free amino acids of each individual tissue should be used in the calculation of its FSR.

The true precursors of protein synthesis are the aminoacyl-tRNAs and so, strictly speaking, their specific activities should be used in FSR calculations. Since it is not practicable to measure these on a routine basis, it is conventional to consider the precursor specific radioactivity to be the same as that of the tissue intracellular free amino acid pool as a whole.

The rate of breakdown of protein in tissues under different conditions is far more difficult to measure than synthesis. The usual technique is to label the protein by injecting a radioactively labelled amino acid, and then to follow the subsequent loss of radioactivity from protein. One of the problems of this technique is re-incorporation or recycling of the label after its release from the labelled protein. These technical problems can be avoided by appropriate choice of the labelled precursor (Waterlow *et al.*, 1978). For example, by using arginine labelled at the guanidine carbon ([6-^{14}C] arginine) for measurements of liver proteins, since there is a high probability that any molecule of arginine which enters the liver pool will exchange its guanidino carbon with unlabelled carbon dioxide through the urea cycle before being recycled into protein.

In the measurement of muscle protein breakdown, the problem of amino acid re-utilisation can largely be overcome by the use of labelled histidine, which is modified after incorporation into the protein as a 3-methyl histidine residue. This molecule has been shown not to bind to tRNA when incubated in an *in vitro* protein synthesising system. It cannot therefore be re-utilised.

These pulse-labelling methods assume that protein breakdown is random, i.e. obeys first order kinetics. However, as has already been stated, recent work by Millward *et al.* (1975) has shown this not to be the case in the myofibrillar proteins in the muscle. Dreyfus *et al.* (1960) had reported earlier that myosin was not degraded randomly but rather had a lifetime of 30 days. Morkin had reported experiments which suggested that both in diaphragm (Morkin, 1970) and heart (Morkin, 1974), myofibrils were renewed by addition of newly synthesised myofilaments to the periphery of the myofibril. If this is the case, turnover cannot be completely random. The implication of non-random myofibrillar turnover is that the loss of labelled amino acids after a pulse dose may vary according to the subsequent treatment. For example, during starvation, when the myofibrillar diameter is known to decrease (Goldspink, 1965), peripheral, highly-labelled filaments will be degraded, causing an acceleration of loss of radioactivity.

The most successful method of measuring breakdown is to measure the fractional rate of synthesis (k_s) and the fractional rate of change of protein mass (kg) of a tissue. The fractional rate of breakdown, k_d, can be calculated by $k_d = k_s - kg$. The sources of error with this approach stem mainly from the difficulty in measuring accurate rates of change of protein mass, unless either very large groups of animals or long experimental periods are used.

The constant infusion technique referred to earlier is not suitable for tissues with fast turnover rates, which are constantly losing or exporting protein, as is the case with liver, mammary gland or gut. In the liver, for example, this method will measure the FSR of only the intracellular proteins and not those that are secreted, like albumin. Rothschild *et al.* (1970) observed that these export proteins remain in the liver for only about 30 minutes, after which they are lost to the plasma. Another reason why the constant infusion technique is unsuitable

for measuring synthetic activities of highly metabolising tissues is the problem of label re-utilisation. In the present study, the continuous infusion technique would not be suitable. Consequently, the so-called 'massive dose' technique was used (Henshaw *et al.*, 1971; McNurlan *et al.*, 1979). A large dose of amino acid is injected. The principle behind this technique is that the massive dose of the precursor amino acid floods the free amino acid pools of all tissues of the animal, so that all compartments (e.g. intracellular, extracellular, aminoacyl-tRNA) reach the same radioactivity almost instantaneously. It is assumed, therefore, that the precursor radioactivity is unchanged over the 10 minute period during which measurements are made. In the original technique, leucine was used as the tracer. The amount injected (McNurlan *et al.*, 1979) was calculated to expand the free amino acid pool to 10-times the normal amount. In the present study, tyrosine was used for technical reasons. The precursor tyrosine pool, however, could be expanded to only 5-times the normal concentrations because of solubility problems.

The massive dose technique assumes that the large amount of amino acid injected does not in itself alter the rate of protein synthesis. It had been argued before that, in response to this large load of the amino acid, which no longer acts simply as a tracer, a potential drawback of producing metabolic changes might occur. The assumption that the large dose of the amino acid does not alter the rate of protein synthesis was tested by McNurlan *et al.* (1979) and Garlick *et al.* (1982) and proved to be valid.

3.2 Materials and Methods

The diets given to the animals were the same as described in Chapter 2, and the animals were treated in exactly the same way.

3.2.1 Protein turnover technique

The injection technique was performed as described by Garlick and Marshall (1972). A 20-gauge disposable needle

was separated from the non-metal component and inserted into a length of narrow-bore polythene tubing, the other end of which was fixed to a hypodermic syringe. On day 15 of lactation, each dam was wrapped in a towel, leaving the tail free, and put into a plastic restraining cage constructed from a two-litre polythene measuring cylinder, pierced with holes to allow free movement of air. The tail was warmed, using heat from an electric light bulb, to improve the visibility of the tail vein. The needle was inserted into the lateral tail vein while the required amount of radioactive tyrosine was injected. The injection was performed within 30 seconds into the unanaesthetised rat. The injected material was L-[U-^{14}C] tyrosine hydrochloride (Amersham International) in a carrier dose of L-tyrosine, as described by McNurlan *et al.* (1979). The dose of tyrosine given was 66 μCi and 0.6 mmol/kg body weight. The amount of carrier was calculated to ensure at least five-times normal tissue tyrosine concentrations occurred after the injection, and that concentrations remained at near constant levels for at least 10 minutes.

As the technique had not been applied to lactating animals before, the latter point was checked by killing animals 2, 5, 10, 20 and 30 minutes after injection. The needle was quickly removed from the tail and the animal decapitated. Blood was collected, allowed to clot and spun in a centrifuge to separate the serum. The liver, mammary and hind leg muscle tissues were removed quickly, frozen in liquid nitrogen and all samples stored at -20°C until ready for analysis.

3.2.1.1 Measurement of free and bound radioactivity in liver and mammary gland protein

The method for the measurement of specific radioactivity of the protein-bound and free tyrosine of liver and mammary tissue was carried out as described by Garlick and Marshall (1972), and is set out below. A flow chart for these measurements is presented in Fig. 3. Measurement of specific radioactivity of muscle required a modification of this method and is described later in Section 3.2.1.2.

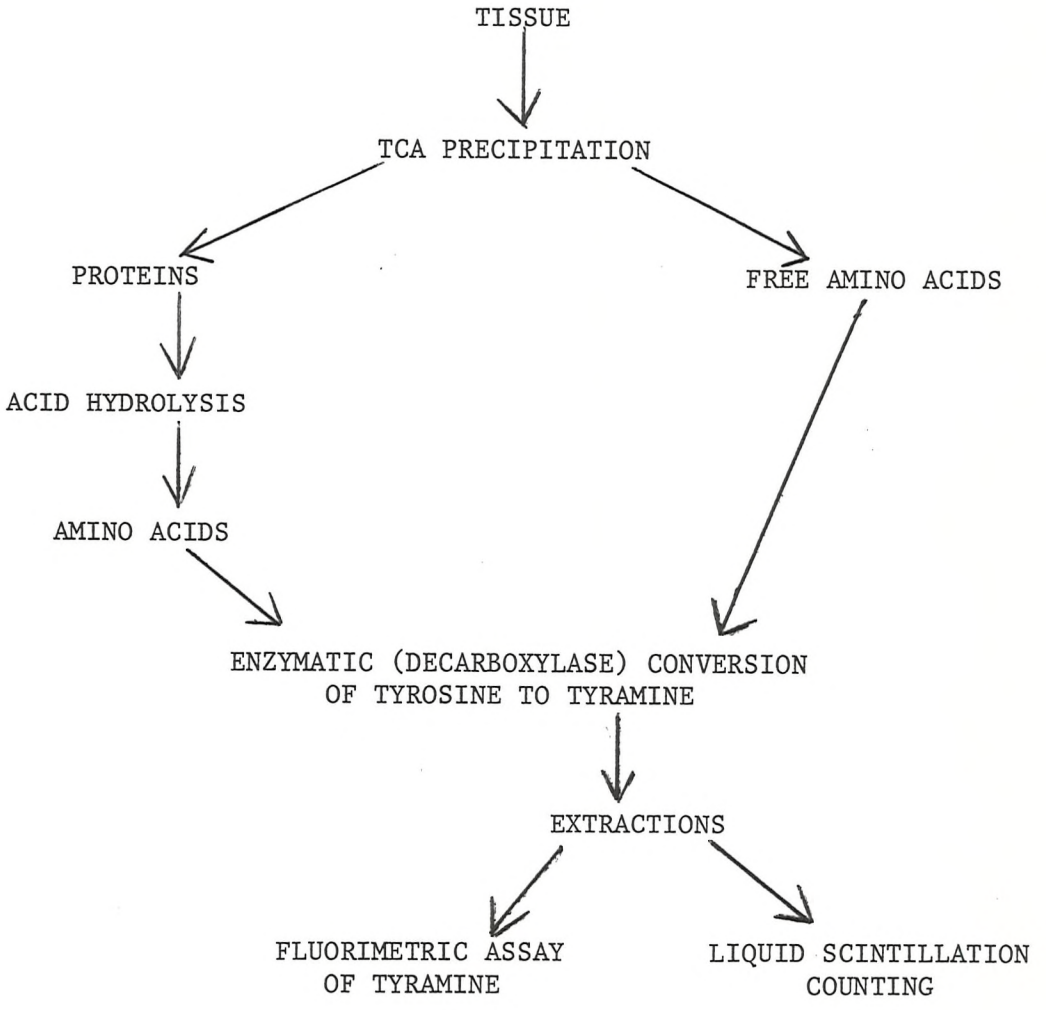


Fig. 3. Flow chart of the method used for measurement of the rate of protein synthesis.

The technique for liver and mammary gland tissue was as follows. Frozen tissue was rapidly weighed and homogenised with an ultra turax homogeniser in ice-cold 10% (w/v) trichloroacetic acid (TCA). The supernatant was washed five times with diethyl ether to remove all traces of TCA. The TCA-precipitated protein was washed six times with 5% TCA to remove all traces of free radioactivity, after which the protein was hydrolysed *in vacuo* in sealed pyrex tubes with 6 M hydrochloric acid (HCl) at 105°C in an oven for 22 h. Hydrochloric acid was removed from the hydrolyzate by repeated evaporation, after addition of water, at 60°C in a vacuum oven, and the resultant solution of amino acids made up to 5 ml with water.

L-tyrosine was estimated as tyramine after reacting with L-tyrosine decarboxylase to avoid possible contamination by D-tyrosine, of very high specific radioactivity, derived from the infusate. Waterlow and Stephen (1968) had shown that, during the infusion of L-[^{14}C] lysine, appreciable errors in the estimate of specific radioactivity of free lysine in tissue would occur if the infusate had racemized to the extent of 0.5%. This is well within the stated limits of purity of the radioactive tyrosine used in the present study. The specific decarboxylase also ensured that the measurement of radioactivity in other tissue compounds was avoided.

The tyrosine assays were carried out as follows. 1.5 ml of the amino acid solution was added to 1.0 ml L-tyrosine decarboxylase in sodium citrate buffer (pH 5.5; 0.5 M), containing 10 mM pyridoxal phosphate (132 mg/50 ml). 1.5 ml of the citrate buffer (without the pyridoxal phosphate) was added, the contents were vortexed very briefly and incubated at 37°C for one hour. After cooling in ice, the tyramine produced was extracted into ethyl acetate by shaking for 30 seconds in the presence of about 2.5 g sodium chloride and anhydrous sodium carbonate mixture (1 : 1) and 2 x 5 ml ethyl acetate. The organic (top non-yellow layer) was carefully removed, using a disposable pipette, and added into 5 ml chloroform in a large

tube, with a glass stopper, and thoroughly shaken. Three ml of a 1 : 2000 aqueous solution of sulphuric acid was added to the mixture, which was shaken immediately for 15 seconds. Duplicate 0.8 ml samples of the upper aqueous layer containing the tyramine were counted for radioactivity with 10 ml tritoscint scintillation fluid in a Beckman liquid scintillation counter. Duplicate 0.8 ml samples of the same solution were assayed for tyramine by the nitrosonapthol fluorometric method of Waalkes and Udenfriend (1955). The method is as follows. To 0.8 ml supernatant (or 50 µl protein hydrolyzate, all made to a volume of 2 ml with distilled water) was added 1.0 ml of nitrosonapthol solution (0.1% 1-nitro-2-napthol in ethanol), 1.0 ml nitric acid : sodium nitrite mixture (50 ml of 1 : 5 nitric acid plus 1.0 ml 2.5% sodium nitrite, mixed just before use). The contents of the tube were vortexed, incubated at 55°C for 30 minutes and cooled in running tap water. Ten ml of ethylene dichloride (dichloroethane) was added, the contents shaken vigorously and the fluorescence of the top layer measured in a fluorimeter with excitation set at 460 nm and emission set at 570 nm. Tyramine was used to obtain a standard curve.

3.2.1.2 Specific radioactivity of muscle protein

The specific radioactivity of muscle tissue was determined using a slightly different technique from that described above for the other tissues. In muscle, only small quantities of label were incorporated into protein due to the fact that the protein synthesis rate is low in this tissue. To measure the low levels of protein-bound radioactivity, it was therefore necessary to dissolve as much protein as possible to obtain a reasonable number of count.

A muscle homogenate in 10% TCA was made and an aliquot centrifuged. The protein precipitate was washed five times with 5 ml of 5% TCA to remove all free radioactivity, and three times with 5 ml of ethanol-ether mixture (3 : 1) to remove the TCA. The precipitate was blotted between filter papers to remove excess solvent and dried at 37°C for 3-4 hours.

Approximately 20 mg of the dried precipitate was dissolved in 1.0 ml of 0.3 M sodium hydroxide, made up to 25 ml with distilled water and the protein content determined, using Folin Ciocalteau reagent (Lowry *et al.*, 1951). To another portion of the dried protein precipitate (about 100 mg), 0.2 ml distilled water and 2 ml NCS (Nuclear Chicago solubiliser) were added and left to dissolve at 37°C for 18 hours. An aliquot of this solution was added to specially prepared NCS scintillation fluid and counted in a Beckman scintillation counter. The results obtained, therefore, were as dpm per weight of protein. The values were converted to dpm/μmol tyrosine to bring them in line with the way in which the results for the other tissues were calculated.

3.2.1.3 Specific radioactivity of serum amino acids

The specific radioactivity measurement of the free amino acid in the serum was determined by precipitating the protein in 200 μl serum with 5 ml absolute alcohol. After centrifugation, the supernatant was transferred to a counting vial, evaporated to dryness over a hot plate and redissolved in 2 ml water. The specific radioactivity was determined as described above for liver and mammary free amino acid pools.

3.3 Results and Discussion

The true protein fractional synthetic rate, FSR or k_s is represented by the equation $k_s = (S_B/\bar{S}_A t) \times 100\%/\text{day}$ where

k_s = FSR = % of protein pool or mass renewed per day.

S_B = specific radioactivity of the protein-bound tyrosine

t = time in days

$\bar{S}_A$ = mean specific radioactivity of tyrosine in the precursor pool, i.e. the free amino acid pool. $\bar{S}_A$ is determined by joining points of S_A at 2 and 10 minutes and extrapolating to zero. $\bar{S}_A$ is then the arithmetic mean of S_A at zero and 10 minutes. However, it can be valid for $\bar{S}_A$ to be determined in this way only if the points joining S_A values at 2, 5, 10, 20 and 30 minutes form a straight line, i.e. that the small rate of loss of label with time is linear.

Moreover, the massive dose technique, like the constant infusion technique, would imply two assumptions for it to be valid :

(i) That the material exchanged between the pools or introduced into them mixes completely and instantaneously with that already present.

(ii) That a constant fraction of the material in each pool is transferred or exchanged in unit time as an exponential or first order process.

The validity of the results obtained from these measurements would therefore depend on how close the assumptions are to what actually takes place in the cell.

It was observed, however, that for all three tissues, muscle, mammary and liver, a straight line joining all the values for S_A could not be drawn. A linear rate of loss of label could not be unequivocally demonstrated because the tissue amino acid pools did not seem to have equilibrated by two minutes. The specific radioactivities at five minutes were higher than at two minutes for all three tissues (Fig. 4). The assumption of instantaneous mixing of the injected amino acid was therefore not strictly correct in this study. The most likely explanation for failure to reach rapid equilibrium in the amino acid pool was that the concentration of the injected tyrosine (labelled plus non-labelled) was insufficient to saturate the tissue amino acid pools. As a result of this problem, the absolute FSR values could not be calculated. However, the values for S_B/S_A at ten minutes could be used to obtain a qualitative measure of protein synthetic rates and enabled general comparisons of the trends of synthetic activities that resulted from variations of dietary proteins.

