UNIVERSITY OF SOUTHAMPTON.

THE EFFECTS OF MATERNAL PROTEIN RESTRICTION IN THE RAT, UPON PROGRAMMING OF BLOOD PRESSURE, RENAL STRUCTURE AND FUNCTION.

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SUBMITTED TOWARDS THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT.

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Epidemiological studies have identified an association between fetal growth impairment and adult cardiovascular disease. This has been examined experimentally using a model of maternal dietary protein restriction during pregnancy in the rat. It has been consistently demonstrated that offspring of dams supplied a low protein diet during pregnancy exhibit blood pressures elevated above those of control animals. It was proposed that hypertension in this, and other models may result from impairment of renal development, and in particular, from a relative nephron deficit. This proposal was addressed in this thesis.

Female Wistar rats were supplied either a 9% casein or an 18% casein diet during pregnancy, and parameters of offspring growth, renal structure and renal function were examined in association with blood pressure.

Exposure to a maternal dietary protein restriction either throughout pregnancy or during discrete weeks of pregnancy elevated the blood pressures of the offspring. Furthermore, it was demonstrated that offspring blood pressure was increased as a consequence of exposure to a maternal dietary protein restriction during pregnancy, regardless of the diet supplied prior to conception. Birthweight was either reduced or unaffected by prenatal exposure to a maternal low protein diet. Postnatally the kidneys were, in general, disproportionately smaller in offspring of 9% casein fed dams, compared with those of controls. Prenatal exposure to a maternal 9% casein diet resulted in offspring with 15% fewer glomeruli than control animals and impairment of nephrogenesis in late gestation. Blood pressure increased with decreasing plasma volume in 9% casein exposed offspring, but not in controls. The 9% casein exposed offspring were also shown to be resistant to the hypertensive effects of salt consumption. Examination of renal blood flow and glomerular filtration rate did not highlight any differences in renal haemodynamics between the experimental groups.

In conclusion, these data suggest that exposure to a maternal low protein diet during gestation impairs renal growth and development. This in turn may alter renal function such that blood pressure is elevated in order that body fluid homeostasis may be regulated efficiently.

Publications from this thesis.

1. Langley-Evans, S.C., Gardner, D.S. and Welham, S.J.M. (1998); Intrauterine programming of cardiovascular disease by maternal nutritional status. *Nutrition*, **14**: 39-47.

2. Langley-Evans, S.C., Welham, S.J.M., Sherman, R.C. and Jackson, A.A. (1996); Weanling rats exposed to maternal low protein diets during discrete periods of gestation exhibit differing severity of hypertension. *Clinical Science*, **91**: 607-615.

3. Langley-Evans, S.C., Sherman, R.C., Welham, S.J.M., Nwagwu, M.O., Gardner, D.S. and Jackson, A.A. (1999); Intrauterine programming of hypertension: the role of the renin-angiotensin system. *Biochemical Society Transactions*, **27**: 88-93.

4. Langley-Evans, S.C., Welham, S.J.M. and Jackson, A.A. (1999); Fetal exposure to a maternal low protein diet impairs nephrogenesis and promotes hypertension in the rat. *Life Sciences*, **64**: 965-974.

5. Langley-Evans, S.C., Sherman, R.C., Welham, S.J.M. and Jackson, A.A. (1997); Fetal exposure to a maternal low protein diet during discrete periods of pregnancy induces hypertension in the rat. *Clinical Science*, **92**: M45.

6. Welham, S.J.M., Jackson, A.A. and Langley-Evans, S.C. (1997); Effect of maternal protein restriction during pregnancy on renal function. *Clinical Science*, **92**: M47.

7. Welham, S.J.M., Jackson, A.A. and Langley-Evans, S.C. (1997); Effect of protein restriction during pregnancy on the renal function of the offspring. 16th International Congress of Nutrition, Montreal.

8. Welham, S.J.M., Jackson, A.A. and Langley-Evans, S.C. (1998) Response of blood pressure and glomerular filtration rate (GFR) to salt in offspring of female rats exposed to a 9% casein diet in pregnancy. *Proceedings of the Nutrition Society*, **57**: 80A.

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Abbreviations.

9% casein	-	Synthetic diet comprising 90g/kg casein.
18% casein	-	Synthetic diet comprising 180g/kg casein.
9%Ca	-	Animals either supplied, or exposed to 9% casein diet.
18%Ca	-	Animals either supplied, or exposed to 18% casein diet.
Week 1	-	Animals exposed to a 9% casein diet during the first week of gestation only.
Week 2	-	Animals exposed to a 9% casein diet during the second week of gestation only.
Week 3	-	Animals exposed to a 9% casein diet during the third week of gestation only.
SBP	-	Systolic blood pressure.
DBP	-	Diastolic blood pressure.
MAP	-	Mean arterial pressure.
MAP	-	DBP+(SBP-DBP)/3
GFR	-	Glomerular filtration rate.
RBF	-	Renal blood flow.
FF	-	Filtration fraction.
PAH	-	Para-amino hippuric acid.
PV	-	Plasma volume.
PVBW	-	Plasma volume corrected for body weight.
TBV	-	Total blood volume.
TBW	-	Body water expressed as a percentage of body weight.
BH_2O	-	Total volume of body water.
PV/ BH_2O	-	Plasma volume expressed as a proportion of body water.
CRL	-	Crown-rump length.
CHD	-	Coronary heart disease.
NIDDM	-	Non-insulin dependent diabetes mellitus.
ACE	-	Angiotensin converting enzyme.
ALP	-	Alkaline phosphatase.
Hct	-	Haematocrit.
SEM	-	Standard error of the mean.

Chapter 1.

General Introduction.

Over the past 10 years, there has been increasing interest in the concept that chronic diseases of adulthood might arise from disturbances of growth during fetal and early postnatal life. This framework of ideas has become known as the "Barker hypothesis". Underlying the hypothesis is the assumption that the metabolic and physiological activity of an individual is "programmed" by the nutrient and hormonal environment to which the fetus and newborn are exposed. There is evidence to suggest that high blood pressure may be one such disease of adulthood which may be programmed in this way. In this thesis I examine the hypothesis that disturbances of renal structure and function during development underlie the development of elevated blood pressure in adult life. The studies have utilised a rat model of maternal protein restriction in pregnancy.

I will now introduce this area of research by first discussing the effects of maternal undernutrition on fetal growth. Following this I will introduce the evidence which suggests that high blood pressure is a "programmable" disease. I will go on to outline potential targets of metabolic "programming" which may play a role in the development of high blood pressure and then discuss the characteristics of the rat model used in this thesis. Finally, I will mention the work to be carried out in this thesis and the reasons why it was chosen.

1.1 Maternal undernutrition during pregnancy impairs fetal growth and results in offspring which have a greater propensity to develop hypertension in adult life.

1.1.1 Maternal undernutrition during pregnancy impairs fetal growth.

Low birthweight is widely used as a simple indication of fetal growth. Possible causes of fetal growth restriction are many and varied, including maternal smoking, alcohol consumption, congenital abnormalities, infections such as rubella, cytomegalovirus,

toxoplasmosis and syphilis, drug abuse (other than smoking and alcohol consumption), placental insufficiency, multiple births and maternal nutritional deprivation (Sadler 1995). A study of the data obtained by the first British perinatal survey (Fedrick & Adelstein 1978) showed that a low birthweight (defined here as less than 2500g) in infants of at least 37 weeks gestational age, without lethal congenital abnormalities and not including macerated stillbirths, could be significantly correlated with the maternal pre-pregnant weight, maternal height, maternal employment (more than 20% of low birthweight infants were delivered of women who had been in gainful employment at the beginning of pregnancy), low maternal social class, previous history of low birthweight, threat of abortion and severe maternal toxaemia. More recently, low maternal weight and weight gain during pregnancy have been demonstrated to be a significant risk factor for fetal growth retardation in studies conducted in the USA (Neggars *et al.* 1997), India (Mavalankar *et al.* 1994), Brazil (Rondo *et al.* 1997; Farraz *et al.* 1990), Guatemala (Villar *et al.* 1986; Neel & Alvarez 1991) and in cross-cultural studies (Kelly *et al.* 1996).

A low maternal, pre-pregnant plasma volume is also significantly associated with low birthweight (Croall *et al.* 1978). A comparison of the birthweights of babies from different cultural backgrounds in the UK (Grundy & Hood 1978) found that babies from Indian and West Indian mothers were generally lighter than those from Irish and British Caucasian mothers. Approximately 15% of the Indian mothers gave birth to babies below the 5th Aberdeen centile for birthweight compared with 3%-5% for the other groups. The effects of altitude may also impinge on fetal growth. Haas and co-workers (Haas *et al.* 1980) showed that Amerindians, who have lived at altitude in Bolivia for many generations, give birth to larger offspring than non-Indians, which were born and grew up at the same altitude. This suggests that the Amerindians have become accommodated to life at altitude and that such accommodation requires a number of generations. Pollution has also been implicated in the impairment of fetal growth (McMicheal *et al.* 1988). It was found that in women living in close proximity to a lead smelter, birthweight declined with increasing lead concentration in maternal blood.

During the period between October 1944 and May 1945 in Holland, a German military blockade resulted in a severe food shortage which reached its worst point in December 1944. Food availability and birth outcome during this period were examined in women

in the Hague (Smith & Mass 1947) and it was found that babies, for whom the period of late gestation fell during the most severe food shortage, were significantly smaller at birth compared with standards of birthweight. In the Gambia, the wet season and dry season represent periods of relative food scarcity and availability respectively. Birth weight has been shown to be significantly compromised during the wet season compared with those born in the dry season (Prentice *et al.* 1981; Prentice *et al.* 1987). Energy supplementation in this population has a beneficial effect on birth weight of babies born in the wet season, but is ineffective on dry season delivered babies (Prentice *et al.* 1987).

Dietary analyses during pregnancy have highlighted a number of interesting trends between maternal diet and birth outcome. High carbohydrate consumption during early pregnancy has been shown to result in lower birthweight (Godfrey *et al.* 1996). This was also observed in the offspring of mothers who had consumed less dairy and meat protein during late gestation. A study of primiparous Aberdeen women and their offspring showed that, during pregnancy, for a daily animal protein intake of less 50g, a higher carbohydrate intake was associated with an increase in offspring blood pressure, although this was unrelated to birthweight (Campbell *et al.* 1996).

Globally, the major factors responsible for fetal growth retardation tend to be maternal undernutrition and socio-economic status (Ebrahim 1984). Approximately 5% of babies born in the developed world and around 40% in developing countries are considered to be of low birthweight. Dietary supplementation of Asian women in Birmingham who failed to show an increase in upper arm skin fold thickness in the first 20 weeks of pregnancy led to a significant increase in the birthweights of their babies (Viegas *et al.* 1982), indicating that the incidence of low birthweight in this population may be due primarily to maternal undernutrition.

1.1.2 How does maternal undernutrition during pregnancy impair fetal growth?

During the period of fetal growth, all the major body components are formed and must be developed to a point which enables survival of the organism outside the uterus. In order that this can occur, the fetus must be adequately supplied with oxygen and other

nutrients and fetal waste products must be disposed of. The supply of particular nutrients to the fetus is also apparently of critical importance during specific stages of gestation. These maternal and placental functions will now be discussed in relation to fetal growth and development.

1.1.3 Embryonic and fetal development.

The intrauterine development of the human occurs in three discrete phases which do not correspond to the three trimesters of pregnancy (Sadler 1995). These are the pre-embryonic period (first three weeks), the embryonic period (third to eighth weeks) and the fetal period (second to third month until birth).

The pre-embryonic period is characterised by the rapid division of the zygote and the ultimate formation of the three basic germ layers (Tam & Beddington 1987). The notochord is also formed in this period and this forms an axis down the midline which will become the basis of the axial skeleton. The neural tube forms just above this. By the end of the pre-embryonic period, primary villi of the trophoblast have formed and acquired a mesenchymal core within which capillaries arise. Once these capillaries have connected with those in the chorionic plate, the villous system of the trophoblast is able to supply nutrients to the developing embryo (King & Mais 1982).

The embryonic period is the stage of the initiation of organogenesis (Sadler 1995). The primary germ layers give rise to all of the major organs, which are present in their initial forms by the end of this period. The ectoderm generally gives rise to structures that will maintain contact with the outside world including the central nervous system, the peripheral nervous system, the sensory epithelia of the ears, eyes and nose, the skin, hair and nails as well as subcutaneous glands, the mammary glands, the pituitary gland and the enamel of the teeth (Carlson 1981). The mesoderm forms the notochord, the somites, from which bone, muscle, cartilage and dermis are derived, the urinary and genital systems, the cardiovascular system, the lining of body cavities and adds to the connective tissue and musculature of the face (Carlson 1991). The endodermal layer primarily gives rise to, in the first instance, the epithelial lining of the primitive gut and the intraembryonic parts of the allantois and vitelline duct and later to the epithelial lining of the respiratory tract, the parenchymal tissue of the thyroid,

parathyroid, liver and pancreas and the epithelial linings of the bladder, urethra, tympanic cavity and auditory tube (Carlson 1981). The fetal period is characterised by the continued development and maturation of the tissues and by the rapid increase in size of the fetus.

Development progresses by a process of differentiation, proliferation and maturation under the control of an array of growth factors and signalling molecules which exert their influence at genetically defined stages (Gilbert 1988). In the human, whilst some organs continue to develop and mature postnatally, such as the lung, others, including the brain, heart and kidney, have completed differentiation by birth and subsequent growth is through hypertrophy of the tissues already present.

The development of the rat follows a similar pattern to that of the human, but is different in several important respects (Gilbert 1988). First, although the brain has completed its hyperplastic phase of development by birth, the kidney has not, and nephrogenesis continues for approximately 10 postnatal days. Also, the short length of gestation and the nature of the reproductive strategy employed by rats, i.e. large litters, results in the production of extremely altricial offspring, which require considerable postnatal care and pose a significant burden on the mother in terms of nutrition.

1.1.4 Fetal and placental growth.

Fetal growth in the human, in terms of body weight, follows a sigmoidal curve (Figure 1.1). The increase in body weight is slow until around 12 to 13 weeks of gestation, at which point it increases exponentially until, approaching term, it begins to plateau. This is also seen in other large mammals, such as sheep, pigs and monkeys (Owens & Robinson 1988). The change in crown rump length follows a more linear course than that of body weight, increasing steadily until birth. Placental growth is also close to linear (Owens & Robinson 1988). The placenta is initially heavier than the fetus and increases in size throughout mid and late gestation, in most mammals. Growth of the placenta has been shown to be mediated by a variety of factors, including growth factors, endothelin I, oncogenes and interferons (Ohlsson 1989; Hay 1992; Fant *et al.* 1992; Loke and King 1990) as well as various steroid hormones and cyclic-AMP (Knoll 1992).

A longitudinal examination of placental growth using ultrasound measurements (Clapp *et al.* 1995) showed placental volume to increase from 130cm^3 at 16 weeks to 375cm^3 at 24 weeks gestation. This rapid increase in placental volume during mid-gestation is paralleled by a substantial increase in the placental villous surface (Baur 1977) which increases from 0.85m^2 at between 12 and 16 weeks to 11.43m^2 between 37 and 41 weeks of gestation with the most rapid increase seen between weeks 17 to 21 and weeks 22 to 26 (Mayhew & Simpson 1994). The microvillous surface area of the placenta also increases rapidly during mid to late gestation, increasing more than 9 fold from week 25 to week 36, at which time it is approximately 94m^2 (Teasdale & Jean-Jacques 1985). It is interesting to note that the microvillous surface area of an adult's small intestine is approximately 200m^2 (Ganong 1991). If we therefore compare the relative nutrient absorptive surface area of adult and fetus (at 36 weeks of gestation) with their body weights, the fetus has an approximately ten-fold greater surface area available for nutrient extraction than does its mother.

1.1.5 The placenta and fetal growth.

The fetus is entirely dependent on the placenta for the acquisition of oxygen and other nutrients. The development of the placenta is such that it is fully capable of supplying whatever the fetus requires before the initiation of the rapid increase in fetal growth. It has been suggested that fetal hormonal status may play a role in the association between supply of nutrients and growth of specific fetal tissues (Chard 1989; Owens 1991). The insulin like growth factor-I (IGF-I) is thought to play a prominent role in this process (Gluckman & Harding 1994; Liu et al. 1994) and has been shown to rise with increasing levels of glucose and amino acids in the maternal circulation. As this occurs, fetal production of IGF-I is stimulated and this enhances uptake of glucose and amino acids by the fetal tissues and relative inhibition of fetal protein catabolism. In situations of chronic maternal undernutrition, maternal and fetal plasma IGF-I levels are lowered (Owens 1989), and it has further been shown that birthweight is directly related to the concentration of IGF-I in umbilical cord blood (Breier *et al.* 1988). The activity of IGF-I is therefore implicated in the orchestration of fetal growth, and is seemingly controlled by the level of maternal supply of substrate, which will, in turn, be controlled by the maternal hormonal and nutritional status. In the sheep, infusion of IGF-I into the fetal circulation led to an increase in the cell number of the placental placentomes (Owens *et*

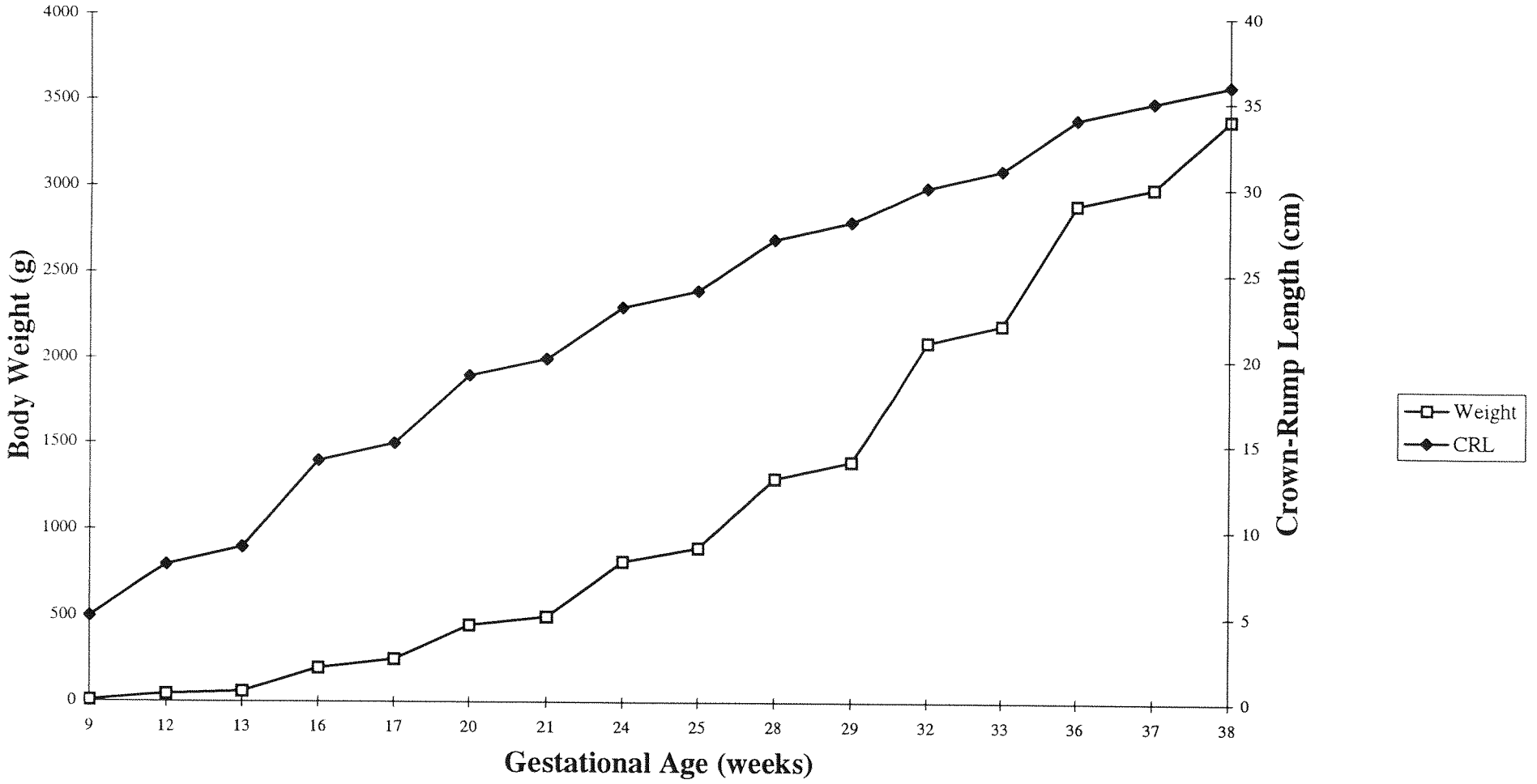
al. 1994) without any significant effect on total placental weight, and enhanced the decline in umbilical resistance to blood flow associated with advancement of pregnancy (Molina *et al.* 1991). IGF-I may therefore have a significant bearing on the metabolism of mother, placenta and fetus.

As pregnancy proceeds, the uterine arterial blood flow increases concomitantly with the increase in fetal weight (Peterson & Behrman 1969) and therefore remains relatively constant if considered in terms of fetal weight. The umbilical blood flow also increases in absolute terms, but, if related to fetal weight, it declines by around 50% from 20 to 40 weeks gestation (Siddiqi *et al.* 1994). It has been suggested that this relative decline in umbilical blood flow represents an attenuation of the placental supply towards late gestation (Elliot & Langer 1994). It may, however, merely represent a change in the mode of supply. There is no compromise in oxygen supply to the fetus (Longo & Ching 1977) and there is a fivefold increase in total glucose transfer to the fetus (Molina *et al.* 1991) from mid-to late gestation. This increase is a function of the expansion of the transport surface area of the placenta rather than an increase in glucose transporter (GLUT-I glucose transporter) density (Morris *et al.* 1985; Hay 1995). The transport of glucose to the fetus is further enhanced in late gestation by a rise in the transplacental glucose concentration gradient (Molina *et al.* 1991), which is probably a result of increased fetal utilisation of glucose and an increase in the fetal insulin concentration.

The metabolic activity of the placenta itself must also be considered when looking at the nutrient demands placed on the mother during pregnancy. At term, the metabolic activity of the placenta by weight is between 5 and 10 times that of the fetus, which is itself higher than the adult (Owens and Robinson 1989). The placenta consumes 30%-60% of the glucose supplied to the uterus at term, a quantity greater than that consumed by highly metabolically active tissues like the adult brain (Bloxam et al. 1987).

Figure 1.1: Growth of the human fetus.

The change in weight and crown rump length (CRL) is shown with advancing gestational age. Values were taken from Sadler (1995).


Body Weight (g)
4000
3500
3000
2500
2000
1500
1000
500
0
Crown-Rump Length (cm)
40
35
30
25
20
15
10
5
0
9
12
13
16
17
20
21
24
25
28
29
32
33
36
37
38
Gestational Age (weeks)
Weight
CRL

The placental utilisation of oxygen and glucose in the sheep is highest during mid-gestation (its own rapid growth phase), consuming around 80% of the uterine supply (Molina et al. 1991) and this declines to around 38% (oxygen) and 54% (glucose) in late gestation (the fetal rapid growth phase). The placenta therefore poses a significant burden on the mother in terms of its own nutrient requirements. This is unsurprising considering the size of its transport epithelium across which nutrients must be actively translocated, a highly energy dependant process.

1.1.6 Substrates for fetal growth.

The three primary requirements for growth in terms of tissue accretion are lipids for the formation of membranes, amino acids for the manufacture of proteins and energy to enable these processes to take place. The energy requirement of the fetus at any stage is dependant upon the context of the process taking place, i.e. the rate of tissue accretion, the functional requirements of the tissue and the type of tissue being laid down. The energy requirement to deposit 1g of fat is 39.7 kJ whereas that needed to lay down 1g of protein is approximately half, at 20.2 kJ. This is reflected in the energy requirements of the fetus throughout gestation. The energy requirement of the fetus per kg of tissue in late gestation is considerably greater than that of the fetus in early and mid-gestation, since there is significant accretion of fat tissue only in late gestation. This accounts for roughly 80% of the fetal energy requirement (Sparks *et al.* 1980). The energy requirement for protein accretion follows a more linear association with fetal weight, and therefore follows an exponential relationship with gestational age (Sparks 1984). However, there is a slight decrease in protein accretion towards the end of gestation (Widdowson 1980).

The principal substrate for energy production by a variety of fetal tissues is glucose. The fetal brain, which at term may account for 10% to 12% of the human fetal body weight is dependant for the most part on glucose (Bissonette *et al.* 1985), with the cerebral utilisation alone possibly using up to 5 or 6 mg glucose/kg body weight/minute (Holliday 1971). The fetal erythrocytes (Oski & Naiman 1965), and renal and adrenal medullas (Pagliara *et al.* 1973) have also been shown to depend upon glucose, as well as, to a certain extent, the fetal heart (Fisher *et al.* 1980).

The fetus seems to be the primary controller of the uteroplacental glucose consumption. A study in sheep in which the maternal and fetal glycaemia were varied independently (Hay 1991) demonstrated that the uteroplacental consumption of glucose was dependent on the fetal plasma glucose concentration rather than the maternal. This may have a protective effect for the mother, as well as enabling the fetus to obtain a greater proportion of the glucose uptake from the placenta. This may further enable the proportional decline in placental glucose utilisation observed towards the end of gestation, since the fetus may exert control over its own plasma glucose concentration and thus divert glucose for the significant requirements made by fat deposition. An alternative source of energy, other than that provided by fat and protein oxidation, is lactate. This is produced by the placenta in late gestation and its production is apparently unaffected by changes in the maternal or the fetal arterial oxygen tension, or in changes in uterine or umbilical blood flows (Sparks *et al.* 1978). It has been shown in both sheep (Burd *et al.* 1975) and humans (Stembara & Hodr 1966) that there is a net uptake of lactate from the placenta into the fetus. However, it has also been shown that a significant proportion of the fetal supply of lactate in late gestation sheep is produced by the fetus (Sparks *et al.* 1982).

Amino acid utilisation by the fetus may be both for tissue accretion and energy production. Amino acids are transported across the placenta via energy dependant transporters and studies have suggested that the regulation of these amino acid transporters may be a function of the levels of amino acids in the fetal blood (Domenech *et al.* 1986; Smith *et al.* 1973). It has also been proposed that a high fetal plasma amino acid concentration may inhibit further placental uptake by competitive binding of amino acids to the reverse faces of the unidirectional transporters (Smith & Depper 1974).

The disposal of amino acids may be dependent upon the types of amino acid available (Battagglia 1978). The majority of the indispensable basic amino acids (e.g. lysine and histidine) absorbed, are deposited as tissue, whereas, some of the acidic amino acids, such as aspartate and glutamate are taken up from the placenta in very low concentrations. In general, the proportion of absorbed amino acids, which are laid down in tissues is very low. There is a large fetal urea production, which is indicative of the use of amino acids for energy production. Tracer studies (VanVeen 1987) have supported this hypothesis. It was also shown that, for some amino acids, such asleucine,

the disposal due to oxidation is proportional to the umbilical uptake in excess of requirements for tissue deposition. IGF-I has been shown to inhibit protein catabolism, as has insulin (Milley 1992). Since plasma glucose concentration stimulates these hormones, it may be the case that with a rise in plasma glucose, amino acid oxidation is superseded by glucose as an energy source for the fetus and protein catabolism is therefore down regulated.

There is some evidence to suggest that there are a variety of fetal to placental amino acid and nitrogen cycles. Ammonia is produced in the placenta by the deamination of amino acids and this is in turn utilised by the fetal liver to synthesise other amino acids (Carter *et al.* 1991). Also, the fetus takes up the amino acids glutamine, glycine and asparagine, and their associated metabolic products, glutamate, serine and aspartate respectively are taken up and used by the placenta (Hay 1991). Whilst serine and glycine are used for protein synthesis and oxidation, the glutamine-glutamate exchange system may form a nitrogen transport mechanism between the fetus and the placenta, similar to that seen in the adult between the liver and the kidney (Haussinger *et al.* 1984).

Lipids are required for a variety of functions in the fetus, as phospholipids for membrane synthesis, as triglycerides for storage, as well as for energy production (Colman & Haynes 1984). It is thought that the human fetus may only be able to support brain growth for 2-3 days without an exogenous supply of lipid (Clandinin *et al.* 1980). The proportion the fetus comprised by lipid is directly related to the ability of the placenta to transport it, so, the human with a fetal fat content of 18% at term has a more highly lipid permeable placenta than does the sheep which is only 3% fat at term (Coleman 1986). It should of course be noted that the maternal diet of the human and the sheep are vastly different in their lipid content and the placental permeability to lipid and, therefore, the lipid content of the fetus may be a function of this.

1.1.7 Nutrient restriction.

Fetal growth is a complex process and requires a sophisticated nutrient delivery system to support it. It is clear that disturbances in maternal nutrition, maternal nutrient homeostasis, placental function, maternal hormonal status and/or fetal utilisation of nutrients may each, either individually or in concert, have potentially catastrophic

effects on fetal growth and development. Some of these are discussed in more detail below.

Optimal fetal growth is dependent upon an adequate supply of nutrients. Disturbances in nutrient supply to the fetus may arise from insufficiency of the maternal diet, maternal factors which alter the supply of nutrients to the placenta, or an impairment of nutrient transfer across the placenta.

1.1.8 Reduced nutrient availability due to inappropriate diet.

1.1.8.1 Experimental evidence.

There have been two main approaches to the experimental imposition of undernutrition in animal models. Either a single nutrient is made limiting and its energy equivalent made up in the diet, thus producing experimental diets of differing nutrient proportions or total food intake is restricted to a proportion of the *ad libitum* food intake of the animal being used. For ruminant animals, the latter method is necessary when considering protein intake as a significant proportion of the dietary nitrogen is supplied by the gut fauna of micro-organisms. However, for animals such as rats, it is possible to produce diets for which *ad libitum* food intake may be maintained whilst varying the content of an individual nutrient.

Numerous studies have indicated effects of nutrient manipulation on fetal growth. Rats fed 30% of the *ad libitum* food intake during pregnancy (Woodall *et al.* 1996) produced offspring whose body weights from birth until 12 weeks of age were significantly lower than those from adequately fed females. The feeding of an 8% protein (by weight) diet to rats during pregnancy produced smaller pups at birth, than those from a 20% protein diet (Desai *et al.* 1996). Altering the carbohydrate source may have direct effects on the birthweights of the offspring in the rat (Soria *et al.* 1996). deBarrio and colleagues (deBarrio *et al.* 1992) studied ewes whose weight was controlled nutritionally prior to conception. Heavy ewes which were subject to a restricted diet during mid-gestation, gave birth to offspring that were relatively short for their weight, whilst offspring of light ewes not subject to any maternal nutritional insult gave birth to relatively long offspring.

Metabolic alterations in the mother and the fetus have been shown to occur in a number of animal studies of undernutrition. Levels of chorionic mammotrophin (a hormone that accelerates the transport of maternal nutrients to the fetus in late gestation) were found to be lowered in female rats fed either a 5% protein diet, or an 18% protein diet with 20% alcohol (by volume) supplied in the drinking water over the final 14 days of pregnancy, compared to control rats fed an 18% protein diet. This study indicated that the maternal nutritional insults induced restriction of fetal nutrient availability in the period of rapid growth (Wunderlich *et al.* 1979). Ewes fed 30% - 40% of the required energy intake (Chandler *et al.* 1985; Leury *et al.* 1990) demonstrated a significant reduction in umbilical uptake of oxygen and glucose. Alterations in the secretion of growth hormone have also been demonstrated in malnourished ewes and their fetuses (Bauer *et al.* 1995). Similarly, a reduction in maternal and fetal IGF-I levels is observed during undernutrition. As has already been mentioned, IGF-I is closely associated with plasma glucose levels and may directly affect the rate of fetal protein catabolism (Owens *et al.* 1993a).

It was proposed by Naismith and Morgan (1976), that pregnancy was characterised by two phases, the anabolic phase and the catabolic phase. These reflected the biphasic nature of maternal protein metabolism. The effect of protein restriction during pregnancy was examined over the anabolic and catabolic phases of gestation in the rat (Mayad-Afshar & Grimble 1983). It was suggested that pregnancy might be characterised by an initial anabolic phase, during which protein is deposited in the maternal tissues in preparation for the later catabolic phase. Protein is subsequently mobilised from the maternal tissue in order to meet the increase in fetal demand towards mid and late gestation. A 5% protein diet was fed to female rats throughout gestation with control rats fed a chow diet containing approximately 18% protein by weight. The females and their offspring were examined at 8 and 12 days of gestation (the anabolic phase) and 17 and 21 days of gestation (the catabolic phase). Maternal liver protein content during the anabolic phase was higher in the chow fed females than the low protein fed group, whilst the muscle protein content of the low protein exposed animals was higher than controls at day 12. During the catabolic phase of pregnancy the maternal liver and muscle protein content was consistently lower in the low protein exposed animals. The rates of muscle protein synthesis and degradation tended to decline more rapidly during the catabolic phase in the low protein exposed group. This

may represent a protective mechanism whereby maternal tissue is relatively spared under conditions of undernutrition. It is also interesting to note that the maternal serum insulin concentrations in the low protein group did not parallel the changes seen in the control animals. The chow fed animals exhibited an elevation of serum insulin during the latter stages of the anabolic phase and during the early catabolic phase, with a sharp decrease in concentrations observed at term. The low protein fed group, however, showed an initially high serum insulin concentration which remained relatively unchanged and declined only in late gestation. The elevation in plasma insulin during the anabolic may have enabled enhanced protein synthesis, whilst its relative lack during the catabolic phase might result in reduced protein synthesis and thus avail the fetus of a larger pool of circulating amino acids.

Maternal undernutrition, in addition to influencing gross parameters of fetal growth, also alters the growth of individual organs. The size, structure and function of the lungs have been shown to be altered as a result of maternal undernutrition (Edelman *et al.* 1986; Gaultier 1991). A significant effect of maternal diet upon pancreatic islet structure and function of rat offspring has been reported (Dahri *et al.* 1991). The offspring of rats fed an 8% protein diet during pregnancy demonstrated a reduced pancreatic cellular proliferation and islet size, as well as depressed pancreatic islet vascularisation and insulin content. Desai and co-workers (1996) showed that offspring of rats fed an 8% protein diet during pregnancy had disproportionately larger lungs and brains, proportionately smaller hearts, kidneys and thymus and had disproportionately smaller pancreases, spleens, livers and tibialis and soleus muscles than the offspring of rats fed a 20% protein diet during pregnancy. Alterations in pancreas, liver, heart, brain and kidney growth have been demonstrated by feeding 6%, 9% and 12% protein diets to rats throughout gestation (Levy & Jackson 1993; Langley & Jackson 1994; Langley-Evans *et al.* 1996b; Langley-Evans & Nwangwu 1998). Rats fed a protein free diet during either days 0 to 6, days 0 to 10 or days 10 to 15 of gestation showed a retardation of fetal cerebral growth until term (Marthens & Shimomaye 1978), and an inhibition of placental growth when compared with offspring of chow fed controls. The effect of undernutrition on the DNA and protein content of fetal and newborn tissues was examined by feeding a 6% protein diet to rats throughout pregnancy as compared 20% protein fed dams (Zeman & Stanbrough 1969). It was found that by day 20 of gestation the protein and DNA concentration was depressed in all of the fetal tissues measured

(liver, kidney, heart, thymus and brain). This was also reflected in the tissues of newborns. It was concluded that the primary effect of a maternal nutritional insult during pregnancy was to decrease the fetal cell number during late gestation. This is a direct hypoplasic effect of maternal under nutrition which in some tissues, most notably the brain and kidney whose own hyperplasic growth in most animals studied is complete at term, may result in a permanent cellular deficit. It is true, however, that the kidney in the rat continues hyperplasic growth for around 10 days postnatally and so this intrauterine insult may not result necessarily in permanent damage (Tufro-McReddie *et al.* 1993).

1.1.8.2 Maternal factors.

Nutrients are delivered from the maternal circulation via the placenta to the fetus. It has been suggested that alteration of the maternal cardiovascular adaptation to pregnancy may significantly affect nutrient delivery to the fetus and therefore have a potentially adverse effect on fetal growth.

During pregnancy, uterine blood flow increases substantially above non-pregnant levels (Blechner *et al.* 1974; Bruce 1976). A combination of the data from several individual studies has strongly suggested there to be a positive linear relationship between maternal plasma volume and uterine blood flow during pregnancy (Rosso 1981). A positive linear relationship has also been demonstrated between maternal plasma volume and offspring birthweight (Croall *et al.* 1978). Thus, it is implied that smaller increases in uterine blood flow (and maternal plasma volume) during pregnancy are associated with an increased risk of fetal growth impairment. Rats fed either 50% of *ad libitum* intake, or a 6% protein diet, during pregnancy were shown to produce significantly smaller offspring by day 21 of gestation (Rosso & Streeter 1979). Those dams fed a 6% protein diet also demonstrated a reduced plasma volume, when corrected for either maternal weight or conceptus weight. Additionally, it was shown that a 50% reduction in food intake during pregnancy resulted in a reduction in placental weight by day 21 and a substantial depression of uterine, ovarian and placental blood flows (Rosso & Kava 1980). It has therefore been suggested that the prohibitive effects of either a maternal food restriction or a maternal protein restriction during pregnancy on fetal growth may arise through depression of the maternal cardiovascular adaptations to pregnancy.

1.1.9 Metabolic programming.

The phenomenon of metabolic programming has attracted much interest in relation to the study of the fetal origins of adult disease. Put simply, metabolic programming is an occurrence whereby fluctuations in the environment of a developing organism permanently alter metabolic and physiological processes within that organism. A clear example may be seen in certain reptiles where the temperature of incubation of the eggs determines the sex of the offspring (Janzen & Paukstis 1991). At one temperature, all offspring will become females. A slight increase of 1 - 2°C results in the production of all males, whilst at a temperature in between these will produce males and females at a ratio of 50:50. This process occurs by the temperature dependent action of the oestrogen aromatase enzyme.

Evidence has accumulated from human and animal studies of maternal nutrition and birth characteristics, which suggests that metabolic programming of elevated blood pressure of the mammalian fetus may occur during periods of maternal undernutrition. This will be discussed below.

1.1.10 Hypertension.

The term hypertension is widely used to describe a condition in which blood pressure is maintained above a pre-defined level. It is a fundamental characteristic of all organisms and their physiological functions to be variable. This innate variation is what drives evolution. The profile of a population's blood pressure is no exception and this may represent one of the major problems in attempting to understand the causes of hypertension. The Fifth Joint National Committee on Detection, Evaluation and Treatment of High Blood Pressure (1993) outlined seven categories of blood pressure (Table 1.1).

Table 1.1: Categories of blood pressure outlined by the Fifth Joint National Committee on Detection, Evaluation and Treatment of High Blood Pressure (1993).

Category	Systolic Blood Pressure (mmHg)	Diastolic Blood Pressure (mmHg)
Optimal	<120	<80
Normal	120-129	80-84
High Normal	130-139	85-89
Hypertension:		
Stage 1	140-159	90-99
Stage 2	160-179	100-109
Stage 3	180-209	110-119
Stage 4	≥210	≥120

Hypertension is defined at a level of blood pressure over which it is thought that there is a significantly greater risk of the development of coronary heart disease (CHD; Stamler 1989), or stroke. It is, of course, necessary for clinicians to define a cut-off point beyond which antihypertensive treatment is administered and a significant increase in the relative risk of CHD would seem a logical one. However, if the blood pressure is considered from the point of view of levels at which antihypertensive treatment leads to significant reductions in the risk of CHD, the profile changes (Rose 1987). Antihypertensive therapy is apparently most effective in reducing the risk of CHD when the diastolic blood pressure is above 104mmHg (Veterans Administration Co-operative Study Group on Antihypertensive Agents 1967 & 1970). Between diastolic blood pressures of 95mmHg and 104mmHg, treatment is still beneficial (Narins 1984), whilst below 95mmHg, antihypertensive therapy appears to have a much lesser effect on long term health (Narins 1984; Hyman & Kaplan 1985). It may also be the case that the rate of increase in blood pressure, as opposed to the blood pressure *per se*, is a greater risk factor for CHD. A combined analysis of a number of studies (MacMahon *et al.* 1990) suggested that an increase in the diastolic blood pressure of 7.5mmHg within the range of 70mmHg - 110mmHg increases the risk of CHD by 29% and the risk of stroke by 42%.

Additional to these problems of definition, is the fact that blood pressure fluctuates throughout the year and also during the course of the day. The blood pressure (both systolic and diastolic) of elderly subjects has been shown to increase with the onset of winter. This, it was suggested, was due to a change in the ambient temperature (Woodhouse *et al.* 1993). It has further been shown that this seasonal change in blood pressure, when present (a study of normotensive Japanese women found no such association - Tsuchihashi *et al.* 1995), is more pronounced in older subjects (Brennan *et al.* 1982). Studies in humans (Mancia *et al.* 1998; Pickering *et al.* 1972; Mancia *et al.* 1983; Staessen *et al.* 1991; Broadhurst *et al.* 1990), dogs (Cowley *et al.* 1973) and rats (Takezawa *et al.* 1994) have all demonstrated significant blood pressure variation over the course of 24 hours, some showing a range of mean arterial pressure (MAP) of 40mmHg to 50mmHg.

From a purely scientific viewpoint, hypertension may be used to describe any level of blood pressure elevated over control values. Thus, if an intervention results in a

significant elevation in blood pressure, then this higher level of blood pressure is described as hypertension when compared with values of blood pressure obtained from a sample not exposed to that intervention. This form of hypertension will be referred to as experimental hypertension throughout the rest of the thesis.

1.1.11 Determinants of hypertension.

In the same way that hypertension is difficult to categorise, it is also difficult to determine its cause. Hypertension has been studied since the 18th century with the early observations of Samual Schaarshmidt on the behaviour of the vasculature in hypertension (Backer 1953). However, despite the work carried out in the intervening 250 years to the present day, it is still a condition with no known absolute cause or causes. In 1982, Bjorn Folkow (Folkow 1982) extensively reviewed the subject and proposed a series of 10 acknowledged facts regarding hypertension. He stated that hypertension is a polygenetic disease which might be affected by environmental factors such as salt intake and psychological factors. He stated that there was a structural adaptation of the heart to raised blood pressure (primarily the left side), by deposition of contractile tissue (cardiac hypertrophy) and that there was an increased systemic resistance to flow, due either to altered activity of "...vascular effector cells..." or a structural adaptation in the vessel walls. Altered states of sensitivity to extrinsic factors, hormones and altered nervous activity and control, resetting of the short term barostat reflex and an undefined role for the kidney, were also acknowledged to contribute to chronic blood pressure elevation.

Essential hypertension or primary hypertension (terms which represent hypertension for which there is no apparent causative mechanism) is generally characterised by a raised systemic vascular resistance and an apparently normal cardiac output. It may be the case, however, that these parameters are not static throughout the course of the disease. In many young borderline (according to the clinical definition shown above) hypertensives, the cardiac output is significantly elevated (Eich 1966; Levenson 1985) which, it is suggested, may be related to an enhanced sympathetic tone. Over time the cardiac output returns to normal and an enhanced systemic vascular resistance maintains the elevation in blood pressure.

Electrolyte status, particularly that of sodium, has been widely related to the development of hypertension. Studies in China (Kesteloot *et al.* 1987; Hsiao *et al.* 1986), Kenya (Poulter *et al.* 1984), Zaire (M'Buyamba-Kabangu *et al.* 1986) and Britain (Khaw 1983) have all demonstrated a direct link between sodium intake, sodium excretion, and blood pressure. Furthermore, it had been shown that in populations with a low sodium intake, there is a low mean blood pressure and little increase in blood pressure with age (Elliott &Marmot 1984). High Body Mass Index (BMI) and a high alcohol intake have also been associated with an elevated blood pressure (Intersalt Co-operative Research Group 1988).

Clearly hypertension is a complicated phenomenon under the influence of a number of factors both extrinsic and intrinsic. Until relatively recently, the ultimate causes of hypertension or an individuals susceptibility to it have been considered to be a consequence of genetic variation, and this is reflected in the variety of animal models (particularly in rats) developed for the study of hypertension.

1.1.12 Rat models of hypertension.

With little exception, rat models of hypertension have been developed by selective breeding of rat strains. This provided a number of sub-populations of rats which display either a blood pressure substantially higher than that seen in a general population of albino laboratory rats, or a susceptibility to increases in blood pressure in response to certain interventions. There are currently 8 such strains of rat available (Proceedings of the 4th International Symposium on Rats with Spontaneous Hypertension and Related Studies 1981): the New Zealand genetically hypertensive rat (GH), the spontaneously hypertensive rat (SHR), the Dahl salt-sensitive rat (DS), the Milan hypertensive rat (MHS), the Munster hypertensive rat (SHM), the Sabra hypertensive rat (SBH), the stroke prone SHR (SPSHR) and the Lyon hypertensive rat (LH). Each of these rat strains exhibit specific characteristics. There are, however, a number of characteristics shared by some or all of these strains.

In all cases, the blood pressure is substantially elevated over normotensive control strains. The DS and SBH strains, however, only show elevations of blood pressure in response to salt consumption. In most strains the heart is heavier than in controls, but

the kidney weight follows no discernible pattern, being increased in some (SHR, SPSHR), unchanged (LH, SBH) and smaller (GH, MHS) in others. There is generally no difference in pulse, although this appears slightly depressed in the MHS and similarly the stroke volume is comparable with that of controls. In all strains, the total peripheral resistance is elevated, and all strains except GH and Dahl salt-resistant (DR) animals, exhibit an additional elevation in blood pressure upon consumption of salt (not measured in LH).

Other rat models of hypertension have examined the effects of hypertensive stimuli on previously normotensive animals, as is the case in artificially induced Goldblatt hypertension. In rats, this is also referred to as two kidney-one clip hypertension. In these models, one of the renal arteries is occluded and the resulting hypertension is due, primarily to the activity of the renin-angiotensin system (Imamura *et al.* 1995; Schricker *et al.* 1994). This model of hypertension is useful in examining the pathophysiology of renovascular hypertension, which is caused by renal artery stenosis, but has a limited role in understanding essential hypertension.

1.1.13 The Barker hypothesis.

The Barker hypothesis expands the concept that fetal growth impairment causes metabolic alterations in the fetus, which may predispose the offspring to the subsequent development of diseases such as hypertension and CHD, in adult life. The hypothesis also suggests that obesity and non-insulin dependent diabetes mellitus (NIDDM) may also be terminal outcomes of fetal growth impairment.

The Barker hypothesis was initiated by early epidemiological studies of cardiovascular disease (Barker & Osmond 1986; Barker *et al.* 1989; Barker & Osmond 1987a; Barker & Osmond 1987b; Osmond *et al.* 1990). These studies highlighted an association between death from ischaemic heart disease or stroke and high infant mortality around the time when the victims of these diseases had been born. In addition, from relationships between maternal mortality during pregnancy and death from ischaemic heart disease and stroke (Barker & Osmond 1987b) and associations between place of birth, and death from cardiovascular disease (Osmond *et al.* 1990) it was shown that the early life experiences of an individual were stronger influences upon the subsequent

development of cardiovascular disease in adulthood than factors such as smoking, diet and place of current residence. Indeed, a dietary analysis of three English towns (Ipswich, Stoke-on-Trent and Wakefield) showed that despite consuming the lowest amount of fat, people in Wakefield had the highest deaths due to ischaemic heart disease (Cade *et al.* 1988).

It was suggested that these relationships might highlight a trend in which infant mortality might be associated with fetal growth impairment and that a fetus compromised *in utero*, which did not die, had increased risk of later heart disease and stroke. A low birth weight, used as a marker of fetal growth impairment, had previously been shown to be associated with elevated blood pressure in adult life in a study of Swedish army conscripts (Gennser *et al.* 1988). Further studies found similar links between birthweight and adult blood pressure in the UK, USA and Australia (Osmond *et al.* 1993; Law *et al.* 1993; Barker *et al.* 1989; Moore *et al.* 1996; Curhan *et al.* 1996; Barker *et al.* 1990), although some found a stronger relationship between adult blood pressure and a low body weight at 1 year of age (Barker *et al.* 1989; Fall *et al.* 1995).

Examination of birth weight alone as a marker of fetal growth retardation is limited to the extent that, apart from factors such as maternal under nutrition, maternal smoking, anaemia etc. birth weight may also be affected by the actual size of the mother (Fedrick & Adelstein 1978; Grundy & Hood 1978) and the condition of the mother prior to conception (Krammer 1987). In view of this, the association between birth weight and placental weight, and birth proportions were also used as markers of fetal growth retardation.

A low birth weight in association with a relatively large placenta (relative to birth weight) was found to be a significant risk factor for the development of elevated blood pressure and that the highest blood pressures were associated with the largest placentas and lowest birth weights (Barker *et al.* 1990; Barker *et al.* 1992; Moore *et al.* 1996; Law *et al.* 1991). Body proportions at birth were also associated with elevated blood pressure in children (Law *et al.* 1991). In this study, it was shown that blood pressure at 4 years of age was positively related to the ratio of body weight and head circumference at birth. The same study group as that used in Barker *et al.* (1990) were again examined in a later study (Barker *et al.* 1992). In this study, ponderal index (weight/length^3) at birth

was examined in relation to placental size and later blood pressure. It was shown that, for placental weights greater than 1.25lb, mean blood pressures rose with decreasing ponderal index, and with an increasing ratio of head circumference to body length. For placental weights less than 1.25lb, mean blood pressure rose with a decreasing ponderal index at birth. It was proposed, therefore, that for a small placenta, long thin babies and those with relatively large heads, had a greater risk of developing elevated blood pressure in adult life, whilst for a large placenta, risk of hypertension was greatest in short, fat babies.

Apart from cardiovascular disease, fetal growth impairment has also been found to be associated with glucose intolerance (Hales *et al.* 1991; Robinson *et al.* 1992), relative glucose intolerance (Dahlquist *et al.* 1996; Forrester *et al.* 1996), greater abdominal fat (Law *et al.* 1994), and raised serum cholesterol and LDL-cholesterol concentrations (Barker *et al.* 1993).

On the basis of these studies a hypothesis regarding fetal growth impairment and adult disease has been proposed (Table 1.2).

1.2 Targets of growth retardation or metabolic programming that might result in an increased propensity to develop an elevated blood pressure in adulthood include central factors (neuronal, endocrine, and metabolic), vascular factors, renal factors.

1.2.1 Central factors.

Central regulators of blood pressure control include neural and endocrine factors. The sympathetic nervous system plays a role in blood pressure control via actions on the heart, the vasculature and the secretion of vasoactive substances. The sympathetic nervous system acting via the baroreflex plays a role in the short-term (minutes to hours) regulation of blood pressure (Brooks & Osborn 1995). Vasoactive hormones produced in response to central nervous system action include, vasopressin (Veelken *et al.* 1989) and angiotensin II, via the stimulation of renin release (Agabiti-Rosei *et al.* 1984). Clearly, alterations in the activity of the sympathetic nervous system influencing

any or all of the above mentioned factors, through a disturbance in fetal growth, may have a significant role in the elevation of blood pressure seen in hypertension.

Alterations in the secretion of, or sensitivity to vasoactive hormones may also be affected by the fetal growth impairing effects of maternal undernutrition. Angiotensin II is of considerable importance in the control of blood pressure (Cowley 1992). The renin-angiotensin system has been shown to be sensitive to a maternal dietary protein restriction in the rat (Langley & Jackson 1994; Langley-Evans & Jackson 1995; Sherman & Langley-Evans 1998). Other vasoactive molecules and hormones which play a role in blood pressure regulation include nitric oxide (Vallance *et al.* 1989), vasopressin (Rascher *et al.* 1985), atrial natriuretic peptide (Richards 1987) and endothelin (Yanagisawa *et al.* 1988). There are clearly a number of centrally acting factors which, if disturbed by exposure to a maternal nutritional deficit *in utero*, may influence the later blood pressure of the offspring.

1.2.2 Vascular factors.

The vasculature itself is another potential target for developmental alterations in response to a maternal dietary protein restriction. The volume and elasticity of the vasculature, as well as its sensitivity to neural control and vasoactive hormones, may additionally influence blood pressure. The level of elastin within the vascular wall partially determines its elasticity and therefore its resistance to changes in lumenal diameter (Burton 1954). Thus, if vascular elastin production is altered in response to intrauterine growth restriction, then this could have a direct effect on the compliance of the blood vessels. This could potentially reduce the efficacy with which the mechanisms of blood pressure control exert their influence.

Table 1.2: Hypothesis for the fetal origins of adult disease (adapted from Barker 1995).

Period of under-nutrition	**1st Trimester**	**2nd Trimester**	**3rd Trimester**
Birth measurements.	Proportionately small babies.	Small and thin babies.	Short babies, normal weight.
Body weight at 1 year.	Reduced.	Normal.	Reduced.
Predisposition in adulthood.	Elevated blood pressure.	Elevated blood pressure. NIDDM.	Elevated blood pressure. High ldl cholesterol.
Most common cause of death.	Haemorrhagic stroke.	Coronary heart disease.	Coronary heart disease. Thrombotic stroke.

Changes in the expression of receptors for vasoactive hormones within the vasculature will also alter the response of the blood vessels to those hormones. This is another potential target of metabolic programming. If, for example, endemic production of nitric oxide within the blood vessels is altered, then vascular tone will be changed in consequence (Vallance *et al.* 1989). Clearly, modulation of the production of, or the sensitivity to, any vasoactive compound via processes occurring as a result of fetal growth retardation might have a significant bearing on blood pressure control in later life.

The control of blood pressure is influenced by a number of different factors including neurological, hormonal and vascular. However, in this thesis the renal involvement in blood pressure control was examined, and this will now be discussed in detail.

1.2.3 Renal factors.

1.2.3.1 The kidney.

The kidney is a major excretory organ of the body, removing potentially toxic compounds, maintaining plasma pH and regulating body fluid volume and electrolyte concentrations (Ganong 1991). The main excretory unit of the kidney is the nephron, which is supplied with a continuous flow of filtered plasma by the filtration action of the glomerulus. The glomerulus is a highly convoluted capillary across which the plasma is filtered. Filtration occurs as a result of the hydrostatic pressure within the glomerulus induced by the relative lumenal diameters of the afferent and efferent arterioles (Bayliss & Blantz 1986). The efferent arteriole (distal to the glomerulus) has a narrower bore than the afferent arteriole (situated in front of the glomerulus) and this induces a hydrostatic pressure within the glomerulus, which forces the filtration of plasma solutes across the glomerular basement membrane (Bayliss & Blantz 1986). Non-charged plasma solutes of less than 4ηm in diameter are freely filtered and filtration of neutral solutes declines with increasing diameter up to a maximum of 8ηm (Ganong 1991). There is a high concentration of negatively charged sialic acid residues in the basement membrane, the presence of which enhances the filtration of cations and impedes the filtration of anions (Ganong 1991).

The filtrate crosses the glomerular basement membrane into the Bowmans capsule and passes into the proximal convoluted tubule (Ganong 1991). In the proximal tubule, glucose, amino acids and various ions are resorbed (Schafer 1984). The filtrate passes into the loop of Henle. As the filtrate moves towards the bottom of the loop, it becomes more concentrated. This occurs as a result of the action of a Na+:K+:2Cl- co-transporter in the thick ascending limb of the loop of Henle (Jamison 1982). The action of this co-transporter results in an increase in the sodium concentration of the extracellular fluid between the closely opposed descending and ascending limbs of the loop. This sets up a concentration gradient of sodium between the tubular fluid of the descending limb and the extracellular fluid, down which sodium passes passively into the descending limb. The concentration gradient is maintained down the length of the descending limb and consequently the concentration of the tubular fluid is greatest at the bottom of the loop (Jamison 1982). The filtrate passes through the distal convoluted tubule and into the collecting duct, from which solutes and water may be selectively absorbed under the action of various hormones, such as arginine vasopressin (AVP) and aldosterone (Morel 1981). The concentrating mechanism enables the rapid resorption of water under the influence of AVP (the action of which is to cause the insertion of water channels in the principle cells of the collecting duct), as there is a significant concentration gradient between the collecting duct and the extracellular fluid at the base of the loop of Henle (Morel 1981).

The rate at which fluid is filtered at the glomerulus, the glomerular filtration rate (GFR) is a function of the hydrostatic pressure within the glomerulus (Bayliss & Blantz 1986). This, is maintained by the relative tone of the afferent and efferent arterioles. Arteriolar tone may be modulated selectively by various vasoactive hormones, for example angiotensin II, and also by sympathetic nervous control (Naver *et al.* 1996; Diboner 1982). The GFR plays a significant role in determining the rate of excretion of some solutes. A high GFR may result in a tubular flow rate, which is too rapid for the complete resorption of some molecules (e.g. glucose; Ganong 1991). Alternatively, a low GFR may result in excessive retention of water and electrolytes.

1.2.3.2 Renal development.

Renal development in the human follows three discrete stages (Sadler 1995). The pronephros develops early and is later followed by the mesonephros, which begins to develop tubular structures, which subsequently degenerate. The metanephric (permanent kidney) forms in the 5th week of gestation and subsequently forms the ureter and structures of the adult kidney (Saxon *et al.* 1986). There are two primary activities that take place during renal development, branching of the ureteric bud and condensation and proliferation of the metanephric mesenchyme to form tubules (Stuart & Nigam 1995). Early studies, using cultured metanephric mesenchyme from the day 11 mouse embryo, demonstrated the ability of certain embryonic tissues to induce tubular formations in the mesenchyme even though separated from it by a filter (Grobstein 1953). These studies highlighted a role in nephrogenesis for factors, which did not require cell to cell contact to be effective. These have been examined using three primary methods, including the culture of metanephric tissue, Madin-Darby canine kidney (MDCK) cells and murine inner medullary collecting duct (IMCD) cells, and knockout mice.

The ureteric bud arises from the mesonephric duct and arborises within the metanephric mesenchyme (Sadler 1995). It has been suggested that the Wilms tumour (WT-1) gene may be the orchestrator of the initiation and subsequent development of the ureteric bud (Pritchard-Jones *et al.* 1990). It is expressed in metanephric mesenchyme, and WT-1 knockout mice fail to form a ureteric bud and subsequently exhibit bilateral renal agenesis (Pritchard-Jones *et al.* 1990). Thus the implication is that ureteric bud initiation is signalled by the metanephric mesenchyme and may be controlled by the WT-1 gene product. The arborisation of the ureteric bud is apparently under the influence of a variety of factors, including proteoglycans, growth factors, extracellular matrix protein (ECM) proteinases and other gene products.

Proteoglycans are protein-carbohydrate complexes containing up to 95% carbohydrate and whose properties more closely resemble those of carbohydrates than proteins (Schwartz 1992). Heparin sulfate proteoglycan (HSP) is expressed in the basement membrane in a pattern that suggests a role in the inhibition of branching (Stuart & Nigam 1995). This view is upheld by studies using MDCK cells (Santos & Nigam

1993). Chondroitin sulfate proteoglycan (CSP) appears to have the opposite effect, in that it promotes branching (Spooner *et al.* 1985). Interestingly, incubation of metanephric rudiments with CSP appears to increase the number of nephrons induced by an elevation in the branching activity of the ureteric bud (Platt *et al.* 1990).

Other extracellular matrix molecules may also play a significant role in the branching morphogenesis of the ureteric bud. Collagen I, fibronectin, laminin and entactin have all been demonstrated to promote branching in culture, whilst fibronectin and vitronectin have an inhibitory effect (Santos & Nigam 1993; Perantoni *et al.* 1991). Fibronectin has been shown to be expressed in the mesenchymal regions where ureteric bud branching occurs (Ekblom 1981), although the integrin receptors for fibronectin have yet to be discovered in the ureteric bud (Korhonen *et al.* 1990). It is suggested that the breakdown of the mesenchymal barrier of ECM proteins is necessary for the invasion of the ureteric bud, and this is supported by the variety of ECM proteinases present (Mignatti & Rifkin 1993). The proteinase plasmin, is activated by plasminogen which is in turn activated by the action of urokinase and tissue type plasminogen activator (u-PA & t-PA respectively). The kidney has been shown to express high levels of u-PA and t-PA (Danglot *et al.* 1986) and in addition, it has been shown that renal tubules of 17.5 day old mouse embryos express u-PA (Sappino *et al.* 1991). In MDCK cells (Montesano *et al.* 1991), administration of serine protease inhibitors prevents the branching morphogenesis response to hepatocyte growth factor (HGF). Also, cells cultured in fibroblast conditioned media demonstrate a large increase in u-PA activity in response to HGF, an effect which is abolished when the media is pre-incubated with anti-HGF (Pepper *et al.* 1992). Thus, ECM proteinases may play a significant role in ureteric bud branching, which is possibly also under the control of growth factors.

Growth factors have been extensively studied in relation to renal development. When placed in a collagen gel, MDCK cells undergo transformations resulting in the formation of cystic type structures. Upon exposure to HGF, these structures become more branched and tube-like and demonstrate cellular polarity (Montesano *et al.* 1991; Santos *et al.* 1993). HGF and its receptors are expressed during renal development and addition of antibodies to these has been shown to result in a disruption of nephrogenesis (Santos *et al.* 1994). HGF is expressed in the metanephric mesenchyme and is thought to stimulate the branching of the ureteric bud (Woolf *et al.* 1995; Rosen *et al.* 1995). Thus

a dual role for HGF has effectively been suggested, in that HGF stimulates the expression of ECM proteinases which may allow the HGF induced arborisation into the metanephric mesenchyme. A significant question raised by this, is that of what initiates the expression of HGF from the metanephric mesenchyme. Despite the apparent significance of HGF in branching morphogenesis, HGF knockout mice do not show any signs of renal dysplasia (Schmidt *et al.* 1995). In addition to HGF, epithelial growth factor (EGF), transforming growth factor-α (TGF-α) and nerve growth factor (NGF) have all been shown to initiate branching morphogenesis in the ureteric bud (Barros *et al.* 1995; Sariola *et al.* 1988).

The MDCK cell-line has proved a useful tool in the examination of post-inductive events in the metanephric mesenchyme. These cells are dependent on calcium to adhere to each other and to demonstrate polarity. A culture medium containing 1.8mM Ca^{2+} allows cell-cell adhesion to occur, and this takes place in a similar way to that seen in the developing metanephric mesenchyme (Stuart & Nigam 1995). Metanephric mesenchyme, upon induction, condenses and forms cystic structures which subsequently elongate to form nephrons. Little is known of how these processes occur.

Of particular interest is the role played by IGF-I in renal development, as this has already been shown to be altered in various models of maternal undernutrition induced fetal growth retardation (Owens *et al.* 1993; Owens *et al.* 1994; Breier *et al.* 1988; Gluckman & Harding 1994; Liu *et al.* 1994). The insulin-like growth factors (IGFs) are hormones, which mediate the growth and proliferation of tissues during fetal and postnatal development (Humbel 1984; Moses *et al.* 1980; Soares *et al.* 1985). The actions of IGFs are mediated via the IGF receptors, of which there are two. IGF-I is preferentially bound to the type 1 receptor, whilst the type 2 receptor preferentially binds IGF-II (Nissley & Rechler 1984). IGFs generally exist in the circulation bound to IGF binding proteins (Baxter & Martin 1989).

Expression of IGF-I and its binding proteins have been demonstrated in the developing kidney (Rogers *et al.* 1991). IGF-I receptors have been found in the epithelia of the ureteric bud (an outgrowth which will subsequently form the ureter, renal pelvis, calyces and collecting ducts - Saxon *et al.* 1986) and metanephric blastema cells (which will subsequently form glomeruli and tubules). At the end of gestation in the mouse, the

IGF-I receptors have been found to be localised in the tubular epithelia (Matsell *et al.* 1994). Culturing of fetal mouse kidneys with anti-IGF-I antibodies demonstrated that IGF-I is essential for renal development (Rogers *et al.* 1995). Liu and co-workers (Liu *et al.* 1993) and Wada and colleagues (Wada *et al.* 1993) demonstrated the inhibitory effect of the addition of antisense oligodeoxynucleotides of IGF-I receptor cDNA on nephrogenesis, in embryonic mouse kidneys. The inhibitory effect was shown to be greater in the early stages of development and this, it was concluded, was as a result of the decline of IGF-I receptor expression as development proceeds.

Damage to the mesonephric kidney has been shown to result in stunting of limb growth in the developing chick embryo, an effect which has been induced by the addition of anti-IGF-I antibodies (Geduspan *et al.* 1993) and it has also been shown that the expression of IGF-I and IGF-I receptors in the mesonephric kidney is consistent with the role of mesonephric derived IGF-I in limb bud outgrowth (Geduspan *et al.* 1992).

Renal development is an involved process, complicated by the numerous factors which play a part. In particular, IGF-I is of interest in the context of maternal undernutrition and fetal renal development as its expression has been shown to be modulated in fetal tissues in response to alterations in maternal nutrition (Owens *et al.* 1993; Owens *et al.* 1994; Breier *et al.* 1988; Gluckman & Harding 1994; Liu *et al.* 1994). Studies examining the impairment of fetal growth and effects on renal development and subsequent renal function have highlighted a potentially causal role for maternal undernutrition. These will be discussed below.

1.2.3.3 Fetal growth impairment, renal development and renal function.

Renal structural alterations have been shown to result from fetal growth impairment in humans (Hinchliffe *et al.* 1992). In a two part study, using non-macerated stillborns, which were both below the 10th centile for birthweight and showed signs of type II (asymmetrical) intrauterine growth retardation (IUGR; disproportionately large head, small abdominal viscera and lack of subcutaneous fat - Usher & McLean 1974) and infants which died in their first year and who had been below the 10th centile at birth for body weight, nephron number was determined. Control infants had been above the 10th centile for birthweight. Both groups of IUGR infants had significantly fewer

than the controls, both being below the 5% prediction limit calculated from the control groups. Konje and co-workers (Konje *et al.* 1996) demonstrated, using ultrasound measurements of fetal renal morphometry, that the kidneys of small for gestational age babies exhibited slower growth in anterio-posterior, transverse and circumference planes between weeks 26 and 34 of gestation.

In rats subjected to either an uterine arterial ligation from day 17 or a 5% protein diet from day 8 of pregnancy, offspring were found to have significantly fewer nephrons at two weeks of age compared to offspring from females fed 22% protein diet throughout pregnancy (Merlet-Benichou *et al.* 1994). All females had been transferred to a standard diet at parturition and therefore, it was concluded that the nephron reduction had occurred as a result of intrauterine conditions. Zeman similarly examined the effect of undernutrition on the glomerular complement of rat offspring from females fed either a 6% protein or a 24% protein diet during pregnancy (Zeman 1968). This study showed that offspring from protein deprived mothers had fewer nephrons per kidney than did the control group. Acid phosphatase activity, a marker for the presence of lysosomes (Strauss 1954) and alkaline phosphatase activity, which is used as a marker of tubular maturity and is located along the brush border of the proximal tubule (Wachstein 1955), were depressed in the kidneys of the maternal low protein group. It was therefore suggested that the functional tubule brush border is diminished in the offspring of low protein fed mothers. This was further highlighted by the histological demonstration that these offspring had shorter proximal tubules with fewer convolutions than the control animals (Zeman 1968).

Zeman and co-workers further examined the result of maternal protein restriction on the renal function of the offspring. Using the same 6% protein and 24% protein diets, water diuresis, osmotic diuresis and inulin clearance (Hall & Zeman 1968) and arginine vasopressin (AVP),glomerular filtration rate (GFR) and the transport maximum of p-amino hippuric acid (TmPAH) were examined (Allen & Zeman 1973) in the offspring. Newborn and 2 day old offspring of protein restricted mothers exhibited a depressed urine production and water diuresis (Hall & Zeman 1968). Osmotic diuresis in the protein restricted offspring was depressed in offspring at 2, 4 and 6 days of age and the inulin clearance in the controls was four times greater than the low protein offspring. Inulin clearance is used to estimate GFR and it was therefore implied that GFR was

greatly reduced in the low protein exposed offspring. The excretion of an AVP load was shown to be impaired in offspring of protein restricted dams at 6 days of age (Allen & Zeman 1973) and these same offspring showed a reduced GFR and TmPAH at 22 days. The transport maximum of PAH represents the maximum plasma concentration at which this molecule may be excreted from the blood. A depression in the GFR may be primarily responsible for the lower TmPAH in the low protein animals, although it may also be reflective of a tubular insufficiency for excretion.

Using a 4% protein maternal diet and a 24% protein diet control Grosvenor and Zeman (Grosvenor & Zeman 1983) examined amino acid uptake in the offspring. Mothers fed the low protein diet gained only 5g throughout pregnancy, indicating the severity of this nutritional manipulation. Uptake of alpha-aminoisobutyric acid, an amino acid analogue which is taken up by tissues via sodium-dependent amino acid transporters (McInnes & Scriver 1979), was found to be enhanced in renal tissue slices from low protein exposed offspring at 21 days of gestation compared to those from controls. Uptake of glycine was not different between the two groups and this was thought to be due to the different time of maturation of the glycine transporters (Baerlocher *et al.* 1971).

Maternal under-nutrition has therefore been shown to retard fetal growth both in terms of gross body weight and dimensions and also in terms of individual organ development. A significant effect of maternal diet-induced fetal growth impairment on the metabolic and specific organ function of the offspring has been demonstrated, particularly with regard to the kidney.

1.2.3.4 The kidney and hypertension.

The renal blood flow (RBF) is widely used, along with the GFR, as a measure of the functional status of the kidney. Both a depressed renal blood flow (Pederson & Kornerup 1976; Ljungman 1982; Bolomey *et al.* 1949) and an enhanced renal blood flow (Bello *et al.* 1965; Bianchi *et al.* 1979) have been reported in patients with essential hypertension. London *et al.* (1984) examined cardiac output in relation to renal blood flow and showed that, at any given cardiac output, the renal blood flow of hypertensive patients was significantly lower than that found in normotensives. It was thus demonstrated that renal blood flow *per se*, may not necessarily appear to be

different in hypertensives unless considered as a function of cardiac output. Renal blood flow has also been demonstrated to decline more rapidly with age in hypertensives than in normotensives (London *et al.* 1984; Bauer *et al.* 1982). Conversely, the GFR has been shown to decline more slowly with age in hypertensive patients (Ljungman 1982; De Leeuw *et al.* 1978), although, in both studies the level of blood pressure was inversely related to the GFR. If this is considered in relation to the age dependant decline in RBF, it may be seen that the filtration fraction, that proportion of the blood passing through the kidney which is filtered at the glomerulus, increases in hypertensives with age, to a greater extent than in normotensives. This reflects an increase in the workload of the kidney in hypertensives.

There is a substantial literature implicating the kidney in the development of hypertension. Transplantation studies have shown that kidneys, from strains of genetically hypertensive rat, induce hypertension in previously normotensive rats. Conversely, transplantation of kidneys from normotensive donors into hypertensive rats reduces the blood pressure of the recipients. This has been demonstrated in Dahl salt sensitive rats (Dahl *et al.* 1974), Milan hypertensive rats (Bianchi *et al.* 1974), Prague hypertensive rats (Heller *et al.* 1993) and the Okamoto strain of spontaneously hypertensive rat (Kawabe *et al.* 1978).