3.3.1 Specific radioactivity of serum free tyrosine

The specific radioactivity of the free tyrosine in the serum following an injection of isotope can be used to give an indication of the plasma tyrosine flux, an index of protein turnover in the whole body. A decrease in specific activity

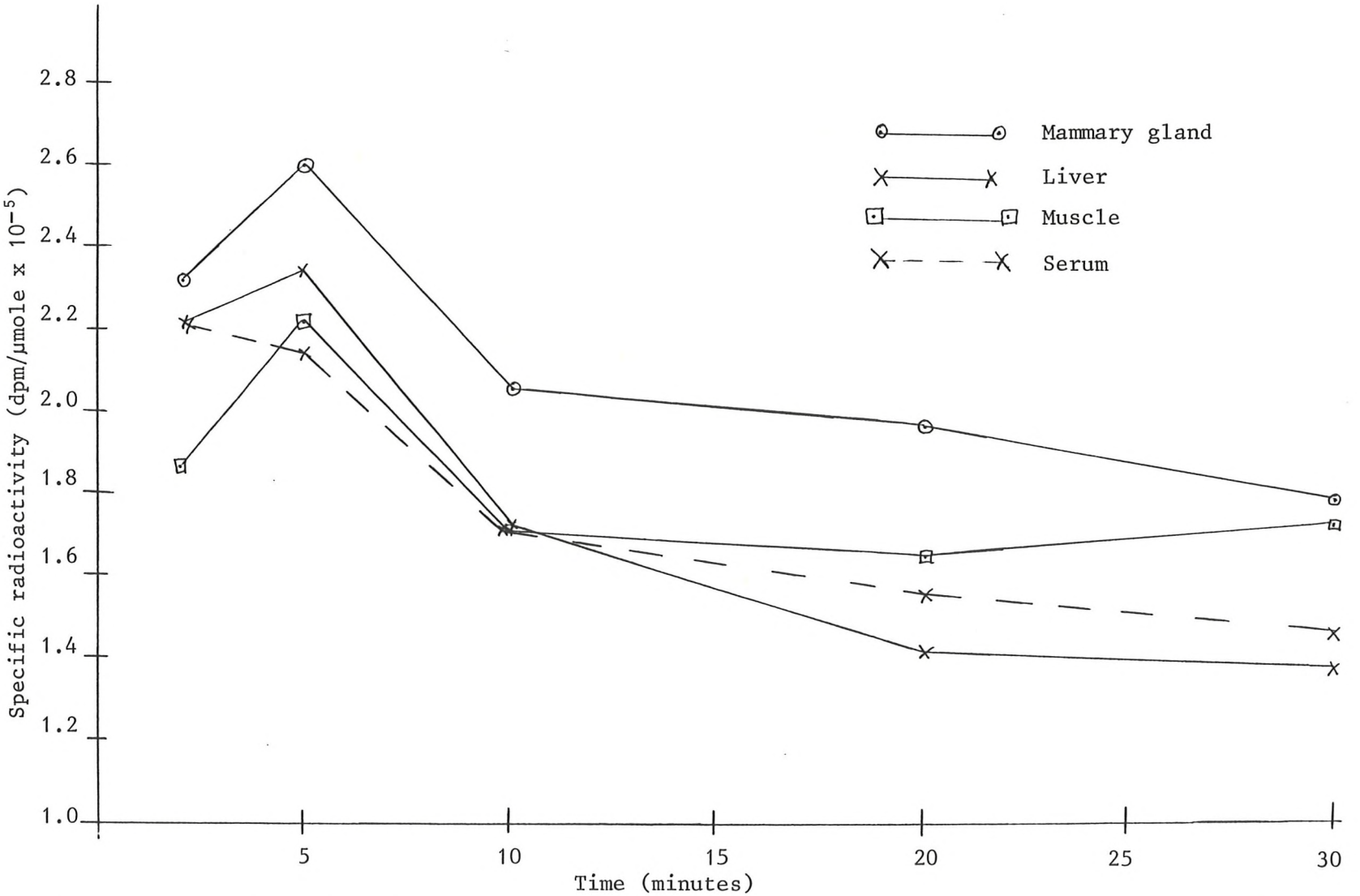


Fig. 4. Specific radioactivity of free amino acid pool in liver, mammary gland, muscle and serum of control rats killed at various times after the injection of [^{14}C]-tyrosine.

is to be expected if an increased amount of unlabelled amino acid is entering the pool, as a result of dietary intake or tissue protein breakdown. Either of these supplies of amino acids could benefit milk protein synthesis. In order to have a general idea, therefore, of how total protein turnover was being affected by the various dietary treatments, the specific radioactivity of the serum free amino acid pool was measured at 2 and 10 minutes. The results are presented in Table 17. The specific activity of tyrosine was highest for the rats on the worst diet, the 10% cereal protein diet, and lowest for the pair-fed group.

It should be obvious that the greater the total body protein turnover, the greater the rate of dilution by unlabelled tyrosine from tissue breakdown, and so the lower the specific radioactivity of the serum free amino acid pool. Conversely, the lower the total body protein turnover, the slower the rate of dilution from tissue breakdown and dietary protein and hence the higher the specific radioactivity of the serum free amino acid pool. The results reflect that the 10% cereal protein-fed group had the least, and the pair-fed group had the highest, total body protein turnover.

3.3.2 Liver 'fractional synthetic rate'

A qualitative assessment of the fractional synthetic rates for the three tissues is presented in Table 18, which shows the S_B/S_A values expressed as a percentage of that of the control group.

As a result of the large fluctuations within groups, there were no statistically significant differences in the liver FSR resulting from changes in dietary protein intakes. The trend that could be seen, however, was that lactation depressed FSR and that a reduced intake in protein tended to increase FSR. Protein quality, however, had no effect on liver protein FSR. The results are in agreement with those of Sampson and Jansen (1983), who, after feeding diets similar in quality to those given in the

Table 17. Specific radioactivity of the free amino acid pool in the serum 2 and 10 minutes after injection (Mean ± SEM).

Number of animals shown in parentheses

Diet	Specific radioactivity (dpm/μmole) ($\times 10^{-5}$)	
	2 min	10 min
20% milk protein(5)	2.21 ± 0.08	1.81 ± 0.15
10% milk protein(5)	2.41 ± 0.06[a]	1.85 ± 0.18
20% cereal protein(6)	2.40 ± 0.05[a]	2.02 ± 0.11
10% cereal protein(6)	2.53 ± 0.04[b,c]	2.09 ± 0.2
20% milk protein (pair-fed)(5)	1.98 ± 0.02[b]	1.68 ± 0.12
20% milk protein (non-lactating)(6)	2.38 ± 0.06[a]	2.28 ± 0.08

Significantly different from the 20% milk protein group:
a, $p < 0.05$; b, $p < 0.005$.

Significantly different from the pair-fed group:
c, $p < 0.005$.

Table 18. Effect of dietary protein variations on the protein 'fractional synthetic rate' (FSR) calculated as SB/SA at 10 minutes in liver, mammary and muscle tissue (Mean ± SEM).

Number of animals shown in parentheses

Diet	Liver (SB/SA)x10^5	Liver SB/SA as % of control	Mammary (SB/SA)x10^5	Mammary SB/SA as % of control	Muscle (SB/SA)x10^5	Muscle SB/SA as % of control
20% milk protein(5)	516 ± 51	100	820 ± 46	100	86 ± 7	100
10% milk protein(5)	702 ± 158	136	909 ± 104	111	88 ± 14	102
20% cereal protein(6)	601 ± 58	116	670 ± 37[a,b]	82	76 ± 11	88
10% cereal protein(6)	500 ± 80	97	500 ± 68[a]	61	64 ± 13	74
20% milk protein(5) (pair-fed)	595 ± 51	115	1412 ± 237[a]	172	64 ± 12	74
20% milk protein(6) (non-lactating)	708 ± 77	137	-		71 ± 8	82

Significantly different from the control group:
a, $p < 0.001$

Significantly different from the pair-fed group:
b, $p < 0.05$

present study, calculated FSR values that did not appear to be significantly different between the different dietary groups.

The outstanding difference between their results and those reported in Table 18 is that in the present study, the pair-fed group had FSR values that were higher than those of the control, though not significantly so. A possible reason for this difference might be that the measurements in the two studies were carried out at different times of day after the rats had finished their food rations. This difference in the fractional synthetic rates between the groups was even more pronounced in the mammary gland, as will be explained below. The lack of a significant difference of liver protein FSR between groups is in line with observations made by other workers, where it has been shown that non-extreme dietary protein restrictions, as was the case in this study, did not significantly alter liver protein synthetic rates.

It appears likely that the overall control of liver protein mass during lactation may be exerted through changes in the rate of proteolysis, as has been observed in non-lactating animals. This conclusion can be reached, since there were substantial losses of protein from livers of all animals receiving inadequate dietary protein intakes (Table 13).

The massive dose method measures total liver protein synthesis, i.e. export as well as intracellular protein. Since the fractional synthetic rate is not decreased by dietary insufficiency but the concentration of serum albumin is, an increase in proteolysis would again be suggested.

Observations on similar lines have also been made by other workers (Garlick *et al.*, 1973), who have shown that insulin (Mortimore & Mordon, 1970), amino acids (Woodside & Mortimore, 1972), and energy supply (Parrilla & Goodman, 1974), all suppress liver protein breakdown.

3.3.3 Muscle 'fractional synthetic rate'

As was the case for the liver, no significant changes were observed in the FSR of the various groups (Table 18). The general trend, however, showed a reduction in the 10% cereal protein-fed rats. One of the problems, of course, is that the massive dose technique is not particularly suited to measure FSRs in tissues with slow rates of protein turnover, like muscle. There is, however, overwhelming evidence in the literature to support the suggestion that a reduction in dietary protein intake or quality results in a fall in FSR. This effect would also be suggested by the results of the present work. The results are again in agreement with those of Sampson and Jansen (1983) in lactating rats, and those of other workers on rats in the pregnant (Mayel-Afshar & Grimble, 1982) and non-lactating, non-pregnant state (Garlick *et al.*, 1975). This reduction in the synthesis rate of the tissue that is the most abundant protein reserve in the animal is a beneficial adaptation. The amino acids not used for muscle protein synthesis could be used to synthesise milk proteins, or other proteins that are more indispensable than the muscle proteins, within the dam.

Fig. 5 shows the muscle FSR and the tibialis weights of the different groups. When the FSRs of the pair-fed group and the 20% cereal protein group are compared with their tibialis weights, it can be seen that, although the FSRs are about the same, the muscle mass of the pair-fed group was higher, though not significantly so. A possible explanation for this observation is that the cereal protein-fed rats had a higher rate of protein breakdown than occurred in the pair-fed group. It is possible that the combination of the low quality of the cereal protein, coupled with the reduced food intake, was a severe enough condition to bring about an elevation of the protein breakdown rate.

The suggested elevated protein degradation in the 10% cereal protein group could also be due to the absence, or presence in low concentrations, of an essential amino acid, such

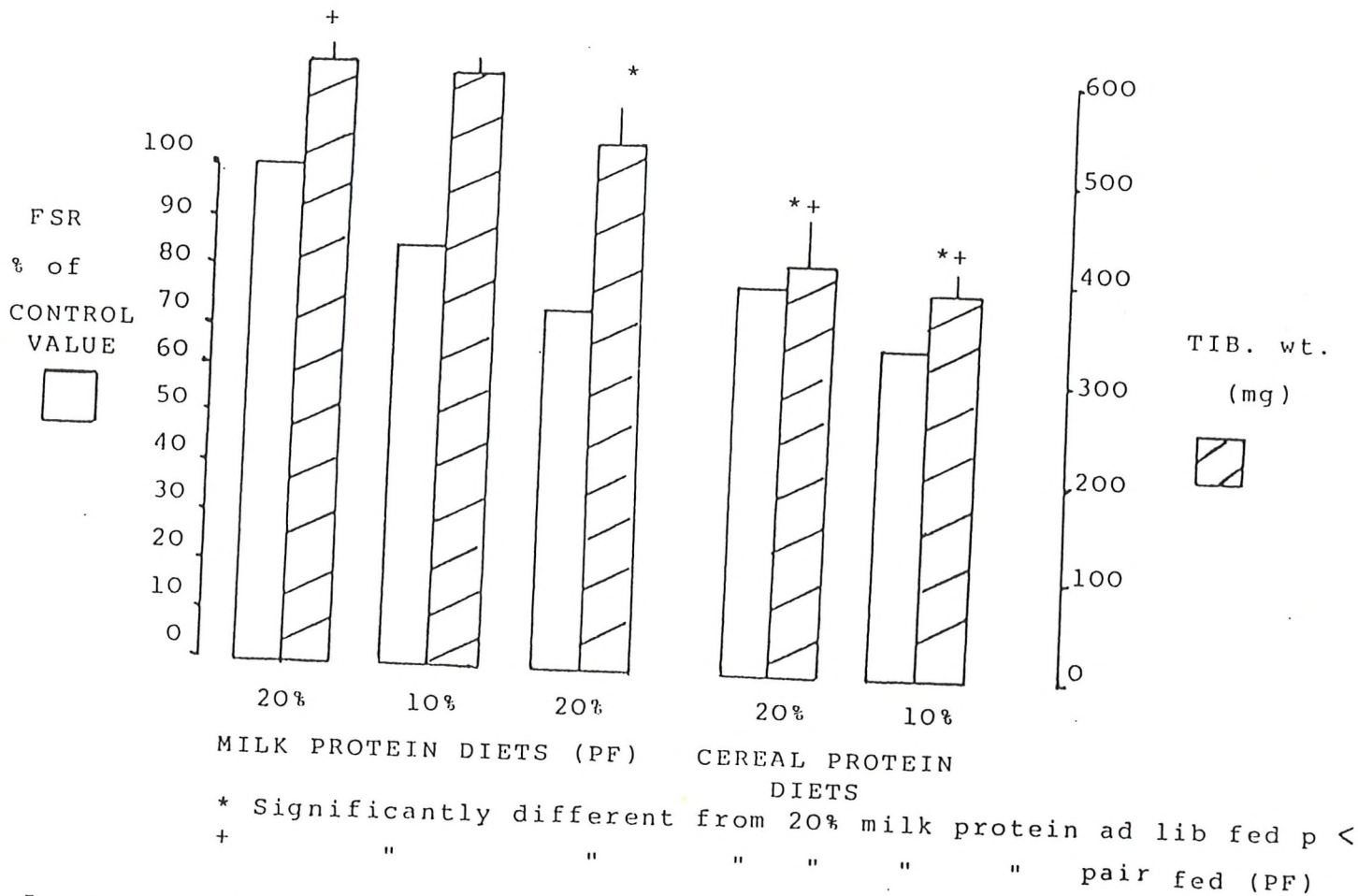


Fig. 5. Fractional synthetic rate of protein in muscle and tibialis muscle weight (Mean ± SEM).

as lysine or tryptophan. This phenomenon has been found in *in vitro* studies: Scornik *et al.* (1980), demonstrated increased rates of protein breakdown in Chinese hamster ovary cells that have been starved of histidine.

It must be remembered that the lactating state is more stressful than the non-lactating, non-pregnant state. It has been reported in the literature that muscle protein breakdown becomes a significant factor only in very severe conditions, such as starvation for four days or a protein-free diet for nine days (Millward *et al.*, 1976). During the process of lactation, a poor diet might produce sufficient nutritional stress for muscle protein breakdown rates to be elevated.

3.3.4 Mammary 'fractional synthetic rate'

The results of this study (Table 18) have been reported elsewhere by Mansaray and Grimble (1983a). There was a significant decrease in the FSR of rats on the cereal diets, and no effect on the FSR of the 10% milk protein group, when compared with the control. The results show clearly that poor protein quality depresses mammary protein synthetic rate, and thus would affect milk protein quantity and milk volume. The latter effect could possibly be by a reduction in the synthesis of the lactose synthetase enzyme, which will affect milk volume via the lactose content. This point is explained in more detail in Chapters 4 and 5. The results also suggest that the mammary gland is more like muscle and less like liver in its response to reduced dietary protein intake, in that reduced FSR occurs.

It is interesting to note that the cellular RNA concentration (RNA/DNA) and the synthetic efficiency (protein/RNA) in the mammary gland behaves in a similar way to that in muscle in response to poor protein quality (Tables 15, 16). Liver RNA, however, shows only a decrease in concentration with no change in efficiency (Table 13).

RNA efficiency is influenced by hormonal factors and the supply of amino acid substrate. Thus, the observation that FSR and RNA efficiency behave in the same way in the mammary gland and muscle should not be unexpected because the mammary gland, like muscle, has to receive raw materials from peripheral blood for the synthesis of milk protein, unlike liver, which receives amino acids direct from the portal system. The difference between the mammary gland and the muscle, however, is that in the mammary gland, the reduced FSR, resulting from the reduced dietary protein quality, is an adaptation 'forced' on the tissue as a result of the significant reductions in the amino acid substrate supply, and so is not a profitable adaptation as far as pup growth is concerned. The decrease in FSR in muscle may, however, be a 'profitable' adaptation for the adequate nutrition of the pups by ameliorating the effects of dietary protein inadequacy.

These results show similar effects to those reported in the pregnant state by Mayel-Afshar and Grimble (1982), that is, that dietary protein inadequacy affects both dams and foetuses, although foetuses are less severely affected than the dam, owing to adaptations in the maternal muscle protein metabolism.

The significant rise in the mammary FSR of the pair-fed rats can be explained by the fact that these rats were both fed and killed in the morning. Meal-feeding results in increased levels of blood glucose, amino acids and insulin. Much of this glucose and amino acids will be absorbed rapidly by the mammary tissue, since this tissue is very efficient in removing nutrients from the blood stream. The high plasma insulin concentrations associated with the post-absorptive state would increase the rate at which these nutrients enter the cell and the rate of protein synthesis. The increased amount of the protein synthetic machinery, as indicated by the cellular RNA concentrations of the meal-fed (pair-fed) group would assist this process.