The kidney is, therefore, widely considered to have a major role in blood pressure control. Disturbances in renal structure and function have been postulated to result in the maintenance of elevated blood pressure.

1.2.3.4.1 Renal functional alterations which may have a direct effect on blood pressure, include the efficiency of sodium and water homeostasis.

More than 30 years ago it was proposed, by Guyton and colleagues, that the role of the kidney in the development of hypertension may proceed by virtue of an alteration in the pressure natriuresis and diuresis phenomena (Guyton *et al.* 1972). The principle of pressure natriuresis is that any increase in blood pressure will cause an increase in the rate of sodium excretion. Pressure diuresis is essentially the same process but relating to

water excretion. Therefore, at a blood pressure elevated above the normal (for that subject) sodium and water will be lost, whilst a depressed blood pressure will lead to sodium and water retention. This phenomenon was related to sodium intake and it was hypothesised that blood pressure is maintained at a level sufficient to enable regulation of the body sodium content by the action of pressure natriuresis. Thus, a low sodium intake would depress blood pressure and reduce the rate of pressure natriuresis. This would allow sodium retention. A high sodium intake would lead to an increase in the blood pressure and an increased sodium excretion, which would occur until the body sodium content had declined to its equilibrium level.

Guyton proposed that hypertension may result if the pressure natriuresis curve shifts to the right (Figure 1.2), i.e. the same body sodium equilibrium point is maintained, but now it requires a higher blood pressure to maintain it (Guyton *et al.* 1980). From this he developed two "Laws of Hypertension". First, he stated that hypertension may only develop if the equilibrium point of sodium intake and excretion is shifted to a higher blood pressure. Secondly, he hypothesised that if the equilibrium point is shifted to a higher pressure, then blood pressure will be maintained around that new level. This proposal is difficult to refute, despite the observation that hypertensive individuals may not demonstrate significant blood pressure elevations with increased salt intake. In such individuals, it may be argued that, not only has their equilibrium point been shifted, but also that the rate of increase of blood pressure and sodium excretion may be steeper. So, for any increase in sodium intake above the equilibrium point, the rise in blood pressure required to excrete the excess may be lower than in normotensives.

This hypothesis has been supported, in many cases indirectly, by virtue of the means by which blood pressure elevation is induced in experimental models. Renal artery stenosis (Norman *et al.* 1978), aldosterone and deoxycorticosterone administration (Hall *et al.* 1984; Cowley *et al.* 1979), reduction of renal mass (Douglas *et al.* 1964) and infusion of vasoconstrictor agents, including AVP (Hall *et al.* 1986) and angiotensin II (Hall *et al.* 1984) all, in general, act by reducing the renal ability to excrete sodium and water at normal blood pressure levels, thus inducing the necessity for an elevation in the blood pressure. Many of the genetically hypertensive rat strains demonstrate a blunted and reset pressure natriuresis (Roman & Cowley 1985; Liu *et al.* 1994; Gross *et al.* 1994). Furthermore, most antihypertensive drugs currently in use, result in the excretion of

sodium and water and lead to a resetting of the pressure natriuresis equilibrium. These include angiotensin converting enzyme (ACE) inhibitors (Mattson & Roman 1991), vasodilators (Kline & Mercer 1987) and calcium channel blockers (Fenoy *et al.* 1992).

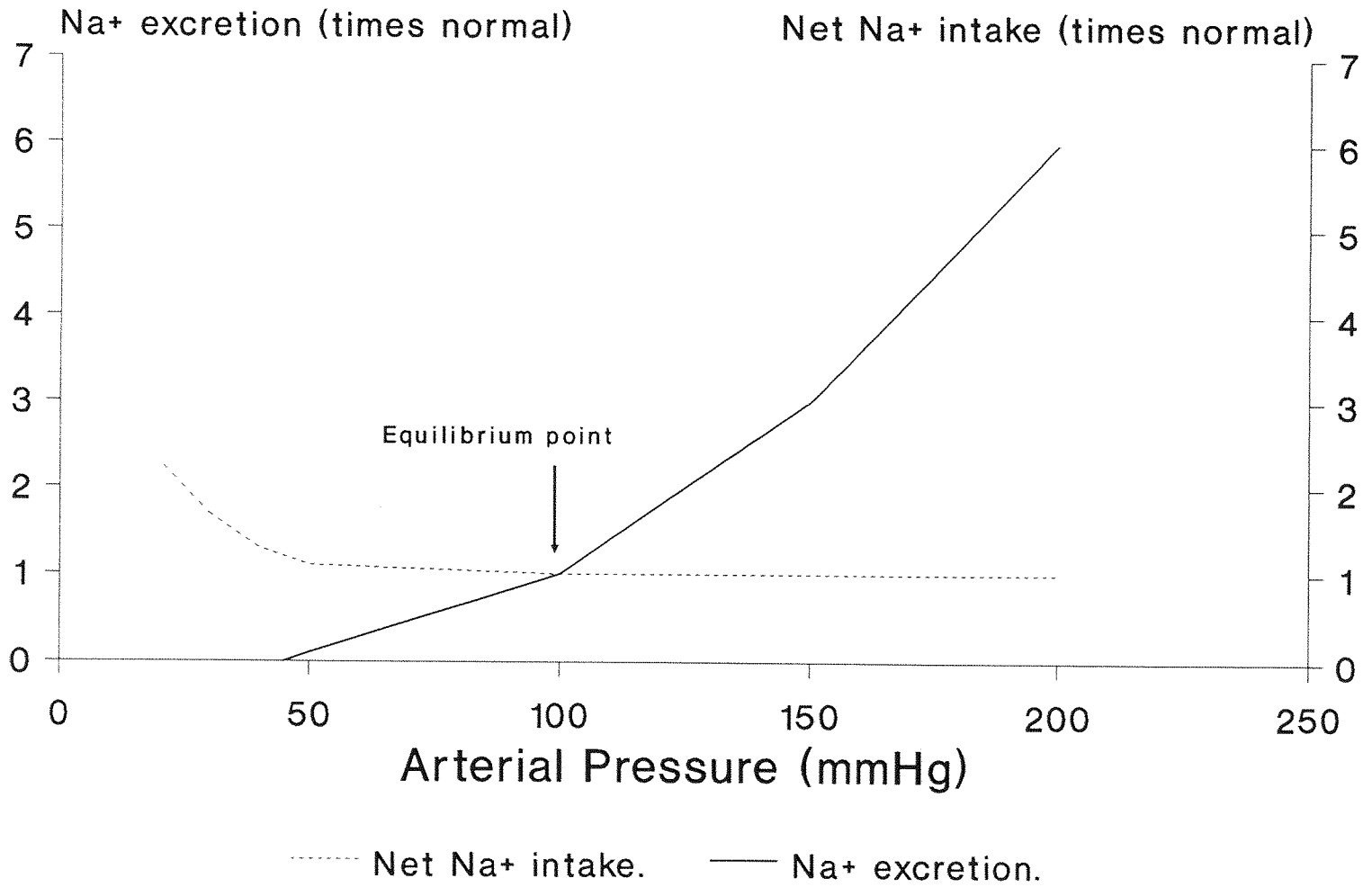
A rise in renal perfusion pressure is required to maintain electrolyte balance after administration of salt and water retaining hormones, such as angiotensin II (Hall *et al.* 1984a) and aldosterone (Hall *et al.* 1984b). If, during administration of these hormones, the renal perfusion pressure is experimentally maintained at pre-administration levels, then significant salt and water retention ensues. It has further been shown that by controlling fluid intake to accommodate for sodium excretion, blood pressure may be maintained at a normal level during long term angiotensin II administration (Krieger & Cowley 1990).

1.2.3.4.2 A relative nephron deficit has been proposed may be causative in the development of hypertension.

Mackenzie and Brenner (1995) proposed another dimension to the role of the kidney in the aetiology of hypertension. It was suggested that a reduced complement of nephrons in the adult kidney might predispose the individual to the development of elevated blood pressure and progressive nephron loss (Mackenzie & Brenner 1995). Extensive renal ablation in rats leads to an increase in blood pressure and glomerular hydraulic pressure, apparently for the purpose of maintenance of normal glomerular filtration and fluid excretion (Brenner 1985). An elevated glomerular hydraulic pressure leads to glomerulosclerosis and therefore further nephron loss, resulting in a cycle of blood pressure elevation and nephron loss (Hostetter *et al.* 1981). This has also been demonstrated in humans subject to renal resection as a result of neoplasms or renal trauma (Novick *et al.* 1991). In many cases, renal donors are found to develop elevated blood pressure with time (Torres *et al.* 1987; Hakim *et al.* 1984). Patients with congenital renal abnormalities, such as those with oligomeganephronia (characterised by very few, hypertrophic nephrons), have also been shown to be subject to glomerulosclerosis (Fetterman & Habib 1969), thus endorsing the view that an initial nephron deficit leads to a more rapid nephron loss.

Figure 1.2: Pressure natriuresis curve.

Adapted from Guyton (1980), this figure shows the proposed relationship between sodium intake, sodium excretion and blood pressure.


Na+ excretion (times normal)
Net Na+ intake (times normal)
7
6
5
4
3
2
1
0
Equilibrium point
0
50
100
150
200
250
Arterial Pressure (mmHg)
Net Na+ intake.
Na+ excretion.

The intra-individual variation in human glomerular complement has been shown to be extremely wide (Moore 1931; Nyengaard & Bendtsen 1992), ranging from 300 000 nephrons to over 1 000 000 nephrons, with an average of approximately 600 000 nephrons. Examination of different human populations has shown that the incidence of hypertension is higher in populations with a lower glomerular complement. In Japan, where hypertension is common (Takahashi *et al.* 1957), it has been demonstrated that people have small kidneys (Tauchi *et al.* 1971), an association that has also been demonstrated in African Americans (Final Report of the Subcommittee on Definition and Prevalence of Hypertension; the 1984 Joint National Committee; Luft *et al.* 1979). In two strains of spontaneously hypertensive rat, the Milan hypertensive rat and the SHR, it has been shown that the glomerular complement is between 10% and 20% lower compared to normotensive controls (Brenner *et al.* 1988) whereas a strain of mouse known to be resistant to hypertension, the PVG strain, has a relatively greater number of glomeruli than controls.

Mackenzie and Brenner hypothesise (Mackenzie & Brenner 1995) that a nephron deficit may affect the individual at two stages during the development of hypertension. Possible sodium retention may initially induce an associated water retention and therefore an increase in blood volume, and/or promote the secretion of hormones, such as angiotensin II or arginine vasopressin. A gradual nephron loss, associated with elevated glomerular hydraulic pressure and subsequent glomerulosclerosis may then follow. It is suggested that the thrifty phenotype hypothesis may be useful in determining the cause of a relative nephron deficit. The thrifty phenotype hypothesis derived from the thrifty genotype hypothesis, which was originally conceived to explain the incidence of diabetes (Neel 1982). This suggested that, the early hunter-gatherers might best make use of an infrequent food supply by a rapid carbohydrate internalisation and conversion to adipose tissue. This would be mediated by a large pancreatic beta-cell secretion of insulin, during feeding. People with this trait, it was suggested, would stand a better chance of survival during conditions of food scarcity. Neel proposed that, in the developed world, where food intake is regular, the presence of this genetic trait would lead to over activation of the beta-cells and lead to their subsequent exhaustion and therefore insufficient insulin production, a trait linked more to non-insulin dependent diabetes. The thrifty phenotype hypothesis (Hales & Barker 1992) was derived from this after the discovery that low birth weight was associated

with non-insulin dependent diabetes mellitus (Hales *et al.* 1991). This suggested that, rather than the altered beta-cell activity being genetically predetermined, it may result from a fetal accommodation to maternal undernutrition. Mackenzie and Brenner (1995) proposed that the reduction of nephrons might also result from a fetal accommodatory response to nutritional deprivation in order that electrolyte conservation and fluid homeostasis might be maintained at as low an energy cost to the individual as possible.

The kidney, therefore, has a significant, if not primary, role to play in the initiation and development of hypertension. This may well be a result of alterations in renal developmental during fetal growth retardation, arising from maternal undernutrition.

1.3 A rat model of hypertension induced by a mild maternal protein restriction.

1.3.1 Introduction.

The rat model to be studied in this thesis was originally described by Levy & Jackson (1993). Female Wistar rats were supplied isocaloric synthetic diets in which the primary variable was the casein content. Four experimental diets were originally used, comprising either 60g, 90g, 120g or 180g casein per kg (6% casein, 9% casein, 12% casein and 18% casein respectively). The 18% casein diet was the control diet and was chosen to reflect the protein level found in standard laboratory rat chow (around 18% by weight). The experimental diets were fed to female Wistar rats weighing between 200g and 250g throughout pregnancy. Numerous studies have now been carried out using a similar model to induce hypertension during postnatal life (Langley & Jackson 1994; Langley-Evans *et al.* 1994; Langley-Evans & Jackson 1996; Langley-Evans 1996; Langley-Evans *et al.* 1996b; Gardner *et al.* 1998; Gardner *et al.* 1997; Langley-Evans *et al.* 1995; Langley-Evans & Jackson 1995; Langley-Evans *et al.* 1996c; Langley-Evans *et al.* 1996d; Langley-Evans *et al.* 1996f; Langley-Evans & Nwangwu 1998).

1.3.2 Maternal low protein diet and growth.

The initial study of Levy & Jackson (1993) found that exposure to a maternal 9% casein diet both before and during pregnancy reduced fetal weight at day 20 of gestation (Levy & Jackson 1993). However, subsequent work (Langley-Evans *et al.* 1996c) found that

such a maternal protein restriction, resulted in enhanced fetal growth between days 14 and 20 of gestation. At full-term (22 days post-conception), the body weights of 9% casein exposed offspring were comparable with those of controls. Langley-Evans and Nwangwu (1998) have further demonstrated increased fetal growth to day 14 by exposure to a maternal 9% casein diet. However, in this study, fetal weight at day 20 of gestation was lower in 9% casein exposed animals compared with control animals. Birthweight in this model shows a variable response to a maternal protein restriction, sometimes lower than in controls (Langley-Evans *et al.* 1994; Langley-Evans & Nwangwu 1998) and sometimes comparable (Langley-Evans *et al.* 1996b; Langley-Evans *et al.* 1996c). The ponderal index of low protein exposed animals was lower at day 20 and higher at term than in controls. Thus, a mild maternal dietary protein restriction was thus shown to impede growth in late gestation in a manner which suggested that protein available to the mother may become a limiting factor in late fetal growth. Postnatally, there is little or no effect of maternal diet on offspring body weight, but offspring of 9% casein fed dams have large hearts (Langley-Evans 1996) and disproportionately small kidneys (Langley-Evans *et al.* 1996c) compared with control animals.

1.3.3 Maternal low protein and glucocorticoids.

Glucocorticoids are 21 carbon steroid hormones synthesised in the adrenal cortex (Granner 1993). The predominant glucocorticoid in the human is cortisol and in the rat, corticosterone. Glucocorticoids (cortisol will be discussed unless otherwise stated) are synthesised from cholesterol in a five stage process, two of which (conversion of cholesterol to pregnenalone and the conversion of 11-deoxy-cortisol to cortisol) occur in the mitochondria whilst the remaining three stages (conversion of pregnenalone to progesterone, progesterone to 17α-hydroxyprogesterone and then to 11-deoxy-cortisol) take place in the cell's lipid bilayer (Harding 1979). Cortisol is not stored and its secretion is under the influence of adrenocorticotrophic hormone (ACTH) which generally follows a diurnal rhythm (Granner 1993). Cortisol action is mediated by binding to the intracellular glucocorticoid receptor. The receptor/ligand complex crosses the nuclear membrane and initiates DNA transcription (Gustafsson *et al.* 1987). Cortisol has many effects including the suppression of the immune system (Munck *et al.* 1984),

control of food intake (King 1988; Honma *et al.* 1983), gluconeogenesis and fat synthesis (Exton *et al.* 1976; Pittner *et al.* 1985).

Glucorticoids have also been shown to play a role in control of vascular tone. Administration of the glucocorticoid receptor antagonist RU38486 in rats was shown to reduce the pressor response to catecholamines (Grunfeld & Eloy 1987) and in isolated rabbit (Fowler & Chou 1991) and dog (Besse & Bass 1966) aortic preparations, deoxycorticosterone, corticosterone and hydrocortisol were found to potentiate the vasoconstrictor response to catecholamines. No potentiation of angiotensin II or vasopressin-mediated constriction was found in these experiments. In Cushings syndrome, which is characterised by elevated cortisol secretion due to adrenal tumours or hyper secretion of ACTH (Forsham & Di Raimondo 1960), pressor responses to angiotensin II and noradrenaline are increased (Saruta 1986) and this may be mimicked by systemic administration of ACTH or cortisol, although here, the responsiveness to angiotensin II is not seen (Kurland & Freedburg 1951; Whitworth *et al.* 1986).

The means through which cortisol may influence vascular sensitivity to vasoactive hormones are either by an effect on their secretion, an effect on their receptor activity, expression and/or the post-receptor signalling pathway, or by an effect on the mechanism by which contraction occurs. This latter effect would presumably enable the potentiation of vasoconstriction to all pressor agents (Walker & Williams 1992). Glucocorticoids have been shown to inhibit phospholipase A_2 and therefore the production of the vasorelaxing hormones prostaglandin E_1 (PGE_1), prostacyclin and eicosanoids (Flower 1988; Axelrod 1983). This has been associated with the potentiation by glucocorticoids of catecholamines by use of the phospholipase A_2/cyclo-oxygenase (both involved in prostaglandin production) inhibitor indomethacin (Handa *et al.* 1984). It was shown that both indomethacin and dexamethasone (a synthetic glucocorticoid analogue) potentiated the pressor response to noradrenaline, leading to the conclusion that perhaps glucocorticoids may act through the pathway of phospholipase A_2/cyclo-oxygenase inhibition. Furthermore, glucocorticoids act upon the renin-angiotensin system, elevating ACE activity, AT_1 receptor expression and angiotensinogen production (Langley-Evans 1997)

Glucocorticoids are metabolised by the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD; Seckl 1993). This enzyme is present in the kidney, where it is required to prevent the innapropriate binding of cortisol to the mineralocorticoid receptors in the distal nephron (Edwards *et al.* 1988). These receptors normally bind aldosterone and enhance sodium uptake, but have a higher affinity for cortisol. If 11β-HSD is absent due to a congenital abnormality (apparent mineralocorticoid excess) or is inhibited, then cortisol binds to the mineralocorticoid receptor and promotes an inappropriate sodium retention, potassium loss and hypertension (Stewart *et al.* 1987; Stewart *et al.* 1988). Glucocorticoids are also prevented from crossing the placenta by 11β-HSD (Seckl 1993) and it has been proposed (Edwards *et al.* 1993) that a relative placental insufficiency of this enzyme may be responsible for over exposure of the fetus to maternal glucocorticoids, leading to the intrauterine programming of hypertension. Rats with the lowest placental activity of 11β-HSD deliver offspring of low birthweight and have relatively large placentas. Treatment of pregnant rats with the 11β-HSD inhibitor carbenoxolone leads to the production of hypertensive offspring (Lindsey *et al.* 1994) with a basal hyperglycaemia and insulin resistance to an oral glucose load. The final week of rat gestation appears to be a period of particular importance for 11β-HSD function, with respect to protection of the fetus from hypertensive effects of glucorticoids (Langley-Evans 1997).

It was proposed (Langley-Evans *et al.* 1996d) that the consumption of a low protein diet during rat pregnancy may affect the expression or action of 11β-HSD in the placenta and therefore allow the passage of maternal glucocorticoids to the fetus, which may impair fetal growth. Indeed, placental activity of 11β-HSD was shown to be reduced by exposure to a maternal 9% casein diet during pregnancy (Langley-Evans *et al.* 1996d). It was additionally shown that the activity of the glucocorticoid sensitive enzyme glutamate synthetase (GS) was elevated in placentas of offspring of 9% casein fed mothers, whilst that of the glucocorticoid insensitive aryl hydrocarbon hydrolase, malate dehydrogenase (MD) and pyruvate kinase (PK) were unaffected by a maternal dietary protein restriction. This suggested that the placentas from these animals were either more sensitive to glucocorticoid exposure, or exposed to a greater concentration of glucocorticoids than in controls. Similar results have been shown at full term and in

adult life in offspring of rats supplied a 9% casein diet during pregnancy (Langley-Evans *et al.* 1996b).

1.3.4 Hypertension and the rat model.

Exposing a female rat to a diet low in protein during pregnancy results in her offspring developing a higher blood pressure during later life. All the studies using this model which have examined blood pressure, have shown an increase in systolic blood pressure of around 15mmHg - 20mmHg in offspring exposed to a maternal 9% casein diet during gestation compared with controls (Langley & Jackson 1994; Langley-Evans *et al.* 1996a; Langley-Evans *et al.* 1996c; Langley-Evans *et al.* 1996d; Langley-Evans *et al.* 1996e; Langley-Evans 1996; Langley-Evans & Jackson 1995; Langley-Evans & Jackson 1996; Langley-Evans & Jackson 1996; Langley-Evans *et al.* 1994; Langley-Evans & Nwangwu 1998). This effect is independent of maternal blood pressure (Langley-Evans *et al.* 1994). Furthermore, it has been shown that not only a maternal dietary protein restriction, but also altering the source of dietary fat (coconut oil instead of corn oil; Langley-Evans 1996) may result in an elevation of blood pressure in the offspring.

Other factors associated with hypertension have also been examined in relation to this rat model and suggested a prominent role for the kidney. Hypertension is often treated with angiotensin converting enzyme (ACE) inhibitors, such as captopril and elanapril (Rose 1991). These compounds prevent the conversion of angiotensin I to angiotensin II (AngII). It has been shown (Langley-Evans & Jackson 1994) that pulmonary ACE activity was increased in offspring of protein restricted females. The efficacy of ACE inhibition was therefore examined in the rat model of maternal protein restriction induced hypertension (Langley-Evans & Jackson 1995). Offspring from 9% casein fed females at 4 weeks of age showed elevated levels of plasma ACE compared to 18% casein fed controls. Administration of captopril to adult offspring induced a slight but not significant decline in systolic blood pressure of 18% casein exposed controls and a significant reduction in the blood pressure of the 9% casein exposed group. The blood pressure of the 9% casein exposed animals did not become significantly elevated above those of the 18% casein controls until 7 - 8 weeks after the cessation of captopril treatment. This work was corroborated by Sherman and Langley-Evans (1998) in a

study which demonstrated that inhibition of ACE using captopril, in rats from 2 - 4 weeks of age over a 2 week period, prevented those exposed to a maternal 9% casein diet *in utero* from developing the elevated blood pressure seen in their non-treated counterparts.

Dietary salt has for many years been suggested as a causal factor in the development of hypertension (Intersalt Co-operative Research Group 1988; Kesteloot *et al.* 1987; Poulter 1984). In the rat, blood pressure has been shown to rise and fall directly with varying concentrations of sodium in an intraperitoneal dialysate (Friedman *et al.* 1990). Its effect on the control animals in this model (Langley-Evans & Jackson 1996) was to elevate their blood pressure to a level equivalent to that of 9% casein exposed animals. However, there was no blood pressure response to a salt loading within the 9% casein exposed group. It has been suggested that the kidneys of the 9% casein exposed animals may require a higher blood pressure in order to maintain sodium homeostasis via the hypothesis of Guyton (1972). Therefore, a mild maternal protein restriction during pregnancy may affect fetal kidney development. This may predispose the offspring to the development of elevated blood pressure in postnatal life.

1.4 Overview of work to be done in the thesis.

1.4.1 Why was the kidney chosen for close examination?

Examination of animals exposed to a maternal 9% casein diet *in utero* highlighted a potential role for the renin-angiotensin system (Langley-Evans & Jackson 1994; Langley-Evans & Jackson 1995) in the pre-natal programming of hypertension. As renin is secreted from the kidney and angiotensin II plays a significant role in renal physiology, it was considered possible that nutritionally mediated structural alterations in the kidney, may be responsible for some of the disturbances seen in the renin-angiotensin system in these animals. Furthermore, the apparent insensitivity of the blood pressures of hypertensive 9% casein exposed offspring to consumption of a salt load (Langley & Jackson 1996), highlighted the possibility that the renal mechanism of sodium balance may be altered in these animals.

1.4.2 Aims of the thesis.

The aims of this thesis were to examine whether exposure to a maternal 9% casein diet *in utero* influences rat blood pressures by disturbing renal development and later renal function. Renal development was assessed by examination of the glomerular complement of low protein exposed, and control animals both during gestation and postnatally. Renal function was examined both directly, using measurements of parameters of renal function (i.e. GFR and RBF), and indirectly by examination of body fluid dynamics. These approaches were chosen in order to address the hypothesis of Brenner and co-workers (1988) which suggests that a relative nephron deficit may predispose an individual to the development of elevated blood pressure in later life.

Chapter 2.

Materials and Methods.

2.1 Animals.

All animal procedures were carried out in compliance with the Animals Act (1986). Wistar rats were bred in the University of Southampton animal housing facility. Both males and females were supplied for breeding purposes. Direct animal experimentation, other than modification of maternal diet during pregnancy and validation studies, was carried out on the offspring of females fed experimental diets during pregnancy. The animal housing facility maintained a diurnal cycle of twelve hours of light and twelve hours of darkness. All the rooms were maintained at a temperature of 23^{o}C. Offspring of females fed experimental diets during pregnancy were weaned onto and maintained on standard laboratory chow (CRM(X) diet - SDS animal products, Wincham, Cheshire).

2.2 Diets.

Animals were either fed standard laboratory chow (CRM(X) diet - S.D.S., Wincham, Northwich, Cheshire.), 9% casein or 18% casein synthetic diets. The standard laboratory chow is shown in table 2.1. The composition of the experimental diets are shown in tables 2.2 and 2.3.

To examine the effects of feeding a low protein diet during pregnancy it was necessary to prepare synthetic diets, a 9% casein and an 18% casein diet. These were mixed, within the University of Southampton facility, according to Langley & Jackson 1994 (Tables 2.2 and 2.3). It was necessary to include methionine as a separate ingredient, in the synthetic diets, because casein is limiting in sulphur containing amino acids. The diets were isocaloric. The energy content of the low protein diet was balanced with that of the 18% casein diet using carbohydrate. Thus, whilst the manipulation of interest in the experiments was a 50% reduction of protein content, in interpreting all data, it should be noted that the energy density of the 9% casein diet was maintained with

additional carbohydrate (14% increase). The ingredients were mixed with water and formed into 40g – 60g balls, which were then placed in an oven at 60^{o}C for approximately three days, or until the balls had hardened. Experimental diets were stored at -12^{o}C prior to use.

2.3 Maintenance of experimental breeding females.

Virgin female Wistar rats were obtained for breeding, weighing approximately 200g - 230g and were housed with a breeding male in a wire bottomed cage. Animals were inspected twice a day and conception was determined by the appearance of a semen plug on paper placed beneath the cage. This was taken as the first day of pregnancy. Immediately at conception, the females were transferred to individual wire mesh cages and were provided an *ad libitum* supply of one of the two experimental diets, in accordance with individual experimental protocols. During pregnancy, animals were inspected daily. Weight gain and food intake were measured throughout pregnancy where stated.

2.4 Parturition and weaning.

At birth, the sex of all offspring was determined and the animals were then weighed. Sex determination in a neonatal rat is accomplished by making a judgement on the distance between the genitalia and the anus (genito-anal distance). Females have a shorter genito-anal distance than males. Offspring were culled to 8 (4 males and 4 females). If, when animals had reached an age where determination of sex is easier (i.e. at weaning), it was found that the original sex determination at term was incorrect, the data regarding birthweights were abandoned for that litter. The mother was transferred to standard laboratory chow within 12 hours of delivery. The offspring remained with the mother for four weeks postnatally, after which time they were housed in single sex groups of two to three animals, in separate wire mesh cages. The offspring were all weaned onto standard laboratory chow unless stated otherwise. All offspring were maintained on standard laboratory chow for the period up to further experimentation unless stated otherwise.

Table 2.1 : The composition of standard laboratory CRM(X) chow (S.D.S., Wincham, Northwich, Cheshire,).

Nutrient	Concentration g/kg
Protein	174
Fat	31
Fibre	123
Pectin	13
Hemicellulose	68
Cellulose	33
Lignin	10
Starches	448
Sugars	47
Calcium	8
Total Phosphorus	6.4
Phytate Phosphorus	2.1
Available Phosphorus	4.3
Sodium	3
Chlorine	3.2
Magnesium	2.2
Potassium	6.4
Iron	110 mg/kg
Copper	16 mg/kg
Manganese	70 mg/kg
Zinc	65 mg/kg
Cobalt	280.9 μg/kg

Table 2.1 (continued).

Nutrient	Concentration (g/kg)
Iodine	603 μg/kg
Selenium	217μg/kg
Fluorine	9 mg/kg
Retinol	5104.1 μg/kg
Vitamin A	16843.4 ig/kg
Cholecalciferol	80.7 μg/kg
Vitamin D_3	3228.6 ig/kg
α-tocopherol	93.8 mg/kg
Vitamin E	103.2 mg/kg
Vitamin B_1	16.8 mg/kg
Vitamin B_2	13.6 mg/kg
Vitamin B_6	18.5 mg/kg
Vitamin B_{12}	83.5 μg/kg
Vitamin C	8 mg/kg
Vitamin K_3	184.9 mg/kg
Folic Acid	4.4 mg/kg
Nicotinic Acid	78.7 mg/kg
Pantothenic Acid	26.1 mg/kg
Choline	951 mg/kg
Inositol	2355.4 mg/kg
Biotin	369.1 μg/kg
β-Carotene	0.9 mg/kg

Table 2.2 : Composition of the 18% casein experimental diet.

Nutrient	Concentration (g/kg)
Casein	180
Starch	425
Sucrose	213
Cellulose	50
Choline Chloride	2
D,L-Methionine	5
Mineral Mix (AIN76)	20
Vitamin Mix (AIN 76)	5
Corn Oil	10

AIN - American Institute of Nutrition.

Table 2.3 : Composition of the 9% casein diet.

Nutrient	Concentration (g/kg)
Casein	90
Starch	485
Sucrose	243
Cellulose	50
Choline Chloride	2
D,L-Methionine	5
Mineral Mix (AIN76)	20
Vitamin Mix (AIN 76)	5
Corn Oil	10

AIN - American Institute of Nutrition.

2.5 *Indirect measurement of systolic blood pressure.*

In humans, blood pressure is determined with an arm cuff sphygmomanometer. This is a bag, which is placed around the arm and into which air is pumped. The cuff is placed around the upper arm and is inflated to a pressure sufficient to occlude the artery and hence prevent blood flow into the lower arm. The pressure in the cuff is slowly released and, by listening for the pulse return with the aid of a stethoscope, the pressure at which the blood flow returns is determined. Indirect measurement of blood pressure in the rat was carried out in a similar fashion using an inflatable cuff placed around the animal's tail. The tail cuff works in a similar way by occluding the tail artery. The blood return is measured by a photoelectric sensor. This method of blood pressure determination provides essentially similar measurements as those obtained by direct cannulation (Bunag & Teravainen 1991; Bunag & Riley 1974; Bunag 1983), and results show a good degree of reproducibility as reported in detail by Sherman and Langley-Evans (1998).

Prior to testing, all animals were placed in a room maintained at 28^{o}C for two hours. Each animal was placed in a perspex restraining tube. Three tubes of differing sizes were available for 3 ranges of animal weight (80g - 150g, 150g - 250g, and 250g - 400g; IITC, USA). The end plate of the restraining tube carried the tail cuff and was attached to the tube with the cuff positioned around the tail. Two tail cuffs were available (3/8" for animals up to 150g and 7/16" for animals from 150g to 400g; IITC, USA). Each animal was allowed a 3 minute "settling period" in order to acclimatise to its surroundings and therefore reduce the stress associated with the measurement. The cuff was inflated to 300mmHg via a Model 229 Blood Pressure Monitor (IITC Life Sciences, California, U.S.A.). The pressure was allowed to decline at a rate of 3mmHg/sec. and the return blood flow was detected by a photo-electric sensor mounted mid-way down the length of the cuff. The pulse rate was recorded, as was the trace of the curve showing the cuff pressure at which the pulse returned. This provided the systolic blood pressure of the animal (Figure 2.1). The blood pressure was measured five times for each animal with no delay between measurements and the three readings that demonstrated a repeatable value were averaged to obtain the animal's systolic blood pressure.

Two pilot studies were carried out to confirm that there was no effect of either time lag between measurements, or training of the animals to the apparatus, on the measurements of blood pressure obtained using this method. Using 11 animals it was determined that there was no effect of leaving a longer period between measurements and no effect of familiarisation of the rats to the procedures (Figure 2.2 & Table 2.4). The coefficients of variation of the blood pressures of either the same animal measured on separate days, or different animals measured on the same day, were approximately 5%. Data obtained from the model 229 BP monitor was sent to a PC with appropriate software (IITC). A pre-set algorithm was used to record and analyse the data obtained without bias. Training of the animals to the procedure has been shown to have no effect on the blood pressure readings obtained (Langley-Evans *et al.* 1996; Sherman & Langley-Evans 1998), thus naive animals were used in all studies.

2.6 Measurement of the Glomerular Filtration Rate (GFR), Renal Blood Flow (RBF) and blood pressure via direct cannulation.

2.6.1 Overview of the renal circulation.

Approximately 25% of the blood pumped from the left ventricle passes directly into the kidney. Its course runs through the renal artery, between the medullary pyramids within the interlobar arteries which run up to the cortex, where they split into the arcuate arteries (or arciform arteries) which run along the bases of the pyramids (Lote 1994). The arcuate arteries then give rise to the interlobular arteries running perpendicular to the arcuate vessels, up towards the capsule and from these, branch vessels give rise to the afferent arterioles, which themselves lead to the glomeruli. Blood passing through the glomeruli enters the efferent arterioles and from here moves into either the peritubular capillary network (which occurs in the outer 2/3 of the cortex) or the network of vessels surrounding the tubules and loop of Henle of the juxtamedullary nephrons, the vasa recta (in the inner 1/3 of the cortex). The venous return flow is via the interlobular veins, through the arcuate and interlobar veins and out through the renal vein. Roughly 90% of the blood entering the kidney passes through the cortex, the remaining 10% services small populations of cells such as fat cells. The majority of

blood in the kidney is therefore filtered rather than supplying metabolically active cells (Lote 1994).

2.6.2 Glomerular filtration.

The filtration of the blood occurs at the glomerulus. As blood passes from the afferent arteriole into the glomerulus, the pressure difference established by the differing relative diameters of the afferent and efferent vessels, causes plasma constituents of a certain size (often modulated by their charge) to be forced across the capillary membrane and into the Bowmans space (Lote 1994d). The glomerular filtration rate (GFR) may be measured by determining the rate of appearance in the urine of a freely filtered compound, which remains in the extracellular fluid, in comparison to its concentration in the blood. Inulin, a complex plant derived polysaccharide is widely used for this purpose, and the clearance of inulin from the blood is used as a measure of the GFR. Inulin is infused at a rate slow enough to prevent its accumulation in the blood. Its concentration is then determined in the blood and the urine. The inulin clearance is then determined using the equation:

$$C_I = \frac{U_I * V}{P_I * T} = \text{GFR (ml/min)}$$

Where C_I is the clearance of inulin, U_I is the urinary concentration of inulin, V is the urine volume produced over time T and P_I is the plasma concentration of inulin.

Figure 2.1: A typical blood pressure trace obtained from an animal using the tail cuff method.