The results for the FSR reported in the present study are in agreement with those reported by Sampson and Jansen (1983) in terms of the fact that a poor quality protein diet resulted in a reduction in the mammary protein FSR, and that the FSR of mammary tissue is higher than that of even the liver, suggesting that the mammary gland might be more active at synthesising protein than the liver. The main difference between the two sets of results is again on the pair-fed group, where values were lower than even in the wheat protein-fed group. The values reported in the present study were significantly higher for the pair-fed group, than for the 20% cereal protein-fed group, and even higher than the controls. In the results of the study of Sampson and Jansen, it is difficult to reconcile the reduced mammary protein FSR of the pair-fed group compared to the wheat gluten-fed group, with the slightly higher absolute protein synthesis which occurred in the pair-fed group. A possible explanation for these observations might be that the pair-fed rats had a higher mammary tissue protein content.

Another difference between the two sets of results is the suggestion of Sampson and Jansen (1983) that there was no diurnal variation in the FSR of their meal-fed group. Meal-feeding is known to vary protein FSR in muscle throughout the day (Garlick *et al.*, 1973). The results of the work reported in this thesis would also imply diurnal variation in the protein FSR of the mammary gland because, if the high values measured for the pair-fed group persisted throughout the day, the amount of protein produced would have been higher than that produced by the control group. This could have resulted in the pup growth of the pair-fed group being higher than the control, which was definitely not the case, as has been seen in Chapter 2.

CHAPTER 4

EFFECTS OF PROTEIN QUALITY AND QUANTITY ON THE COMPONENTS OF THE LACTOSE SYNTHETASE COMPLEX OF RAT MAMMARY GLAND

CHAPTER 4

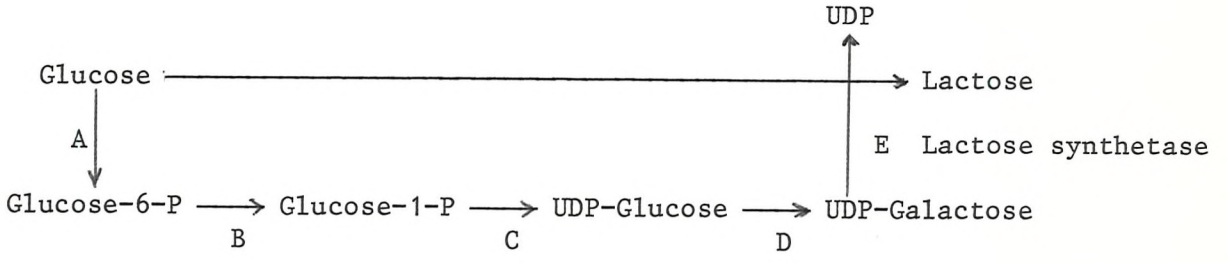
EFFECTS OF PROTEIN QUALITY AND QUANTITY ON THE COMPONENTS OF THE LACTOSE SYNTHETASE COMPLEX OF RAT MAMMARY GLAND

4.1 Introduction

Of the three major milk components, i.e. fat, protein and lactose, only lactose exerts a significant osmotic pressure because of its comparatively small molecular weight. Lactose is thought to play a crucial role in milk formation by drawing water into the Golgi vesicles, where it becomes temporarily located (Linzell & Peaker, 1971).

The synthesis of lactose from glucose involves five enzyme catalysed steps. The first four are common to several metabolic pathways (Fig. 6). The fifth enzyme, lactose synthetase, is unique to mammary tissue and the enzyme is also present in milk. It catalyses the conversion of UDP-galactose and glucose to lactose and UDP, in the rate-limiting step of the biosynthetic pathway.

Lactose synthetase is composed of two dissociable proteins, the A and B proteins (Brodbeck & Ebner, 1965), both of which are required for enzyme activity (McKenzie *et al.*, 1971; Fitzgerald *et al.*, 1970a; Brodbeck & Ebner, 1965). The B protein has been shown to be the milk-specific protein, α-lactalbumin (Brodbeck *et al.*, 1967), and it has no enzymic activity of its own. The A protein, galactosyl transferase, has been shown to catalyse the transfer of galactose from UDP-galactose to N-acetylglucosamine, either free or covalently attached to a protein (Brew *et al.*, 1968). This latter reaction gives a protein-bound N-acetyl-lactosamine group, which is an important step in the serial attachment of monosaccharides to form the carbohydrate moiety of many glycoproteins (McGuire *et al.*, 1965). Galactosyl transferase, in the presence of α-lactalbumin, modifies its



A Hexokinase
B Phosphoglucomutase
C UDP-glucose pyrophosphorylase
D UDP-glucose epimerase
E Lactose synthetase

Fig. 6. Pathway for lactose synthesis from glucose.

substrate acceptor specificity to include glucose, and the characteristics of the transfer of N-acetylglucosamine are affected in a complex way. Thus, the α-lactalbumin allows the synthesis of a new product, lactose, by lowering the Michaelis constant (K_m) of the A protein for glucose (Klee & Klee, 1970), and by increasing the maximal velocity (V_{max}) of the reaction (Morrison & Ebner, 1971). The Michaelis constant of galactosyl transferase for glucose in the absence of α-lactalbumin is about 1.5 molar, but in the presence of α-lactalbumin falls to values in the millimolar range (Fitzgerald *et al.*, 1970b). Alpha-lactalbumin, therefore, has a controlling activity on the specific activity of galactosyl transferase and has thus been designated a 'specifier' protein (Brew *et al.*, 1968). Alpha-lactalbumin also controls the rate of lactose synthesis, both during mammary development and also in hormonally-stimulated mammary organ cultures (Turkington *et al.*, 1968a).

The reactions catalysed by the two enzymes can be summarised as follows :

(i) UDP-galactose + N-acetylglucosamine

$$\xrightarrow[\text{Cofactors: tris-HCl, pH 7.4; } MnCl_2 + ATP]{\text{Galactosyl transferase}} \text{N-acetyllactosamine + UDP}$$

(ii) UDP-galactose + glucose

$$\xrightarrow[\text{Cofactors: tris-HCl, pH 7.4; } MnCl_2 + ATP]{\text{Lactose synthetase}} \text{Lactose + UDP}$$

A single form of α-lactalbumin has been isolated from goat, pig, sheep and human milk (Schmidt & Ebner, 1971). Two forms of α-lactalbumin, however, have been separated from bovine milk by DEAE cellulose chromatography (Barman, 1970). The major component has a molecular weight of 14,435 and the minor component (15%) has a molecular weight of 16,800 and is a glycoprotein containing 11-12 sugar residues per molecule. The two components have the same amino acid composition and both are active in the lactose synthetase assay. In fact, Tanahashi *et al.* (1968) demonstrated

that the crude preparations of the A and B proteins from the milk of cow, sheep, goat and human were interchangeable, as far as the ability to convert galactosyl transferase to lactose synthetase. On a qualitative basis, the A and B proteins of these species were also interchangeable. Tanahashi *et al.* (1968) reported, however, that, while milks from ruminants reacted with antisera to bovine α-lactalbumin, milks from non-ruminants did not. Thus, there appears to be an immunological difference between the α-lactalbumins of ruminants and non-ruminants. Schmidt and Ebner (1971) have shown, using amino acid analysis, spectral properties, electrophoretic mobility, N- and C-terminal amino acids and tryptic peptide maps, that the α-lactalbumin isolated from the milk of the pig, goat, sheep and human differed slightly in structure.

Qasha and Chakrabartty (1978) have also been able to separate two species of α-lactalbumin, α-lactalbumin$_1$ and α-lactalbumin$_2$, from rat milk and have purified them to homogeneity by gel filtration, followed by DEAE-cellulose chromatography. They found that α-lactalbumin$_1$ was a bigger molecule, in contrast to other known α-lactalbumins, and has a molecular weight of 21,500. α-lactalbumin$_2$ was found to have a molecular weight of 16,000. Both α-lactalbumins were found to be active in the lactose synthetase assay and both are glycoproteins.

The onset of lactation in the goat has been reported to be preceded by the appearance of mammary and urinary lactose, well before the end of pregnancy (Fleet *et al.*, 1975). These workers showed that four phases in the milk secretory activity of the goat could be distinguished, namely :

(i) The dry period at about mid-pregnancy (weeks 4-10), when there was little or no fluid in the gland. Any fluid present resembled extracellular fluid rather than milk in appearance and composition, except for a greater immunoglobulin concentration.

(ii) The period of pre-colostrum formation in the glands (weeks 12-15 of pregnancy, i.e. 7-10 weeks pre-partum), when the udder volume increased and fluid could be obtained from the teat. This fluid changed in composition from a resemblance to cellular fluid to that of milk, in that there were marked increases in the lactose, potassium and phosphate, and decreases in pH, sodium, chloride and bicarbonate. The immunoglobulin concentration continued to rise and was quite high near term.

(iii) There was the period of formation of true colostrum, two to three days before parturition and two to three days after, which was characterised by a dramatic 3-11 fold increase in the citrate concentration.

In the rat and human, however, the appearance of lactose in the mammary tissue occurs at about the time of parturition.

There is controversy about whether the level of galactosyl transferase or that of α-lactalbumin is more important in affecting the amount of lactose synthesised under various hormonal and nutritional circumstances. Kuhn *et al.* (1980) have suggested that, in laboratory animals, α-lactalbumin appears to be the major agent in the regulation of lactose synthesis during lactogenesis, but not necessarily limiting at other times; whereas the increase in the amount of galactosyl transferase seems largely to account for the rising yield of lactose during lactation.

The increase in galactosyl transferase activity could therefore account broadly for the increasing amount of lactose synthesis. It is, however, by no means clear what stimulus is responsible for this increase in activity. Although the withdrawal of progesterone at the end of pregnancy exposes the enzyme to the inductive influence of placental lactogen and prolactin, the profiles of galactosyl transferase activity in the mammary gland and of prolactin concentration in the plasma during lactation are not strikingly similar. Moreover, it has

been reported that the galactosyl transferase activity of rabbit mammary gland was scarcely affected 6-7 days after hypophysectomy had taken place, even though milk production was virtually abolished (Jones & Cowie, 1972). These workers also concluded that the decline in the effective concentration of α-lactalbumin was one factor contributing to the decreased lactose synthetase activity after hypophysectomy.

In mouse mammary gland, the increased concentrations of α-lactalbumin coincides with the onset of lactation, in a way that the preceding increase in galactosyl transferase does not (Turkington *et al.*, 1968b).

The coincidence of the appearance of α-lactalbumin and lactogenesis has been documented more closely in the late-pregnant rat, especially where lactogenesis has been initiated experimentally by ovariectomy and hysterectomy which mimics the natural stimulus for lactogenesis. Both in the whole animal and in mammary explants, the induction of α-lactalbumin requires prolactin or placental lactogen, and is retarded by progesterone, two features that characterise lactogenesis in the natural state (Turkington & Hill, 1969).

Human lactation is similar to that in the rat, since lactogenesis is accompanied by a surge of α-lactalbumin synthesis.

However, if the concentration of α-lactalbumin in milk (about 1 mg/ml or more) is any guide to its concentration in the Golgi lumen (where the galactosyl transferase protein is situated and where the lactose synthetase reaction takes place), it is possible that it would always fully saturate the transferase. Thus, Wilde and Kuhn (1979) showed that mammary homogenates showed no loss of endogenous lactose synthetase activity when prepared from rats with reduced food intake (76% of control food intake) in which lactose synthesis *in vivo* has fallen considerably. In other words, factors other than the fall in lactose synthetase activity *in vivo* might, to some extent, explain the reduced rates of lactose synthesis. However, the fact that Wilde and Kuhn (1979) did not observe a reduction in the *in vitro* lactose synthetase activity might also be because the dietary restriction in their study was not as severe as in the study reported in this

thesis. As will be seen from Table 19, lactose synthetase activity was significantly reduced in the 6% milk protein group.

There is debate about which of the two proteins, α-lactalbumin or galactosyl transferase, is the major influence in the amount of lactose synthesised during normal lactation. Lactose synthetase activity has been shown to increase with the concentration of α-lactalbumin *in vitro* (Brew, 1970; Fitzgerald *et al.*, 1970b). Kuhn (1968) demonstrated that, when purified bovine α-lactalbumin was added to a lactose synthetase assay medium, a greater activity of lactose synthetase was observed at all times, including the low but definite activities found during the days before parturition. A positive correlation has been shown between lactose and α-lactalbumin concentrations in an intra-species study (Ley & Jennes, 1970). More recently, a significant positive correlation has been reported between the concentrations of α-lactalbumin and lactose for the first 20 days of lactation (Nicholas *et al.*, 1981). These workers also showed a significant negative correlation of the two components after the cessation of lactation at day 20. Moreover, Kulski *et al.* (1977) have also shown that α-lactalbumin concentrations fell after parturition from previous levels and lactose concentrations rose to higher levels four days after birth. It seems, therefore, as if the effect of α-lactalbumin concentration on lactose concentration in humans, goats, rats and possibly other mammals is only true during lactation and would not apply during involution or around parturition. A possible explanation for this observation, at least as far as lactose is concerned, is that the secretory cells are 'leaky' at around parturition, during involution or pregnancy. This means that the movement of lactose is via the paracellular or shunt pathway in which substances move between cells rather than through cells (Linzell & Peaker, 1973; 1974). This pathway is probably through the 'tight' junctions or zonulae occludentes, which connect neighbouring mammary cells. These

cell junctions are believed to be truly tight only during established lactation. This would mean, for example, that after birth in the rat the tight junctions will be truly tight and no more lactose will be lost to the extracellular fluid, leading to a rise in lactose concentration in the milk.

Wilde and Kuhn (1979) reported a 13-fold increase in the rate of lactose synthesis per gram of mammary tissue between parturition and day 16 of lactation in the rat; the increase in lactose synthetic activity reflecting a greater tissue content of galactosyl transferase.

Lactose synthetase activity is known to be under hormonal control. Studies using mouse mammary tissue culture have shown that insulin alone brings about cell division but that the daughter cells remained undifferentiated. Insulin and cortisol bring about cell division and differentiation and, in the presence of insulin, cortisol and prolactin, acting synergistically, the synthesis of the two components of lactose synthetase, and hence secretion of milk occurred (Lockwood *et al.*, 1966; Turkington *et al.*, 1968c; Palmiter, 1969). High progesterone concentration in the blood, as is the case during pregnancy, inhibits both prolactin secretion and its effects on the mammary cells and therefore inhibits the synthesis of lactose synthetase (Brew, 1970). In Chapter 6, the results of the effects of various protein diets on the levels of insulin and corticosterone will be presented.

With regard to the effect of maternal nutrition on lactation, the evidence presented in Chapter 1 seems to suggest that malnutrition affects lactation by bringing about small changes in milk nutrient concentration but, more importantly, by a reduction in milk volume. As stated earlier, lactose is the main osmotic component of milk. The effect of malnutrition on milk volume may therefore be via the effect on lactose synthesis. Searching through the scientific literature, very little research appears to have been carried out on the effect of diet manipulation on the lactose synthetase activity or on the α-lactalbumin content of milk and the mammary gland.

The purpose of this study, therefore, was to measure α-lactalbumin concentration and lactose synthetase and galactosyl transferase activities to see whether the changes observed in lactational performance during malnutrition could be explained by alteration in the activity and amount of the constituents of the lactose synthetase enzyme.

The α-lactalbumin levels were determined in two different ways. Firstly, by an indirect method in which the percentage stimulation of the lactose synthetase activity was determined in rat mammary homogenates, upon the addition of a known amount of α-lactalbumin; secondly, by using a more direct method, in which an attempt was made to separate the various proteins present in milk, using high performance liquid chromatography (HPLC).

4.2 Materials and Methods

The diets fed were the same as described in Chapter 2 and the animals were treated in the same way.

On day 15 of lactation, the dams were killed by decapitation and the abdominal mammary tissue rapidly removed and deep frozen. The mammary tissue was homogenised in cold 0.02 M Tris-HCl buffer, pH 7.4, containing 0.01 M $MnCl_2$ and 0.001 M β-mercapto-ethanol.

4.2.1 Enzyme assays

Lactose synthetase activity (E.C.2.4.1.22) and N-acetylglucosamine galactosyl transferase (E.C.2.4.1.38) activity were measured in whole tissue homogenates, as described by Vonderhaar (1977). The principle of the technique is outlined below :

(i) Galactosyl transferase activity :

$$\text{UDP-[}^{14}\text{C]-galactose} + \text{N-acetylglucosamine} \xrightarrow[\text{Cofactors: } MnCl_2 + ATP]{\text{Galactosyl transferase}} \text{N-acetyl lactosamine} + \text{UDP}$$

(ii) Lactose synthetase activity :

$$\text{UDP-[}^{14}\text{C]-galactose + glucose} \xrightarrow[\text{Cofactors: } MnCl_2 + ATP]{\text{Lactose synthetase}} \text{Lactose + UDP}$$

In essence, the reaction mixture, which contained the UDP-[^{14}C]-galactose (Amersham International) and cofactors, was mixed with the tissue homogenate and an appropriate galactose acceptor (glucose for lactose synthetase assay and N-acetyl-glucosamine for galactosyl transferase assay), or water to obtain a correction factor for non-enzymic hydrolysis of the radioactive UDP-galactose. The mixture was incubated at 37°C for 30 minutes and the reaction was stopped by cooling in ice and addition of ice-cold water. The mixture was poured into a Dowex column (Anion exchange resin, 1 x 8-200), in which unreacted UDP-[^{14}C]-galactose was adsorbed and [^{14}C]-labelled lactose (or N-acetyllactosamine as the case may be) was eluted. The column was washed with unlabelled lactose to ensure that all the radioactive product was eluted. Enzyme activity was expressed as nmol product formed per 30 minutes per mg protein at 37°C.

4.2.2 Effect of exogenous bovine α-lactalbumin on lactose synthetase activity

The effect of added bovine α-lactalbumin (10 mg/g tissue) on lactose synthetase activity was measured to find out whether any decrease in lactose synthetase activity observed in mammary tissue homogenates was due to a deficiency of the B protein of the enzyme complex.

4.2.3 Quantitation of milk α-lactalbumin by HPLC

High performance liquid chromatography (HPLC) was used to attempt to identify and quantify the α-lactalbumin present in milk and also to see how the concentration was affected by the various dietary conditions. The procedure involved the use of various buffers, with different ionic strengths and pHs to determine the appropriate conditions to separate milk proteins.

An HPLC (Varian model 5,000) apparatus, together with a protein analysis column (model I-125 Waters Associates, Massachusetts, USA) was used.

The optimal conditions obtained for separation were : 20 mM sodium citrate buffer, pH 6.0, flow rate of 0.5 ml/min and absorbance set at 280 nm. The citrate buffer was prepared, using glass distilled water. The buffer was filtered to remove particulate matter and degassed before use. Milk (or serum) from each sample was diluted (1 : 25 with buffer) and 100 µl put into the HPLC column. Traces of the spectra of each sample were made by a recorder. Examples of the separation of peaks obtained are shown in Fig. 7.

Since the α-lactalbumin peak could not be distinguished from a number of peaks in the milk spectrum resulting from the HPLC separation, an attempt was made to identify this peak by single radial immunodiffusion, as described by Hudson and Hay (1980), using fractions corresponding to the different HPLC peaks.

4.2.4 Procedure for radial immunodiffusion

Agar (2% w/v; Oxoid) in barbitone acetate buffer (0.082 M, pH 8.2; Oxoid) was boiled and cooled to 56°C. Antiserum to purified bovine α-lactalbumin (Sigma), raised from rabbits, was diluted (1 : 10 phosphate buffered saline) and warmed to 56°C. The two solutions were then mixed (1 ml diluted serum per 1 ml agar). This mixture was poured into petri dishes and allowed to set. A hole of about 8 mm diameter was punched at the centre of the agar and an aliquot from the eluates from each HPLC peak was carefully put into the hole. This procedure was repeated, using different concentrations of the antiserum (1 : 20, 1 : 50, 1 : 100. 1 : 500 and 1 : 1000 dilutions). The petri dishes were covered, put in a container with moist cotton wool and left in a 37°C room for 48 h.

4.3 Results and Discussion

4.3.1 Lactose synthetase and galactosyl transferase activities

The results are presented in Table 19 and have already been reported by Mansaray and Grimble (1983b; 1984). They showed

Table 19. Effects of diet on lactose synthetase and galactosyl transferase activities, and of exogenous α-lactalbumin on lactose synthetase activity.

Number of animals shown in parentheses

Diet	Lactose synthetase	Galactosyl transferase	Stimulation by α-lactalbumin (%)
20% milk protein(b)	28.9 ± 4.0	13.3 ± 2.3	8.9
10% milk protein(b)	24.2 ± 2.2	13.1 ± 1.0	13.5
6% milk protein(b)	14.5(a) ± 2.7	14.9 ± 1.4	56.0(a)
20% milk protein(b) (pair-fed)	21.1 ± 3.1	11.4 ± 1.2	4.8
20% cereal protein(b)	11.6(a,b) ± 1.6	12.3 ± 1.4	57.0(a,b)
10% cereal protein(b)	11.0(a) ± 1.8	11.7 ± 1.6	66.1(a)

Values significantly different from *ad libitum* 20% milk protein group: a, $p < 0.05$.

Values significantly different from pair-fed group: b, $p < 0.5$.

Enzyme activity expressed as pmole product formed/30 min/mg protein at 37°C

that lactose synthetase activity was significantly reduced in the 6% milk protein group and in the two cereal protein groups. The results therefore suggested that inadequate or poor quality dietary protein could have an effect on the lactose synthetase activity and therefore on the amount of lactose produced by the mammary gland.

However, the varying degrees of malnutrition used in the study had no effect on galactosyl transferase activity. Thus, the reductions in lactose production, observed under these conditions and reported in Chapter 5, were not due to changes in the galactosyl transferase part of the lactose synthetase complex. Reduced activity must have been due, therefore, to changes in α-lactalbumin. This possibility was confirmed by the stimulatory effect of exogenous α-lactalbumin in the cereal protein and 6% milk protein groups.

The substantial increase in the percentage stimulation of the lactose synthetase activity in groups where these activities were low would suggest therefore that the reduction in the lactose synthetase activity, caused by inadequate dietary protein, could be due to a decreased production of the α-lactalbumin part of the lactose synthetase complex. The observation that galactosyl transferase activity is unaffected by the dietary changes further strengthens this conclusion. Jones and Cowie (1972) also came to the same conclusion that the α-lactalbumin concentration was the factor contributing to the decreased lactose synthetase activity which they observed in rabbits after hypophysectomy. It follows, therefore, that although Kuhn *et al.* (1980) had suggested that the rise in lactose production seen in normal lactation is due to an increase in galactosyl transferase concentration in mammary gland, the response is not true when lactose production is reduced by malnutrition.

4.3.2 Measurement of α-lactalbumin

An attempt to obtain a direct measurement of mammary cell α-lactalbumin concentration via the concentration of this material in milk by HPLC proved unsuccessful.

A number of protein peaks appearing in milk are serum proteins. Thus, the separation of serum proteins was to help eliminate those peaks which were not α-lactalbumin. The concentrations of these proteins in milk and plasma are usually the same. Spectra of two milk samples from the 20% milk protein and 20% cereal protein groups, respectively, are presented in Fig. 7. The first three peaks on these spectra were probably casein peaks because, when the supernatant of a pH 4.6 precipitation was run in the HPLC column, those three peaks were eliminated (Casein is known to precipitate at pH 4.6). Running bovine α-lactalbumin in the HPLC did not help identify rat α-lactalbumin because the bovine α-lactalbumin peak did not superimpose on any of the peaks from rat milk. Moreover, there was no single peak from all the rat milk samples which seemed to correlate with the earlier indirect measurement of α-lactalbumin in mammary gland.

The single radial immunodiffusion technique was employed to see whether any immunoprecipitation would occur between bovine anti-α-lactalbumin and any of the eluates from the various milk protein peaks. No precipitation was observed, even when a wide range of concentrations of the anti-serum was used. The reason for this, of course, was that bovine anti-α-lactalbumin does not cross react with rat α-lactalbumin. Unfortunately, the immunoprecipitation reaction was attempted before Schmidt and Ebner's paper (1971) was found in the literature. This paper showed that antisera to ruminant α-lactalbumin does not cross react with non-ruminant α-lactalbumin like the rat's. As a result, therefore, the direct measurement of rat α-lactalbumin could not be achieved. However, the optimal conditions for the separation of milk

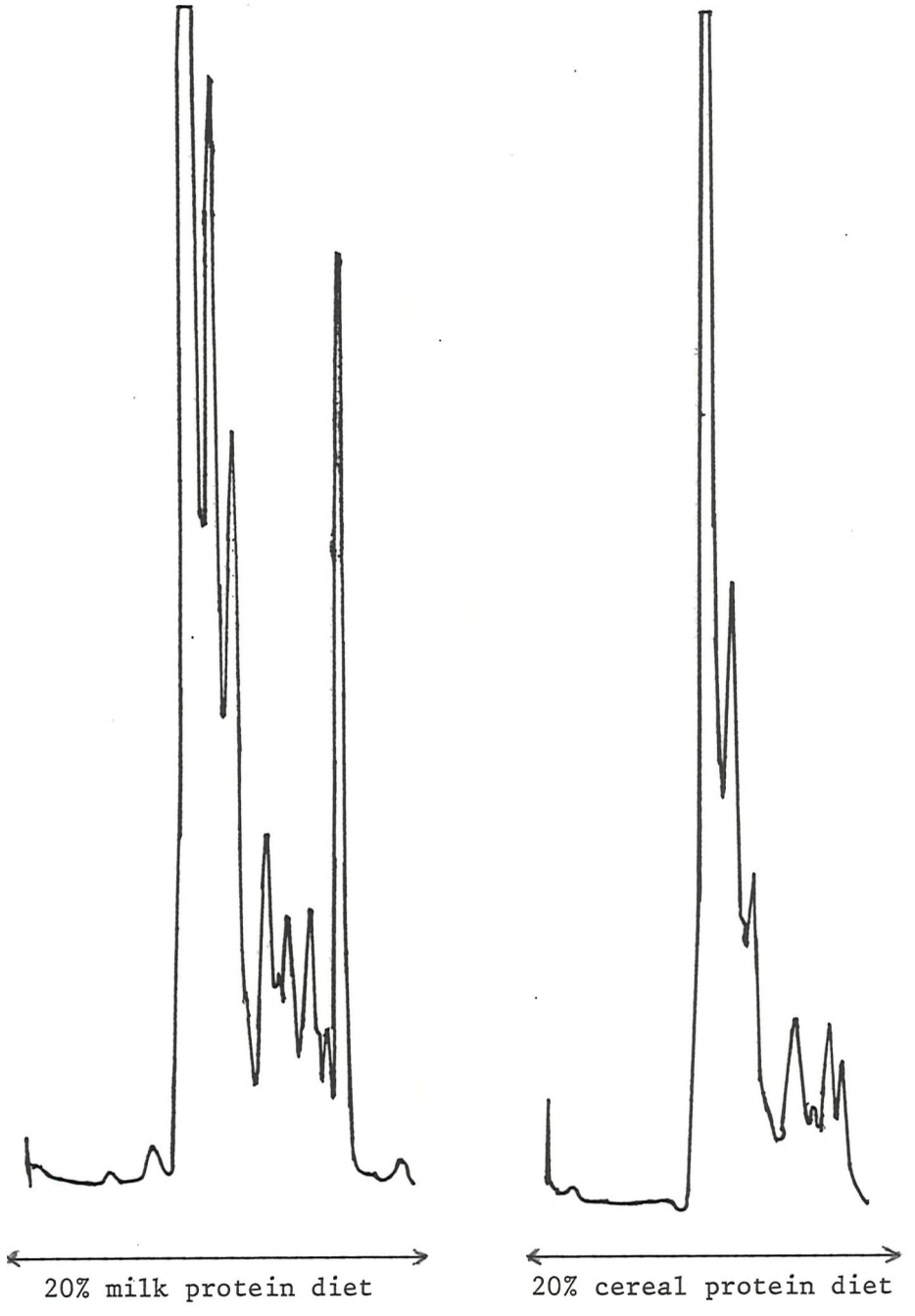


Fig. 7. HPLC trace of separation of protein in milk of rats fed 20% milk protein or 20% cereal protein diets.

proteins by HPLC were established. Further attempts to identify rat α-lactalbumin could therefore find this separation technique useful.

CHAPTER 5

EFFECTS OF PROTEIN QUALITY AND QUANTITY ON RAT MILK VOLUME AND COMPOSITION

CHAPTER 5

EFFECTS OF PROTEIN QUALITY AND QUANTITY ON RAT MILK VOLUME AND COMPOSITION

5.1 Introduction

There are substantial differences in the relative amounts of components between rat and human milk. Rat milk has a higher lipid and protein content and a lower lactose content than human milk; typical values are shown in Table 20. In addition, at peak lactation, the rat produces 115-140 ml of milk per kg body weight, compared to 12-23 in humans. This means that, for its size, the nutritional demands for lactation are considerably higher in the rat than in the human. It is likely, therefore, that dietary changes will have more pronounced effects on the composition and rate of secretion of milk in the rat.

In this chapter, the effects of the various diets on the volume and composition of rat milk are examined. The milk components measured were :

(i) Lactose, which is the main milk carbohydrate.

(ii) Total protein.

(iii) Non-protein nitrogen.

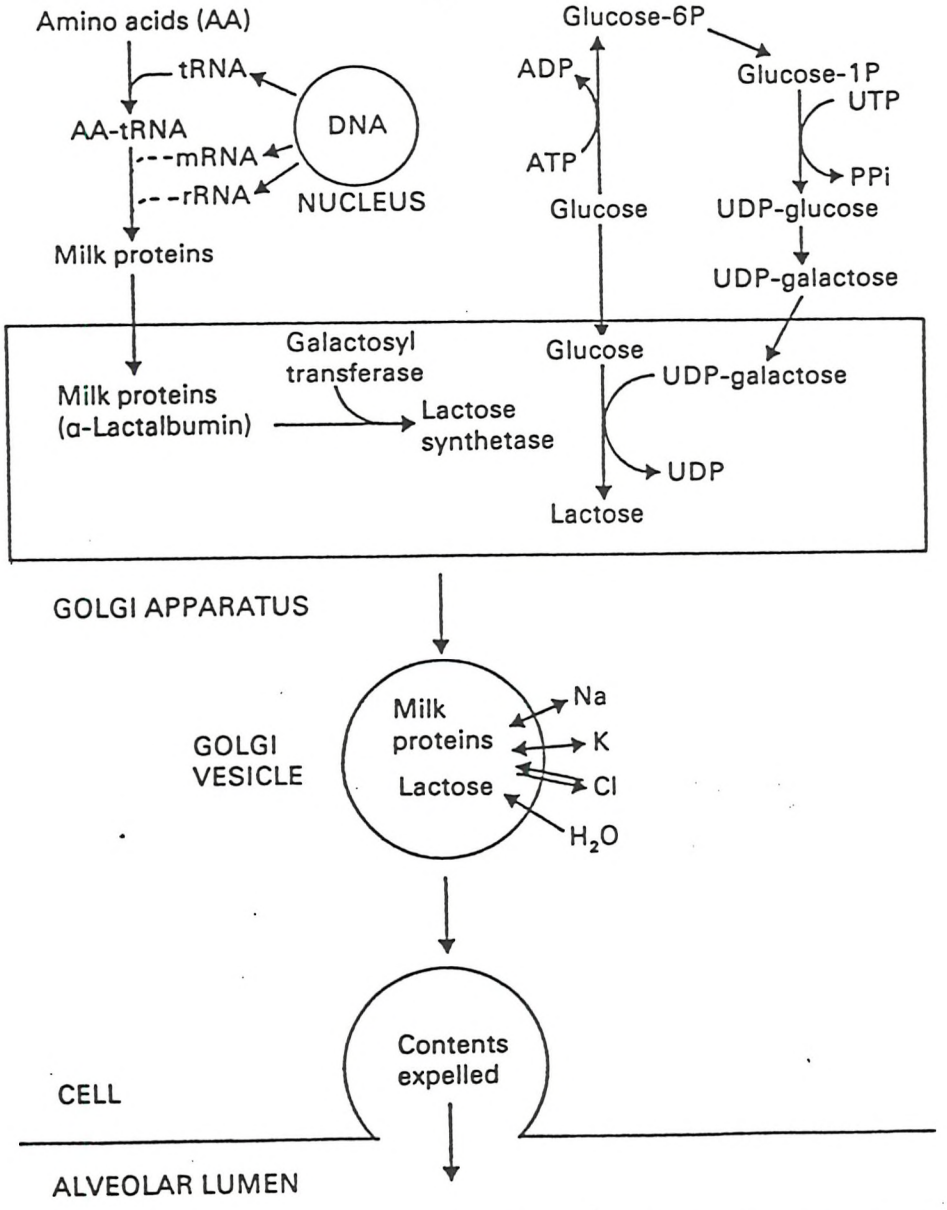
It must be mentioned that, although milk fat was not examined in the present study, this component is, quantitatively, the most important in milk because it supplies most of the energy requirements of the pups. Like in humans, poor nutrition would reduce both the milk volume and its fat concentration.

5.1.1 Mammary gland metabolism

Fig. 8 shows the general pathways for the synthesis of milk nutrients in the secretory cells of the mammary gland. Milk proteins, mainly caseins, α-lactalbumin and lactoferrin, are synthesised from amino acid precursors extracted from the blood. These proteins are extruded into the lumen of the

Table 20. Representative values for macronutrient composition and daily yield of human and rat milk.

Macronutrient and milk yield	Human	Rat
Lactose (g/ℓ of milk)	70	26
Lipid (g/ℓ of milk)	38	103
Protein (g/ℓ of milk)	10	84
Daily yield of milk (ml/24 h)	700	40



A schematic outline of the processes for the synthesis and secretion of milk protein and lactose in the secretory cells of the mammary gland. From Thomas, P.C. (1983).

Fig. 8.

endoplasmic reticulum, and passed on to the Golgi apparatus where they are accumulated. The Golgi apparatus is also the site for lactose synthesis, since the galactosyl transferase component of the lactose synthetase system is situated here. Lactose synthetase, as has already been explained in Chapter 4, is the last and rate-determining step in the pathway by which glucose is converted to lactose. Both milk proteins and lactose are secreted from the lactating cell into the alveolar lumen by the same route. Non-milk-specific proteins, like serum albumin, pass directly into the lumen from the blood.

The total production of each milk nutrient is determined by its concentration and the milk volume. The volume is determined by the water content. The water content, in turn, is regulated by the milk osmotic components, such as sodium, potassium and chloride ions and lactose. As has already been mentioned in Chapter 4, under most circumstances, lactose yield can be regarded as the determinant of milk water and therefore milk volume.

Under starvation conditions, the lactating rat is faced with two choices: either to continue to produce milk at a normal rate, with the consequent danger of complete depletion of its carbohydrate and fat reserves, or, alternatively, to decrease lactation, possibly losing its young, but maintaining some nutrient reserve. It appears that survival of the mother, rather than the pups, could be more important. As the rat has a high fertility rate, this is a sensible choice.

Alteration of the nutritional status of lactating rats can have profound effects on the metabolism of the mammary gland. Starvation of lactating rats for 24 h results in a dramatic decrease in the glucose uptake by the mammary gland, as measured by arteriovenous differences, without any significant change in the arterial glucose concentration (Robinson & Williamson, 1977; 1978). This change in glucose

utilisation is accompanied by output of lactate and pyruvate by the gland, whereas, in the fed state, these substrates are taken up by the gland. Mammary gland blood flow also decreases during starvation, so that the decrease in rate of removal of glucose by the gland is likely to be even greater than that indicated by the arteriovenous differences.