As the cuff is inflated, the trace of the pulse is lost (first vertical line). The pressure in the cuff slowly declines and the cuff pressure at which the pulse reappears (second vertical line) represents the systolic blood pressure of the animal.

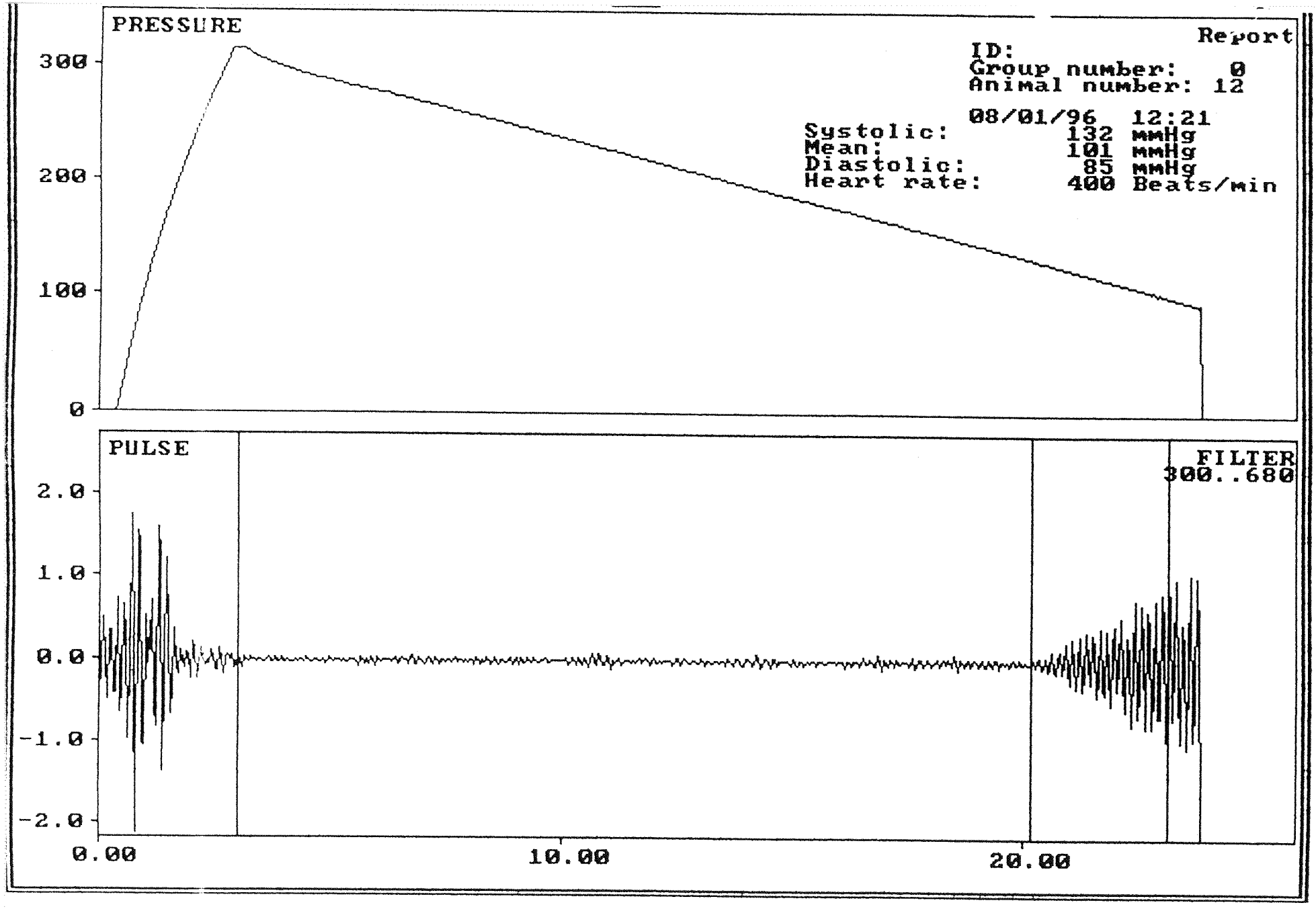

PRESSURE
Report
ID:
Group number: 0
Animal number: 12
08/01/96 12:21
Systolic: 132 mmHg
Mean: 101 mmHg
Diastolic: 85 mmHg
Heart rate: 400 Beats/min
300
200
100
0
PULSE
FILTER
300..680
2.0
1.0
0.0
-1.0
-2.0
0.00
10.00
20.00

Figure 2.2: Systolic blood pressures of 5 rats measured immediately, and at 1, 2 and 5 minute intervals after enclosure within the restraining tube.

Five female Wistar rats at 20 weeks of age were used for the determination of systolic blood pressure. Blood pressure was measured immediately upon enclosure within the restraining tube and at 1, 2 and 5 minute intervals subsequently. Values are shown as means ± SEM. One-way ANOVA indicated no effect of restraint time upon blood pressure (P=0.414).

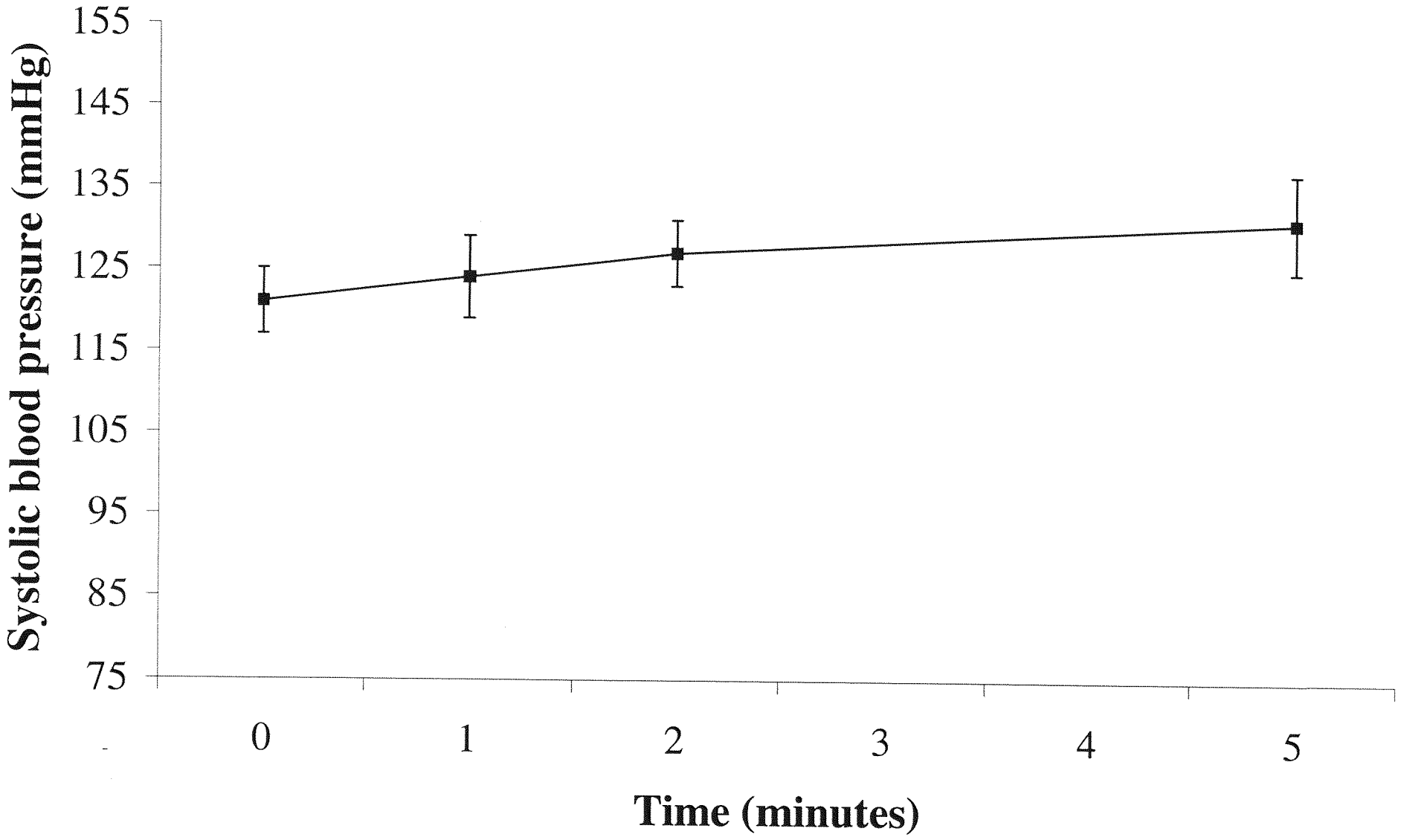

Systolic blood pressure (mmHg)
155
145
135
125
115
105
95
85
75
0
1
2
3
4
5
Time (minutes)

Table 2.4: Systolic blood pressure of animals measured over 2 weeks to determine any effect of familiarity with the procedure on blood pressure.

	Systolic blood pressure (mmHg).	
Animal.	**Baseline.**	**Week 1.**
1	123±1	126±2
2	132±4	132±2
3	130±4	134±2
4	108±1	100±4
5	119±3	124±3
6	94±2	98±11

Female Wistar rats at 7 weeks of age (baseline) had their systolic blood pressures measured using the tail cuff method. The same animals had their blood pressures determined again one week later (Week 1). Values are shown as means ± SEM. There were no differences in blood pressure as a result of familiarity with the procedure. Sherman and Langley-Evans (1998) demonstrated similar results.

The renal blood flow (RBF) is measured in a similar way using para-amino hippuric acid (PAH), of which approximately 90% is eliminated from the body via filtration and tubular secretion in a single pass through the kidney. This is taken into account in the determination of PAH clearance by multiplying the figure obtained by 1.11 (Ganong 1991). The clearance of PAH provides values for the renal plasma flow and this may be converted to RBF by dividing by the value for the haematocrit, thus accounting for the volume of blood cells.

$$RBF = \frac{U_{PAH} * V}{P_{PAH} * T} * 1/Hct = C_{PAH} * 1/Hct$$

Where C_{PAH} is the clearance of PAH and Hct is the haematocrit.

2.6.3 Protocol.

Only female animals were used in these experiments, as the male urethra is extremely difficult to cannulate and this would have impeded urine collection. Each animal was weighed and 1mg/kg body weight of pentabarbitone sodium (Sagatal) was administered via intra-peritoneal (i.p.) injection. Once the animal was completely anaesthetised, it was placed on its back on a sheet of insulating plastic and the trachea was cannulated with a plastic catheter (16 gauge; H.G.Wallace, UK) and held in place with cotton ligatures. Care was taken in placement of the tracheal catheter and this could be determined by the depth of breathing. The animals body temperature was measured by a rectal thermometer pushed 5-6cm through the rectum into the lower intestine and body temperature maintained at between 36^{o}C and 37^{o}C by an overhead lamp. Artificial maintenance of body temperature was necessary because sodium pentabarbitone anaesthesia impairs temperature homeostasis. The urethra was catheterised (0.4mm internal diameter (ID), 0.8mm outer diameter (OD); Portex, UK) for the collection of urine. The right carotid artery was then cannulated (0.4mm ID, 0.8mmOD, Portex, UK) for the measurement of blood pressure. The carotid artery cannula was connected to an NaCl (9g/L) filled plastic dome (Linton Instruments, Diss, Norfolk), which was connected to a pressure transducer (Linton Instruments, Diss, Norfolk). The signal from the pressure transducer was connected via an amplifier to a chart recorder.

The left jugular vein was then cannulated (0.4mm ID, 0.8mmOD, Portex, UK) for the slow infusion of ^{14}C-Inulin and ^{3}H-PAH (Amersham, UK), for the measurement of glomerular filtration rate and renal blood flow respectively. The infusate comprised a solution of NaCl (9g/L), bovine serum albumin (40 mg/ml), 0.16μCi/ml ^{14}C-Inulin and 0.36μCi/ml ^{3}H-PAH. The left femoral vein was cannulated (0.4mm ID, 0.8mm OD, Portex, UK) for blood sampling. A baseline blood sample of 200μl was taken and prepared for analysis (see below). The abdomen was gently massaged to evacuate the bladder of urine, which was also prepared for analysis. The rate of label infusion was 7.2ml/hr via the left jugular vein. This provided an infusion rate of ^{14}C-inulin of 1.16μCi/hr and ^{3}H-PAH of 2.6μCi/hr. This infusion rate allowed a measurable volume of urine to be produced, but was not too great so as to affect the blood pressure. Blood pressure was monitored throughout the experiment and the measurement abandoned if blood pressure was unstable.

Once the infusion had begun the animal was left for 30 minutes in order to allow a diuresis to be initiated. At the end of the 30 minute period, the urine collected was measured gravimetrically and the collection pot was replaced. In a pilot study, two animals had been cannulated and it was found that a diuresis was not initiated until at least 30 minutes after the start of the infusion. Urine production tended to plateau around 90 minutes after the start of the infusion. Blood and urine samples were taken every 15 minutes. For each urine sample, the lower abdomen was gently massaged to completely evacuate the bladder. All urine volumes were determined gravimetrically. For each blood sample, 200μl was taken and stored on ice in a heparinised 1.9ml eppendorf tube. Two samples were taken from this for the determination of haematocrit. Seven samples were taken over the course of the experiment, unless otherwise stated. Administration of drugs during the cannulation procedure is discussed in individual chapters.

The entire cannulation procedure was technically difficult. It took some time to acquire sufficient skill to enable reliable measurements to be obtained. Careful monitoring of blood pressure was necessary to ensure a constant renal perfusion pressure. Furthermore, it was found that very thorough heparinisation of all cannulae was vital to prevent blood from coagulating in the lumens.

Once all the samples had been taken, the animal was terminally anaesthetised with an intravenous injection of sodium pentabarbitone (Sagatal). The left kidney was removed and weighed. All radioactive animal tissue was disposed of by maceration. Blood samples were spun in a microfuge (Eppendorf) for 10 minutes to obtain plasma and haematocrit tubes were spun in a haematocrit spinner. Haematocrit was determined using a haematocrit gauge.

The radioactivity of inulin and PAH in urine and plasma were measured using a liquid scintillation counter (Beckman). Urine samples (100µl) and plasma samples (50µl) were pipetted into scintillation vials and 4ml of scintillation fluid (Optiphase Hisafe) was added to all tubes. The vials were transferred to the scintillation counter which determined the ^{14}C and ^{3}H in each sample. From these data, concentrations of inulin and PAH were determined in plasma and urine samples and the GFR and RBF calculated as previously described (section 2.6.2).

In both experiments in which GFR and RBF were determined, problems were encountered with the ^{3}H-PAH label. Estimates of RBF in several animals were extremely low and judged incorrect as they approximated the values obtained for GFR. Post-experimental examination of the label using thin layer chromatography suggested that the some of the label had become dissociated and thus determination of the radioactivity would not provide a valid estimate of RBF. Because of this, all data for RBF were selected according to the value of filtration fraction (FF) that they provided. The FF is the proportion of blood plasma that is filtered at the glomerulus and can be determined by dividing the value for GFR by that of RBF. If any value for FF exceeded 0.5 (i.e. if more than 50% of the blood passing through the kidney is filtered), then the value for RBF for that animal was abandoned. This is discussed in the appropriate chapters.

2.7 Determination of glomerular complement.

Kidneys were removed when animals were culled and fixed in buffered formalin over 24 hours. The fixed kidneys were then dehydrated using the following procedure. The kidneys were removed from formalin, placed in 70% alcohol and left overnight.

Kidneys were placed in 90% alcohol solution for 1 hour and the solution was replaced with fresh 90% alcohol and left for 1 hour. Kidneys were placed in absolute alcohol (ethanol) for 2 hours. The ethanol was then replaced with fresh ethanol and left for 2 hours. The ethanol was again replaced with fresh ethanol and left for a further hour. Kidneys were finally placed in chloroform overnight. The kidneys were then embedded in wax blocks.

For embedding, kidneys were taken out of chloroform and placed in molten, filtered mounting wax and left for 1 hour. The kidneys were then transferred to clean wax for 1 hour. The procedure was then repeated and finally, the kidneys were placed on their sides in the bottom of a plastic well. Fresh wax was poured around the kidneys and this was left to set overnight. Note, it was important that the kidneys were placed on their sides as this enabled sectioning in the saggital plane.

Serial 5μm thick saggital sections were cut on a microtome (Baird & Tatlock). One section in 50 was retained for histological examination of fetal and/or neonatal kidneys, and for kidneys of animals at 4 weeks of age, 1 section in every 100 was taken. Sample sections were floated in a water bath kept at 40^{o}C. This stretched the sections and prevented folds appearing when mounted. Individual sections were then collected onto non-coated microscope slides (Gold Star, Chance Propper Ltd., UK) and left to dry overnight. The dry sections were stained with haematoxylin and eosin (Table 2.6) and were mounted in DPX mounting fluid (R.A.Lamb, UK). Slides were covered with a glass cover slip (Chance Propper Ltd., UK) and left in a fume cupboard overnight to set.

Using a magnification of 100x (Zeiss binocular microscope, Zeiss, Germany), the glomeruli in each section were counted. This was repeated for each section to validate the estimate. The numbers of glomeruli for each section were averaged to give a mean number of glomeruli per section. This was then multiplied by the number of sections that had been taken from that kidney (n.b. each kidney had been completely sectioned) and divided by 20 to give an estimate of the glomerular complement of that kidney. The product of the mean glomeruli per section and the number of sections was divided by 20 in order to prevent including each glomerulus in the final figure more than once. The maximum glomerular diameter was approximately 100μm. This was determined using a

10mm*10mm graticule inserted into the eyepiece and comparing each glomerulus with the grid. Each square in the grid represented 100μm*100μm and the maximum glomerular diameter was determined visually using this grid. Thus, it was estimated that glomeruli had a diameter of 100μm on average and therefore each would be visible in 20 sections, hence the product of mean number of glomeruli and total number of sections was divided by 20. This method had previously been shown to provide a good estimate of glomerular complement after 2 kidneys had been completely sectioned and the glomeruli from 1 section in every 25 had been determined (Table 2.5). Data obtained using this method compared well with nephron numbers in rat kidneys reported by Zeman (1968) and Merlet-Bernichou *et al.* (1994) and with numbers reported by Howard and Beech (personal communication) using kidneys removed from animals in our colony (our data (Table 2.5) = 11648 - 14827, Howard & Beech data = 13644±940 (n=14)). The latter used highly accurate stereological techniques, considered the Gold Standard for estimates of nephron number (Howard 1997).

2.8 Determination of tissue protein content.

Tissue samples were homogenised in Tris-HCl buffer (10mM, pH7.4; Appendix) at a concentration of 10mg tissue/ml. Bovine serum albumin (BSA) standards were prepared in 0.1M NaOH (range: 0.2mg/ml - 1mg/ml). 10μl of standard or sample were added to 4ml plastic tubes and to each tube was added 200μl of Bradford reagent (Appendix). Tubes were transferred to a water bath and incubated at 37°C for 30 minutes. The absorbance was then read on a spectrophotometer at a wavelength of 560ηm. All samples and standards were assayed in duplicate.

Table 2.5: Number of glomeruli in 2 completely sectioned kidneys.

	Kidney 1.	Kidney 2.
No. of Sections	870	1000
Mean glomeruli/section	268±9	297±6
Median no. of glomeruli	253	308
Minimum no. of glomeruli	60	84
Maximum no. of glomeruli	660	414
Total glomeruli/20	11648	14827
Mean x No. of sections/20	11658	14850

Two kidneys from female rats at 4 weeks of age were serially sectioned and their glomerular complement determined as described in section 2.7.

2.9 *Determination of tissue DNA content.*

The DNA content of tissues was measured using an assay based upon that described by Richards (1974). Tissue samples were prepared as for the protein assay (section 2.8). To 250µl of 2M NaOH was added 250µl of tissue sample (10mg tissue/ml) or DNA standard (10 - 100µg/ml Tris-HCL buffer). This was incubated at 37°C for 1 hour. 1ml of 4M perchloric acid (PCA; Sigma, Poole, UK) was added and the solution was incubated at 70°C for 20 minutes. At the end of the incubation, samples were allowed to cool to room temperature. 0.6ml of DPA reagent (Appendix 1) was then added and the solution was left to stand at room temperature for 24 hours. 1.5ml of amyl acetate (Sigma, Poole, UK) was then added and the samples were mixed well. Samples were centrifuged at 2000g for 1 minute and the organic blue phase was removed and its absorbance measured on a spectrophotometer at 600ηm in a glass cuvette. All samples were assayed in duplicate. This assay is extremely sensitive to slight changes in the protocol and on occasion samples had to be re-assayed.

2.10 *Determination of tissue alkaline phosphatase (ALP) activity.*

ALP activity was determined using a commercially available kit (ALP optimised E.C. 3.1.3.1 colorimetric test, Sigma, UK). The Sigma "optimised" kit employs a method recommended by the German Society for Clinical Chemistry (1970 & 1972). The method allows the measurement of p-nitrophenol (which has an absorbance maximum at a wavelength of 405ηm), which is cleaved from the substrate, p-nitrophenyl phosphate by ALP in the reaction shown below:

$$\text{p-nitrophenyl phosphate} + H_2O \xrightarrow{\textbf{ALP}} \text{p-nitrophenol} + \text{phosphate}$$

Tissue samples were prepared as for the protein assay (section 2.8). 5 ml of Reagent A (ALP Reagent A contained diethanolamine buffer (pH 9.8; 1.214mol/L) and Mg^{2+} (0.607mmol/L)) was mixed with 25 ml of Reagent B (ALP Reagent B contained p-nitrophenyl phosphate (60.8mmol.L)) to make the Start Reagent. This was maintained

at 30°C throughout the experiment to avoid the necessity of using a temperature conversion factor. To a 4ml plastic tube containing 3ml of Start Reagent, was added 0.05ml of sample. Tubes were mixed by inversion, transferred to a cuvette and the absorbance was immediately read at a wavelength of 405 ηm on a spectrophotometer, against water as a blank. The solution was transferred back to the 4ml tube and incubated at 30°C. Absorbance readings were carried out after exactly 1, 2 and 3 minutes from the initial reading.

ALP activity was determined using the following equation:

$$\text{ALP (U/L)} = \frac{\Delta A \text{ per minute} * TV * 1000.}{18.45 * LP * SV}$$

Where: ΔA per minute = Change in absorbance per minute at 405ηm.

TV = Total volume (ml)

SV = Sample volume (ml)

18.45 = Millimolar absorptivity of p-nitrophenol at 405ηm.

LP = Light path (1-cm) = 1.0

1000 = Conversion of units per ml to units per litre.

All samples were assayed in duplicate.

2.11 Determination of plasma volume.

Plasma volume was determined using the Evans Blue method described by Rosso and Streeter (1979). Rats were anaesthetised using sodium pentabarbitone (Sagatal; 1mg/kg). The left femoral vein was then cannulated using plastic tubing (0.4mm I.D., 0.8mm O.D.; Portex, UK). A baseline blood sample of 0.2ml was removed for the determination of haematocrit and 4mg/100g body weight of Evans Blue dye (Sigma, Poole, UK) was injected intravenously. After 5 minutes, 1ml of blood was removed and the animal was terminally anaesthetised. In a pilot study, examination of different time delays between injection and sampling in 2 animals, demonstrated that collection of the final blood sample provided equivalent plasma volume estimates at 5, 10, 15, 30 and 60

minutes after the injection of the Evans Blue dye. Haematocrit tubes were spun in a haematocrit spinner for 10 minutes and the haematocrit determined using a haematocrit gauge. The final blood sample was collected into a 1.9ml Eppendorf tube and spun in a microfuge (Eppendorf). Plasma was collected and its absorbance was measured on a spectrophotometer at a wavelength of 620ηm. Evans Blue standards were made up in distilled water (0.5 - 10mg/ml).

The plasma volume was determined using the following equation:

$$\frac{X\ (mg/ml)}{Y\ (mg/ml)} * Z\ (ml) = \text{Plasma Volume (ml)}$$

Where:

X	=	Concentration of Evans Blue dye added.
Y	=	Plasma concentration of Evans Blue dye.
Z	=	Volume of fluid injected (containing Evans Blue dye at a concentration of X mg/ml)

Table 2.6: Staining procedure for haematoxylin and eosin.

Stage.	Solution.	Time.
1	Xylene	5 mins.
2	Xylene	5 mins.
3	Absolute Alcohol	2 mins.
4	Absolute Alcohol	2 mins.
5	70% Alcohol	2 mins.
6	70% Alcohol	2 mins.
7	Tap Water	1 min.
8	Harris Haematoxylin	10 mins.
9	Tap Water	2 mins.
10	Acid Alcohol	4 secs.
11	Tap Water	max. 5 mins. - to "blue"
12	Eosin	5 mins.
13	Tap Water	rinse
14	70% Alcohol	2 mins.
15	70% Alcohol	2 mins.
16	Absolute Alcohol	1 min.
17	Absolute Alcohol	1 min.
18	Xylene	2 mins.
19	Xylene	2 mins.

Harris haematoxylin was supplied by Sigma (Poole, UK).

Acid alcohol was prepared as described in Appendix 1.

2.12 Statistics.

Statistics employed within this thesis were carried out using the SPSS statistical computer program. These included one-, two- and three-way ANOVA, Students t-test, Tukeys honestly significant test, regression and correlation analyses.

In all the studies described in this thesis, the number of offspring examined is used as the *n* number for each experimental group. It must be born in mind, however, when considering the results and the statistical analyses performed, that the true *n* number could be regarded as the number of litters within each dietary group rather than the combined number of animals from all litters within dietary groups. There are arguments in favour of either approach. For practical reasons, the nature of the experiments is such that the number of litters must be restricted. Nevertheless, each of the offspring enjoys its own prenatal and postnatal experience. Any increase in *n* will increase the likelihood that statistically significant differences will be shown for group means. Therefore, to an extent, a judgement is needed to assess whether statistical significance is indicative of biological significance. Indeed, some measurements may appear similar in a statistical sense despite being different in a biological sense. In these situations, where there is uncertainty, it may be necessary to increase the numbers to provide adequate confidence.

Chapter 3.

Nephron deficit and elevated blood pressure following mild intrauterine undernutrition.

3.1 Introduction.

It is now well established that impaired fetal growth and development increases the risk of developing hypertension in adulthood. Maternal undernutrition during pregnancy may be responsible for this growth impairment (Barker *et al.* 1989; Osmond *et al.* 1993; Law *et al.* 1993). Epidemiological studies which have examined the relationship between birthweight (as a marker of fetal growth retardation) and adult blood pressure, consistently demonstrate that adult blood pressure declines as weight at birth increases.

Maternal undernutrition during human pregnancy is a significant contributor to fetal growth retardation (Smith & Mass 1947; Prentice *et al.* 1981; Campbell *et al.* 1996; Godfrey *et al.* 1996). This has also been examined in the rat by the imposition of a variety of protein restricted synthetic diets (6%, 9%, 12% casein by weight compared with an 18% casein control diet) during pregnancy (Levy & Jackson 1993; Langley & Jackson 1994). These studies demonstrated that maternal protein restriction both prior to and during pregnancy impaired fetal growth (Levy & Jackson 1993) and resulted in offspring with blood pressures significantly elevated above those of control animals (Langley & Jackson 1994).

The rat studies of Levy and Jackson (1994) and Langley and Jackson (1994), imposed the experimental diet for 2 weeks prior to conception and throughout pregnancy. There is some evidence to suggest that the condition of the mother prior to human pregnancy may influence the development of the fetus, regardless of the conditions during pregnancy (Croall *et al.* 1978). It was therefore necessary to determine whether the consumption of experimental low protein diets prior to conception in this rat model, had a significant effect on the blood pressure of the offspring and whether the blood pressure effect is observed if exposure to these diets occurs only during gestation.

Intrauterine factors leading to a nephron deficit may lead to an elevation in adult blood pressure (Brenner *et* al. 1988; Mackenzie & Brenner 1995). A nephron deficit might result from a genetic predisposition, disease, or possibly as a consequence of maternal undernutrition during pregnancy. We therefore examined the kidneys of offspring exposed to a maternal low protein diet during gestation and determined their nephron complement.

Three preliminary studies were performed. Study 1 examined whether feeding female rats a 9% casein diet for 2 weeks prior to conception was necessary for the manifestation of the hypertensive effect of a maternal 9% casein diet. Offspring blood pressures were measured at 7 weeks of age. Study 2 examined the effect of exposure to a maternal 9% casein diet on fetal body and organ weights at day 20 of gestation. In study 3, the glomerular complement of offspring of rats supplied a 9% casein or an 18% casein diet during pregnancy, was determined at 4 weeks of age.

3.2 Protocol.

3.2.1 Study 1:

Twenty virgin female Wistar rats weighing between 200g and 230g were allocated to four groups, with five animals in each group, according to the dietary protocol (Table 3.1). For two weeks prior to conception, one group was supplied a 9% casein diet (9%Ca), two groups were fed standard laboratory chow (SDS, Wincham, Cheshire) and the remaining group was supplied an 18% casein diet (18%Ca - control). The animals were then mated and transferred to either the 9% casein or the 18% casein experimental diets for the duration of pregnancy according to the experimental protocol (Table 3.1). Food intake and body weights of the pregnant females were measured daily, using the same set of scales, both prior to and during pregnancy. At parturition, females were transferred to standard laboratory chow and birthweights were recorded. At 7 weeks of age, the systolic blood pressures of the offspring were determined using the tail cuff method (Chapter 2, section 2.5).

Table 3.1: Dietary protocol for study 1.

Experimental group.	n	Diet prior to pregnancy.	Diet during pregnancy.
18%Ca - 18%Ca	5	18% casein	18% casein
9%Ca - 9%Ca	5	9% casein	9% casein
Chow - 18%Ca	5	Chow	18% casein
Chow - 9%Ca	5	Chow	9% casein

Female Wistar rats were fed either standard laboratory chow (SDS, Wincham, Cheshire), an 18% casein diet or a 9% casein diet for two weeks prior to conception. At conception animals were transferred to either the 18% casein or the 9% casein diets.

3.2.2 Study 2:

An additional group of female Wistar rats were supplied either a 9% casein diet (n=3) or an 18% casein diet (n=4) throughout pregnancy. Animals were fed upon chow prior to conception. At day 20 of gestation, females were sacrificed by terminal anaesthesia (Pentabarbitone sodium), offspring were removed and measurements of fetal body weight, kidney weight, heart weight, body length and width (abdominal) were made. Length was taken as the naso-anal length of the offspring and width was measured at the level of the widest part of the abdomen with the fetus laid on its back. Both were measured using a pair of callipers.

3.2.3 Study 3:

Four female Wistar rats (200g - 220g) were mated and individually housed. Two females were fed an 18% casein diet throughout pregnancy and the remaining pair of animals were fed a 9% casein diet throughout pregnancy. At parturition, females were transferred to standard laboratory chow. At 4 weeks of age, female offspring were sacrificed (18% casein - 7 animals; 9% casein - 8 animals), their left kidneys were removed and were used for the determination of nephron complement (Chapter 2, section 2.7). The right kidneys were used for determination of protein and DNA content (Chapter 2, section 2.9).

3.2.4 Statistics.

One and two-way ANOVA were used in all analyses and either TUKEY's or Students T-Tests were used to determine differences between individual groups. The statistical package SPSS was used in all analyses.

3.3 Results.

3.3.1 Study 1:

Maternal food intake and weight gain were measured before and during pregnancy. Food intake did not differ between dietary groups either prior to conception or during pregnancy. Within each group however, the final week of pregnancy was characterised by a significant ($P<0.05$) decrease in food intake (13% - 35%; Figure 3.1).

Maternal weight gain with advancing pregnancy was unaffected by maternal diet. With the exception of the 18%Ca-18%Ca animals, weight gain was greater in every week of pregnancy than during the two weeks prior to conception ($P=0.000$; Figure 3.2). Females of the 18%Ca-18%Ca group exhibited a similar weight gain in the first week of pregnancy to that of the pre-pregnant period. These animals did, however, take longer to conceive than those of other groups and thus the weight of these animals at the start of pregnancy was significantly greater than those of all other groups ($P<0.05$). Maternal weight gain increased in all groups during the final week of pregnancy compared to the previous two weeks (Figure 3.2). This was significant ($P<0.05$) in all groups, except in the Chow-9%Ca group in which the maternal weight gain in the final week of pregnancy was similar to week 1 and week 2. The weight gain of 18%Ca-18%Ca animals was significantly greater than all other groups in the final week of gestation ($P=0.03$).

Additionally, data on weight gain and food intake during pregnancy were grouped according to the diet supplied during pregnancy (Table 3.2). This was done in order to enable a larger sample size to be examined. Thus, data for 9%Ca-9%Ca and Chow-9%Ca animals were combined, as were data for Chow-18%Ca and 18%Ca-18%Ca groups. Animals supplied an 18% casein diet exhibited a significantly greater weight gain in the final week of pregnancy than animals fed on 9% casein ($P=0.006$) and this was maintained when weight gain was corrected for initial body weight ($P=0.016$; Table 3.2

These data for maternal weight gain were also expressed as a function of food intake (i.e. weight gain (g)/food intake (g)). This analysis showed that 18% casein exposed animals

acquired more weight per gram of food consumed (a measure of the efficiency with which food is utilised) than 9% casein supplied animals during the final week of pregnancy (P=0.000; Table 3.2). One data point was removed for this analysis as it was greater than 2 standard deviations away from the mean, hence the discrepancy in the *n* number in the table. These combined data showed no effect of maternal diet on food intake (P=0.540), or food intake corrected for body weight (P=0.906; Table 3.2).

Birthweights of all pups measured within 12 hours of delivery were similar in all of the dietary groups (Table 3.3). The number of offspring shown in the table vary considerably because a number of the females either were not pregnant, or ate their offspring. The group most affected were the 18%Ca-18%Ca animals, of which only 3 delivered and one of these litters was not reached within 12 hours of delivery, hence the data for this group represent only 2 litters. The data from animals which did not deliver were excluded from all of the above analyses. Both groups of females supplied chow prior to conception and those fed a 9% casein diet both prior to and throughout pregnancy delivered significantly heavier male offspring than female offspring (P<0.05).

The systolic blood pressures of the offspring were measured at 7 weeks of age using the tail cuff method (Materials and Methods 2.5). Within dietary groups, the systolic blood pressures of male and female offspring were similar and thus the data were pooled (Table 3.3). The blood pressures of the offspring of animals supplied a 9% casein diet during pregnancy were significantly higher (11 - 12mmHg; P<0.05) than those exposed to an 18% casein diet. The diet supplied prior to conception had no effect on the offspring's blood pressure.

3.3.2 Study 2:

Offspring of females exposed to a 9% casein diet during pregnancy were heavier than control fetuses at day 20 of gestation (P=0.008; Table 3.4). Controlling for body weight demonstrated that at day 20 of gestation, low protein exposed animals had disproportionately large hearts compared to 18% casein exposed control fetuses (P=0.037).

Naso-anal length and width (abdominal) were measured with a pair of callipers. Low protein exposed offspring were of similar length and width to controls, and determination of the Lee index (weight/length^3) indicated that there was no low protein induced impairment of fetal truncal growth (Table 3.4).

Placental weights were also measured. It was found that absolute values for placental weight did not vary between the groups, but a maternal protein restriction appeared to impair placental growth when corrected for offspring body weight (P=0.034).

3.3.3 Study 3:

At 4 weeks of age, kidneys of female offspring were used for the determination of their nephron complement. Kidneys of female offspring exposed to a maternal 9% casein diet throughout pregnancy contained 15% fewer glomeruli than control animals (P=0.037; Table 3.5). Analyses of protein and DNA content showed no apparent differences in total cell number (P=0.371), total protein content (P=0.669) or the quantity of protein within each cell (P=0.284; Table 3.5).