Experiments performed *in vitro*, using rat isolated alveoli, suggest that glucose metabolism in starvation is inhibited at the stage of pyruvate oxidation. This suggestion is confirmed by the finding that pyruvate dehydrogenase in mammary glands from starved rats is inactivated (Krankel & Reinauer, 1976; Baxter & Coore, 1978). The changes resulting from starvation, together with decreased lactose synthesis (Carrick & Kuhn, 1977), may be part of an overall mechanism by the gland to spare body glucose supplies, when glucose availability is limited (Williamson & Robinson, 1977).

Although none of the rats on the experimental diets in the present study were suffering from starvation, some, like the cereal groups, had significantly reduced food intakes. Therefore, the effects resulting from starvation, that have been described above, might to some extent be experienced by some of these groups. It is also possible that rats on an inadequate dietary protein diet, in terms of quality and quantity, would bring about alterations in the metabolism of the mammary gland that would result in a reduction in the volume and components of the milk which they produced. Lunn, Whitehead and Baker (1976) demonstrated that, when male rats were fed either a control diet or a low protein diet in isoenergetic amounts, the total concentration of free amino acid in the plasma, liver and muscle were unchanged, although there were large changes in the individual amino acids. Essential amino acids were disproportionately depleted in the tissues of rats on the low protein diet, whereas the concentrations of non-essential amino acids were increased.

This, of course, would result in a reduction in the amount of protein synthesised in various tissues. This effect will be more intensely expressed in tissues like the mammary gland, which have a fast rate of protein synthesis. This is, indeed, the case, as can be seen from the protein synthesis results in Chapter 3.

It was important in the present study to examine the effects of the various diets on the volume and composition of rat milk at identical stages of lactation, because it is known that these parameters have a developmental pattern. The concentrations of some milk macronutrients vary with stage of lactation, as reported by Keen *et al.* (1981).

5.1.2 Milk protein and non-protein nitrogen content

Keen *et al.* (1981) showed that the protein concentration in rat milk is stable during the first week of lactation, followed by a small increase during mid-lactation and a small decrease during late-lactation. This variation of milk protein concentration with the stage of lactation has also been observed in human studies. Colostrum has the highest protein concentration, followed by transitional milk and mature milk, which have a decreasing protein concentration with time.

While early work indicated that the protein content of rat milk was not affected by dietary protein intake (Mueller & Cox, 1946), recent work has suggested that its concentration may be lower than normal in rats fed a protein-deficient diet (Crnic & Chase, 1978). Moreover, a more recent report demonstrates that maternal dietary protein intake can affect the protein concentration of human milk (Forsum & Lonnerdal, 1980), in contrast with the views expressed in earlier reports (Lonnerdal *et al.*, 1976).

In addition to milk protein, milk also contains nitrogen in other forms known collectively as non-protein nitrogen (NPN). The non-protein nitrogen in milk would include

compounds like free amino acids, small peptides, urea, hypoxanthine, creatinine, creatine and uric acid. The relative amounts of free amino acids in human milk is much larger than that in, for example, cow's milk, being 5% and 0.8%, respectively (Svanberg *et al.*, 1977). Although free amino acids constitute only a minor proportion of the total amino acid content of human milk, and thus contribute very little to the protein quality of milk, they may serve other important functions, such as zinc absorption across the intestine (Wapnir *et al.*, 1983). Differences in free amino acid composition are apparent between species; for example, taurine, only a minor component in cow's milk, is the second most abundant amino acid in human milk, and is considered to be essential in early infancy (Sturman *et al.*, 1977).

Harzer *et al.* (1984) have reported that the total non-protein nitrogen content of milk is not affected by diet, although Svanberg *et al.* (1977) have also reported that well-nourished Swedish mothers had a significantly lower milk NPN concentration than that of poorly-nourished Ethiopian mothers.

5.1.3 Milk carbohydrate content

With regard to carbohydrate in milk, Kuhn (1972) identified two forms of lactose in the rat, that is, free lactose and the trisaccharide N-acetylneuraminyl lactose (or neuraminlactose), which would not have the same osmotic effect as the free lactose weight for weight. He demonstrated that neuraminlactose comprised over 30%, on a molar basis, of the total milk carbohydrate by day four of lactation, although by day fifteen it was barely detectable (Chalk & Bailey, 1979). It would be assumed, therefore, that the neuraminlactose component did not affect the lactose measurement in the present study, since the measurements were carried out on milk from rats on their fifteenth day of lactation. As far as the effect of diet on lactose is concerned, it has been shown that a reduction in maternal food intake results in decreased concentrations of this component in rat milk (Mutch & Hurley, 1974) but not in that of humans (Lonnerdal *et al.*, 1976).

5.2 Materials and Methods

The diets, the animals and the treatments which they received were the same as described in Chapter 2.

5.2.1 Milk volume measurements

Milk volume was measured on the fourteenth day of lactation by a modification of the method described by Rath and Thenen (1979), in which the accumulation of tritiated water (^3H_2O) in pups is measured following the administration of ^3H_2O to their dams. The dams were injected intraperitoneally with 0.2 ml of ^3H_2O (10 mCi/ml). Immediately after the injection, the pups were separated from their dams and the dams' drinking water replaced with tritiated water (17 mCi/ml). The pups were replaced after one hour and allowed to stay with the mother for 24 h. Two pups from each dam were then killed, homogenised (ultraturax homogeniser) in ice-cold distilled water and the homogenate made up to 500 ml. An aliquot was centrifuged and a known volume of the supernatant (400 µl) was counted for [^{3}H] in tritoscint scintillation fluid. Blood was obtained from the dam by making a small slit in the tail, collected in heparinised capillary tubes, centrifuged and 4 µl of the plasma counted for [^{3}H] in tritoscint. Milk volume was calculated from the radioactivity of the dam's plasma and the pup's homogenate and volume. A flow diagram of the method described is shown in Fig. 9.

This method assumes that all the radioactivity in the pup comes from milk. It also assumes that all or most of the radioactivity in the pup stays in body water. The validity of the latter assumption was tested by estimating the radioactivity that was lost in the urine of pups or incorporated into body lipid.

Urine was collected by gently squeezing the pup's abdomen with moist cotton wool and the absorbed radioactivity counted. To estimate the radioactivity incorporated into fatty acids, pups were sacrificed and the alimentary tract and bladder removed. The alimentary tract was emptied and returned to the carcass. Carcasses were dried in an oven at 105°C for 48 h to remove all water. The dry carcass was minced with three times its weight

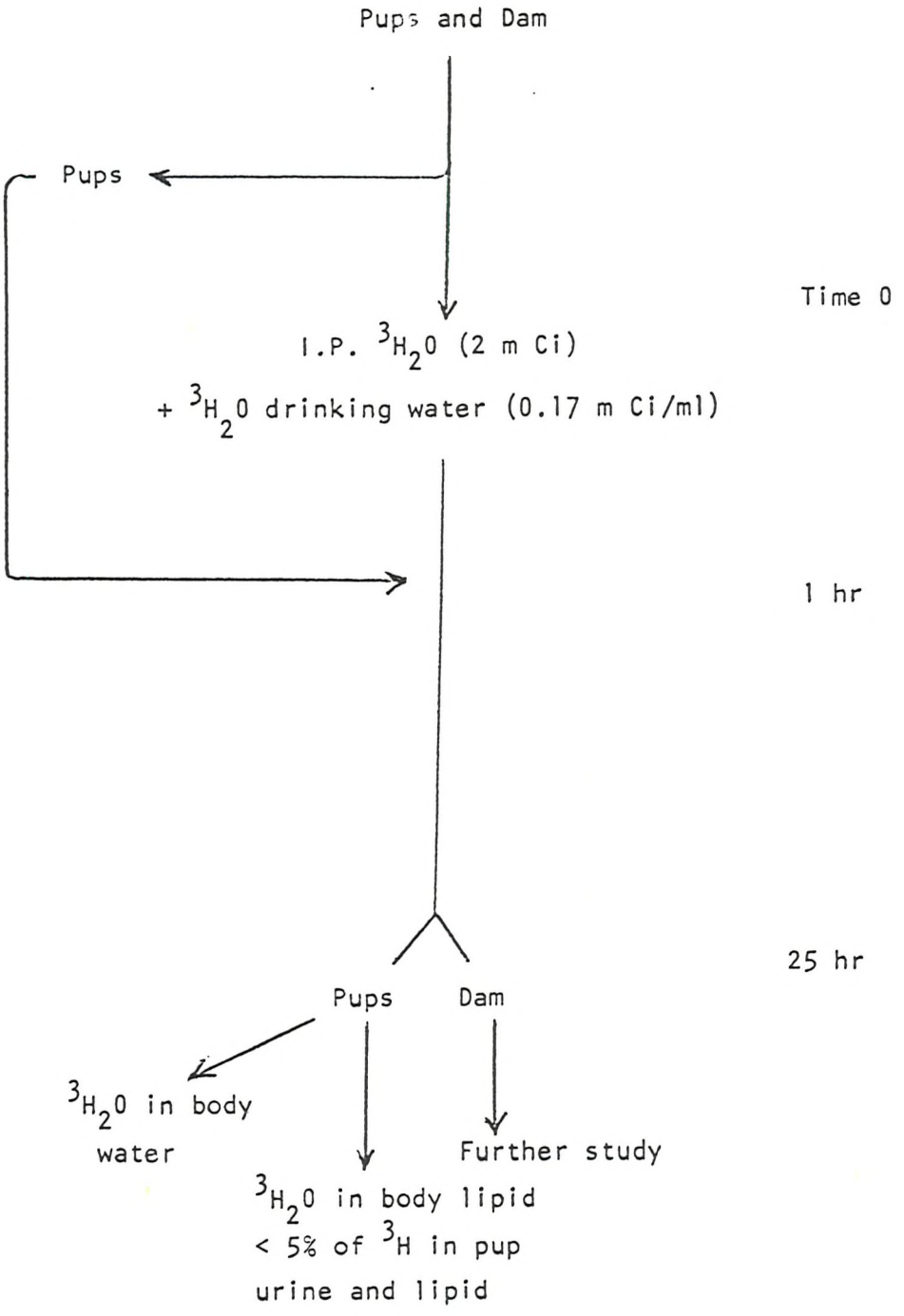


Fig. 9. Milk volume measurement.

of anhydrous sodium sulphate, the fat extracted with chloroform : ethanol (2 : 1) and the radioactivity in the fat counted (Miller *et al.*, 1979).

The milk volume measurement technique also assumes that all or most of the radioactivity in the dam's milk is present in milk water. The validity of this assumption was tested by determining the radioactivity of an aliquot of milk before and after it was heated to dryness to remove all water. The results are shown in Table 21.

The results show that the technique used for the milk volume measurements, as described in this thesis, was valid since each pup lost less than 1% of the radioactivity from body water into the urine, about 2% into carcass fat and there was less than 5% in milk solids.

5.2.2. Milk protein, non-protein nitrogen and lactose measurements

5.2.2.1 Lactose

Protein was precipitated from a known volume of milk, in a glass centrifuge tube, using an equal volume of 1.0 M TCA. After thorough mixing, protein was separated by centrifugation. For the lactose assay, the supernatant was neutralised with 1.0 M sodium hydroxide and lactose determined by an enzymic method using a Boeheringer kit. The principle of this assay involves the hydrolysis of lactose to glucose and β-galactose by the enzyme β-galactosidase. Beta-galactose is then oxidised by nicotinamide adenine dinucleotide (NAD) to galactonic acid and NADH by the enzyme β-galactose dehydrogenase (Gal-DH) as follows :

(i) Lactose + H_2O $\xrightarrow{\beta\text{-galactosidase}}$ Glucose + β-galactose

(ii) β-galactose + NAD^+ $\xrightarrow{\text{Gal-DH}}$ Galactonic acid + NADH

The amount of NADH formed in reaction (ii) is stoichiometric with the amount of lactose. The increase in NADH is measured by means of its absorbance at 365 nm.

Table 21. Demonstration of the validity of milk volume technique.

Distribution of $^{3}H_2O$	
Pup carcass :	
Total (dpm x 10^{-7})	4.21 ± 0.77
Percentage of total $^{3}H_2O$ in pup	97.76 ± 0.07
Pup fat :	
Total (dpm x 10^{-5})	9.35 ± 1.63
Percentage of total $^{3}H_2O$ in pup	1.98 ± 0.06
Pup urine :	
Total (dpm x 10^{-5})	1.06 ± 0.28
Percentage of total $^{3}H_2O$ in pup	0.25 ± 0.03
Milk solids :	
dpm x 10^{-3}/µl milk	1.32 ± 0.13
Percentage of total in milk	4.45 ± 0.48

5.2.2.2 Total non-protein nitrogen (NPN)

Non-protein nitrogen was determined in the supernatant from the TCA precipitation by the ninhydrin reaction (Yemm & Cocking, 1955).

5.2.2.3 Total protein

Total protein was measured from the TCA precipitate, using the Folin Ciocalteau reagent, as described by Lowry *et al.* (1951).

5.2.3 Sucrose feeding

Since the rats on the cereal diets had reduced food intakes and lactated poorly (Tables 11, 22 and 23), it was reasonable to assume that lactation might be improved by increasing the energy intake while the protein intake remained unchanged. The way in which this was achieved was to replace the dam's drinking water with a 10% sucrose solution. The control for this experiment was a group of lactating dams receiving a 20% cereal protein diet and water. An additional group, receiving a 20% milk protein diet and water, was included to examine whether seasonal variations in lactational performance occurred in well-fed animals. These animals are referred to later as 'standards'. The milk volume and composition of these rats were determined using the methods described above.

5.3 Results and Discussion

5.3.1 Milk protein, NPN and milk water content

Daily total protein production and concentration is shown in Table 22, together with NPN concentration and milk water content.

5.3.1.1 Milk protein concentration

While the protein concentrations of the rats on the milk protein diets are not significantly different from one another, the values for the cereal groups are significantly reduced. At the same time, it is also seen that the pair-fed

Table 22. Effects of diet on milk protein, non protein nitrogen and milk water (Mean ± SEM).

Number of animals shown in parentheses

Diet	Protein (g/ℓ)	Total protein (g/dam/d)	Non-protein nitrogen (g N/ℓ)	Milk water (ml/ℓ)
20% milk protein(6)	88.1 ± 7.0	4.65 ± 0.35	0.232 ± 0.03	777.9 ± 9.2
10% milk protein(6)	90.8 ± 7.2	4.04 ± 0.38	0.203 ± 0.012	742.3 ± 14.6
6% milk protein(6)	98.0 ± 10.6	2.28 ± 0.27[d]	0.191 ± 0.025	678.8 ± 28.5[b]
20% milk protein(6) (pair-fed)	145.0 ± 6.4	5.08 ± 0.41	0.195 ± 0.016	627.1 ± 14.1[d]
20% cereal protein(6)	23.3 ± 3.7[d,f]	0.53 ± 0.13 [d,f]	0.409 ± 0.022[c,f]	724.8 ± 14.9 [b,f]
10% cereal protein(6)	27.1 ± 6.2[d]	0.64 ± 0.14[d]	0.368 ± 0.016[c]	758.4 ± 13.8[a]

Significantly different from 20% milk protein group:
a, $p < 0.05$; b, $p < 0.01$; c, $p \leq 0.005$; d, $p < 0.001$.

Significantly different from pair-fed group:
f, $p \ll 0.001$.

group has a significantly higher protein concentration than the control. This last observation falls in line with the results for the milk water (Table 22), which show that the pair-fed group had the least diluted milk, and also could be explained in part by the fact that there was an increased protein fractional synthetic rate in mammary tissue of the pair-fed group. It can also be seen that the protein synthetic rate of the mammary gland (Table 18) correlated very well with milk protein concentration (Table 22).

When the total amount of milk protein produced per day is examined, it can be seen that the pair-fed group had a slightly higher value than the control group, thus showing positive adaptation to reduced food intake where a good quality protein is present in the diet. This suggests that protein was not the limiting factor in the slightly reduced pup growth in the pair-fed group. The limiting factor was probably carbohydrate energy and hence glucose supplies to mammary tissue, as will be shown below by the reduced lactose content of milk. There could also have been a reduction in the milk lipid content. However, this was not measured in the present study.

5.3.1.2 Non-protein nitrogen concentrations

The results show that there is no significant difference between all the milk protein groups, but that values for the cereal groups were significantly higher. This observation finds a parallel in the study reported earlier in which the NPN content of human milk in well-nourished and poorly-nourished populations were compared (Svanberg *et al.*, 1977). The milk of poorly-nourished women had higher NPN contents.

There are two possible explanations for this phenomenon. Firstly, that the lower quality protein in the cereal groups resulted in incomplete use of the amino acids for milk protein or body protein synthesis. A greater amount of

dietary amino acids would therefore be available for gluconeogenesis, resulting in an enhanced production of urea. This urea could pass into milk and thus increase the NPN concentration. The second explanation could be that the proportions of amino acids extracted from the blood by the mammary gland, following a meal of cereal protein, would not be efficiently used for milk protein synthesis because of their imbalance (Table 12). Those not incorporated might pass unchanged into the milk.

These results therefore suggest that poor quality dietary proteins affect milk quality by significantly reducing total milk protein and increasing non-protein nitrogen concentration.