Figure 3.1: Food intake of females prior to and throughout pregnancy.

Female Wistar rats were supplied either an 18% casein diet, a 9% casein diet or standard laboratory chow (SDS, Wincham, Cheshire) for two weeks prior to conception and either 18% casein or 9% casein diets during pregnancy. The figure shows the mean daily food intake for the periods before conception (Week 0) and the three weeks of pregnancy (Week 1, Week 2 and Week 3). Values are expressed as means ± SEM. For 18%Ca-18%Ca, n=3; 9%Ca-9%Ca, n=4; Chow-18%Ca, n=4; Chow-9%Ca, n=5. * represents a significant difference between week 3 and the two other weeks of pregnancy within dietary groups ($P<0.05$).

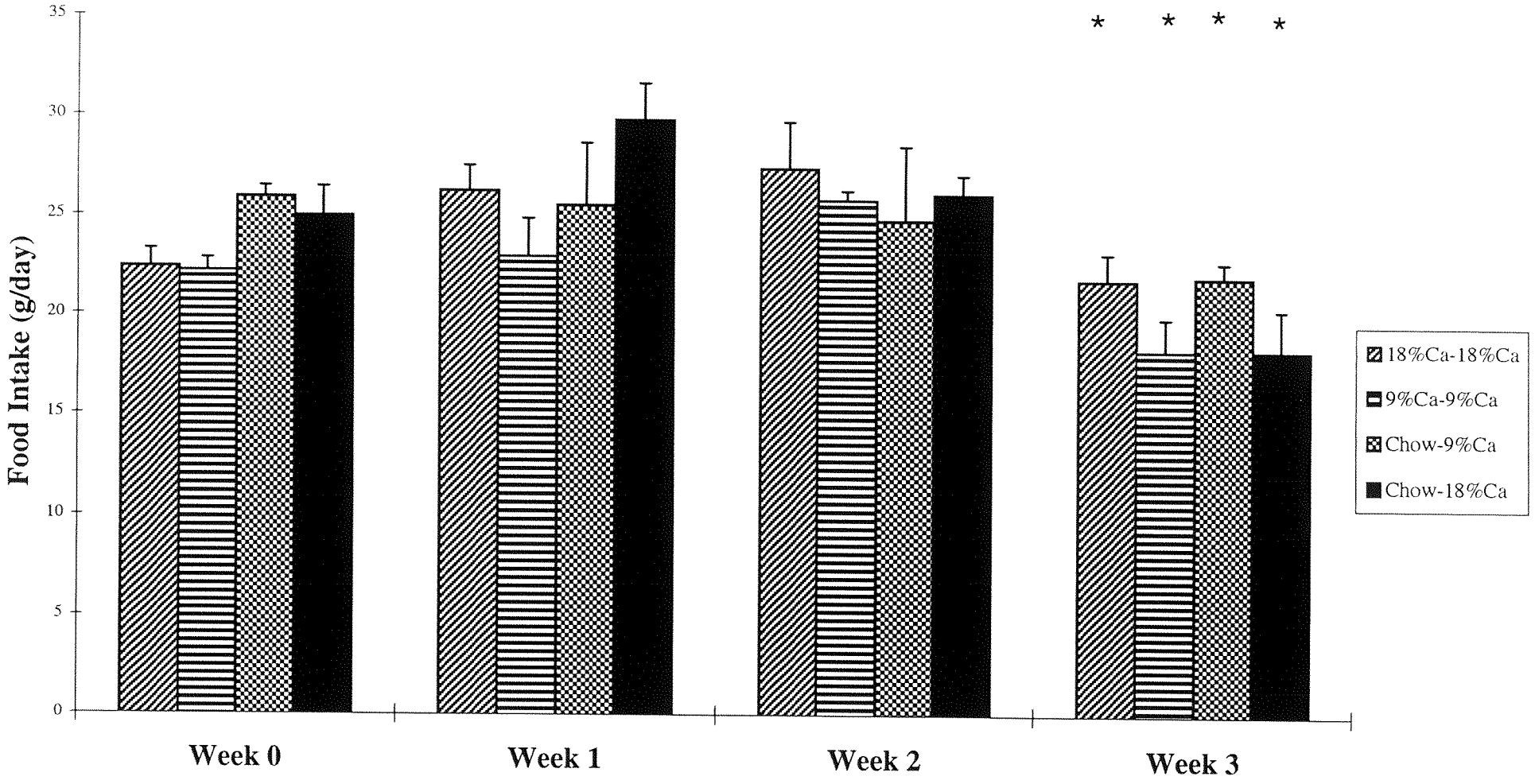

Food Intake (g/day)
35
30
25
20
15
10
5
0
* * * *
Week 0
Week 1
Week 2
Week 3
18%Ca-18%Ca
9%Ca-9%Ca
Chow-9%Ca
Chow-18%Ca

Figure 3.2: Weight gain of females prior to and throughout pregnancy.

Female Wistar rats were supplied either an 18% casein diet, a 9% casein diet or standard laboratory chow (SDS, Wincham, Cheshire) for two weeks prior to conception and either 18% casein or 9% casein diets during pregnancy (18%Ca-18%Ca, 9%Ca-9%Ca, Chow-18%Ca, Chow-9%Ca respectively; Table 3.1). The figure shows the mean daily weight gain for the periods before conception (Week 0) and the three weeks of pregnancy (Week 1, Week 2 and Week 3). Values are expressed as means ± SEM. n = 5 for each group. † represents a significant difference compared to Weeks 0 to 2 within dietary groups ($P<0.05$). * represents a significant difference compared to Week 0 within dietary groups ($P<0.05$). ‡ represents a significant difference compared to 18%Ca-18%Ca controls in Week 3 ($P<0.05$).

One-way ANOVA analyses showed that the rate of weight gain increased with advancing pregnancy in 9%Ca-9%Ca, Chow-18%Ca and Chow-9%Ca groups ($P=0.000$ in all groups).

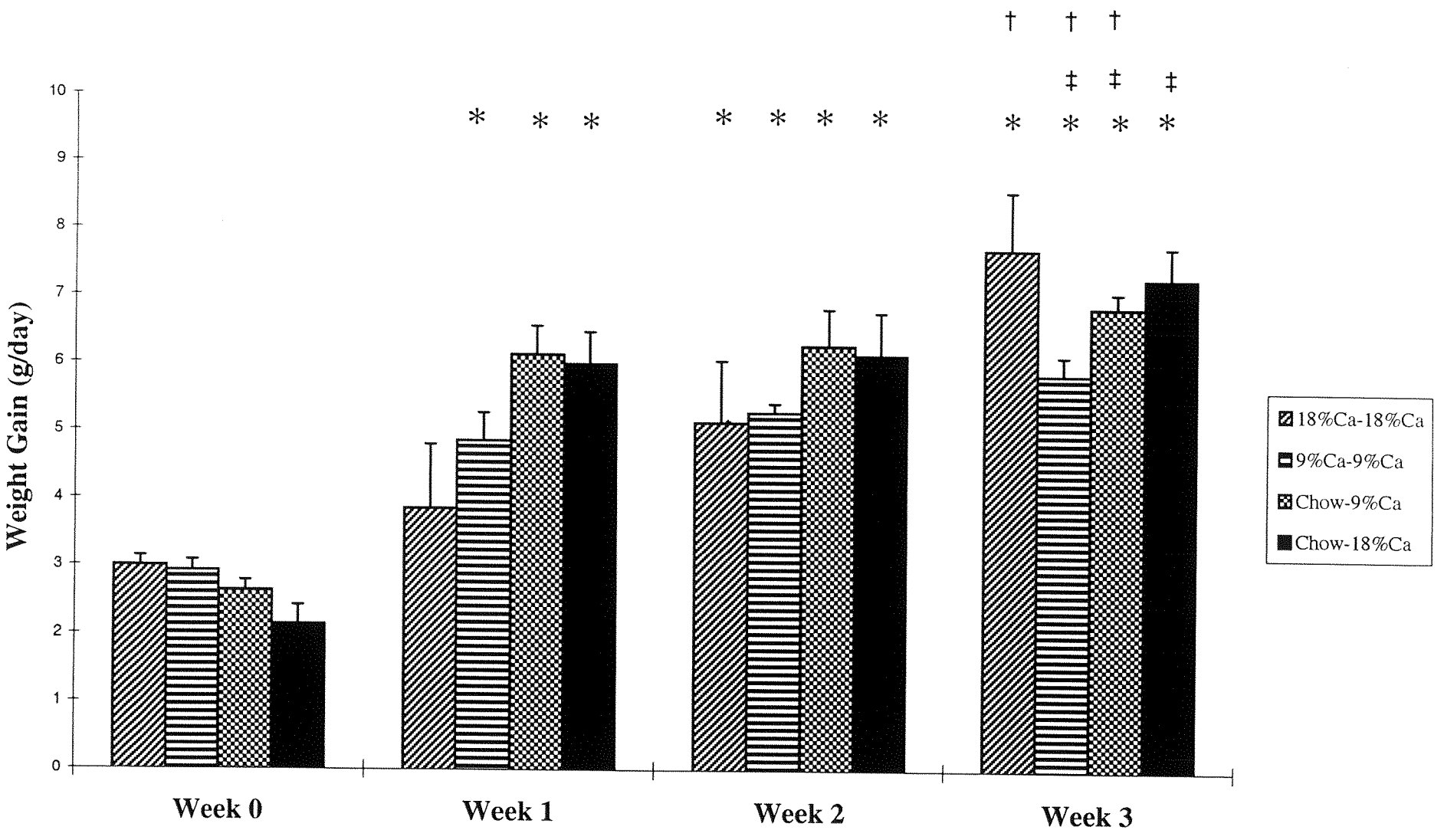

Weight Gain (g/day)
0
1
2
3
4
5
6
7
8
9
10
Week 0
Week 1
Week 2
Week 3
18%Ca-18%Ca
9%Ca-9%Ca
Chow-9%Ca
Chow-18%Ca

Table 3.2: Weight gain, food intake and feeding efficiency (food intake/weight gain) of animals supplied either a 9% casein or an 18% casein diet during pregnancy.

Female Wistar rats were supplied either an 18% casein diet, a 9% casein diet or standard laboratory chow (SDS, Wincham, Cheshire) for two weeks prior to conception and either 18% casein or 9% casein diets during pregnancy. Data are shown as means ± SEM. * - significantly different to 18% casein fed controls ($P<0.05$). Within dietary groups: + - significantly different to Week 0; ‡ - significantly different to Week 1; § - significantly different to Week 2 (all at $P<0.05$ level).

	n	Week 0	Week 1	Week 2	Week 3
Weight gain (g/day)					
18% casein	7	2.58±0.27	4.89±0.47^{+}	5.98±0.44^{+}	7.74±0.40^{+}‡§
9% casein	9	2.56±0.27	4.95±0.48^{+}	5.65±0.24^{+}	6.26±0.25^{+}‡§*
Weight gain/body weight (g/100g/day)					
18% casein	7	1.11±0.12	1.66±0.20	1.79±0.15^{+}	2.00±0.09^{+}
9% casein	9	1.09±0.12	1.75±0.17^{+}	1.76±0.06^{+}	1.74±0.05^{+*}
Weight gain/food intake (g/g)					
18% casein	7	0.10±0.01	0.18±0.02^{+}	0.22±0.02^{+}	0.45±0.02^{+}‡§
9% casein	9	0.11±0.02	0.20±0.02^{+}	0.23±0.02^{+}	0.32±0.02^{+}‡§*
Food intake (g)					
18% casein	7	24.51±1.11‡§	27.63±1.60	27.18±1.16	20.58±1.54‡§
9% casein	9	24.80±0.80	25.19±1.39	24.53±1.65	19.52±0.91^{+}‡§
Food intake/body weight (g/g)					
18% casein	7	0.11±0.01	0.09±0.01^{+}	0.08±0.00^{+}	0.05±0.00^{+}‡§
9% casein	9	0.11±0.00	0.09±0.00^{+}	0.08±0.01^{+}	0.05±0.00^{+}‡§

Table 3.3: Birthweights and systolic blood pressures at 7 weeks of age.

Maternal diet.	n	Male birthweight (g)	n	Female birthweight (g)	n	Male and Female SBP (mmHg)
Chow-18%Ca	27	5.0±0.1‡	37	4.6±0.1	35	136±4
18%Ca-18%Ca	7	4.8±0.2	6	4.9±0.2	19	132±3
Chow-9%Ca	17	5.1±0.1‡	22	4.8±0.1	30	148±5*+
9%Ca-9%Ca	14	5.1±0.1	18	4.3±0.1	22	143±3*+

Female Wistar rats were supplied either an 18% casein diet, a 9% casein diet or standard laboratory chow (SDS, Wincham, Cheshire) for two weeks prior to conception and either 18% casein or 9% casein diets during pregnancy. The table shows the birthweights of all offspring assessed within 12 hours of delivery and the systolic blood pressure (SBP) of male and female offspring at 7 weeks of age. * represents a significant difference compared to 18%Ca-18%Ca controls ($P<0.05$). + represents a significant difference compared to Chow-18%Ca controls ($P<0.05$). ‡ represents a significantly greater birthweight than females within the same dietary group.

Table 3.4: Fetal body and organ weights at day 20 of gestation.

	18% Casein		9% Casein	
	n	Mean±SEM.	n	Mean±SEM.
Body weight (g)	29	3.74±0.12	23	4.25±0.15*
Length (mm)	29	43±1	23	45±1
Width (mm)	29	13±1	23	13±1
Lee index (g/cm^3)	29	0.046±0.001	23	0.047±0.001
Kidney weight (mg)	29	17±1	23	19±1
Heart weight (mg)	29	20±1	19	24±1*
Kidney weight/body weight (mg/g)	29	4.35±0.18	23	4.22±0.15
Heart weight/body weight (mg/g)	29	4.56±0.37	19	5.53±0.17*
Placental weight (g)	18	0.62±0.02	11	0.65±0.02
Placental weight/body weight (g/g)	18	0.13±0.01	11	0.12±0.00*

Female Wistar rats were mated and supplied either a 9% casein diet (n=3) or an 18% casein diet (n=4) throughout pregnancy. At day 20 of pregnancy, females were sacrificed and offspring removed for the determination of parameters of fetal growth. Values are expressed as means ± SEM. * represents a significant difference compared to 18% casein exposed controls ($P<0.05$).

Table 3.5: Glomerular complement, DNA and protein content of the kidneys of female offspring at 4 weeks of age.

	n	18% casein	n	9% casein
Total glomeruli per kidney.	7	15314±818	8	13074±409*
Protein (mg/g tissue)	5	72.85±3.39	8	75.02±3.06
DNA (mg/g tissue)	5	10.99±1.62	8	9.46±0.86
mg DNA / mg protein	5	0.15±0.01	8	0.13±0.01

Female Wistar rats were fed either an 18% casein diet or a 9% casein diet throughout pregnancy. The left kidneys of female offspring at 4 weeks of age were used for the determination of their glomerular complement, protein and DNA content. Values are expressed as means ± SEM. * represents a significant difference compared to 18% casein exposed controls ($P<0.05$).

3.4 Summary of results.

1. The feeding of synthetic (18% or 9% casein) diets had no effect upon maternal food intake. In all groups, food intake was significantly greater in the final week of pregnancy than in the two preceding weeks.

2. Weight gains in pregnancy were greater than in the pre-pregnant period.

3. The feeding of a low protein diet either prior to and during pregnancy, or during pregnancy alone had no effect upon the birthweights of the offspring.

4. Offspring of dams fed a low protein diet during pregnancy had significantly higher blood pressures than those of offspring of controls, at 7 weeks of age. The hypertensive effect of the low protein *in utero* was not modified by the plane of maternal nutrition prior to pregnancy.

5. Maternal protein restriction increased heart weight and reduced placental weight corrected for fetal weight at day 20 of gestation.

6. Offspring of females fed a low protein diet during pregnancy had fewer glomeruli per kidney than controls, at 4 weeks of age. The protein and DNA content of their kidneys did not significantly differ, suggesting that the number and size of cells in the kidneys of offspring from both dietary groups were similar.

3.5 Discussion.

In this study the impact of nutrition in female rats prior to conception, on the blood pressure of the offspring was assessed. Maternal weight gain, food intake and food efficiency were also examined and markers of fetal growth were additionally determined.

Food intake was unaffected by maternal dietary regimen. In all groups, maternal food intake decreased in the final week of pregnancy in the face of the greatest increase in body weight. The values for food intake compare well with previously published data in this model (Levy & Jackson 1993; Langley-Evans *et al.* 1994), although these published studies did imply that maternal food intake was either slightly reduced (Langley-Evans *et al.* 1994) or slightly increased (Levy & Jackson 1993) in animals supplied a 9% casein diet, compared with those fed a control diet.

During the last day of rat pregnancy, maternal food intake decreases substantially, and the females tend to stop eating altogether a few hours prior to delivery. This may be a reason why absolute food intake during the final week of pregnancy appears to decline. Other factors may also contribute to this. During gestation, thermogenesis and the level of physical activity are both reduced (Illingworth *et al.* 1986). Furthermore, food efficiency has been shown, in the rat, to increase during pregnancy (Child 1920). These additional factors may contribute to the late gestation decline in food intake seen in this study.

Laboratory rat chow typically contains 18% to 19% protein by weight. Rat pregnancy can be maintained on very low protein intakes (6% protein - Zeman 1968, Levy & Jackson 1993), but severe dietary protein restriction compromises the survivability of the fetuses and the offspring (Langley & Jackson 1994). Exposure to a 7% protein diet during gestation has been shown to adversely affect the survivability of offspring, regardless of the diet supplied to the mother throughout lactation (Venkatachalam & Ramanathan 1964). A maternal 9% casein diet provides approximately 50% of the protein normally consumed by rats maintained on standard laboratory chow (Clarke *et al.* 1977). Such a maternal protein restriction has been shown to reduce birthweight slightly (Langley-Evans *et al.* 1994) or not at all (Langley-Evans *et al.* 1996b; Langley-Evans *et al.* 1996c). Litter size is unaffected (Langley-Evans *et al.* 1994; Langley & Jackson 1994; Levy & Jackson 1993) and neither maternal food intake or weight gain are altered by supply of a 9% casein diet during pregnancy (Langley-Evans *et al.* 1994; Present study). Were data of maternal weight gain and food intake, birth weight and litter size to be examined independently of maternal diet, they would fail to highlight any adverse intrauterine conditions. Thus, a maternal 9% casein diet imposes a very

mild insult in terms of physical growth, but still results in offspring with elevated blood pressures.

It is important to note that dietary energy availability was not compromised in either of the experimental diets. Several studies have suggested in both animals and humans that food consumption is directly associated with energy availability, such that food is consumed in order to satisfy energy requirements. Early rat studies employing calorie dilution, in which the calorie content of experimental diets was adjusted by changing the proportion of cellulose (Adolph 1947), indicated that the volume of food consumed provided energy proportional to that which was used by the animal. Similar conclusions were drawn from studies examining the effect on food consumption, of placing rats in a cold environment, thus inducing an elevation of thermogenic activity and therefore energy consumption (Stevenson 1954). Satiety and appetite should also be mentioned here. It has been shown that the energy content of a foodstuff is "learned" by an individual and subsequent intake of that food is varied according to the energy requirements of that individual (Booth *et al.* 1976). However, the palatability of food also plays a role in intake. If a meal is varied in terms of taste, colour and texture, the ability to regulate intake according to energy requirements is hampered (Rolls *et al.* 1984). Clearly, in this study, the use of a single diet would not raise issues of palatability, and therefore it may be reasonably assumed that food intake is regulated according to the requirements of the animal.

The acceleration of maternal weight gain during the final week of pregnancy is attributable to the rapid growth of the fetus. The greatest rise in fetal body weight occurs during the final week of gestation. Consequently, late gestation must potentially represent the phase of development most sensitive to nutrient restriction, since a greater quantity of nutrients are required at this time than earlier in gestation. An interesting question would be perhaps to ask whether metabolic disturbances of offspring, such as an elevated blood pressure, are induced by a maternal protein restriction acting solely in the final week of gestation, or whether such effects might occur earlier on in development (see Chapter 4).

Since maternal food intake was similar in both dietary groups, it is fair to assume that those animals supplied a low protein diet during pregnancy consumed approximately half the protein of animals supplied the 18% casein diet during pregnancy. We may not conclude that fetal protein supply was halved in low protein exposed animals. It may be the case that a low protein diet alters the partitioning of nutrients between the mother and the fetus. Indeed, it has been shown (Berg 1965) that female rats which actually lose body weight due to a dietary restriction during pregnancy, may still produce viable litters (albeit of low birthweight), implying a degree of parasitism of the fetus on the mother. The experiments of Berg (1965) do, however, indicate a significant degree of litter reabsorption when maternal body weight is compromised. It appears that offspring body weights at birth are largely maintained within a narrow range, as long as the mother is able to maintain some degree of weight gain during pregnancy (Berg 1965). Nutrient partitioning in the fetus is under the influence of the insulin-like growth factors (IGF). Expression of fetal IGF's has been shown to be sensitive to a maternal dietary protein restriction during pregnancy (Owens *et al.* 1989). Thus, although there are apparent metabolic consequences of exposure to a maternal low protein diet during gestation, the fetus may, by it's own actions, actively acquire much of what it requires.

Birthweight compared well with previously published values (Langley-Evans *et al.* 1994; Langley-Evans *et al.* 1996c). It was demonstrated that a mild maternal protein restriction did not significantly alter birthweight, and this highlighted the potential danger inherent in using such a gross marker of fetal growth from which to draw conclusions regarding the intrauterine environment. This study has shown that both physiological and developmental alterations, as evidenced by data obtained for blood pressure and renal structure (discussed below), may occur in response to a maternal dietary manipulation independently of changes in fetal growth. Thus, it is clear that subtle changes during development may have lasting consequences for the individual. These physiological alterations may represent an accommodation of the offspring to undernutrition, rather than being a mere consequence of a restricted nutrient supply.

The combination of data sets from all females supplied either a 9% or an 18% casein diet throughout pregnancy regardless of pre-mating diet, enabled comparison of food intake and weight gain in a way that was more statistically meaningful since the sample

size was increased. With the data combined, it was apparent that a maternal 9% casein impaired both maternal weight gain and food efficiency in the final week of pregnancy. The decline in maternal weight gain might represent either an impairment of maternal growth, an impairment of weight gain of the conceptus, or both. Further, maternal body composition may be altered by feeding a 9% casein diet. Lean tissue and fat tissue differ in weight (i.e. $1cm^3$ of lean tissue weighs more than the same quantity of fat tissue) and thus, disturbances in maternal weight gain may reflect alterations in the type of tissue that the mother, or fetus, is laying down. In addition, a change in the maternal body water content could also alter maternal weight.

At day 20 of gestation, offspring of females fed a 9% casein diet during pregnancy were significantly heavier than control animals, implying that a maternal protein restriction had, in fact, enhanced fetal growth. This is consistent with findings published by Langley-Evans *et al.* (1996c) and Langley-Evans and Nwangwu (1998). These studies demonstrated an acceleration of fetal growth in 9% casein exposed animals from day 14 of gestation. However, at term, offspring were of comparable birthweight and therefore the rate of growth in the final stage of gestation had significantly declined in low protein exposed offspring. In the present chapter, values of maternal weight are presented as weight gain per day. Given that the rate of increase of body weight of the fetuses is actually declining in the low protein group, then the decline of the maternal weight gain in this group may be partially due to changes in fetal growth (regardless of their actual body weight) as opposed to a restriction of maternal weight increase. Other studies by Langley-Evans and co-workers (Langley-Evans *et al.* 1996c) indicate similar patterns of fetal growth. At day 12 of gestation, the body weights of low protein exposed animals are similar to control animals, but at day 14, the body weights of these animals rise rapidly to around 150% of controls. This then declines and remains at around 105% - 110% of control body weights between days 16 - 20 of gestation and finally, at term, body weights of offspring from protein restricted dams are generally lower then controls. From the available data, it would seem therefore, that the fetal growth rate of low protein exposed animals, when compared to control animals, is characterised by an early acceleration, followed by an almost equally rapid decline.

Fleming *et al.* (personal communication) have suggested that the distribution of cells in the 4 day old rat embryo between the inner cell mass (embryonic tissue) and the outer cell mass (placentogenic tissue), is altered in response to maternal protein restriction. Low protein exposure allocates a greater proportion of cells to the outer cell mass compared to the inner cell mass at this age in offspring exposed to a maternal 9% casein diet, compared to controls. These data are in contrast to those of Leese and co-workers (Leese *et al.* 1994) which found no effect of a maternal 6% casein diet at days 4 and 5 of gestation on gross morphology of the embryo. Data on fetal weight prior to day 10 of gestation in this model is unavailable at present. It may be proposed that an animal with fewer embryogenic cells than controls early on in development, might initially lag behind in terms of growth. In the rat, intrauterine life is very short and, for the fetus, it is imperative to achieve a certain level of development by birth (i.e. the newborn must be able to support its own respiration, excretion, fluid homeostasis, digestion, immune function etc.). It may be the case that fetal growth is accelerated in low protein exposed offspring as soon as possible (apparently at or near day 14) in order that certain developmental endpoints might be achieved. This is, of course, speculative and clearly further embryonic and fetal investigations need to be carried out in order to address this question. However, such disturbances of intrauterine growth, particularly the late gestation decline in growth, might have serious implications for the development of certain organs, particularly the kidney, within which nephrogenesis is occurring from late gestation until around 10 days postnatally (Tuffro-McReddie *et al.* 1993). The kidney is also widely regarded as a significant contributor to adult blood pressure and therefore any developmental impairment of this organ, may have a causative role in the elevation of blood pressure seen in this model in response to a maternal protein restriction (Chapters 5 and 6).

The growth of the fetal heart was enhanced by a maternal dietary protein restriction. This may be important in the development of elevated blood pressure in postnatal life since a larger heart may enable a greater stroke volume, one of the three contributors to blood pressure (stroke volume, heart rate and peripheral resistance). At weaning (around 4 weeks of age), however, no effect of maternal dietary protein restriction has been found on heart weight (Langley-Evans *et al.* 1996c). The early difference observed in the present chapter, may be a consequence of the decline in the growth rate of low

protein exposed animals during late gestation and be unrelated to later cardiovascular outcome. The study also highlighted an impairment of renal growth, corrected for body weight, at day20 of gestation and at weaning in response to a maternal low protein diet. This is suggestive of a role for the kidney in the aetiology of hypertension in this model.

The blood pressure of offspring exposed to a maternal low protein diet was shown to be elevated above control values independently of the nature of the maternal diet prior to mating. It can thus be concluded that the hypertensive effect of a maternal protein restriction is exerted during pregnancy and that the plane of nutrition on entering pregnancy has no role in the programming mechanism. This does not preclude the possibility that a more severe dietary protein restriction (e.g. 4% - 6% protein) prior to conception may impinge upon the fetus but the present results suggest that a mild maternal protein restriction prior to conception does not. The degree of blood pressure elevation of low protein exposed offspring at 7 weeks of age was around 15 - 20 mmHg in males and 10 - 15 mmHg in females above controls, which was comparable with other studies using the same rat model and method of blood pressure determination (Langley-Evans *et al.* 1996a; Langley-Evans *et al.* 1996b; Langley-Evans *et al.* 1996c; Langley-Evans *et al.* 1996d; Langley-Evans *et al.* 1996e).

There were no differences in cell size or cell number between the kidneys of offspring of 9% and 18% casein fed females, as indicated by tissue DNA and protein content. However, histological analysis showed that kidneys of offspring exposed to a 9% casein diet *in utero* possessed fewer glomeruli than those of control animals. This suggests that low protein exposed offspring had fewer nephrons than controls.

These observations are apparently contradictory in that, how is it possible that the kidneys of the low protein exposed animals, which possess fewer nephrons than controls, can have the same number of cells as those of control animals, since most of the kidney comprises nephrons? There are two main points to consider; first the variability of the DNA and protein assays, and secondly, the possibility that the cells within the kidneys of low protein exposed animals may have undergone a degree of hypertrophy. The results from the two assays inevitably possessed a degree of variability. When the sample size is relatively small, as in this case, such variability may

mask any differences in cell number which may exist between the two groups. It is also possible, however, that renal cells of the low protein exposed animals are actually larger than those of controls. Indeed, examination of the data shows that, although there are no statistically significant differences between the two groups, the kidneys of 9% casein exposed animals had more protein (9% casein - 75.02mg/g tissue; 18% casein - 72.85mg/g tissue) and less DNA than control animals (9% casein – 9.46mg/g tissue; 18% casein – 10.99mg/g tissue). This may suggest that, with larger sample sizes, it may be shown that the kidneys of 9% casein exposed animals do possess fewer, larger cells than those of controls.

Values for glomerular complement compare well with the published literature (Merlet-Benichou *et al.* 1994) and reflect previous findings which indicate that a maternal dietary protein restriction during pregnancy impairs nephrogenesis in the offspring (Zeman 1968; Merlet-Benichou *et al.* 1994). These published studies employed a more severe dietary protein restriction than that used in this experiment (5% - 6% protein). The data described in this chapter further suggest that despite the mild nature of the dietary insult, a maternal 9% casein diet has a substantial effect on fetal growth and development. Uniquely the experiments demonstrate that a manipulation of the maternal diet both modifies renal morphometry and elevates blood pressure in the weanling offspring. Examination of non-macerated stillborn human babies showed that infants which were small for their gestational age possessed fewer nephrons than those of appropriate size for their gestational age (Hinchliffe *et al.* 1992). Additionally, it has been shown using ultrasound measurements, that human fetuses which are small for gestational age exhibit impaired renal growth (Konje *et al.* 1997; Konje *et al.* 1996). The kidneys of these fetuses appear long and narrow compared with those of appropriately sized fetuses. The hypothesis has been proposed that individual risk of hypertension may be inversely related to nephron number (Brenner *et al.* 1988). Indeed, the genetically hypertensive SHR rats have been shown to possess fewer nephrons than normotensive Wistar-Kyoto rats (Brenner *et al.* 1988). The data obtained in this study support this hypothesis and, in addition, suggest that maternal undernutrition may play a major role in the determination of the nephron complement of the offspring.

The physiological mechanism whereby a nephron deficit might result in an elevation of blood pressure is, as yet, undefined, although it has been postulated that blood pressure might be elevated in order to enable kidneys with a reduced nephron complement to maintain efficient sodium and body fluid homeostasis (Brenner *et al.* 1988). Examination of nephrogenesis during fetal and early postnatal life may help to elucidate this mechanism as different populations of nephrons develop at different stages (Tuffro-McReddie *et al.* 1993). Furthermore, measurements of renal function such as the filtration capacity of the kidneys and the plasma volume relationships of low protein exposed animals should provide an insight into whether renal structural anomalies translate to an impairment of function. Other important questions arising from this study include whether the primary hypertensive effects of a maternal protein restriction occur during any particular stage of gestation, and whether this hypertensive effect is purely a consequence of altered fetal growth parameters (i.e. a morphological change) or is perhaps programmed by some other endocrine or metabolic mechanism.

In conclusion, this study has shown that the hypertensive effect of a maternal low protein diet is exerted during pregnancy, and that the plane of nutrition prior to pregnancy does not modulate this effect. At day 20 of gestation, the hearts of low protein exposed animals are larger than those of controls, suggesting a possible role for the heart in the development of elevated blood pressure in this model. Most interestingly, the kidneys of low protein exposed offspring possess fewer glomeruli than controls, supporting the hypothesis that a nephron deficit may predispose the animal to elevated blood pressure.

Chapter 4.

Critical timing of undernutrition: effects on renovascular programming.

4.1 Introduction.

In chapter 3 it was demonstrated that offspring of females fed a 9% casein diet during pregnancy had higher systolic blood pressures than those from 18% casein diet exposed animals. Exposure to the experimental low protein diet prior to conception was not necessary for the elevation of blood pressure of the offspring.

Previously published work (Langley-Evans *et al.* 1994; Langley-Evans *et al.* 1995a) examined the effect of a maternal dietary protein restriction, imposed throughout rat pregnancy upon blood pressures of the resulting offspring. However, many examples of metabolic programming in the natural world demonstrate that there is invariably a critical period of action for the programming stimuli. For example, the phenomenon of temperature dependent sex determination seen in many reptiles occurs only during the period of gonadal development (Janzen & Paukstis 1991). It is feasible therefore, that the unfavourable effects of maternal protein restriction might be exerted on the conceptus during a discrete phase of development.

Potential contributors to the blood pressure elevation of 9% casein exposed offspring include renal and cardiac effects, the renin-angiotensin system, sympathetic activity, vascular dysgenesis or perhaps inappropriate release or sensitivity to a variety of hormones such as atrial natriuretic peptide (ANP), aldosterone, corticosterone or endothelin. The tissues and cells involved in these processes develop and mature at different stages of gestation. Exposure to a maternal low protein diet during a discrete developmental period may specifically compromise one or more of the cardiovascular control mechanisms.

The aim of the present study was to examine the effect of a maternal 9% casein diet imposed during discrete weeks of pregnancy, on renal structure, function and biochemistry

of the offspring. This was examined in 2 separate studies. In the first study the effect of exposure to a maternal 9% casein diet during discrete periods of pregnancy on nephrogenesis was examined. Study 2 addressed the effects of exposure to a maternal 9% casein diet during discrete periods of pregnancy on offspring blood pressure at 4 weeks of age and offspring renal function at 19 weeks of age.

4.2 Protocol.

4.2.1 Study 1.

Twenty virgin female Wistar rats (200g - 230g) were mated and individually housed. Animals were allocated to five separate groups (Table 4.1) such that 4 animals were fed an 18% casein diet throughout pregnancy (18%Ca), 4 were fed a 9% casein diet throughout pregnancy (9%Ca), and the remaining 3 sets of 4 animals were fed a 9% casein diet during either the first (Week 1), second (Week 2) or third (Week 3) week of pregnancy. These rats were fed an 18% casein diet for the other 2 weeks of pregnancy.

Two females from each group were sacrificed at day 20 of pregnancy and the remaining animals were sacrificed at term. The left kidney from each fetus or newborn was removed and used either for determination of glomerular complement (Chapter 2, section 2.7) or measurement of protein (Chapter 2, section 2.8), DNA (Chapter 2, section 2.9) and alkaline phosphatase (ALP) activity (Chapter 2, section 2.10).

4.2.2 Study 2.

Twenty-five virgin female Wistar rats weighing between 200g and 230g were mated and individually housed. Day 0 of pregnancy was taken from the appearance of a vaginal plug. The animals were allocated to five groups of 5 animals and supplied either a 9% casein diet or an 18% casein diet (Chapter 2, section 2.2), according to the experimental protocol (Table 4.1). Two groups were supplied either a 9% casein (9%Ca) or an 18% casein (18%Ca) diet throughout pregnancy. The remaining 3 groups were supplied a 9% casein diet for either the first (Week 1), second (Week 2) or third (Week 3) week of pregnancy

only and an 18% casein diet for the remaining 2 weeks. At parturition, all females were transferred to standard laboratory chow (Materials and Methods 2.2).

Birthweights were measured within twelve hours of delivery and the litters culled to 8 animals (4 males and 4 females where possible). The offspring remained with the mothers until weaning. At 4 weeks of age, the offspring had their systolic blood pressures measured using the tail cuff method (Materials and Methods 2.4). Body weights were recorded, and all animals except two females from each litter were sacrificed for measurement of organ weights (lung, liver, spleen, heart and kidney). The remaining two females from each litter were retained for renal function measurements. Female animals were chosen for renovascular measurements because the female urethra is more readily cannulated than that of the male.

Basal renal blood flow (RBF), glomerular filtration rate (GFR) were measured under anaesthesia in the remaining offspring at 19 weeks of age (Materials and Methods 2.6). The diuretic furosemide was then administered intraperitoneally at a concentration of 10mg/kg body weight, in order to examine the effects of a pharmacologically induced fluid depletion upon renal haemodynamics. At the end of the experiment, the left kidney was removed and weighed.

4.3 Results.

4.3.1 Study 1.

The results of measurements of glomerular complement and those of renal biochemical measurements are shown in Figure 4.1 and Table 4.2. Maternal diet had a significant effect on the glomerular complement of offspring kidneys at day 20 ($P=0.000$) and full term ($P=0.009$). At day 20, the kidneys of Week 3 offspring contained a greater number of glomeruli than all other groups ($P<0.01$). Kidneys of Week 1 offspring and 18%Ca controls had fewer glomeruli than both the 9%Ca animals and the Week 2 group ($P<0.05$; Figure 4.1). At term, the kidneys of 18%Ca controls, 9%Ca and Week 1 animals contained more glomeruli than both the Week 2 ($P<0.05$) and the Week 3 animals ($P<0.05$). There were no

differences in glomerular complement between the Week 1, 18%Ca and 9%Ca animals at this time point (Figure 4.1).

Examination of the change in glomerular number within dietary groups from day 20 to full-term by two-way ANOVA showed that maternal dietary regimen had a significant effect on late gestation nephrogenesis (P=0.000). The 18%Ca controls more than trebled (261% increase) their glomerular complement, with the Week 1 animals quadrupling theirs (324% increase). However, the glomerular complement of the 9%Ca animals only doubled (103% increase) whilst that of the Week 2 animals increased by only one third (38% increase). The Week 3 animals appeared to actually lose glomeruli. It is clear from the data that the late gestation acceleration of nephrogenesis was impaired by exposure to a maternal 9%Ca diet either during mid- (Week 2; P=0.014) or late- (Week 3; P=0.000) gestation.

Protein and DNA concentrations were measured in kidneys at day 20 and term. Measurement of DNA in 9%Ca animals at day 20 provided unreliable results and were thus, not included. There were no differences in protein concentration (day 20 P=0.143 & full-term P=0.371), DNA concentration (day 20 P=0.408 & full-term P=0.371) or DNA per mg protein (day 20 P=0.647 & full term P=0.264) as a result of maternal dietary manipulation (Table 4.2).

Table 4.1: Allocation of animals to dietary groups.

	Study 1	Study 2	Week of pregnancy		
GROUP	**n**	**n**	**Week 1**	**Week 2**	**Week 3**
18%Ca	4	5	18% casein	18% casein	18% casein
9%Ca	4	5	9% casein	9% casein	9% casein
Week 1	4	5	9% casein	18% casein	18% casein
Week 2	4	5	18% casein	9% casein	18% casein
Week 3	4	5	18% casein	18% casein	9% casein

In both studies, either a 9% casein diet or an 18% casein diet (Materials and Methods 2.2) was supplied to pregnant females. At parturition, all females were transferred to standard laboratory chow (CRM(X), S.D.S., Wincham, Cheshire).

Alkaline phosphatase (ALP) was assayed in kidneys of animals from all dietary groups at day 20 of gestation and at full-term. ALP was assayed as it is considered a useful marker of the maturity of nephrons (Desalu 1966). As ALP exists in the brush border of the proximal tubule (Wachstein & Bradsh 1955), measurements of its activity may be modulated both by the number of proximal tubules and the length of the tubules. When expressed per mg tissue, ALP activity was shown to be modulated by maternal diet at day 20 ($P=0.000$) and term ($P<0.05$). At day 20 the ALP activity of the kidneys of the Week 1 animals was significantly higher than in all other groups, and that of the Week 2 animals was greater than the 9%Ca, 18%Ca and Week 3 animals ($P<0.05$; Table 4.2). At full-term kidneys of the 18%Ca exposed animals had a greater ALP activity per mg tissue than those of the Week 3 animals ($P<0.05$).

When ALP activity was expressed per mg protein, there were no differences between dietary groups at either day 20 ($P=0.073$) or term ($P=0.936$; Table 4.2). There was no influence of maternal diet on the effect of time on ALP activity (Two-way ANOVA - $P=0.759$). However, in both 18%Ca controls and Week 3 animals, Students T-tests demonstrated that ALP activity significantly increased from day 20 to full term (18%Ca - $P<0.005$; Week 3 - 0.011).

ALP activity was also expressed as a proportion

4.3.2 Study 2.

4.3.2.1 Growth parameters:

There were no differences in birthweight between males or females attributable to the effects of maternal diet (Table 4.3). However, male offspring from the 18%Ca, the 9%Ca and the Week 2 groups were consistently heavier than their female littermates ($P<0.05$).

Body and organ weights of 9%Ca and 18%Ca animals at 4 weeks of age were similar in both males (Table 4.4) and females (Table 4.5). The males (Table 4.4) of Week 1, Week 2 and Week 3 groups were heavier than control (18%Ca) males, and kidney, liver, lung and

heart weights of these animals were also greater than those observed in 18%Ca animals ($P<0.05$). The spleens of the Week 1 and Week 2 males were heavier than in controls (18%Ca). The female offspring from all five dietary groups were similar in terms of body weight and the weights of all the major organs examined (Table 4.5).

In order to determine any variation in the allometry of organ growth, the organ weights were expressed as proportions of the body weight and compared between dietary groups in males and females (Tables 4.6 & 4.7). When organ weights were expressed in this manner, only the kidneys were found to vary in size between the groups (One-way ANOVA: $P<0.05$ - males; $P<0.01$ - females). Amongst the males, the kidneys of the, Week 1, Week 2 and Week 3 animals were disproportionately smaller than those of the 18%Ca animals ($P<0.05$). Kidneys of 9%Ca males tended to be smaller than those of control males (NS, $P=0.074$). The kidneys of the females of the Week 1, Week 2 and Week 3 groups were all disproportionately smaller than those of the 9%Ca group ($P<0.05$). Only the females of the Week 1 group had kidneys which were smaller in proportion to body weight than in 18%Ca controls ($P<0.05$).

At 19 weeks of age, body weight was again measured in the remaining female animals and the left kidney was weighed after measurement of renal haemodynamics. Body weight was similar in all groups, whilst the kidney weights of the 9%Ca animals were lower than controls ($P<0.05$; Table 4.8). The kidney weights of the 9%Ca animals as a proportion of body weight were also reduced relative to controls ($P<0.05$). Kidney weight in both absolute terms and corrected for body weight, was unaltered by single week exposure to a low protein *in utero*.

Figure 4.1: Glomerular complement of kidneys of animals exposed to a maternal low protein diet during pregnancy at day 20 of gestation and term.

Female Wistar rats were supplied either an 18% casein diet throughout pregnancy (18%Ca), a 9% casein diet throughout pregnancy (9%Ca), or a 9% casein diet during either the first (Week 1), the second (Week 2) or third (Week 3) of pregnancy. Females were either sacrificed at day 20, or at parturition. Fetal kidneys were removed and used for determination of glomerular complement. Mean values for glomerular complement are shown for animals at day 20 and at term ± SEM. Two-way ANOVA showed a significant effect of maternal diet and stage of development on the glomerular complement ($P<0.001$). * - different to 18% Ca ($P<0.05$). ‡ - different to all groups ($P<0.05$). † - different to 9%Ca and Week 2 ($P<0.05$). § - different to Week 1 and 9%Ca ($P<0.05$). At day 20 *n* numbers were as follows 18%Ca (16); 9%Ca (12); Week 1 (10); Week 2 (11); Week 3 (6). At term the *n* numbers were 18%Ca (20); 9%Ca (14); Week 1 (9); Week 2 (17); Week 3 (12).

Maternal diet.	**Glomerular complement±SEM (n)**	
	Day 20	**Term**
18% Casein	1342±165 (16)	4839±538 (20)
9% Casein	2166±283 (12)	4402±394 (14)
Week 1	1152±99 (10)	4885±818 (9)
Week 2	2070±171 (11)	2853±202 (17)
Week 3	5733±754 (6)	3474±415 (12)

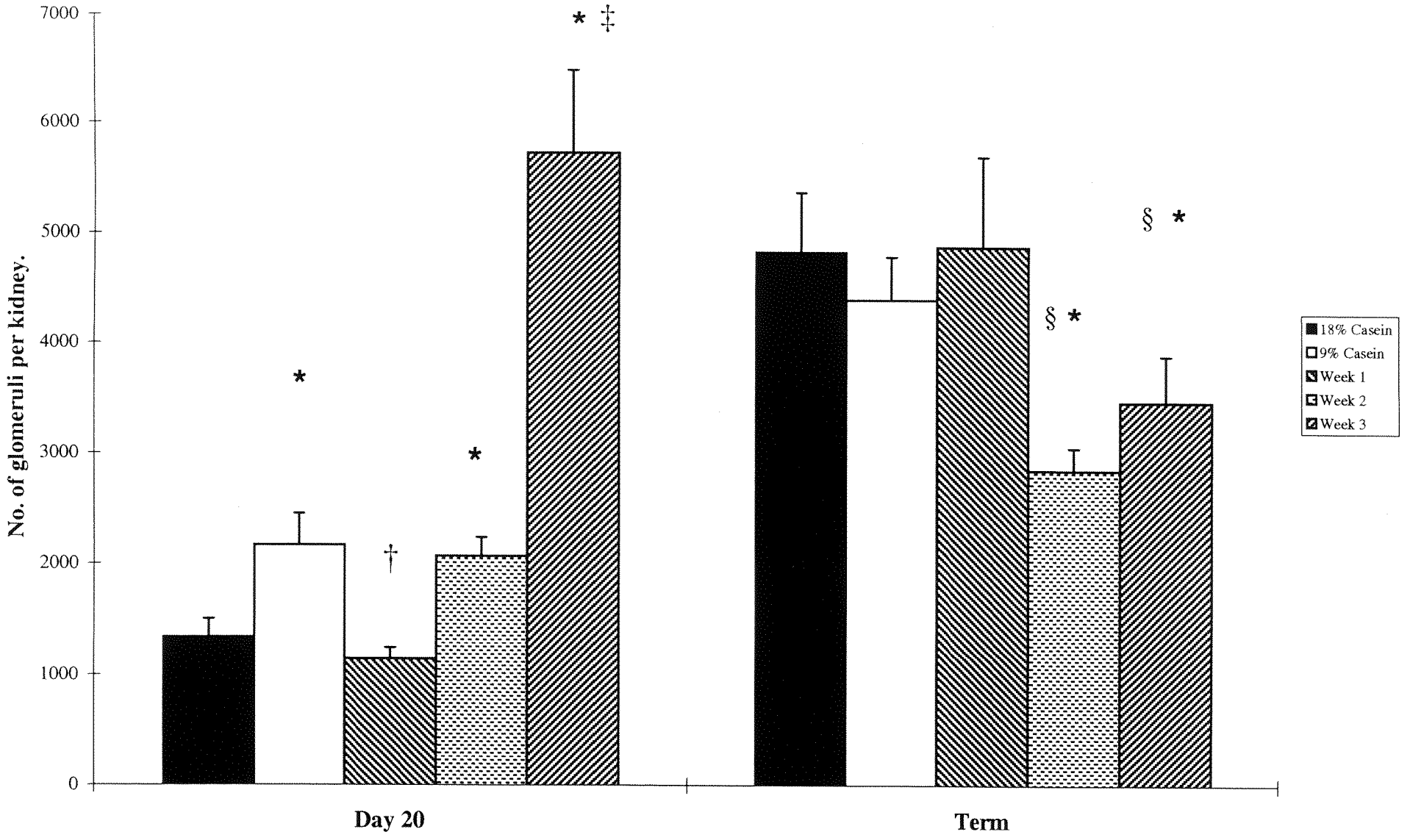

7000
6000
5000
4000
3000
2000
1000
0
No. of glomeruli per kidney.
* ‡
*
†
*
§ *
§ *
Day 20
Term
18% Casein
9% Casein
Week 1
Week 2
Week 3

Table 4.2: Renal biochemical measurements in offspring at day 20 and term.

Measurements of protein content, DNA content and alkaline phosphatase (ALP) activity were measured in kidneys of offspring from each of the 5 dietary groups. Values are shown as means ± SEM. + represents a significant difference relative to 18%Ca controls at full term. ‡ represents a significant difference relative to 18%Ca, 9%Ca and Week 3 animals ($P<0.05$). § represents a significant difference relative to all other groups ($P<0.05$). † represents a significant difference compared with the same dietary group at day 20 of gestation ($P<0.05$). Twoway ANOVA showed no significant effects of maternal diet and age on protein content ($P=0.880$), DNA per mg tissue ($P=0.431$), DNA per mg protein ($P=0.376$), ALP per mg tissue ($P=0.089$) or ALP per mg protein ($P=0.759$).

	n	Day 20	n	Term
Protein (mg/g tissue)				
18%Ca	5	257±44	6	335±13
9%Ca	12	188±18	6	258±23†
Week 1	9	248±23	6	314±29
Week 2	7	217±19	7	296±35
Week 3	5	252±7	6	279±29
DNA(mg/g tissue)				
18%Ca	4	39±7	3	35±1
9%Ca	4	30±6	-	-
Week 1	5	33±5	4	38±4
Week 2	4	35±8	4	44±20
Week 3	4	35±7	6	33±4
mg Protein/mgDNA				
18%Ca	4	7.0±1.0	3	10.0±0.7
9%Ca	4	8.1±1.0	-	-
Week 1	5	8.8±1.0	4	9.3±0.5
Week 2	4	7.5±0.8	4	7.5±2.0
Week 3	4	7.3±0.6	6	8.7±1.2
ALP units/mg tissue.				
18%Ca	5	60±4	6	152±7†
9%Ca	12	59±3	6	125±14†
Week 1	9	111±15§	6	158±20†
Week 2	7	99±11‡	8	126±5†
Week 3	6	65±6	7	117±4†+
ALP units/mg Protein.				
18%Ca	5	0.25±0.04	6	0.45±0.04†
9%Ca	12	0.36±0.06	6	0.51±0.07
Week 1	9	0.44±0.05	6	0.52±0.07
Week 2	7	0.47±0.06	8	0.52±0.12
Week 3	6	0.26±0.02	7	0.45±0.05†

4.3.2.2 *Blood pressure:*

Systolic blood pressure was measured in all offspring at 4 weeks of age. One-way ANOVA indicated that all animals exposed to a 9% casein diet at any stage during pregnancy had blood pressures significantly higher than those of control animals ($P<0.05$; Figure 4.2). In males and females the highest blood pressures were seen in offspring exposed to a 9% casein diet throughout pregnancy (Increase relative to control: 26mmHg - Males; 28mmHg - Females). The greatest effect of a single week exposure to a maternal 9% casein diet was seen in the Week 3 animals ($P<0.05$) in both males (16mmHg higher) and females (20mmHg higher). In males, Week 1 blood pressures were 15 mmHg above controls ($P<0.05$) whilst Week 2 blood pressures were 9mmHg above those of 18%Ca animals ($P<0.05$). In the females, Week 1 blood pressures were 11mmHg higher than in controls, whilst Week 2 animals had blood pressures elevated above controls by 15mmHg.

4.3.2.3 *Renovascular parameters:*

Renal haemodynamic parameters were determined in 19 week old female offspring. All animals were cannulated for the measurement of glomerular filtration rate (GFR), renal blood flow (RBF) and blood pressure both before and after the administration of the diuretic furosemide. Blood pressure was continuously measured throughout the infusion in order to verify a stable perfusion pressure.

Measurements of renal haemodynamics in the rats prior to furosemide administration indicated that maternal diet had no effect upon either renal blood flow (RBF) or glomerular filtration rate (GFR). GFR measurements were similar in all groups of animals (1.99 - 2.18 ml/min; Table 4.9). RBF was shown not to differ between control animals and those exposed to a maternal 9% casein diet during any stage of pregnancy (Table 4.9).

Table 4.3: Birthweights of male and female offspring.

Dietary group.	n	Males	n	Females
18%Ca	19	5.17±0.10*	13	4.77±0.15
9%Ca	21	5.21±0.10*	17	4.73±0.14
Week 1	27	5.22±0.10	29	5.21±0.09
Week 2	26	5.45±0.11*	30	4.94±0.07
Week 3	11	5.31±0.26	17	4.99±0.19

Female Wistar rats were mated and supplied either an 18% casein diet throughout pregnancy (18%Ca), a 9% casein diet throughout pregnancy (9%Ca), or a 9% casein diet during the first (Week 1), second (Week 2) or third (Week 3) week of pregnancy and an 18% casein diet during the remaining two weeks. Birthweights were measured within 12 hours of delivery. Values are expressed as means ± SEM. Significant differences ($P<0.05$) between males and females within groups, are denoted by *.

Table 4.4: Body weights and organ weights of male offspring at 4 weeks of age.

	Maternal dietary group.				
	18%Ca	**9%Ca**	**Week 1**	**Week 2**	**Week 3**
n	12	7	18	16	11
Body weight (g)	67±2	79±7	93±4*	89±5*	80±3*
Lung weight (g)	0.58±0.02	0.67±0.06	0.83± 0.04*‡	0.77±0.04*	0.76±0.03*
Liver weight (g)	2.95±0.11	3.38±0.22	4.10±0.18*‡	4.00±0.24*	3.54±0.15*
Heart weight (g)	0.37±0.01	0.42±0.03	0.52±0.02*‡	0.50±0.02*	0.47±0.02*
Spleen weight (g)	0.32±0.02	0.37±0.03	0.45±0.02*‡	0.46±0.05*	0.36±0.03
Kidney weight (g)	0.34±0.01	0.37±0.03	0.43±0.01*‡	0.41±0.02*	0.38±0.01*

Female Wistar rats were mated and fed either an 18% casein (18%Ca) or a 9% casein (9%Ca) diet throughout pregnancy, or a 9% casein diet during the first (Week 1), second (Week 2) or third (Week 3) week of pregnancy, with the remaining weeks being supplied an 18% casein diet (Table 4.1). At 4 weeks of age, the offspring had their systolic blood pressures measured and those not kept for later renal function measurements were sacrificed and organ weights measured. Values are expressed as means ± SEM. * represents a significant difference relative to 18%Ca controls ($P<0.05$), ‡ represents a significant difference relative to 9%Ca controls.

Table 4.5: Body weights and organ weights of female offspring at 4 weeks of age.

	Maternal dietary group.				
	18%Ca	**9%Ca**	**Week 1**	**Week 2**	**Week 3**
n	5	5	9	8	7
Body weight (g)	83±5	92±6	90±4	81±4	77±4
Lung weight (g)	0.86± 0.1	0.82± 0.04	0.81± 0.04	0.76± 0.03	0.68± 0.03
Liver weight (g)	3.42± 0.23	4.16± 0.43	4.04± 0.19	3.52± 0.19	3.50± 0.22
Heart weight (g)	0.43± 0.02	0.47± 0.02	0.49± 0.02	0.45± 0.02	0.46± 0.03
Spleen weight (g)	-	-	0.45± 0.02	0.34± 0.02	0.36± 0.02
Kidney weight (g)	0.43± 0.03	0.48± 0.03	0.40± 0.02‡	0.39± 0.02‡	0.37± 0.02‡

Female Wistar rats were mated and fed either an 18% casein (18%Ca) or a 9% casein (9%Ca) diet throughout pregnancy, or a 9% casein diet during the first (Week 1), second (Week 2) or third (Week 3) week of pregnancy, with the remaining weeks being supplied an 18% casein diet (Table 4.1). At 4 weeks of age, the offspring had their systolic blood pressures measured and those not kept for later renal function measurements were sacrificed and organ weights measured. Values are expressed as means ± SEM. ‡ represents a significant difference relative to 9%Ca controls.

Table 4.6: Organ weights of male offspring at 4 weeks of age expressed as proportions of body weight.

	Maternal dietary group.				
	18% casein	**9% casein**	**Week 1**	**Week 2**	**Week 3**
n	12	7	18	16	11
Lung weight/body weight (g/100g)	0.88± 0.03	0.86± 0.06	0.90± 0.03	0.88± 0.02	0.95± 0.04
Liver weight/body weight (g/100g)	4.41± 0.06	4.31± 0.01	4.43± 0.06	4.47± 0.08	4.40± 0.09
Heart weight/body weight (g/100g)	0.57± 0.02	0.54± 0.01	0.56± 0.01	0.57± 0.02	0.58± 0.02
Spleen weight/body weight (g/100g)	0.48± 0.02	0.47± 0.02	0.49± 0.02	0.51± 0.04	0.44± 0.04
Kidney weight/body weight (g/100g)	0.51± 0.02	0.47± 0.01	0.47± 0.01*	0.47± 0.01*	0.46± 0.01*

Female Wistar rats were mated and fed either an 18% casein (18% Casein) or a 9% casein (9% Casein) diet throughout pregnancy, or a 9% casein diet during the first (Week 1), second (Week 2) or third (Week 3) week of pregnancy, with the remaining weeks being supplied an 18% casein diet. At 4 weeks of age, the offspring had their systolic blood pressures measured and those not kept for later renal function measurements were sacrificed and organ weights (Lung; Liver; Heart; Spleen; Kidney; Body weight) measured. Values are expressed as means of organ weights as percentages of body weight ± SEM. * represents a significant difference relative to sex matched 18% Casein controls ($P<0.05$).

Table 4.7: Organ weights of female offspring at 4 weeks of age expressed as proportions of body weight.

	Maternal dietary group.				
	18% casein	**9% casein**	**Week 1**	**Week 2**	**Week 3**
n	5	5	9	8	7
Lung weight/body weight (g/100g)	1.05± 0.15	0.91± 0.08	0.90± 0.03	0.95± 0.04	0.88± 0.04
Liver weight/body weight (g/100g)	4.15± 0.22	4.49± 0.19	4.47± 0.07	4.38± 0.10	4.51± 0.08
Heart weight/body weight (g/100g)	0.52± 0.03	0.51± 0.02	0.55± 0.02	0.56± 0.02	0.59± 0.03
Spleen weight/body weight (g/100g)	-	-	0.50± 0.03	0.43± 0.03	0.47± 0.03
Kidney weight/body weight (g/100g)	0.52± 0.01	0.52± 0.01	0.44± 0.01*∞‡	0.49± 0.01‡	0.47± 0.02‡

Female Wistar rats were mated and fed either an 18% casein (18% Casein) or a 9% casein (9% Casein) diet throughout pregnancy, or a 9% casein diet during the first (Week 1), second (Week 2) or third (Week 3) week of pregnancy, with the remaining weeks being supplied an 18% casein diet. At 4 weeks of age, the offspring had their systolic blood pressures measured and those not kept for later renal function measurements were sacrificed and organ weights (Lung; Liver; Heart; Spleen; Kidney; Body weight) measured. Values are expressed as means of organ weights as percentages of body weight ± SEM. * represents a significant difference relative to sex matched 18% Casein controls ($P<0.05$), a superscripted ‡ represents a significant difference relative to sex matched 9% Casein controls.

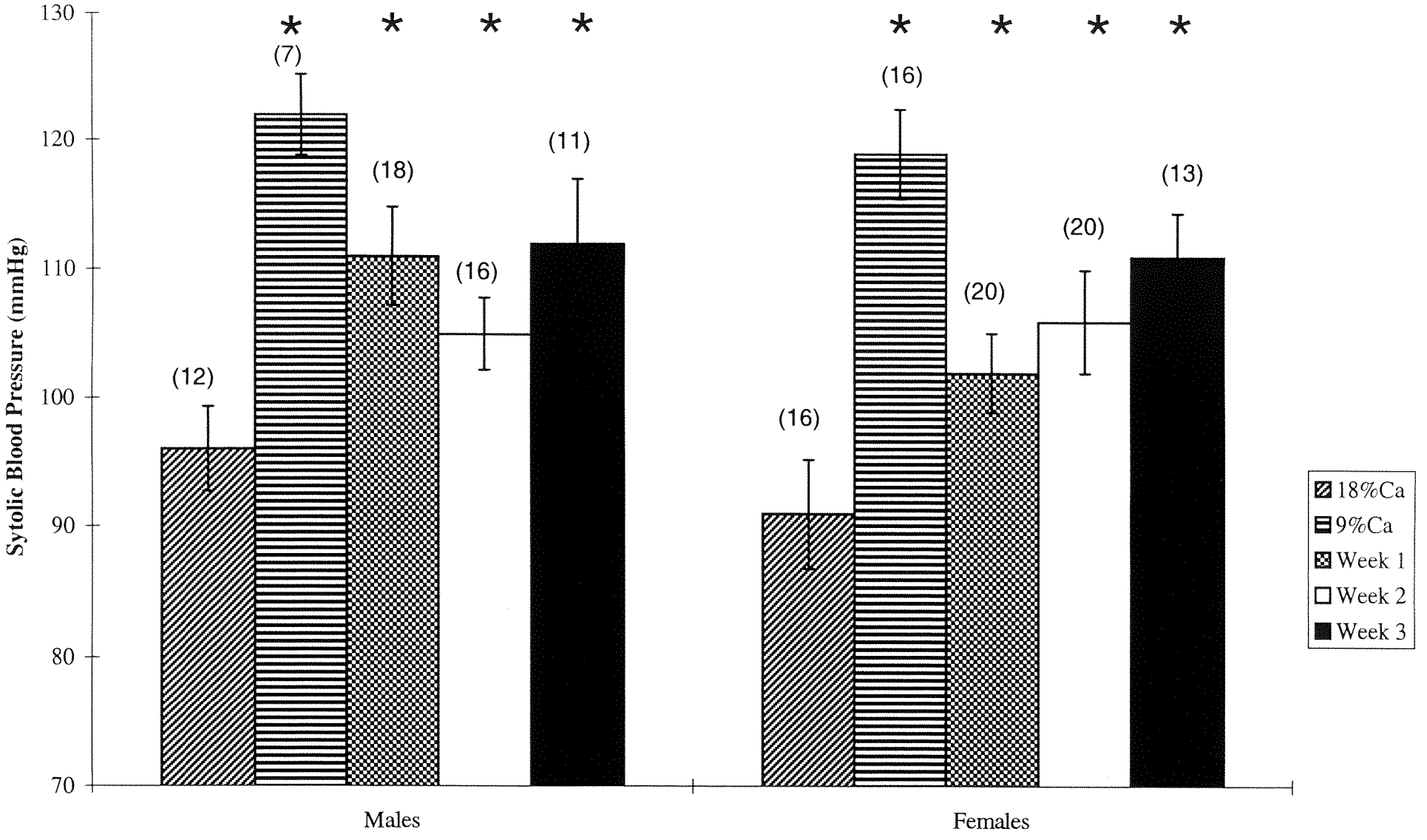
Table 4.8: Body weights, kidney weights and kidney weight to body weight ratios of female animals at 19 weeks of age.

	n	Body Wt. (g)	Kidney Wt. (g)	KW/BW (%)
18%Ca	9	270±3	1.00±0.02	0.37±0.01
9%Ca	7	274±6	0.94±0.02*	0.34±0.01*
Week 1	8	275±9	0.99±0.03	0.36±0.01
Week 2	5	267±5	1.02±0.04	0.38±0.01
Week 3	5	267±7	0.91±0.04	0.35±0.01

Female Wistar rats were mated and fed either an 18% casein (18%Ca) or a 9% casein (9%Ca) diet (Materials and Methods 2.2) throughout pregnancy, or a 9% casein diet during the first (Week 1), second (Week 2) or third (Week 3) week of pregnancy, with the remaining weeks being supplied an 18% casein diet (Table 4.1). At 19 weeks of age body and left kidney weights were determined after renovascular measurements had been made. These are expressed above as means ± SEM. * represents a significant difference ($P<0.05$) compared to 18%Ca controls.

Figure 4.2: Systolic blood pressures of male and female offspring at 4 weeks of age.

Female Wistar rats were mated and fed either an 18% casein (18%Ca) or a 9% casein (9%Ca) diet (Materials and Methods 2.2) throughout pregnancy, or a 9% casein diet during the first (Week 1), second (Week 2) or third (Week 3) week of pregnancy, with the remaining weeks being supplied an 18% casein diet. At 4 weeks of age, the offspring had their systolic blood pressures measured using the tail cuff method (Materials and Methods 2.4). The figure shows mean systolic blood pressures of male and female offspring $\pm$ SEM. * indicates a significant difference relative to sex matched 18%Ca controls ($P<0.05$). The n number for each group are shown in parentheses.


Sytolic Blood Pressure (mmHg)
130
120
110
100
90
80
70
(12)
(7)
(18)
(16)
(11)
(16)
(16)
(20)
(20)
(13)
*
Males
Females
18%Ca
9%Ca
Week 1
Week 2
Week 3

The technically difficult measurements of RBF were hampered by problems associated with the ^{3}H-PAH label. Some of the estimates for RBF were very low which may have been due to dissociation of the label prior to administration. Values obtained for GFR were comparable to published values (Tenstad & Williamson 1995). Consequently, values for RBF were rejected if the filtration fraction (FF) exceeded 50%. This resulted in a low sample size in the 9%Ca group. Animals from each dietary group were not studied during separate experimental periods and it is thus unclear as to why the 9%Ca group was more affected than any other.

Furosemide was administered 90 minutes from the start of label infusion. There was a trend for both GFR and RBF to decrease in response to furosemide administration, but this was not significant (Table 4.10; Figure 4.3; Figure 4.4). The Week 3 animals were the only group in which a significant reduction in GFR in response to furosemide was observed ($P=0.001$). Filtration fraction (FF) either decreased slightly (18%Ca, 9%Ca, Week1 and Week 3) or remained stable (Week 2) following furosemide administration.

Urine samples were taken every fifteen minutes throughout the cannulation procedure for the determination of GFR and RBF. Comparison of the volumes of urine produced both prior to and after the administration of furosemide indicated maternal diet had no effect on urine production (Table 4.10). In all groups, there was a significant effect of furosemide on urine production ($P<0.05$). Furosemide increased the formation of urine by 3 to 4 fold in all groups (Table 4.10).

Table 4.9: Glomerular filtration rate (GFR), renal blood flow (RBF) and filtration fraction (FF) in animals at 19 weeks of age.

	n	GFR (ml/min)	n	RBF (ml/min)	FF (GFR/RBF%)
18%Ca	7	2.09±0.09	4	5.10±1.17	36±5
9%Ca	6	1.99±0.07	3	7.98±0.86	28±2
Week 1	6	2.16±0.15	6	7.24±0.54	30±3
Week 2	6	2.16±0.17	5	5.91±0.80	36±3
Week 3	6	2.18±0.13	5	6.12±0.55	36±3

Female Wistar rats were mated and fed either an 18% casein or a 9% casein diet (Materials and Methods 2.2) throughout pregnancy (18%Ca & 9%Ca respectively), or a 9% casein diet during the first (Week 1), second (Week 2) or third (Week 3) week of pregnancy, with the remaining weeks being supplied an 18% casein diet. At 19 weeks of age female offspring from each dietary group were anaesthetised and cannulated for the measurement of renal blood flow (RBF) and glomerular filtration rate (GFR). Values are shown as means ± SEM for GFR and RBF prior to the administration of furosemide.

Table 4.10: Glomerular filtration rate (GFR), renal blood flow (RBF) and filtration fraction (FF) in animals at 19 weeks of age after administration of furosemide.

	n	GFR (ml/min)	n	RBF (ml/min)	FF (GFR/RBF%)
18%Ca	7	1.83±0.07	4	4.63±1.88	29±6
9%Ca	6	2.08±0.08	3	8.03±1.91	27±3
Week 1	6	1.91±0.11	6	7.07±0.88	27±4
Week 2	6	1.93±0.14	5	4.86±0.50	36±3
Week 3	6	1.61±0.06	5	5.52±0.57	31±3

Female Wistar rats were mated and fed either an 18% casein or a 9% casein diet (Materials and Methods 2.2) throughout pregnancy (18%Ca & 9%Ca respectively), or a 9% casein diet during the first (Week 1), second (Week 2) or third (Week 3) week of pregnancy, with the remaining weeks being supplied an 18% casein diet. At 19 weeks of age female offspring from each dietary group were anaesthetised and cannulated for the measurement of renal blood flow (RBF) and glomerular filtration rate (GFR). Values are shown as means ± SEM for GFR and RBF after the administration of furosemide.

Figure 4.3: The change in renal blood flow (RBF) in response to Furosemide administration.

Female Wistar rats were mated and fed either an 18% casein or a 9% casein diet (Materials and Methods 2.2) throughout pregnancy (18%Ca & 9%Ca respectively), or a 9% casein diet during the first (Week 1), second (Week 2) or third (Week 3) week of pregnancy, with the remaining weeks being supplied an 18% casein diet. At 19 weeks of age female offspring from each dietary group were anaesthetised and cannulated for the measurement of renal blood flow (RBF) and glomerular filtration rate (GFR). Values are shown as the mean change in RBF after furosemide administration $\pm$ SEM. Two-way ANOVA showed no effect of maternal diet and administration of furosemide on RBF (P=0.986). Sample sizes in each group were; 18%Ca n=4, 9%Ca n=3, Week 1 n=6, Week 2 n=5, Week 3 n=5.

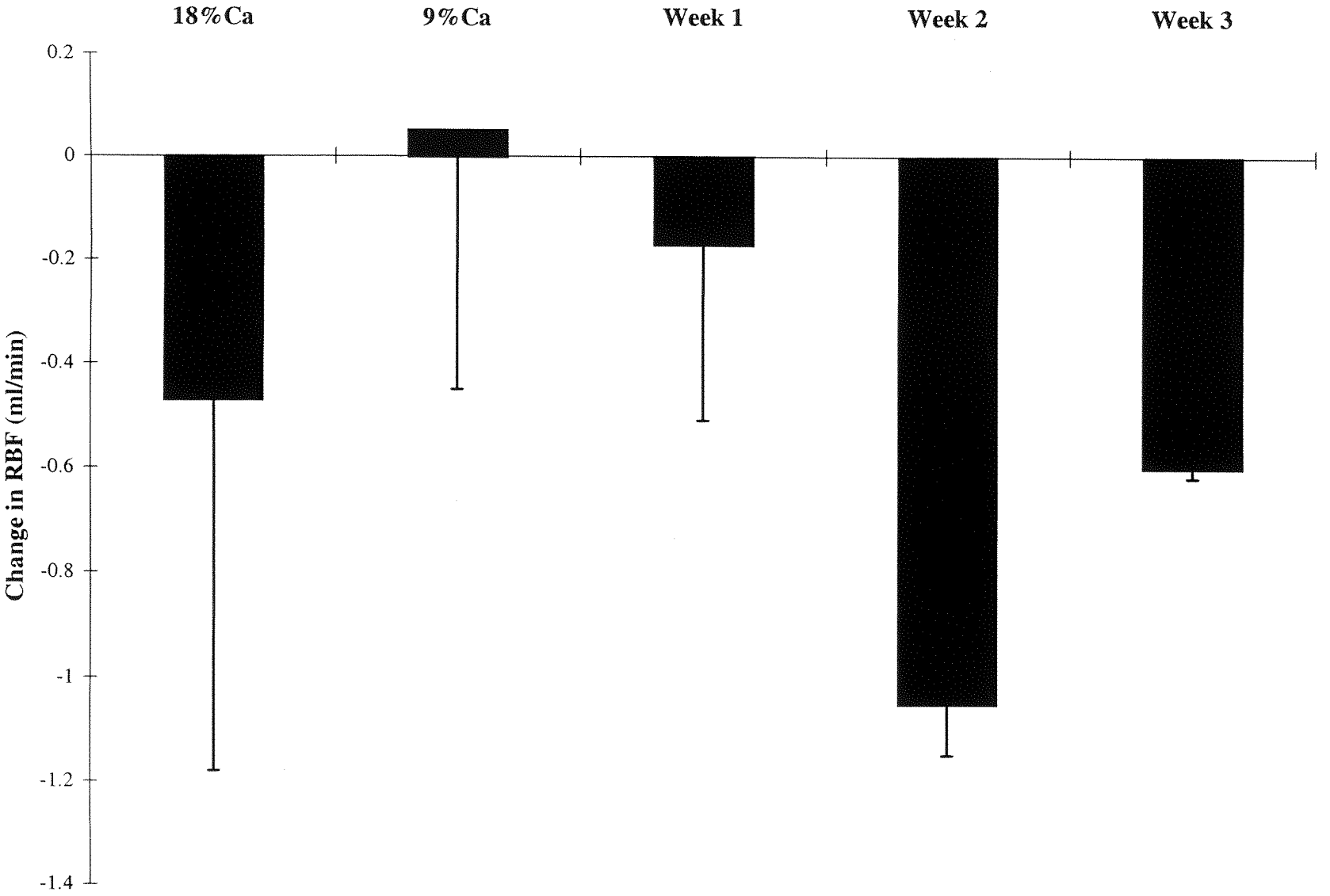

18%Ca
9%Ca
Week 1
Week 2
Week 3
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
-1.4
Change in RBF (ml/min)

Figure 4.4: The change in glomerular filtration rate (GFR) in response to Furosemide administration.