5.3.2 Milk lactose concentration and milk volume

Milk lactose content was determined, not only because lactose is an important milk component in terms of the energy it provides, but also because lactose, being a major osmotic component of milk, could have an influence on the milk volume. It has already been mentioned that, at least in humans, a poor diet affects milk volume more than any other parameter. A significant reduction in the milk volume obviously would result in a reduction in the total production of all the milk nutrients.

The results for the milk lactose content and volume are shown in Table 23. These results have already been reported by Mansaray and Grimble (1984). They show that lactose production was reduced in all groups which had reduced food intakes, irrespective of the protein quality or quantity. It has been pointed out earlier in this chapter that starvation reduces the amount of glucose extracted by the mammary gland for lactose and lipid synthesis. It is therefore possible that the reduced food intakes, observed in all the experimental groups, would have this effect to a lesser extent and thus bring about a reduction of the lactose content.

Table 23. Volumes and lactose concentrations of milk from rats on various protein diets (Mean ± SEM). Number of animals shown in parentheses.

Diet	Lactose concentration (g/ℓ)	Total lactose (g/dam/d)	Milk volume (ml/dam/d)	Pup growth (g/pup/d)
20% milk protein (6)	33.4	1.77	52.8	1.92
10% milk protein (6)	30.8	1.39	44.5(a)	1.57(a)
6% milk protein (5)	23.4(a)	0.54(a)	23.0(a)	0.65(a)
20% milk protein (pair-fed) (5)	24.1(a)	0.83(a)	34.7(a)	1.23(a)
20% cereal protein (6)	22.6(a)	0.44(a,b)	19.6(a,b)	0.64(a,b)
10% cereal protein (6)	21.8(a)	0.50(a)	23.0(a)	0.59(a)

Values significantly different from *ad libitum* 20% milk protein group: a, $p < 0.05$.

Values significantly different from pair-fed group: b, $p < 0.05$.

Protein quality and quantity also had some influence on lactose production. Comparison of the values from groups fed 6% milk protein and 20% pair-fed milk protein diets show that protein quantity affected total lactose synthesis. Comparison of the 20% pair-fed milk protein and 20% cereal protein groups shows that protein quality also had an effect.

The results also show that milk volume was reduced to a greater extent in the two cereal groups and the 6% milk protein group than in the pair-fed group, showing that quality and quantity of dietary protein have an influence on the milk volume. Table 23 also shows that the reduced total lactose production observed is caused by reductions in both volume and concentration. Lactational performance, that is pup growth, parallels milk volume.

5.3.3 Correlation of lactose synthetase and lactose concentration

In Chapter 4, it was stated that lactose synthetase was the rate-limiting enzyme in the synthetic pathway for lactose synthesis (Fig. 5). To obtain an indication of whether the reduced lactose synthetase activity that was obtained in some of the groups (Table 20) had any significance as far as actual lactose production by the rat, the daily lactose production was correlated with the lactose synthetase activity. Fig.10 shows that there is a highly significant correlation, suggesting that this is the case.

5.3.4 Correlation of lactose concentration and milk volume

The reductions in total lactose production were due partly to reduced milk volume and partly to reduced concentrations of this component. As lactose is an important osmotic component of milk, the reduced volumes observed could be related to lower lactose concentrations. This possibility was investigated by correlating the two variables. A highly significant positive correlation was found, suggesting that there could be a link between milk volume and lactose concentration in circumstances where lactose production is impaired by diet (Fig.11). It may also be

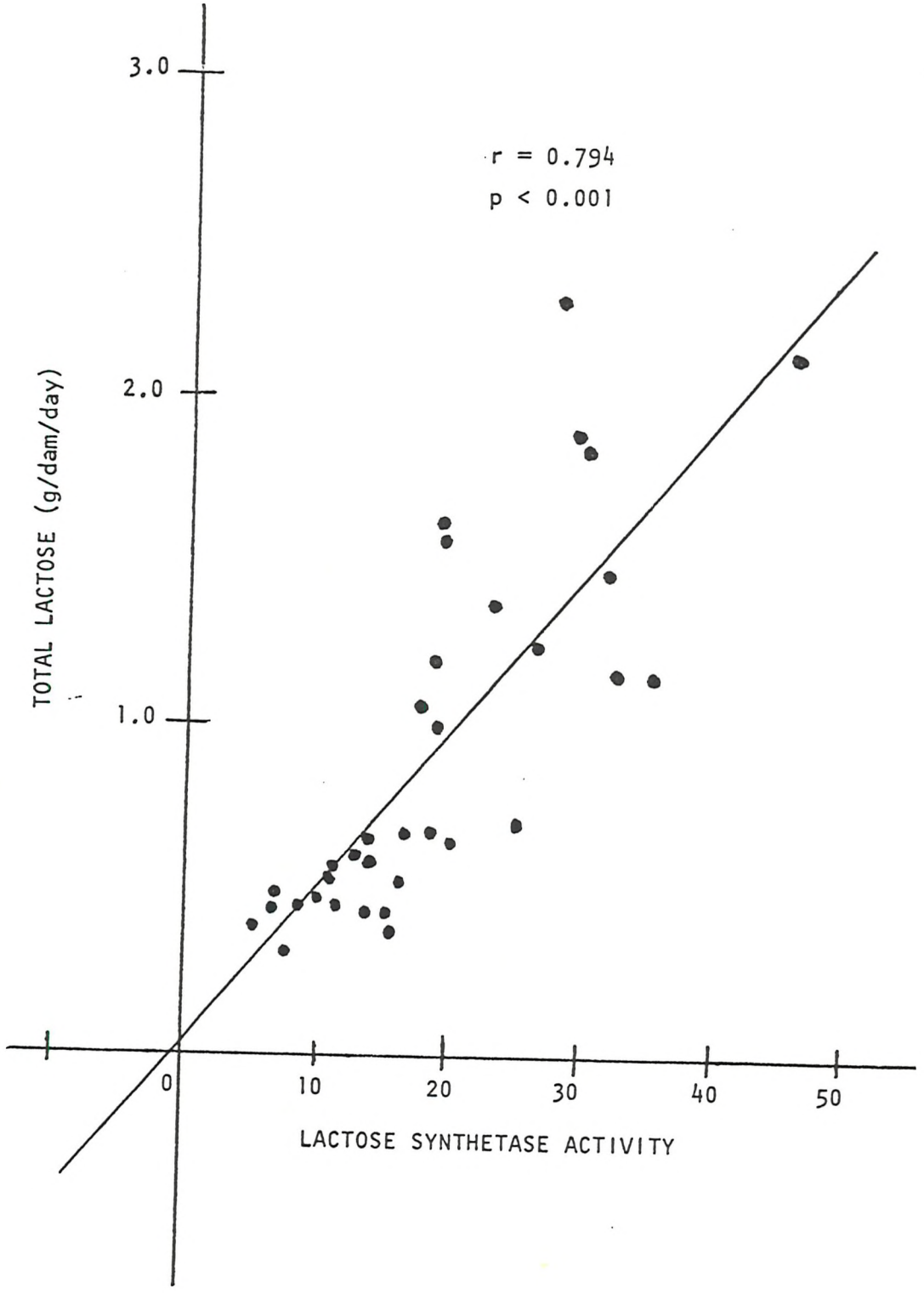


Fig. 10. Correlation between lactose synthetase activity and total lactose.

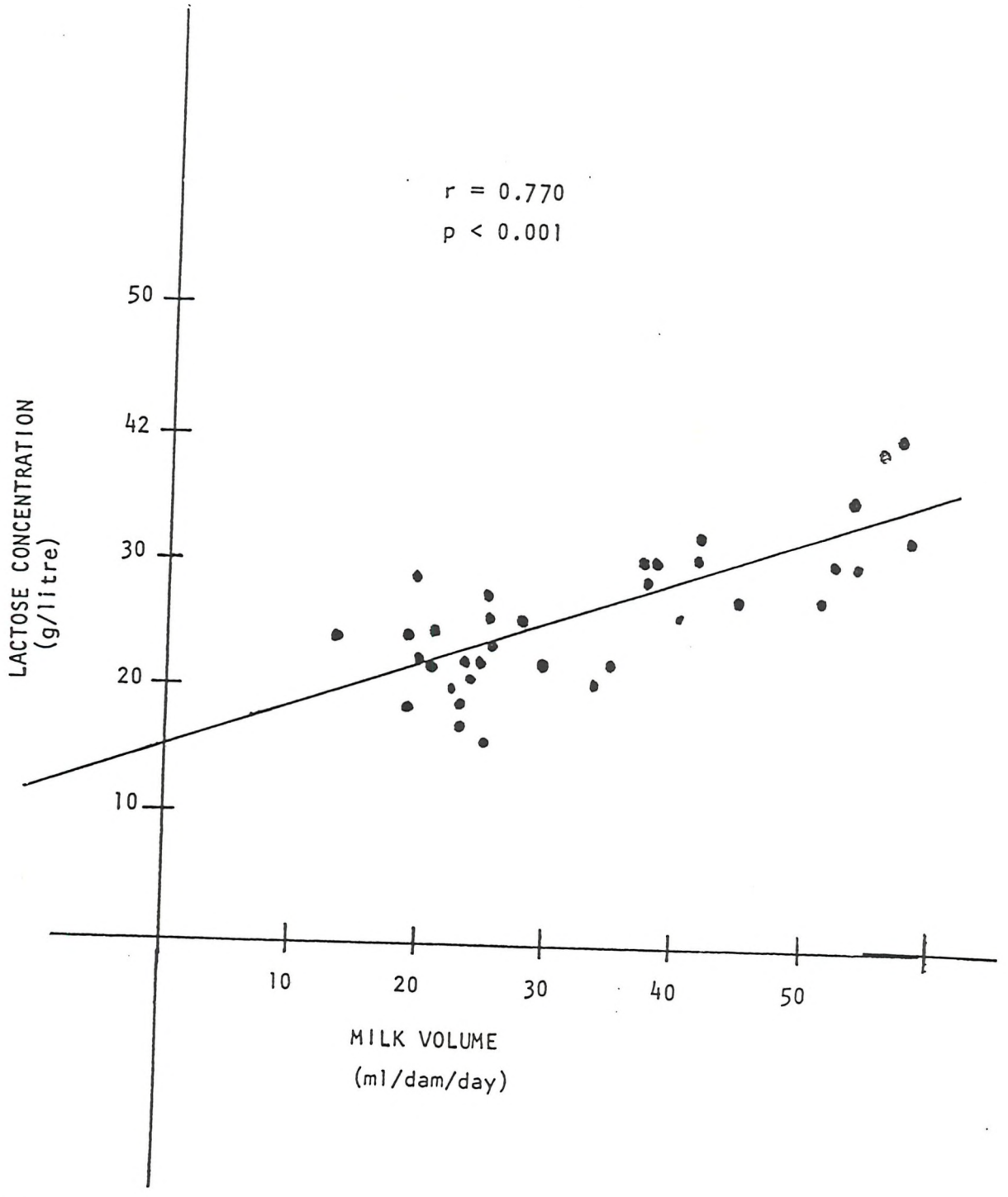


Fig. 11. Correlation between milk volume and lactose concentration.

concluded that changes in the activity of lactose synthetase, brought about by diet, may ultimately have an effect upon milk volume.

5.3.5 Effects of sucrose feeding on energy intake, pup growth, milk volume and composition in 20% cereal protein fed rats

The results are presented in Table 24 and they show that the rats receiving a 20% cereal protein diet and water consumed 335 kJ/day of food, while those on the 20% cereal protein diet given 10% sucrose solution consumed 356 kJ/day of total energy. The latter group took 314 kJ in the form of the food pellets and only 42 kJ in the form of sucrose. The protein intakes of the two groups did not differ by much.

There were no significant differences between the groups in any of the parameters measured. This observation did not come as a surprise, since the energy intakes of the two groups were virtually the same. In that respect, this series of experiments did not show what they were intended to demonstrate, which was to examine whether an increase in energy intake would improve lactational performance in animals receiving poor-quality dietary protein.

It was, however, observed in this supplementary study, that the lactational performance (pup growth) was slightly improved, when compared to the values in Table 11. This was probably due to slightly improved protein and lactose concentrations and milk volumes (compare Table 24 with Tables 22 and 23). It must be pointed out, however, that the rats on 20% milk protein diet, which were used as 'standards' in the sucrose feeding experiments, also had slightly improved lactational performance, and milk volume and composition. A possible reason for these small differences is that these experiments were carried out at different times of year, and thus imply that lactation shows seasonal variation. Improved lactational performance was observed when the experiments were carried out in early spring (February and March), whereas the lower lactational performance was observed in summer (June and July).

Table 24. Effects of sucrose feeding on energy intake, pup growth, milk volume and composition on rats given a 20% cereal protein diet.

Number of animals shown in parentheses

	20% cereal protein diet (6)	20% cereal protein + sucrose diet (6)	20% milk protein diet ('standard') (6)
Energy intake (kJ/d)	335	356	523
Pup growth (g/d)	0.7	0.8	2.0
Milk protein (g/litre)	40	38	116
NPN (mg/litre)	0.311	0.324	0.145
Milk volume (ml/rat/d)	24	23	67
Milk lactose (g/litre)	25.5	25.1	39.1

CHAPTER 6

EFFECTS OF DIETARY PROTEIN ON SERUM HORMONE CONCENTRATIONS

CHAPTER 6

EFFECTS OF DIETARY PROTEIN ON SERUM HORMONE CONCENTRATIONS

6.1 Introduction

In Chapter 1, it was mentioned that endocrine adaptations during lactation could maximize the use of the mother's available nutrients to support lactation. It was also mentioned that these adaptations could serve to buffer short-term variations in the maternal nutrient intake. The serum levels of some of the hormones that are believed to be important during lactation were therefore measured to see how they would be affected by variations in dietary protein quality and quantity.

It is generally accepted that milk secretion is initiated and maintained by the action of a number of hormones, which include prolactin, insulin, corticoids, glucagon and thyroid hormones. Oxytocin may influence the completeness of milk removal at the time of milking or nursing.

Hormones play a role in lactation, either by acting on the mammary gland directly, or by regulating the pool of metabolic precursors available for milk synthesis.

It has been suggested that variations in milk yield are due, in part, to variation of secretion of these hormones. For example, in situations where prolactin is secreted in less than optimal amounts, the milk yield is limited by the extent of the deficiency. If the administration of a hormone stimulates lactation in intact animals, it may be concluded that the endogenous hormone is being secreted in suboptimal quantities (Grosvenor & Turner, 1959a,b). However, in itself, such a response may be pharmacological and unrelated to the normal properties of the hormone. Despite this complication, experiments with intact animals have provided useful information concerning the hormonal control of lactation.

In the present study, serum levels of insulin and corticosterone were measured to see how they would be affected by the amount and quality of protein in the diet. An attempt was made to measure serum prolactin levels but this was not successful. Prolactin was also injected into lactating rats, receiving 20% milk protein and 20% cereal protein diets, to examine whether this would result in an improvement in lactational performance.

6.1.1 Role of insulin in lactation

The results of *in vitro* and *in vivo* studies have demonstrated that insulin is essential for maintenance of normal lactation (Martin & Baldwin, 1971a,b). However, the central anabolic role of insulin in intermediary metabolism makes it difficult to distinguish between those actions of the hormone directed at the mammary gland and those effects mediated via its influence on the overall metabolism. Much of the information on the role of insulin on lactation has been obtained, therefore, from *in vitro* experiments.

It is now generally believed that, during lactation, insulin appears to act in two ways (Baldwin & Louis, 1975). Firstly, it appears to be essential for the maintenance or survival of mammary alveolar cells of non-ruminants, like the rat. If low circulating levels of insulin are produced and maintained by treatment of rats with alloxan, or rats are injected with anti-insulin serum, there is a fall in the number of alveolar cells (Walters & McClean, 1968). Secondly, insulin exerts a short-term acute action on secretory cell metabolism. Insulin insufficiency will result in :

(i) decreased oxidation of glucose by the pentose phosphate pathway;

(ii) decreased synthesis of fatty acids from glucose;

(iii) decreased synthesis of lactose, fat and casein.

Isolated mammary cells from pregnant mice have been used to demonstrate insulin-stimulated transport of the non-metabolisable amino acid, α-amino isobutyric acid (AIB) (Oka & Topper, 1972). Stimulation of RNA synthesis has also been demonstrated after a 2 hour period of incubation of these cells with insulin (Turkinton, 1970).

In non-lactating females, the effect of insulin on mammary tissue is different. Insulin does not stimulate the growth of mammary tissue in mature virgin rats. Moreover, organ explants from virgin animals do not demonstrate insulin-stimulated amino acid transport or DNA synthesis before 24-48 h in culture (Friedberg *et al.*, 1970). Similarly, isolated mammary cells from virgin mice do not show insulin-stimulated amino acid transport (Oka & Topper, 1971). Since pregnancy and prolactin administration are associated with the development of responsiveness to insulin by mammary cells, it has been suggested that prolactin may sensitize the cells to insulin (Krug *et al.*, 1972).

Daily subcutaneous insulin injections into rats between the 5th and 17th days of lactation resulted in increased milk yield and lactose, which was attributed to a general increase in the synthetic capacity of the mammary gland, as indicated by increased mammary DNA (Raskin *et al.*, 1973). More recent studies have, however, shown that there appears to be no consistent relationship between rates of lactose synthesis *in vivo* and plasma insulin concentrations (Carrick & Kuhn, 1978; Wilde & Kuhn, 1979) and that injection of insulin does not restore lactose synthesis depressed by short-term (6 h) food withdrawal (Carrick & Kuhn, 1978). Moreover, rats treated with alloxan, at about the time of lactogenesis, showed no decrease in mammary gland lactose content, despite low insulin concentrations (Kyriakou & Kuhn, 1973). Insulin injections, however, are known to reverse *in vivo* the marked inactivation of pyruvate dehydrogenase or the decreased lipogenesis associated with starvation.