Female Wistar rats were mated and fed either an 18% casein or a 9% casein diet (Materials and Methods 2.2) throughout pregnancy (18%Ca & 9%Ca respectively), or a 9% casein diet during the first (Week 1), second (Week 2) or third (Week 3) week of pregnancy, with the remaining weeks being supplied an 18% casein diet. At 19 weeks of age female offspring from each dietary group were anaesthetised and cannulated for the measurement of renal blood flow (RBF) and glomerular filtration rate (GFR). n = 6 for 9%Ca, Week 1, Week 2 and Week 3 groups, n = 7 for 18%Ca controls. Values are shown as the mean change in GFR after furosemide administration $\pm$ SEM. Two-way ANOVA showed no effect of maternal diet and administration of furosemide on GFR (P=0.477). Sample sizes in each group were; 18%Ca n=4, 9%Ca n=3, Week 1 n=6, Week 2 n=5, Week 3 n=5.

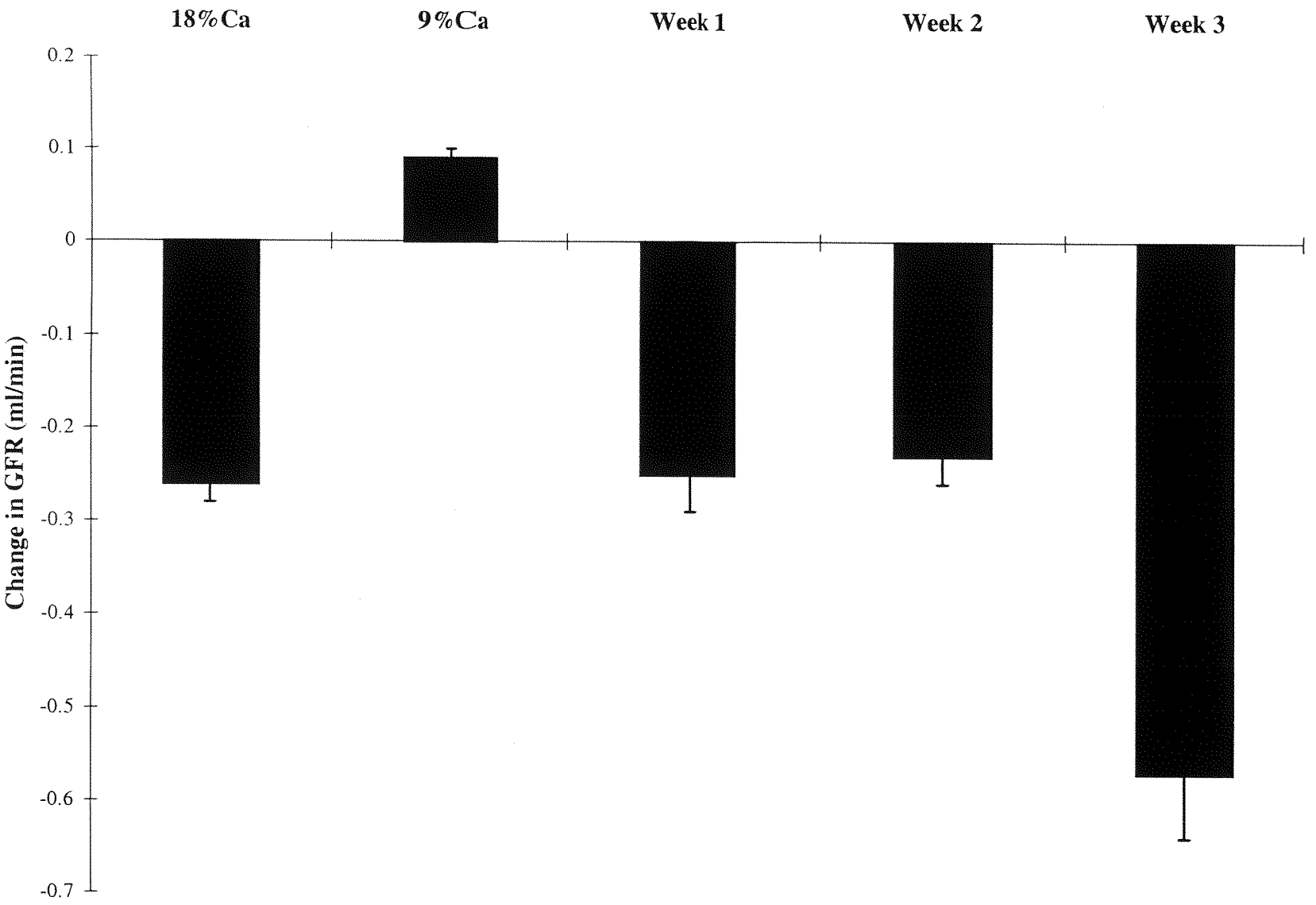

18%Ca
9%Ca
Week 1
Week 2
Week 3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
Change in GFR (ml/min)

Table 4.11: Urine production before and after the administration of Furosemide.

	n	Urine prod. before F (µl/min)	Urine prod. after F (µl/min)	Change in urine prod. (%increase)
18% Ca	7	14.62±2.40	206.19±22.94*	1405±147
9% Ca	6	21.86±4.15	225.10±11.96*	1120±187
Week 1	6	15.18±2.86	179.24±25.46*	1359±433
Week 2	6	24.82±6.57	136.98±30.59*	595±109
Week 3	6	23.17±4.72	167.07±20.65*	749±144

Female Wistar rats were mated and fed either an 18% casein or a 9% casein diet (Materials and Methods 2.2) throughout pregnancy (18%Ca & 9%Ca respectively), or a 9% casein diet during the first (Week 1), second (Week 2) or third (Week 3) week of pregnancy, with the remaining weeks being supplied an 18% casein diet. At 19 weeks of age female offspring from each dietary group were anaesthetised and cannulated for the measurement of renal blood flow (RBF) and glomerular filtration rate (GFR). Urine production (Urine prod.) before and after administration of furosemide (F) is expressed as mean ± SEM. * represents a significant difference ($P<0.05$) compared to pre-furosemide levels. Two-way ANOVA showed no effect of maternal diet and administration of furosemide on urine production ($P=0.633$). An effect of furosemide alone was highlighted with a one-way ANOVA ($P<0.001$). One-way ANOVA both before ($P=0.205$) and after ($P=0.479$) the administration of furosemide showed no effect of maternal diet on urine production.

4.4 Summary of Results.

4.4.1 Growth parameters.

	9% CASEIN			
	Throughout pregnancy	**Week 1**	**Week 2**	**Week 3**
Birthweight	↔	↔	↔	↔
Glom. D20	↔	↔	↔	↑
Glom. Term	↔	↔	↓	↓
Male BW 4 weeks	↔	↑	↑	↑
Female BW 4 weeks	↔	↔	↔	↔
Male kidney weight / body weight	↔	↓	↓	↓
Female kidney weight / body weight	↔	↓	↔	↔
Female BW 19 weeks	↔	↔	↔	↔
Female kidney weight / body weight 19 wks.	↓	↔	↔	↔

The table shows significant differences ($P<0.05$) compared to 18%Ca animals for parameters of offspring growth. ↑ = a significant increase relative to controls. ↓ = a significant decrease relative to controls. ↔ = equivalent to control values. BW = Body weight. Glom. = Glomerular complement

4.4.2 *Renovascular parameters.*

	9% CASEIN			
	Throughout pregnancy	**Week 1**	**Week 2**	**Week 3**
Systolic blood pressure (male)	↑	↑	↑	↑
Systolic blood pressure (female)	↑	↑	↑	↑
GFR 19 wks	↔	↔	↔	↔
RBF 19 wks F	↔	↔	↔	↔
FF 19 wks	↔	↔	↔	↔
GFR 19 post F	↑	↔	↔	↓
RBF 19 post F	↔	↔	↔	↔
FF post F	↔	↔	↔	↔

The table shows significant differences ($P<0.05$) compared to 18%Ca animals for renovascular parameters. ↑ = a significant increase relative to controls. ↓ = a significant decrease relative to controls. ↔ = equivalent to control values. GFR - Glomerular filtration rate. RBF - Renal blood flow. FF - Filtration fraction. F - Furosemide.

4.5 *Discussion.*

The duration of rat gestation is 21 - 22 days. In this time, the fetus largely completes organogenesis and reaches a point where it is able to survive on orally acquired nutrition and is able to regulate its body fluids. The first 7 days of gestation result in an implanted embryo. Initiation of organogenesis occurs in the following 7 days and in the final week of gestation, organogenesis continues concomitantly with tissue maturation. Some continued development of organs (hyperplasia) occurs for several days postnatally, such as in the kidney, but by around postnatal day 10, organogenesis is complete and growth is exclusively due to tissue hypertrophy. In the rat, the average litter size is approximately 12 - 14 offspring. This represents a significant increase in nutritional demand on the mother both towards the end of gestation and throughout the suckling period. For offspring of body weight on average 4g - 5g at birth, the combined conceptus weight is in the region of 20% of the mother's weight. In the human, the offspring weight at birth represents approximately 5% of the maternal body weight.

This study was primarily performed to address two questions. First, is the hypertensive effect of a maternal low protein diet exerted during a specific period of gestation? Secondly, does the apparent nephron deficit of low protein exposed animals (data shown here and in Chapter 3) impair the renal function of these animals? In attempting to answer these questions, a number of other features of offspring exposed to a low protein diet during gestation were also examined.

As in chapter 3, the birthweights of offspring were apparently unaffected by the maternal dietary regimen, although males from the 18%Ca, 9%Ca and Week 2 groups were heavier at birth than their female littermates. The Barker hypothesis (Barker 1995) asserts that fetal growth retardation evidenced by either small babies, or babies with a relatively high or low ponderal index (weight/length^3), are more prone to elevated blood pressure in adult life (Law *et al.* 1991). Such growth retardation is suggested to be a result of maternal undernutrition during different stages of pregnancy (Godfrey *et al.* 1996; Campbell *et al.* 1996; Prentice *et al.* 1981; Prentice *et al.* 1987). Data from this and previous studies of birthweight in this model suggest that the birthweight in rats may not provide a particularly good marker for fetal growth impairment, consequent to

a mild maternal protein restriction. Rat pregnancy lasts only three weeks, and during only two weeks is the fetus implanted. Also, the rat has a litter of, on average, around 12 offspring and the offspring are born with a degree of hyperplasic development still to be achieved (i.e. the kidney). This is not the case in humans, and the fact that gestation is so much longer in the human indicates that a comparison of the achievement of a certain degree of growth of rats and humans at birth should be treated with caution. Disturbances of intrauterine growth in the rat have been shown in this model (Langley-Evans *et al.* 1996c; Langley-Evans & Nwangwu 1998). Langley-Evans *et al.* (1996c) showed that from day 14 to day 20 of gestation, the weight of 9% casein exposed offspring was greater than controls, but at term, weight was lower than in control animals. This suggested a severe growth retardation at the end of gestation, when demands on nutrients would be greatest. Interestingly, the 9% casein exposed animals at day 20 of gestation were long and thin compared to controls, whilst at term, they had become shorter and wider relative to control animals. Langley Evans and Nwangwu (1998) confirmed these findings at day 14 of gestation. There is evidence, therefore, for intrauterine growth retardation in rats exposed to a mild protein restriction during gestation, although terminal outcome measures such as birthweight are not good markers of this. However, as discussed in chapter 3, maternal dietary protein restriction during pregnancy may result in an elevated offspring blood pressure without necessarily impeding overall fetal growth. This highlights the necessity of studying the underlying mechanisms of the fetal origins of adult hypertension using animal models and not relying exclusively on epidemiological evidence based around birth anthropometry.

Values obtained for glomerular complement at day 20 of gestation compare favourably with the literature (Zeman 1968; Merlet-Bernichou *et al.* 1994). Comparisons of glomerular complement at day 20 of gestation and at full-term indicated a significant influence of maternal nutritional status. At day 20, offspring of 18% casein fed females appeared to have the lowest number of glomeruli whilst those of animals supplied a 9% casein diet during the final week of pregnancy (Week 3) had by far the highest glomerular complement (4 times higher than controls). At full-term, this situation had changed such that control animals and those exposed to a maternal 9% casein diet during the first week of gestation (Week 1) had significantly more glomeruli than those of the Week 2 and Week 3 groups. By far the most interesting observation, however,

was the change in glomerular complement from day 20 to term. This effectively showed the rate at which glomeruli were acquired in late gestation, and therefore the rate of nephrogenesis, between these two time points.

Most groups, with the exception of the Week 3 animals underwent a significant acquisition of glomeruli between day 20 and term. Control animals more than trebled their glomerular complement and that of the Week 1 animals quadrupled. However, the glomerular complement of 9% casein exposed animals only doubled whilst that of the Week 2 animals increased by only a third. These data paralleled changes in growth of fetuses exposed to a maternal low protein diet as presented in Chapter 3 and that discussed elsewhere (Langley-Evans & Nwangwu 1998). These studies established that fetal growth rate over the last two days of gestation was markedly attenuated by exposure to a 9% casein diet.

The fact that even at full-term the absolute numbers of glomeruli in the 9% casein exposed and control animals were comparable, may be of importance because of the types of nephron that they represent. There are two types of nephron in the kidney; the juxtamedullary nephrons, which are situated at the edge of and within the medulla, and the cortical nephrons, exclusively situated within the cortex. The cortical nephrons comprise around 70% of the total nephron complement of the kidney, with the remainder being juxtamedullary nephrons (Lote 1994a). The juxtamedullary nephrons are characterised by possession of long loops of Henle and they primarily fulfil the function of creating a hypertonic medulla by the resorption of sodium, potassium and chloride along the loop of Henle. The resorbtive actions of the loop enable the concentration of sodium within the tubular fluid and adjacent interstitial fluid, to increase as it moves down the loop, until it reaches a maximal concentration at the turn of the loop (Lote 1994b). This concentrating mechanism provides the means for efficient water resorption from urine in the collecting duct. For example, if it is necessary to retain water in the body, then water channels are inserted into the walls of the principle cells of the collecting ducts under the action of arginine vasopressin. Water moves from the urine into the medulla by diffusion and is resorbed within the body due to the large concentration gradient between the lumen of the collecting duct and the medulla, induced by the concentrating activity of the loop of Henle.

The cortical nephrons provide the primary filtration capacity of the kidney by virtue of their large number (Lote 1994c).

During fetal development, the juxtamedullary nephrons are the first to appear (Abrahamson 1991) with cortical nephrons developing later. In this study we have shown that early nephrogenesis is not greatly impaired, but the rate of nephrogenesis declines towards term and is impaired for a period postnatally. This would imply that the juxtamedullary nephrons are relatively unaffected by a maternal protein restriction, whilst the main impairment of nephrogenesis affects the development of cortical nephrons. This in turn implies that in the adult kidney of offspring exposed to a maternal 9% casein diet, the proportion of juxtamedullary nephrons is higher than in control animals. This may be of importance when considering the hypothesis of Brenner and colleagues (Brenner *et al.* 1988) which suggests that nephron complement may be inversely related to blood pressure. It is possible that a greater proportion of juxtamedullary nephrons enhances the sodium, and perhaps the water, resorbtive ability of the kidneys such that, according to the principle of pressure natriuresis (Guyton *et al.* 1972), blood pressure is elevated in order to maintain sodium balance. It is also interesting to note that in ageing populations, those demonstrating an elevation of blood pressure with age, also show a significantly greater degree of glomerulosclerosis than non-hypertensive individuals (Kasiske 1987). The majority of glomeruli in which sclerosis occurs in these people are those serving cortical nephrons, with very few sclerotic juxtamedullary glomeruli present. Further, it has been suggested that there is an increase in the filtration of, primarily, the cortical glomeruli (Reckeloff *et al.* 1990) and a decrease in the renal plasma flow of this population of glomeruli with ageing (Hollenberg *et al.* 1974).

Nephrogenesis between day 20 and full term was shown to vary considerably as a result of exposure to a maternal 9% casein diet during discrete weeks of gestation. The glomerular complement of animals exposed to a maternal low protein diet during the first week of gestation (Week 1) appeared to parallel that of control fetuses fairly closely. The rate of nephrogenesis between day 20 and full term was severely impaired by exposure to a low protein diet in week 2 of gestation. At day 20, kidneys of these fetuses contained more glomeruli than those of control animals suggesting an enhanced

growth similar to that seen in terms of body weight (Langley-Evans & Nwangwu 1998). This rate of growth apparently could not be maintained, despite the fact that the mothers had been transferred back to the control diet for the final week of gestation. This implies that either growth retardation or a programming stimulus had occurred either at the time of the nutritional insult or shortly afterwards. The second week of gestation in the rat sees the appearance of the pronephros, which precedes the development of the mesonephros and finally the true kidney, the metanephros. It is perhaps possible that such a nutritional insult at a critical time might impinge on the development of the pronephros and result in a downstream effect on the metanephros.

In the Week 3 group the number of glomeruli appeared to decline from day 20 to term. This may suggest either, that nephrons are lost between these time points, perhaps via apoptosis, or the data may merely show an apparent nephron loss as a consequence of variability within the method used to determine glomerular complement. It is also the case that the sample size of the Week 3 animals at day 20 is quite low compared with those of other groups. Despite these problems, it seems fair to assume that the rate of nephrogenesis in the Week 3 group was severely impaired by the imposition of a maternal 9% casein diet. Previous observations of fetal growth (Chapter 3; Langley-Evans *et al.* 1996b; Langley-Evans *et al.* 1996c; Langley-Evans *et al.* 1996d; Langley-Evans & Nwangwu 1998) suggests that a maternal low protein diet might initially enhance fetal growth and then induce a late gestation growth retardation. In keeping with this assertion, the data in this study, showing a degree of late gestation impairment of nephrogenesis, supports the view that fetuses exposed to a maternal protein restriction in early to mid-gestation may accommodate to the nutritional insult. Such an insult imposed in the final week, however, may come too late for such an accommodation to occur and thus result in a more severe impairment of nephrogenesis. Exposure to a maternal 9% casein diet during the final week of gestation only, resulted in offspring with the highest blood pressures of all the offspring exposed to a maternal low protein diet during specific weeks. Unfortunately, data on the glomerular complement of such offspring at 4 weeks of age are not yet available, and so conclusions as to the long-term effect of a maternal protein restriction during individual weeks of gestation on the nephron makeup of these animals may not be drawn. It is clear however from the present data that such a nutritional insult has significant effects

on renal development up to term. It is important to point out that, although animals exposed to a maternal low protein diet throughout gestation, possessed roughly similar numbers of nephrons to control animals at both days 20 and 22 of gestation, the reduced rate of nephron acquisition between these time points suggests that the reason for the possession of fewer nephrons than controls at 4 weeks of age (Chapter 3) is likely due to a late gestation (and perhaps early postnatal) restriction of nephrogenesis.

The protein and DNA content of kidneys from offspring of the different dietary groups were not influenced by maternal diet at either time point. It may be concluded, therefore, that the kidneys of all groups have approximately the same number of cells and that the cells are all of similar size. Thus, differences in the glomerular complement suggest that there may be differences in the proportions of cell types within the kidneys of animals exposed to a maternal 9% casein diet during the second and third weeks of gestation and those exposed throughout gestation.

Histochemical analysis of alkaline phosphatase (ALP) activity has shown that, pre-natally, as the fetus matures, its activity covers an increasing area of the kidney (Zeman 1968). ALP is expressed along the brush border of the proximal convoluted tubule (Wachstein 1955) and has been suggested to indicate the differentiation of the proximal convoluted tubule (Desalu 1966). Thus, determination of ALP activity in kidneys of day 20 and full term animals was used as an indicator of renal maturity. The ALP activity increased greatly in all groups from day 20 to term when expressed as a proportion of tissue weight, highlighting the maturation of nephrons at this time. At day 20, kidneys of Week 1 and Week 2 groups demonstrated a high level of ALP activity compared with other groups. This may reflect an enhanced rate of renal maturation in these animals. At full term, only kidneys of the Week 3 group showed any effect of maternal diet, in that they demonstrated a depressed ALP activity compared with controls. These data further point to the suggestion made earlier, that the fetus may accommodate to the effects of a maternal dietary protein restriction. A maternal 9% casein diet represents a relatively mild nutritional insult. Zeman showed that a maternal 6% casein diet resulted in offspring whose kidneys demonstrated little or no ALP activity implying a severe retardation of maturation (Zeman 1968). It may be the case here that exposure to a mild maternal protein restriction is effective in depressing ALP activity if it occurs in late

gestation, and that earlier exposure allows the fetus to accommodate without impairment of morphological development. This may explain why offspring exposed to a 9% casein diet throughout gestation demonstrated no dietary induced effect on ALP activity. These data parallel those found for glomerular complement and rate of nephrogenesis. An interesting observation was found when mean ALP activity was corrected for mean glomerular number. Offspring of the Week 1 group showed a very high ALP activity when corrected for glomerular number relative to all other groups at day 20 of gestation. Although this may not be tested statistically, it may suggest that there is a greater proportion of mature nephrons in the kidneys of the Week 1 animals at day 20 compared with other groups. At day 22 the values for ALP activity corrected for glomerular number demonstrated no notable differences.

At 4 weeks of age, offspring exposed to a maternal 9% casein diet *in utero* were heavier than controls. This was most noticeable in animals exposed to a maternal protein restriction during the first or second week of pregnancy. The thrifty phenotype hypothesis (Hales & Barker 1992), suggests that offspring exposed to a nutritional deprivation during gestation, may adapt in some way to enable their survival after birth. Consequences of this may include non-insulin dependent diabetes and elevated blood pressure. Examining these data in view of this hypothesis, it may be the case that a maternal low protein diet is "detected" by the fetus which then adapts to enable more rapid postnatal growth, whilst conditions are favourable. However, male offspring of females supplied a 9% casein diet throughout pregnancy did not differ significantly in body weight to controls at 4 weeks of age, which may suggest that either exposure throughout gestation, prevents this adaptation, or exposure during different phases of gestation have opposing effects.

Comparison of organ weights at 4 weeks of age showed that only the kidney was sensitive to exposure to a maternal low protein diet. Other workers (Zeman *et al.* 1968; Merlet-Bernichou *et al.* 1994) have also shown a significant reduction of kidney size and glomerular complement in offspring of female rats supplied a low protein diet during pregnancy. These studies imposed a severe protein restriction (4% - 6% casein). In the present study, the nutritional insult was relatively mild (9% casein) and yet an effect on kidney weight was still observed. Nephrogenesis occurs within the third week

of gestation and for around 10 days postnatally (Tufro-McReddie *et al.* 1995). The nutrient requirements of the fetus are greatest in the final week of gestation and a protein restriction at this time might well impair nutrient availability to the fetal kidney. Exposure to a maternal 9% casein diet during the first and second weeks of pregnancy also impaired renal growth (in terms of kidney weight corrected for body weight at 4 weeks of age), however, this did not persist into adult life (19 weeks of age) in contrast to those exposed to a 9% casein diet throughout gestation. These data suggest a late gestation restriction of nutrients being associated with the apparent renal growth retardation. Human studies (Konje *et al.* 1997; Konje *et al.* 1996; Hinchliffe *et al.* 1992) have shown that growth retarded fetuses show considerable disturbances of renal growth and glomerular complement. Thus the kidney appears to be extremely sensitive to nutrient restriction during gestation.

The blood pressures of all animals exposed to a maternal 9% casein diet at any time during gestation were elevated above those of control animals. It was noteworthy that those offspring from dams supplied a 9% casein diet throughout pregnancy had by far the highest blood pressures of all groups. The second highest blood pressures were seen in the Week 3 animals and this may reflect a possible renal sensitivity to a maternal protein restriction. However, it is interesting to note that animals exposed to a maternal 9% casein diet during the first and second weeks of gestation also had blood pressures elevated above controls. The data imply that the period of gestation most sensitive to the hypertensive effects of a maternal 9% casein diet is the final week and that additional exposure prior to this time may act synergistically to further elevate blood pressure. This was addressed by Langley-Evans (1998). This study compared the effect of exposure to a maternal 9% casein diet during either the first two weeks only, or the last two weeks only of gestation. It was found that, whilst the blood pressures of all offspring exposed to a maternal low protein diet were elevated above controls, the greatest effect was seen in those animals exposed to a maternal dietary protein restriction during the first two weeks of gestation only. This suggests that early gestational exposure to a maternal dietary insult may be of critical importance in the programming of metabolism.

When the blood pressure data in this chapter are considered alongside those for nephrogenesis between day 20 and full term, it can be seen that, among groups exposed to a maternal 9% casein diet for a single week of gestation, the blood pressure increased with a declining rate of nephrogenesis. The increase in blood pressure of the Week 1 animals occurred despite a rate of nephrogenesis comparable with controls, suggesting that an alternative mechanism other than simply a reduced nephron complement may play a role in the elevation of blood pressure in low protein exposed offspring. In the second week of gestation, the renal progenitor cells are forming and a nutritional insult at this time might affect the development of this cell mass. In the first week, the embryo just reaches implantation. Fleming and co-workers (unpublished) have shown that in the 4 day embryo the proportion of cells in the inner cell mass (embryogenic tissue) compared to that of the outer cell mass (placentogenic tissue) is lower in 9% casein exposed embryos than controls. The implications of this are uncertain, however these data suggest that the rate of subsequent development is potentially impaired and that accommodation may be being made at this early stage to enable greater sequestration of substrates from the mother as development advances.

Renal growth retardation in this model may, therefore, be associated with elevated blood pressure. Guyton and co-workers (Guyton et al. 1972) proposed that blood pressure is elevated in an organism in order to enable efficient sodium balance. Brenner (Mackenzie & Brenner 1995) has suggested that a reduction in the glomerular complement of the kidneys results in elevated blood pressure because these kidneys are less able to excrete sodium and thus require a higher blood pressure to do so. Indeed, other rat models of hypertension indicate that kidneys of rats with elevated blood pressure tend to have fewer glomeruli than controls, whilst a hypertension resistant mouse strain possesses a greater nephron complement (Brenner *et al.* 1988). Human studies have shown that individuals from populations resistant to hypertension possess proportionately larger kidneys than members of hypertension prone populations. Taken together with the present data, these data suggest that a maternal protein restriction might impair nephrogenesis to an extent where the kidneys are unable to excrete sodium effectively and the animal's blood pressure rises in order to maintain sodium balance. Clearly the sodium handling capabilities of low protein exposed animals requires further investigation (see Chapters 5 and 6).

Measurements of renal haemodynamics were technically difficult. The values for GFR compare well with published values (Tenstad & Williams 1995; Anderson *et al.* 1994; Walker *et al.* 1983), as do those for RBF, although these are at the low end of previously derived values (Walker *et al.* 1983; Barthelmebs *et al.* 1994). This may explain the relatively high values obtained for the FF. Since the values for GFR are comparable to published values and they remained stable prior to the administration of furosemide, the reason for the low estimate of RBF may well be due to dissociation of the label. The ^{3}H-PAH label had a limited useful life span (approximately 3 months at -20°C). Examination of the label once the experiment had been completed using thin layer chromatography (TLC) showed that a proportion (between 13% and 33%) of the label had become dissociated. Administration of a label in which an unknown proportion had become dissociated would inevitably lead to an inaccurate estimate of RBF.

GFR was similar in all study groups before the administration of furosemide. Clearly the GFR is maintained in order to keep renal function within certain limits. As the elevation in blood pressure in offspring of 9% casein fed females has been shown to be maintained into adult life (Langley-Evans *et al.* 1996a), then it would appear that a higher blood pressure is required to achieve the same GFR as controls. The RBF was unaffected by manipulation of the maternal diet. These data reflect those found by Iglesias-Barreira and colleagues (1996) who employed the infusion of microspheres to measure RBF. Thus, prior to the administration of furosemide, renal haemodynamics of all low protein exposed animals were similar to controls. In Chapter 3 it was shown that animals exposed to a maternal 9% casein diet throughout gestation possessed fewer glomeruli at 4 weeks of age than control animals. Thus, it may be concluded that renal function is maintained in 9%Ca animals despite possessing fewer functional units.

The administration of furosemide almost instantaneously induced a marked diuresis in all animals. Furosemide is a diuretic that acts at the loop of Henle blocking the Na^{+}:K^{+}:Cl^{2-} transporter. Its chemical name is 4-Chloro-N-furfuryl-5-sulphamoylanthranilic acid ($C_{12}H_{11}ClN_2O_5S$), with a molecular weight of 330.7 and is almost insoluble in water, but is soluble in ethanol and other organic solvents. Other than its inhibition of the Na^{+}:K^{+}:Cl^{2-} transporter, furosemide also has a variety of

systemic effects. Under conditions of high dosage or prolonged exposure to furosemide, hyponatraemia, hypokalaemia and/or hypochloraemic alkalosis may result (Naranjo *et al.* 1978; Lowe *et al.* 1979). Intravenous infusion has been shown to transiently increase heart rate, arterial blood pressure, and systemic vascular resistance in patients with chronic congestive heart failure (Francis *et al.* 1985). Animal studies have shown furosemide to both increase renal vascular resistance (Tenstad *et al.* 1995) and decrease renal vascular resistance (Barthelmebs et al. 1994; Barthelmebs *et al.* 1995) under different experimental conditions. Additionally, furosemide has been shown to induce deafness (Schwartz *et al.* 1970) and renal calcium excretion in patients with hypoparathyroidism (Gabow *et al.* 1977).

The diuretic effect of furosemide was clear from the substantial increase in urine production seen after administration. Prior to furosemide treatment, GFR was unrelated to urine production (P=0.441) whilst afterwards, there was a significant positive relationship between GFR and urine production (P=0.041). It would be interesting to examine the mode of accommodation of these animals to a more long-term treatment with furosemide. This may provide a clue as to the nature of the mechanisms employed by animals exposed to a maternal protein restriction by which fluid homeostasis is maintained. Thus, although structurally altered, the basic renal haemodynamics and response to diuresis is maintained in the hypertensive, low protein exposed rats.

From this study, a number of conclusions may be drawn. Prenatal renal development and postnatal renal growth are sensitive to maternal dietary protein restriction during pregnancy and appears to be unique among the major organs in this respect. Offspring blood pressure, similarly, is sensitive to a maternal low protein diet and is elevated in consequence to such a maternal diet imposed during any discrete week of gestation. This elevation of blood pressure may be an accommodation by the offspring to ensure that normal renal function is maintained. Renal function may be examined in other ways to those used in this study. The kidneys control body fluid homeostasis, and therefore, examination of the plasma volume and body water relationships may highlight areas of renal inefficiency. Also, examination of the effects of salt loading on blood pressure and renal haemodynamics would be useful in obtaining a wider view of the renal function of these animals.

Chapter 5.

Plasma volume and body water relationships in offspring of rats supplied a low protein diet during pregnancy.

5.1 Introduction.

Previous chapters have demonstrated that the morphometric development of the kidney is impaired by a maternal low protein diet, whilst renal haemodynamic functions apparently are not. The hypothesis of Brenner and co-workers (Brenner *et al.* 1988) proposes that a renal nephron deficit may result in an elevation of blood pressure in order to maintain sodium homeostasis.

The observed elevation in blood pressure may merely be a consequence of an alteration in the plasma volume relationships of the animals. We may assume that both dietary groups have a comparable ability to reabsorb fluid from the nephron since renal function is apparently not compromised. In both control and experimental animals at equilibrium, the volume of fluid reabsorbed is equivalent to that filtered. If the cardiovascular capacity of low protein exposed animals is reduced, then the rate of fluid resorption may exceed the volume available to contain it. Alternatively, an increase in plasma volume due to impairment of renal fluid handling may represent a simple mechanism by which blood pressure is elevated in the rat model of programmed hypertension.

In the present chapter, total body water and plasma volume were determined in the offspring of rats fed control or low protein diets during pregnancy. To profile possible changes with time, rats were studied between 4 and 8 weeks of age.

5.2 Protocol.

Eight female Wistar rats were mated and fed on either a 9% casein or an 18% casein diet throughout pregnancy (n=4 per group). Offspring were weaned at 4 weeks of age. The offspring were then allocated to three groups according to age, 4 weeks, 6 weeks

and 8 weeks within each dietary group. Systolic blood pressure was measured using the tail cuff method at each age (Chapter 2, section 2.5). The animals were then weighed and plasma volume was measured using the Evans Blue method (Chapter 2, section 2.11). The animals were sacrificed by neck dislocation and carcasses were stored at -20°C prior to drying for body water analysis. Total body water was determined by desiccating at 80°C to a constant weight and determining the difference between the start weight and final weight.

One-, two- and three-way ANOVA analyses were carried out as appropriate and Tukeys and Students T-Test were applied as post-hoc tests.

5.3 Results.

The body weight, plasma volume and body water were determined in male offspring exposed to a maternal 18% casein diet at 4 (n=6), 6 (n=4) and 8 (n=7) weeks of age, female offspring exposed to a maternal 18% casein diet at 4 (n=5), 6 (n=6) and 8 (n=4) weeks of age, in male offspring exposed to a maternal 9% casein diet at 4 (n=6), 6 (n=6) and 8 (n=8) weeks of age and in female offspring exposed to a maternal 9% casein diet at 4 (n=7), 6 (n=5) and 8 (n=5) weeks of age.

Body weight increased significantly in all groups with advancing age ($P<0.001$). Maternal diet had no effect on offspring body weight ($P=0.341$). In both dietary groups, males were heavier than their female counterparts at 6 and 8 weeks of age ($P<0.05$; Figure 5.1). Plasma volume (PV), measured in animals at 4, 6 and 8 weeks of age (Figure 5.2), was not modulated by maternal diet in males or females. PV was also comparable between dietary groups at all ages when corrected for body weight (PVBW; Figure 5.3). Measurements of haematocrit were used to calculate a value for total blood volume. Similar to the results for plasma volume, the total blood volume (TBV; Figure 5.4) and the TBV corrected for body weight (Figure 5.5) was unaffected by the maternal diet in males and females at any age.

The total body water corrected for body weight (TBW) of all animals was relatively stable at approximately 70% of body weight over the ages studied (Table 5.1). Two

groups however, did exhibit a decline in percentage body water with advancing age. Although the decline was small, one-way ANOVA showed that it was highly significant for both the 18%Ca females (P=0.000) and the 9%Ca males (P=0.001). At 4 weeks of age, female rats exposed to a low protein had a significantly lower total body water content than control females (P<0.05), whilst male offspring of the low protein group had a significantly higher water content than control males (P<0.05; Table 5.1). By virtue of age related changes, these differences were no longer observed at 8 weeks of age.