Despite the fact that insulin is essential during lactation, most of the evidence in the scientific literature suggests that serum insulin concentrations are generally lower in lactating than in non-lactating animals (Agius *et al.*, 1979; Flint *et al.*, 1981). Indeed, high-yielding dairy cattle have lower serum insulin concentrations than low-yielding animals (Hart *et al.*, 1978). This may be due to the fact that insulin is a major lipogenic hormone in adipose tissue; thus, high concentrations result in preferential utilisation of lipid precursors by non-mammary tissues, with a resultant decrease in milk yield (Hove, 1978). The presence of lower insulin concentrations during lactation is beneficial because, at parturition, the number of insulin receptors on adipocytes decreases (Flint *et al.*, 1979), whilst the number increases on mammary epithelial cells (O'Keefe & Cuatreacasas, 1974). Both of these changes appear to be the result of the decrease in serum progesterone at the end of pregnancy (Flint, 1982). The decreased insulin concentration during lactation would mean that the uptake of nutrients would be maintained at a high level in the mammary gland but at lower levels in other tissues like the adipose tissue.

The low plasma insulin levels are maintained during lactation by high insulin receptor numbers. In other words, the mammary gland acts as a 'sink' for insulin. The fact that the lactating mammary gland is a major site for insulin and glucose uptake was demonstrated by the finding that, when pups were removed from their mother for about 24 h, the blood glucose increased by 0.6 mmol/ℓ and the plasma insulin rose three-fold (Agius *et al.*, 1979). This finding also implies that suckling is important in maintaining a high number of insulin receptors on mammary tissue.

Despite the fact that most investigators have reported lower insulin concentrations during lactation, a few papers have demonstrated a significant increase in blood insulin levels during lactation (Sutter-Dub *et al.*, 1974; Grimble, 1981). Grimble (1981)

also reported a significant decrease in insulin levels in rats fed a poor quality cereal protein diet, when compared with the control group fed a milk protein based diet. Sutter-Dub *et al.* (1974) suggested that, since progesterone antagonises the activity of insulin, the elevated insulin levels observed during lactation, despite normal glucose levels, are largely a consequence of the increased concentration of progesterone observed during lactation.

6.1.2 Role of corticosterone in lactation

Glucocorticoid administration has the ability to cause a high rate of glycogen synthesis in the liver (Long *et al.*, 1940). It is now clear that glucocorticoids may variously increase the availability of glucose by enhanced peripheral protein catabolism, increased gluconeogenesis and by the inhibition of peripheral glucose utilisation (Munck, 1971). However, deposition of glucose as glycogen results from activation of glycogen synthase and inactivation of glycogen phosphorylase (Horn-Brook *et al.*, 1966; Von Holt & Fister, 1964).

Hormonal regulation of gluconeogenesis may be exerted directly on the liver or kidney, or indirectly by control of the output of gluconeogenic substrates from peripheral tissues, such as muscle and adipose tissue.

Recent experiments by Caldwell *et al.* (1977), utilising perfused hind quarters from fasted, non-lactating rats, have shown that adrenalectomy causes a marked reduction in the release of glucogenic and branched chain amino acids from skeletal muscle, and that glucocorticoid therapy reversed this effect. In perfusion studies of longer duration, Rannels *et al.* (1976) demonstrated that cortisol treatment of adrenalectomised rats depressed muscle protein synthesis but, in contrast to earlier suggestions, found no changes in protein degradation with adrenalectomy or steroid treatment.

Earlier investigations into the effect of adrenalectomy on lactation in the rat showed a decrease in milk production, which was greater than could be accounted for by reduced food intake (Cowie, 1961). More recently, Korsrud and Baldwin (1972a,b,c) and Plucinski and Baldwin (1976) carried out a comprehensive study into the effect of adrenalectomy on the activity of several enzymes concerned with milk fat, lactose and protein synthesis in rat mammary gland during mid-lactation. These workers found that cortisol therapy was required to maintain the action of succinic dehydrogenase, citrate cleavage enzyme, UDP-glucose phosphorylase, UDP-glucose epimerase, galactosyl transferase, acetyl-CoA carboxylase, lipoprotein lipase and triglyceride synthetase. It has also been observed that glucocorticoid binding proteins in the mammary gland, i.e. glucocorticoid receptors, increased during lactation (Wittliff *et al.*, 1976). These findings support the view that glucocorticoids may play an important role in the regulation of several key enzymes involved in milk nutrient synthesis.

Other workers, however, have failed to establish a convincing difference in basal plasma corticosterone between lactating and non-lactating rats (Stern *et al.*, 1973); neither has any correlation been observed between corticosterone and milk yield (Simpson *et al.*, 1973; Ota *et al.*, 1974). What, in fact, has been suggested is that, at parturition and throughout the period of lactation, corticosterone levels in the rat increase to those of virgin controls, from low levels during pregnancy. However, the activity of the corticosterone binding globulin (CBG) significantly decreases at parturition. This protein binds glucocorticoids and thus renders them biologically inactive. It also makes them unavailable for inactivation. Thus, the levels of the free corticosterone, which would be the biologically active form, would be increased during lactation (Gala & Westphal, 1965).

Suckling is known to stimulate the secretion of ACTH and corticosterone in rats (Stern *et al.*, 1973). Ota *et al.* (1974) studied the release of corticosterone in relation to

prolonged lactation. This study demonstrated that, although milk secretion was maintained by a frequent renewal of litters, there was a loss of suckling-induced corticosterone release at the stage of lactation when the young would normally be weaned. This loss was accompanied by a fall in milk yield, suggesting that, although the basal concentration of corticoids may be a factor limiting lactation in rats, their release by suckling may be more important for the maintenance of milk secretion.

6.1.3 Role of prolactin in lactation

It is generally accepted that prolactin is the key hormone in the initiation and maintenance of lactation. Lactogenesis involves the increased synthesis of prolactin which, together with insulin and corticosterone, constitute the positive aspect of the lactogenic complex of hormones. The negative aspect of the complex is the removal of progesterone-induced inhibition of lactation.

The continuation of milk secretion in the rat depends on the frequent suckling stimulation applied by the pups. Thus, milk secretion quickly abates if the suckling stimulus is withdrawn (Ota & Yokoyama, 1965). Conversely, continued suckling by foster pups, past the normal weaning time, results in prolonged, although reduced, milk secretion (Nicoll & Meites, 1959). Prolactin concentrations are known to increase in rats at suckling (Amenomori & Nellor, 1969) and the capacity of the rat to maintain high circulating levels of prolactin, in early and mid-lactation, is frequently dependent on the frequency of suckling (Grosvenor & Mena, 1974). Separation of mother from pups leads to a rapid fall in plasma prolactin.

In rats, the amount of prolactin rises on the 22nd day of pregnancy, immediately before parturition, and remains high as long as the suckling stimulus is effective (Amenomori *et al.*, 1970). Bromocriptine (2-Br-α-ergocriptine-methane-sulphonate) is a drug that specifically inhibits prolactin secretion (Shaar & Clemens, 1972), and its administration to lactating animals

is usually accompanied by a decrease in serum prolactin levels. Bromocriptine injection is known to severely reduce milk production (Taylor & Peaker, 1975).

There are conflicting reports in the scientific literature about the galactopoietic effect of prolactin injections in the rat. Thatcher and Tucker (1970) showed that prolactin and growth hormone are not markedly galactopoietic during prolonged lactation. However, in the study of Kumaresan *et al.* (1966), rat dams were injected every 12 hours from day 7 to 19 of lactation with 1, 2 or 3 mg prolactin per day. Milk yields were estimated on days 14, 16, 18 and 20 of lactation by measurements of litter weight gains during a 30 minute nursing period, following 10 hours of isolation from the mother. These workers reported that 1 mg prolactin significantly increased milk yield on days 18 and 20 of lactation; 2 mg on days 16, 18 and 20; and 3 mg on days 14, 16, 18 and 20 of lactation. They observed maximal stimulation on day 20 with 2 mg and 3 mg of hormone, with 88% and 82% increase over controls, respectively.

Prolactin is believed to have its influence on lactation by increasing RNA synthesis, which in turn will result in an increase in protein synthesis. DNA synthesis does not seem to be affected. The action of prolactin in stimulating casein synthesis is inhibited by the inclusion in the incubation medium of actinomycin D. This substance prevents DNA-dependent RNA synthesis. It would appear, therefore, that prolactin acts at the level of transcription, inducing the production of mRNA molecules, which initiate the synthesis of milk proteins in the endoplasmic reticulum. Prolactin would thus seem to be responsible for the increased RNA/DNA ratio at parturition and for the appearance of milk proteins.

Studies on pregnant mice mammary explants have shown that the induction of lactose synthetase requires the presence of the lactogenic hormones, corticosterone, insulin and prolactin. However, when, in addition to the lactogenic hormones, progesterone

was added to the incubation medium, the synthesis of α-lactalbumin, the B protein in the lactose synthetase complex, was inhibited but that of galactosyl transferase, the A protein, was unaffected (Turkington *et al.*, 1968). Furthermore, progesterone had no effect on the induction of casein synthesis in the explants. Moreover, assays of A and B proteins in mammary tissue, taken from mice during pregnancy and lactation, showed that the increase in concentration of the A and B proteins was asynchronous. The A protein was synthesised long before parturition and the B protein synthesised later at around parturition. Thus, it has been suggested that the reason for the delay in the increase in B protein activity is the high concentrations of progesterone which are present in blood until just before parturition (Turkington *et al.*, 1968).

6.2 Materials and Methods

The serum used in the assays described was obtained as outlined in Chapter 2.

6.2.1 Hormone assays

6.2.1.1 Insulin

A radioimmunoassay technique was used, as described by Hales and Randle (1963), using a kit from Wellcome Reagents Limited (Beckenham, England). The essential principle of the assay is the reaction of a limited, fixed quantity of anti-insulin serum raised from a rabbit, with a mixture of the sample of insulin to be assayed, together with a constant amount of radioactive insulin. The reaction is allowed to take place for a given length of time, after which the antibody-bound insulin is separated from the free insulin and the distribution of the radioactivity determined. The binding of labelled insulin to antibody is progressively inhibited by increasing amounts of unlabelled insulin, owing to competition for specific binding sites on the antibody. The concentrations of insulin in serum samples under test are determined by reference to a standard curve prepared at the same time.

6.2.1.2 Corticosterone

This hormone was measured by a radioimmunoassay, as described by Fahmy *et al.* (1975).

6.2.1.3 Prolactin

An attempt was made to determine prolactin levels, using a modification of the double antibody radioimmunoassay described by Bolton and Hunter (1973). The assay involved :

(i) Iodination of prolactin (National Institute of Arthritis, Diabetes, Digestive Diseases and Kidney (USA) with [^{125}I] using Bolton-Hunter reagent, N-hydroxysuccinimide ester [^{125}I] iodinated p-hydroxyphenylpropionic acid (New England Nuclear, Boston, Massachusettes, USA). The iodinated prolactin was purified by exclusion gel chromatography, using a Sephadex G-50 column.

(ii) Incubation of the iodinated prolactin with the first antibody, anti-prolactin antibody (Nordic Immunological Laboratories, Stilburg, The Netherlands) for 48 h.

(iii) Incubation of the mixture from (ii) above with the second antibody, normal rabbit serum, for 24 h. After centrifugation, the precipitate was counted for [^{125}I] in a gamma counter.

6.2.2 Prolactin injections

An equivalent of about 10 units prolactin (Sigma), in 0.2 ml 0.9% saline solution, was injected into each rat daily from day 9 to 13 of lactation. These injections were given to rats on the 20% cereal and 20% milk protein diets. Controls were injected with 0.2 ml of 0.9% saline solution. Rats on the two diets mentioned above, but with no injections given, were used as 'standards', to see whether stress from injection had any effect on the parameters that were measured. Food intake, pup growth and maternal weight changes were recorded from day 2 to 14 of lactation. Milk volume measurements were determined on day 14 of lactation and milk composition was determined, as described in Chapter 5.

6.3 Results and Discussion

6.3.1 Dam's energy intake

The results are presented in Fig. 12 and show that prolactin injection had no effect on energy intake.

6.3.2 Pup growth

Pup growth is presented in Fig. 13. The pup growth values are divided into two periods, namely, between days 2 and 8, when there were no injections, and between days 8 and 13, when daily injections of prolactin or saline were given. The results show that prolactin had no effect on lactational performance. Reasons for the lack of effect of exogenous prolactin could be :

(i) That prolactin is not limiting in the groups on diets with reduced pup growth.

(ii) The amount of prolactin injected was inadequate to improve lactational performance. It has already been mentioned that, when Kumaresan *et al.* (1966) injected 1 mg prolactin, which was equivalent to 24 units, lactational performance was shown to improve only from day 18 of lactation.

(iii) Furthermore, Kumaresan *et al.* (1966) showed an improvement in lactational performance on day 14 of lactation only after injecting 3 mg (about 72 units) prolactin. The measurement in the present study was terminated on day 14 of lactation.

It cannot therefore be claimed with absolute certainty that inadequate prolactin was responsible for the reduced lactational performance in the experimental groups.

6.3.3 Maternal body weight changes

The results are presented in Table 25. Prolactin injections had no effect.

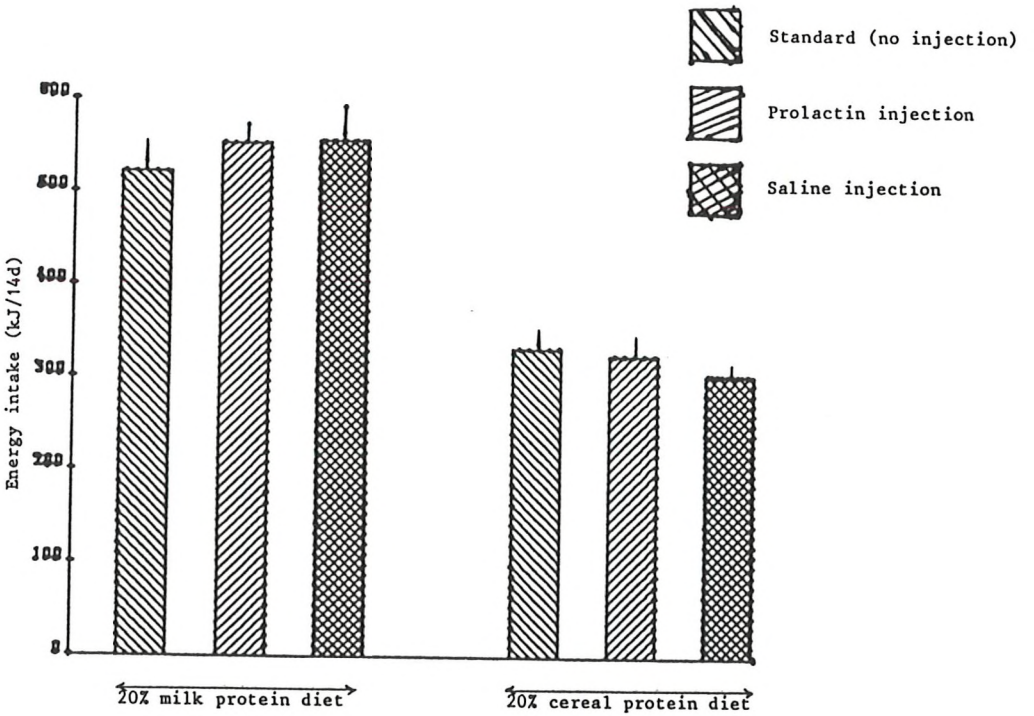


Fig. 12. Dam's energy intake.

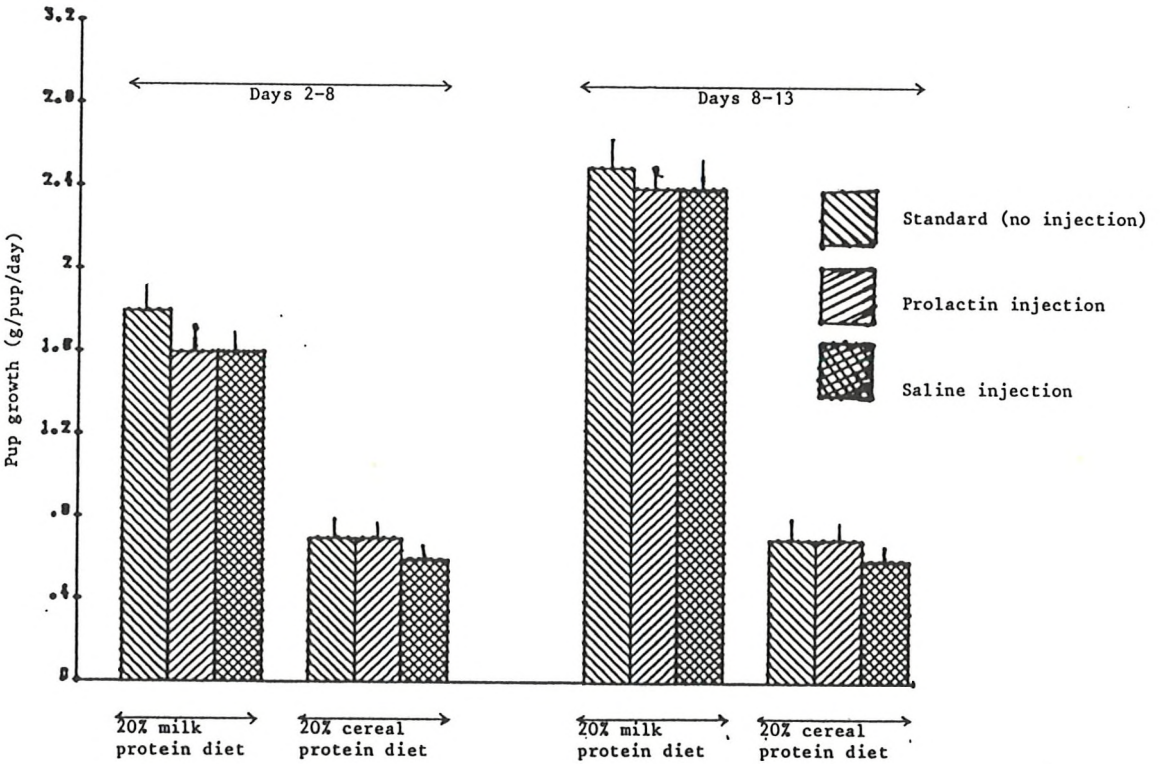


Fig. 13. Growth of pups of lactating rats on various treatments and various protein diets.

Table 25. Body weight changes (day 2-14) and milk composition of lactating rats on various treatments and diets (Mean ± SEM). Number of animals shown in parenthesis.

	20% milk protein diet			20% cereal protein diet		
	Control (5)	Prolactin (5)	Saline (5)	Control (5)	Prolactin (5)	Saline (5)
Body weight changes (g)	16 ± 3	16 ± 5	4 ± 8	-35 ± 5	-39 ± 7	-34 ± 7
Milk volume (ml/rat/day)	58 ± 6	56 ± 5	57 ± 3	24 ± 2	22 ± 1	21 ± 1
Lactose concentration (g/ℓ)	39 ± 10	38 ± 1.1	39.7 ± 2.1	25.5 ± 1.7	22.7 ± 1.8	24.4 ± 1.2
Protein concentration (g/ℓ)	116.3 ± 14	127.5 ± 3.2	124.5 ± 9.9	40.0 ± 7.7	43.7 ± 6.4	41.5 ± 6.1
Non-protein nitrogen (mg/ℓ)	145 ± 16	190 ± 8[a]	175 ± 17	311 ± 29	376 ± 23	373 ± 22

Significantly different from 20% milk protein group: a, $p < 0.05$.

Note: No comparison was made between milk and cereal protein groups because this information was presented earlier.

6.3.4 Milk composition

The results for milk volume, milk lactose, protein and non-protein nitrogen concentrations are presented in Table 25. Prolactin injections had no effect on any of these parameters, except for a small increase in NPN concentration. This observation suggests that prolactin, at least in the amounts injected, may not have an effect on milk composition.

6.3.5 Serum insulin concentrations

The results are presented in Table 26. Most workers (Jones *et al.*, 1984; York *et al.*, 1984) have reported values of between 1 and 4 ng/ml insulin for non-lactating virgin rats and about half these values during lactation. However, Grimble (1981) reported insulin concentrations of 6.2 ng/ml, and Sutter-Dub *et al.* (1974) reported 1.7 ng/ml for lactating rats, and 2.9 ng/ml and 1.14 ng/ml by the two authors, respectively, for non-lactating rats. The values reported in this thesis for the non-lactating rats are therefore in the range of values reported in the scientific literature. Although most workers have reported lower insulin levels in lactation, compared with the non-lactating non-pregnant state, the two reports cited above have shown that insulin levels are higher during lactation. The values reported in this thesis for the lactating animals, however, are far higher than any that have been reported before. A possible explanation for this observation is the experimental procedure applied to the lactating animals before serum samples were taken. Animals were injected with sodium pentabarbitone and oxytocin, after which they were milked for at least 10 minutes. The effects of the chemicals injected, together with the stress due to injections and milking, may have dramatically increased insulin concentrations.

Nutritional status affected insulin concentrations, as can be seen in Table 26. Insulin concentrations of lactating rats on the 6% milk protein and cereal protein diets were significantly lower than those on the control diet. The reduced insulin concentrations would result in reduced rate of uptake of

Table 26. Serum insulin and corticosterone concentrations.

Number of animals shown in parentheses

Diet	Insulin (μg/ℓ)	Corticosterone (μg/ℓ)	$\frac{\text{Insulin}}{\text{corticosterone}} \times 10$
20% milk protein (5)	51.5 ± 10.4	528 ± 44	1.07 ± 0.28
10% milk protein (5)	33.2 ± 3.5	365 ± 48[a]	1.05 ± 0.25
6% milk protein (5)	15.4 ± 3.3[b]	343 ± 45[b]	0.54 ± 0.15
20% cereal protein (5)	14.3 ± 3.9[b,d]	341 ± 35[b,d]	0.40 ± 0.08[a,e]
10% cereal protein (5)	9.6 ± 3.0[b]	367 ± 48[a]	0.27 ± 0.08[a]
20% milk protein (pair-fed) (5)	55.0 ± 16.4	432 ± 76	1.69 ± 0.54
20% milk protein (non-lactating) (5)			

Significantly different from 20% milk protein group:
a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.001$.

Significantly different from pair-fed group:
d, $p < 0.05$; e, $p < 0.025$.

glucose and amino acids by all tissues, including muscle and mammary tissues. This, of course, would lead to reduced rate of synthesis of macromolecules in these tissues. The decrease in insulin concentrations would be an appropriate adaptation during lactation only in tissues like muscle. The reduced uptake of nutrients by muscle would ensure increased amounts of precursor nutrients available for milk synthesis. However, the reduced rate of nutrient uptake by the mammary gland would be an inappropriate adaptation as far as lactational performance is concerned.

Insulin concentrations are affected by a reduction in voluntary food intake. In the present study, food intakes were reduced in 6% milk protein and both cereal groups.

A comparison of insulin values of the pair-fed and 20% cereal protein group shows the effect of reduced protein quality, since both groups received the same amount of dietary energy. The values for the pair-fed and 20% cereal protein groups are significantly different. Those for the pair-fed group were significantly higher. This observation implies that the hormonal status of lactating rats is affected by the dietary protein quality.

The increased insulin concentrations in the pair-fed group could enhance anabolic activity in the mammary gland, thus improving lactational performance.

6.3.6 Serum corticosterone concentrations

The results (Table 26) show that lactation does not affect the concentration of this hormone. This observation is in agreement with other reported studies (Gala & Westphal, 1965; Grimble, 1981). The levels of the hormone, reported in Table 26, are also in agreement with these and other studies (Shargill *et al.*, 1983). However, the results show a significant difference between the control and the 6% milk and cereal protein groups. As has been pointed out earlier, this effect could result in reduced milk nutrient production in the three experimental groups mentioned above because a reduction in corticosterone levels by

adrenalectomy, results in a decrease in the activities of enzymes concerned with milk nutrient synthesis (Korsrud & Baldwin, 1972a,b,c).

The corticosterone concentrations for the pair-fed group were significantly higher than those for the 20% cereal protein group, suggesting that dietary protein quality has an effect on the concentration of this hormone. However, the low corticosterone levels reported in this thesis for the rats on poor quality protein diets might not have been low enough to bring about reductions in all the enzyme activities reported by Korsrud and Baldwin (1972b). These workers reported, among other things, a reduction in the galactosyl transferase activity resulting from adrenalectomy, a finding which was not observed in the study reported in this thesis (Table 19).

6.3.7 Insulin/corticosterone ratio

Since insulin and corticosterone have opposite effects on muscle tissue metabolism, a calculation of the relative proportions may give an indication of the anabolic state of the animals. Thus, the higher the ratio, the higher the anabolic state and the better the lactational performance.

The results presented in Table 26 show that the rats in the pair-fed group were in the most anabolic state. This view is supported by the high rate of protein turnover in the liver and mammary gland of these animals (Table 18), but not by the lactose synthetase and galactosyl transferase values (Table 19). The ratios for the rats on the cereal and 6% milk protein diets were lower than that for the controls, suggesting that the animals on the three experimental groups had a lower state of anabolism. This view is again supported by the reduced protein turnover in liver and mammary tissue and lactose synthetase activities reported earlier (Tables 18, 19).

6.3.8 Serum prolactin concentrations

This experiment was not successful. The problem was probably that the prolactin antigen was not successfully iodinated by the radioactive iodine, with the result that there was no

radioactivity in the antigen antibody precipitate. The experiment could not be repeated due to constraints of time. It is suggested, however, that this study could be continued by repeating the measurements of prolactin concentration in the various groups of rats.

The decreased lactational performance observed in the present study can be explained partly by the changes observed in the hormones measured. In particular, the changes in insulin concentrations,found in the 6% milk protein and both cereal protein groups,correlate well with reduced pup growth, poor milk nutrient content and low protein fractional synthetic and lactose synthetase activities. The influence of the decreased concentration of corticosterone, however, is more debatable, since, as stated earlier, a large reduction in the concentration of this hormone is necessary to impair lactation.

The maternal hormonal changes, resulting from consumption of the various dietary protein diets, were obviously not helpful in allowing mobilisation of nutrients from maternal stores to support lactation, when diet was inadequate, since pup growth was poor. However, when the insulin and corticosterone concentrations are closely examined, it is seen that in the 6% milk protein and both cereal protein groups, insulin concentrations fell by about 300% and corticosterone concentrations by only 50%. This change in the hormonal profile is such as to favour release of amino acids from muscle, which could be used for mammary protein synthesis. However, the degree of change was ineffective, since lactational performance and lactose synthetase activity were equally poor. Maybe, in species like humans, where lactational stress is not as intense as in rats, this hormonal adaptation would be effective.

CHAPTER 7

GENERAL DISCUSSION

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GENERAL DISCUSSION

In the study reported in this thesis, an attempt was made to examine the interactions between dietary protein quality and quantity, and lactational performance. This was done by measuring maternal tissue compositional, enzymatic and hormonal adaptations and relating these changes to milk composition and the success at lactation.

It should be pointed out that the reduced energy intakes of rats on the lowest concentration of good quality protein and both cereal groups could have influenced the results to some extent. It is only by comparing data from the pair-fed milk protein group with that of the 20% cereal protein group that the effect due entirely to reduced protein quality can be revealed.

A poor quality or a low quantity of a good quality dietary protein resulted in a substantial decrease in the mammary tissue weight. Although the mammary glands were not always completely removed from the rats in all cases, rough estimates of the mammary gland weights from rats on the various diets showed very obvious differences. The mammary gland weights from rats receiving an inadequate amount of dietary protein were always significantly less than those from rats on adequate protein diets. These changes would imply a significant reduction in the functional capacity of the gland. The detailed compositional, enzymatic and protein turnover studies carried out have examined the nature of this reduced capacity.

It has already been mentioned that, in the rat, about 20% of mammary proliferation takes place during the first five days of lactation. The observations mentioned above, on the

effects of dietary protein on the functional capacity of the gland, have a parallel with those made by Morgan and Naismith (1980), who showed that, in young non-lactating rats, a high dietary protein concentration results in greater hyperplastic growth in tissues than a low dietary protein concentration.

In the present study, reduced dietary protein quality and quantity also resulted in decreased mammary protein synthetic capacity (RNA/DNA) and protein turnover. The liver and muscle protein synthetic activities were also reduced in rats fed inadequate protein diets. There was a reduction in the secretory activity of the liver in all groups with a reduced energy intake. Differences in the amount and quality of protein did not seem to have much effect on the quantity of serum albumin secreted by the liver. The adaptations of the liver and the muscle would make available an additional source of milk nutrient precursors. However, since it was also observed that the protein synthetic activity and turnover of the mammary gland were reduced, these adaptations to inadequate dietary nutrients were insufficient to ensure adequate mammary synthetic activity or successful lactational performance. A more successful adaptation occurred in the case of rats pair-fed a good quality protein diet. The adaptations of the muscle and liver resulted in substantially increased mammary protein synthetic activity and turnover.

The comparatively better lactational performance of the pair-fed group could be explained, in part, by the fact that this group had the highest whole body protein turnover. The high turnover could enhance the distribution of amino acids within the animal, so that tissues like the mammary gland, which have a high capacity for extracting amino acids from blood, would benefit.

The results presented in this study suggest that the mammary and muscle tissues have similar adaptations to inadequate dietary protein intake. The protein fractional synthetic rate, the protein synthesising capacity (RNA/DNA) and the protein efficiency (protein/RNA) are all reduced in both mammary and muscle tissues

in animals on inadequate protein diets. The liver is not affected in the same way. These differences might be the result of the way in which nutrients are supplied to these tissues. Both muscle and mammary tissues receive nutrients from the systemic circulation, whereas the liver receives additional nutrients directly from the intestines via the hepatic portal vein.

Lactose is an important milk component because it is generally accepted that its concentration in the mammary gland is a major determinant of the volume of milk produced. The activity of the lactose synthetase complex, the enzyme that is believed to influence lactose content in the mammary tissue, was therefore measured. The activity of lactose synthetase was significantly reduced in rats fed diets that were poor in protein quality or low in qauntity.

As the enzyme is composed of two components, that is, galactosyl transferase and α-lactalbumin, it was possible to pinpoint where the dietary effects were acting by measuring both galactosyl transferase and lactose synthetase activities, and the effect of α-lactalbumin on both activities.

The galactosyl transferase component was not affected by the various protein diets, but the α-lactalbumin component of the complex probably was. In other words, the poor diets affected lactose synthetase activity by a specific action on the α-lactalbumin component. Following from these observations on lactose synthetase, it is possible, therefore, that the reduced synthetic activity observed in the mammary glands of rats on inadequate dietary proteins was not a result of uniform reduction in the synthesis of all proteins. The synthesis of some proteins, like α-lactalbumin, was affected by the poor diets more than the synthesis of others, like galactosyl transferase. The effect of the poor diets on α-lactalbumin synthesis would, of course, bring about a reduction in lactose synthetase activity. The reduced lactose synthetase activity would result in reduced mammary lactose concentration, which, in turn, would result in decreased milk volume and thus poor lactational performance.

Dietary protein adequacy also had a profound effect on milk composition and volume. The amount of milk protein produced per day was significantly reduced in rats fed poor quality protein or good quality protein in small quantities. These responses could have resulted from the reduced mammary protein synthetic activity mentioned earlier. This further reinforces the conclusion from the findings mentioned earlier that the adaptations which took place in the non-mammary tissues of malnourished animals were inadequate to ensure successful lactational performance. However, in the pair-fed group, these adaptations were more than adequate to maintain milk protein synthesis. In the rats on poor quality protein diets, the amino acids that were not used for milk protein synthesis appeared in milk in the form of non-protein nitrogen. This milk component was significantly elevated in the rats on cereal diets.

Lactose content and concentration were also significantly affected by dietary protein inadequacy. Lactose concentration and daily production were reduced in all groups which had reduced food intakes, that is, the 6% milk protein, cereal protein groups and also in the group pair-fed the 20% milk protein diet. These observations imply that, while rats on the pair-fed diet were able to adapt their protein metabolism by mobilising amino acids from 'body-reserves', like muscle, similar adaptations were not possible in the case of lactose production, because there are relatively small body reserves of carbohydrate to be mobilised. Moreover, gluconeogenic activity would be limited because of the high demand for amino acids by the mammary gland.

The rats in the 20% cereal protein and pair-fed groups consumed equal dietary protein and energy. However, the pair-fed rats produced more milk, lactose and protein per day than the 20% cereal protein group. Moreover, the tibialis and body weights of the pair-fed dams, at the end of the experimental period, were higher. This observation suggests that energy was less efficiently utilised in rats on poor quality diets. This

suggestion would be in agreement with the finding made by Stock (1972), who fed diets of varying protein quantity to non-lactating rats. More energy, in the form of heat, was lost by the animals fed low dietary protein concentrations.

It would appear that changes in hormone concentrations may have played a part in the adaptations referred to earlier. An inadequate protein quality or quantity in the diet resulted in reduced insulin concentrations. This adaptation would result in a reduced rate of uptake of milk nutrient precursors by the mammary epithelial cells, and also to a reduced rate of mammary synthetic activity. In contrast, the pair-fed rats, which were receiving a high concentration of good quality protein, had elevated insulin concentrations, which would explain the high mammary anabolic activity of rats in this group. This high insulin concentration was probably not maintained throughout the day in the pair-fed animals because, if it was, the lactational performance would have been as successful as the control.

As far as the non-mammary tissues were concerned, the reduced insulin concentrations in animals on inadequate protein diets produced an adaptation in the right direction as it resulted in reduced nutrient utilisation by these tissues. Thus, more milk precursors would be made available to the mammary gland.

Inadequate dietary protein also reduces corticosterone concentrations. However, this reduction was not enough to affect the activity of all mammary enzymes concerned with milk synthesis. The activity of galactosyl transferase was not affected but that of lactose synthetase was significantly reduced. However, when the cumulative effects of the adaptations of insulin and corticosterone are considered, it is seen that animals on inadequate protein diets adapted their metabolism in such a way as to bring about mobilisation of 'muscle protein reserves', and thereby provide additional nutrients for the mammary tissue.

However, since the lactational performance of these rats was still very poor, it could be concluded that the adaptations in hormonal levels were not enough to bring about the desired effects of improved lactational performance.

The study reported in this thesis would suggest generally that, when dietary protein is grossly inadequate, both in terms of its quality and quantity, rats adapt their metabolism appropriately to have the overall effect of mobilising nutrients from body reserves in order to improve milk nutrient synthesis. However, since the lactational demands of rat pups are very high, the maternal metabolic adaptations prove to be inadequate. In other words, the adequacy of the diet is crucially important for the rat to lactate successfully, since the majority of substrates for milk synthesis will come from this source. In humans, however, where the lactational stress is not as intense as the rat, these adaptations could probably be adequate. This observation could explain why there have been many reports of lactating women being successful at lactation, despite consuming diets which are inadequate by international dietary standards.

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