As values of PV and total volume of body water (BH_2O; this differs from TBW in that it represents the total volume of water contained within a carcass and thus is not corrected for body weight) did not highlight any consistent differences between dietary groups, data from all animals were combined and plotted against body weight (Figure 5.6). Both measurements were shown to be strongly positively related to body weight (PV - P=0.000, r^2=0.948; BH_20 - P=0.000, r^2=0.995). The relationship between the two curves was examined and an expression for the change in plasma volume corrected for body water with increasing body weight was derived (Figure 5.7). This relationship essentially shows the change in the PV as a proportion of the BH_2O with increasing body weight. This manipulation was carried out in order to examine whether the relative proportions of body compartments varied with age, and if so, whether this might mask any differences that might be seen due to maternal diet. The raw data were also used to validate this expression by dividing PV by BH_20 and plotting against body weight (Figure 5.8). Both figures showed an initial rapid decline in the proportion of body water comprising plasma volume. This quickly levelled off at a body weight of around 110g. Subsequent to this point, the curve tended towards an asymptote at a value of PV/BH_20 of approximately 4.59% (i.e. where PV represents 4.59% of total body water) if the curve is extrapolated to an infinite body weight. Within the normal range of rat body weights, the curve tended towards a value of PV/BH_20 of just over 5%. The data (Figure 5.8) from all animals appeared to fit the mathematically derived curve well, as did those of 18% casein and 9% casein exposed offspring examined individually.

Blood pressure was measured at 4, 6 and 8 weeks of age and values obtained were examined in relation to the PV/BH_20 for each animal using regression analysis (Figures

5.9 & 5.10). In the 9%Ca animals, it was found that blood pressure increased with a decreasing PV/BH_20 ($P=0.008$, $r^2=0.331$; Figure 5.9). The 18%Ca animals also showed a negative trend of blood pressure against PV/BH_20 but this did not attain significance ($P=0.189$; Figure 5.10).

Figure 5.1: Body weights of offspring at 4, 6 and 8 weeks of age.

Data obtained for body weight are shown for 18% casein (18%Ca) and 9% casein (9%Ca) exposed males (M) and females (F). Values are expressed as means ± SEM. * represents a significant difference ($P<0.05$) compared to females within dietary groups.

	Body Weight (g).			
	18%Ca-M	**18%Ca-F**	**9%Ca-M**	**9%Ca-F**
4 Weeks	68±5	73±6	74±5	77±6
6 Weeks	145±31	128±12	152±31	120±15
8 Weeks	218±21	167±5	197±32	143±21

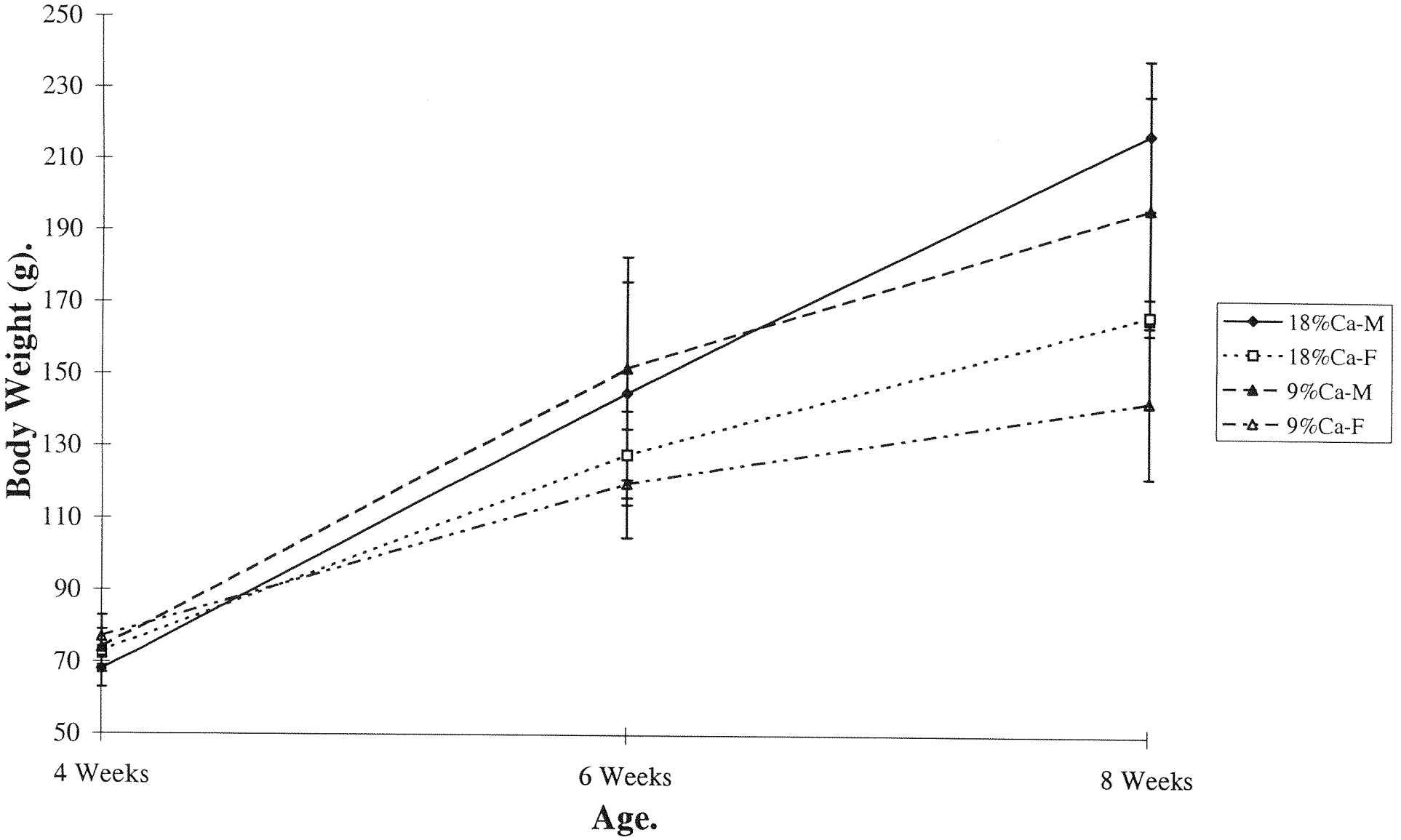

250
230
210
190
170
150
130
110
90
70
50
Body Weight (g).
4 Weeks
6 Weeks
8 Weeks
Age.
18%Ca-M
18%Ca-F
9%Ca-M
9%Ca-F

Figure 5.2: Plasma volume of offspring at 4, 6 and 8 weeks of age.

Data obtained for plasma volume are shown for 18% casein (18%Ca) and 9% casein (9%Ca) exposed males (M) and females (F). Values are expressed as means ± SEM.

	Plasma Volume (ml)			
	18%Ca-M	**18%Ca-F**	**9%Ca-M**	**9%Ca-F**
4 Weeks	3.35±0.4	3.39±0.3	3.49±0.4	3.67±0.3
6 Weeks	5.26±0.5	5.26±0.5	6.12±0.9	5.12±0.5
8 Weeks	8.2±0.9	6.53±0.4	7.38±1.9	5.91±0.4

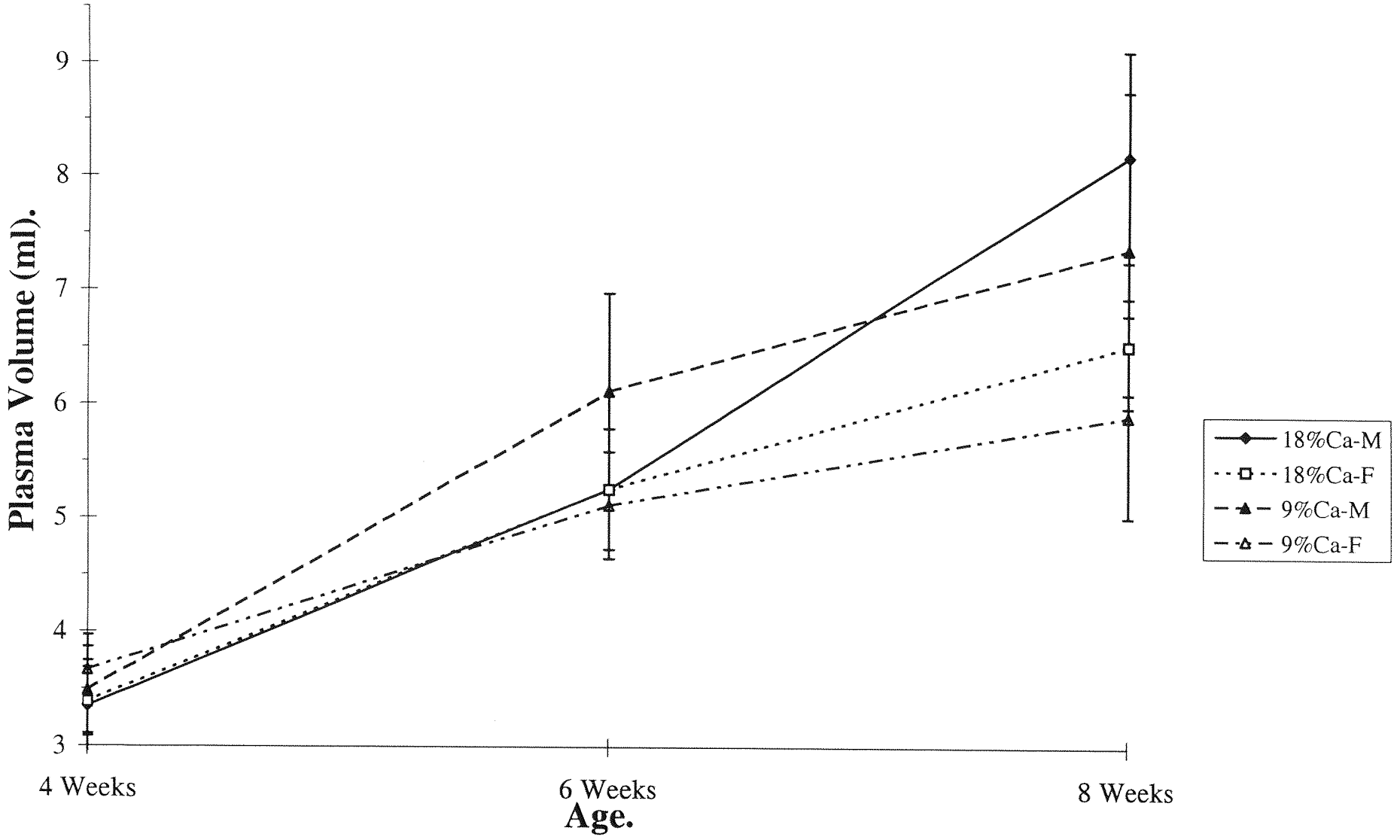

Plasma Volume (ml).
9
8
7
6
5
4
3
4 Weeks
6 Weeks
8 Weeks
Age.
18%Ca-M
18%Ca-F
9%Ca-M
9%Ca-F

Figure 5.3: Plasma volume corrected for body weight of offspring at 4, 6 and 8 weeks of age.

Data obtained for plasma volume corrected for body weight are shown for 18% casein (18%Ca) and 9% casein (9%Ca) exposed males (M) and females (F). Values are expressed as means ± SEM.

	Plasma Volume / Body Weight (%)			
	18%Ca-M	**18%Ca-F**	**9%Ca-M**	**9%Ca-F**
4 Weeks	4.91±0.4	4.68±0.4	4.72±0.7	4.8±0.3
6 Weeks	3.8±0.3	4.11±0.2	4.09±0.4	4.29±0.5
8 Weeks	3.74±0.2	3.91±0.2	3.74±0.4	4.15±0.2

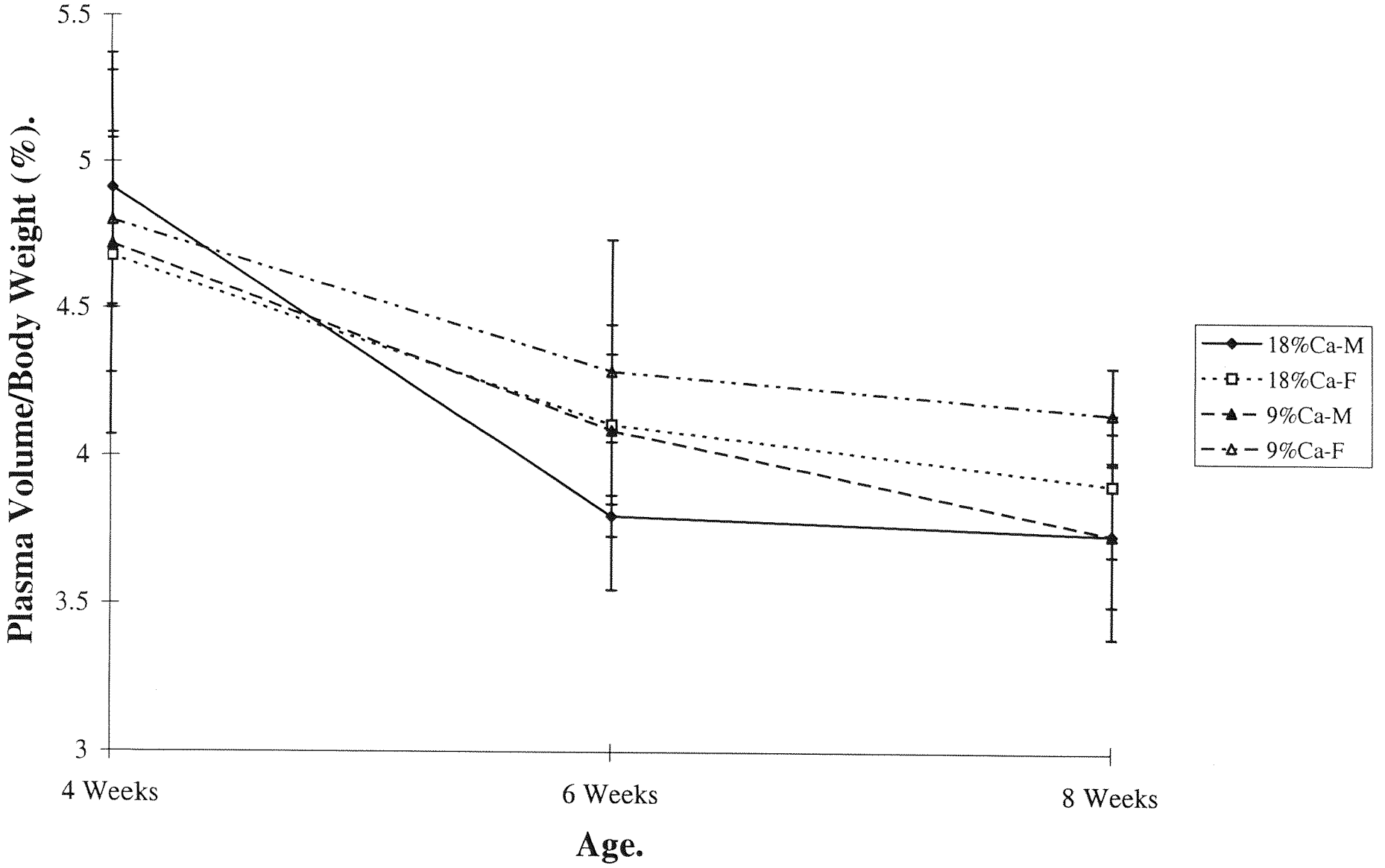

Plasma Volume/Body Weight (%).
5.5
5
4.5
4
3.5
3
4 Weeks
6 Weeks
8 Weeks
Age.
18%Ca-M
18%Ca-F
9%Ca-M
9%Ca-F

Figure 5.4: Total blood volume of offspring at 4, 6 and 8 weeks of age.

Data obtained for total blood volume are shown for 18% casein (18%Ca) and 9% casein (9%Ca) exposed males (M) and females (F). Values are expressed as means ± SEM.

	Total Blood Volume			
	18%Ca-M	**18%Ca-F**	**9%Ca-M**	**9%Ca-F**
4 Weeks	5.39±0.7	5.47±0.5	5.65±0.6	5.96±0.6
6 Weeks	9.2±1.5	8.76±0.8	10±1.3	8.43±0.3
8 Weeks	13.77±1.6	10.97±0.7	12.51±2.6	9.82±1.4

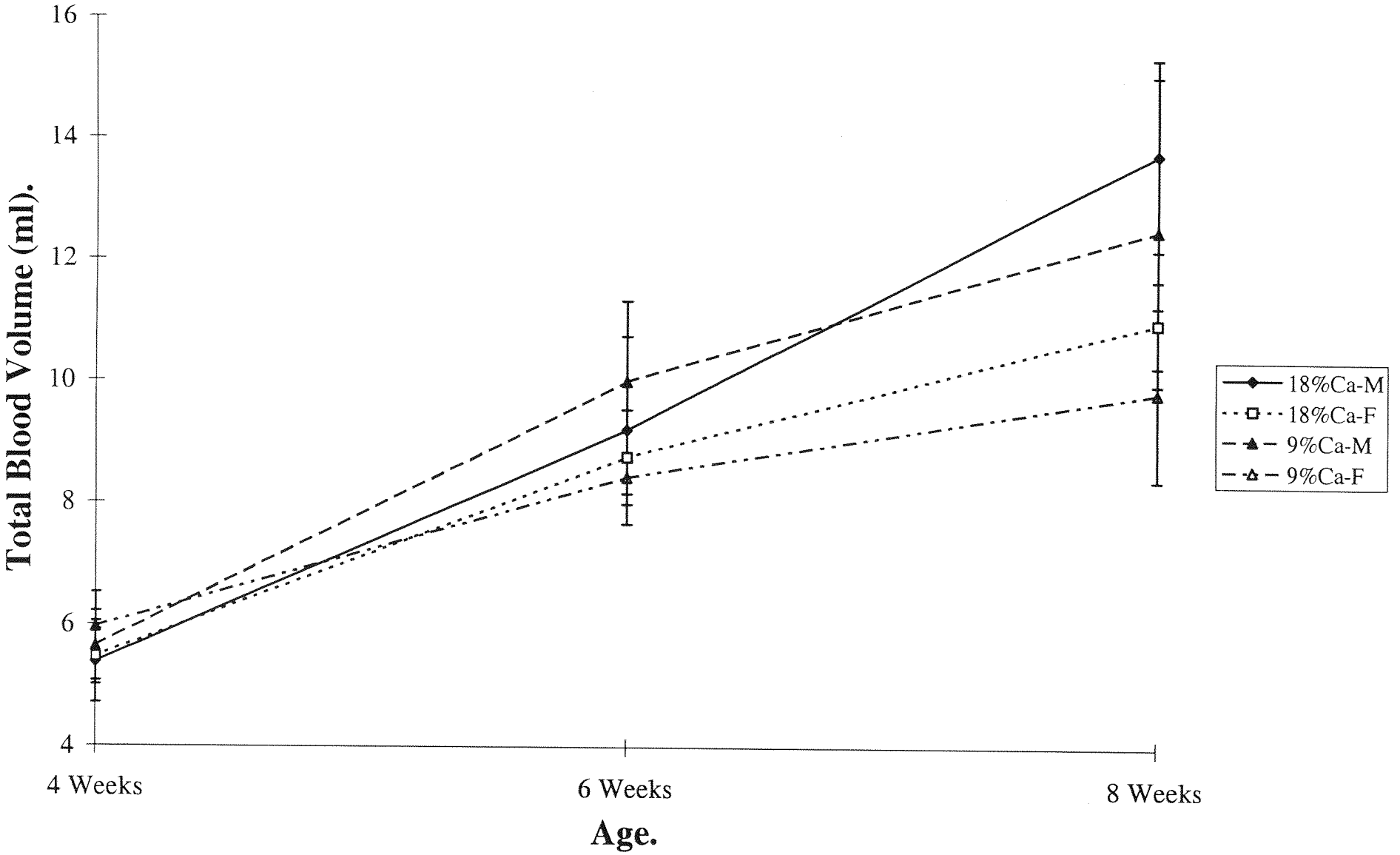

16
14
12
10
8
6
4
Total Blood Volume (ml).
4 Weeks
6 Weeks
8 Weeks
Age.
18%Ca-M
18%Ca-F
9%Ca-M
9%Ca-F

Figure 5.5: Total blood volume corrected for body weight of offspring at 4, 6 and 8 weeks of age.

Data obtained for total blood volume corrected for body weight are shown for 18% casein (18%Ca) and 9% casein (9%Ca) exposed males (M) and females (F). Values are expressed as means ± SEM.

	Total Blood Volume / Body Weight (%)			
	18%Ca-M	**18%Ca-F**	**9%Ca-M**	**9%Ca-F**
4 Weeks	7.91±0.8	7.56±0.9	7.66±1.0	7.78±0.5
6 Weeks	6.42±0.4	6.84±0.4	6.68±0.6	7.88±0.7
8 Weeks	6.32±0.4	6.57±0.5	6.31±0.6	6.89±0.3

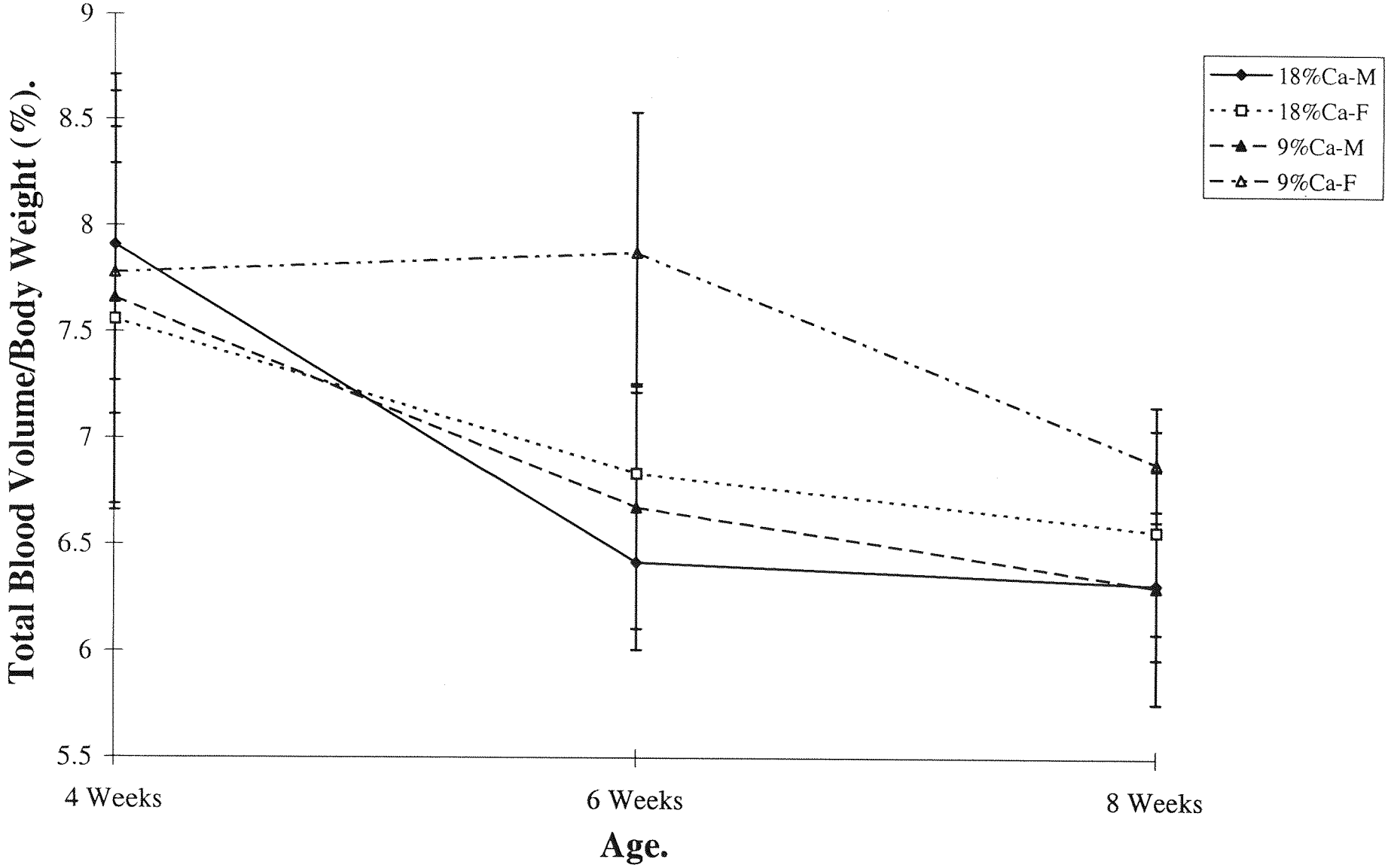

Total Blood Volume/Body Weight (%).
9
8.5
8
7.5
7
6.5
6
5.5
4 Weeks
6 Weeks
8 Weeks
Age.
18%Ca-M
18%Ca-F
9%Ca-M
9%Ca-F

Table 5.1: Total body water content of animals at 4, 6 and 8 weeks of age.

	Total Body Water (% of BW)					
		4 weeks		6 weeks		8 week
	n	Mean±SEM	n	Mean±SEM	n	Mean±SEM
18%Ca-M	6	72.34±1.90	4	71.73±1.52	7	71.31±2.06
18%Ca-F	5	74.69±1.13	6	72.02±1.26	4	69.04±1.24‡
9%Ca-M	6	74.82±1.89*	6	71.72±1.94	8	70.84±1.53‡
9%Ca-F	7	72.27±1.58†	5	72.14±2.44	5	71.48±1.62

Data obtained for total body water are shown for 18% casein (18%Ca) and 9% casein (9%Ca) exposed males (M) and females (F) at 4, 6 and 8 weeks of age. * represents a significant difference ($P<0.05$) relative to 18%Ca-M. † represents a significant difference ($P<0.05$) relative to 18%Ca-F. ‡ represents a significant difference ($P<0.05$) relative to the same group at 4 weeks of age. Three-way ANOVA indicated that total body water corrected for body weight was influenced by an interaction of age, sex and maternal diet ($P=0.001$). Offspring of 18% casein fed mothers demonstrated a sex dependent change in total body water corrected for body weight with advancing age ($P=0.011$).

Figure 5.6: Plasma volume (PV) and body water (BH_20) plotted against body weight.

Female Wistar rats were supplied either a 9% casein or an 18% casein diet throughout pregnancy. The offspring were used for determination of plasma volume and body water content at 4, 6 and 8 weeks of age. The figure shows the curves of plasma volume and gross body water content plotted as dependant variables of body weight. Data from all animals are included as there were no consistent differences in either variable between dietary groups or sexes with advancing age. Both PV and BH_20 showed a close positive relationship with body weight (PV - P=0.000, r^2=0.948; BH_20 - P=0.000, r^2=0.995).

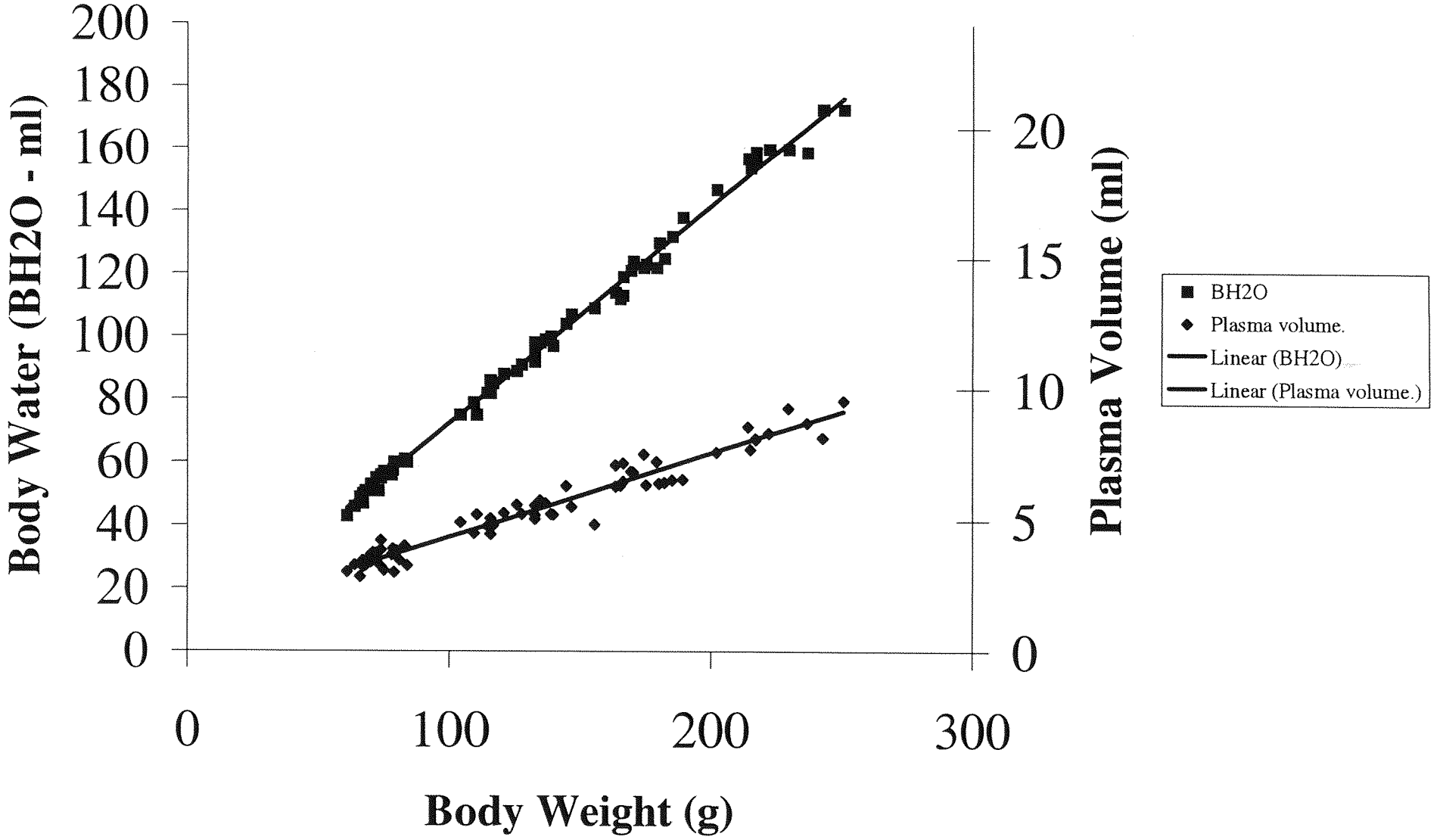

Body Water (BH2O - ml)
200
180
160
140
120
100
80
60
40
20
0
Plasma Volume (ml)
20
15
10
5
0
0
100
200
300
Body Weight (g)
BH2O
Plasma volume.
Linear (BH2O)
Linear (Plasma volume.)

Figure 5.7: Mathematically derived plot for the change in plasma volume as a proportion of body water with increasing body weight.

Female Wistar rats were supplied either a 9% casein or an 18% casein diet throughout pregnancy. The offspring were used for determination of plasma volume and body water content at 4, 6 and 8 weeks of age. From the equations of PV versus body weight and BH_20 versus body weight, an expression was derived for the change in PV/BH_20 with increasing body weight. A lower limit of 60g body weight is shown because this is the lowest value for animals, at 4 weeks of age, in this study.

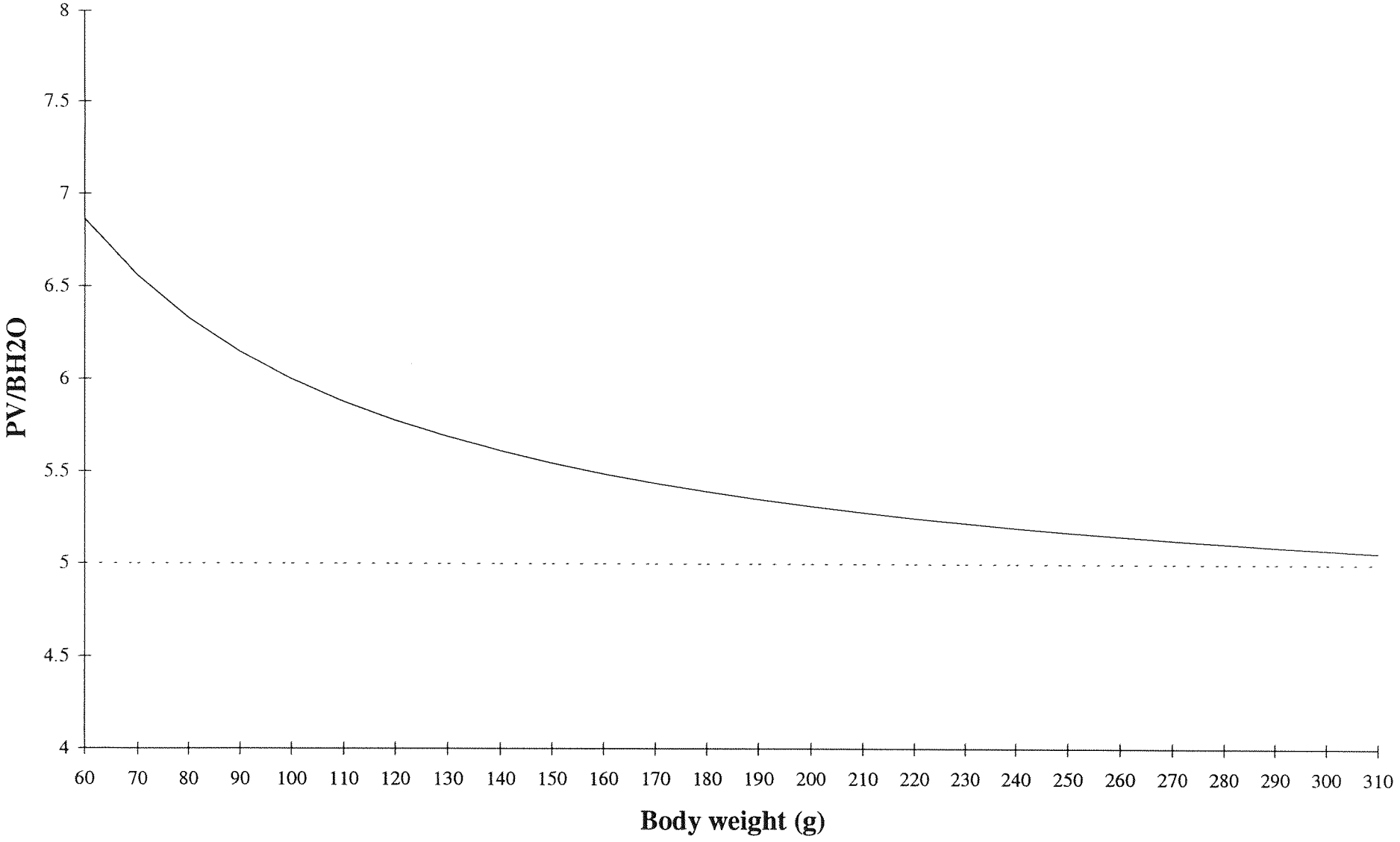

8
7.5
7
6.5
6
5.5
5
4.5
4
PV/BH2O
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220
230
240
250
260
270
280
290
300
310
Body weight (g)

Figure 5.8: Values for PV/BH_20 obtained from study data plotted against body weight.

Female Wistar rats were supplied either a 9% casein or an 18% casein diet throughout pregnancy. The offspring were used for determination of plasma volume and body water content at 4, 6 and 8 weeks of age. The figure shows values obtained for the expression PV/BH_20 from study data. The curve shown is a quadratic line of best fit obtained from the SPSS "curve fit" function. Statistical analysis showed a significant relationship between PV/BH_20 and body weight (P=0.000)

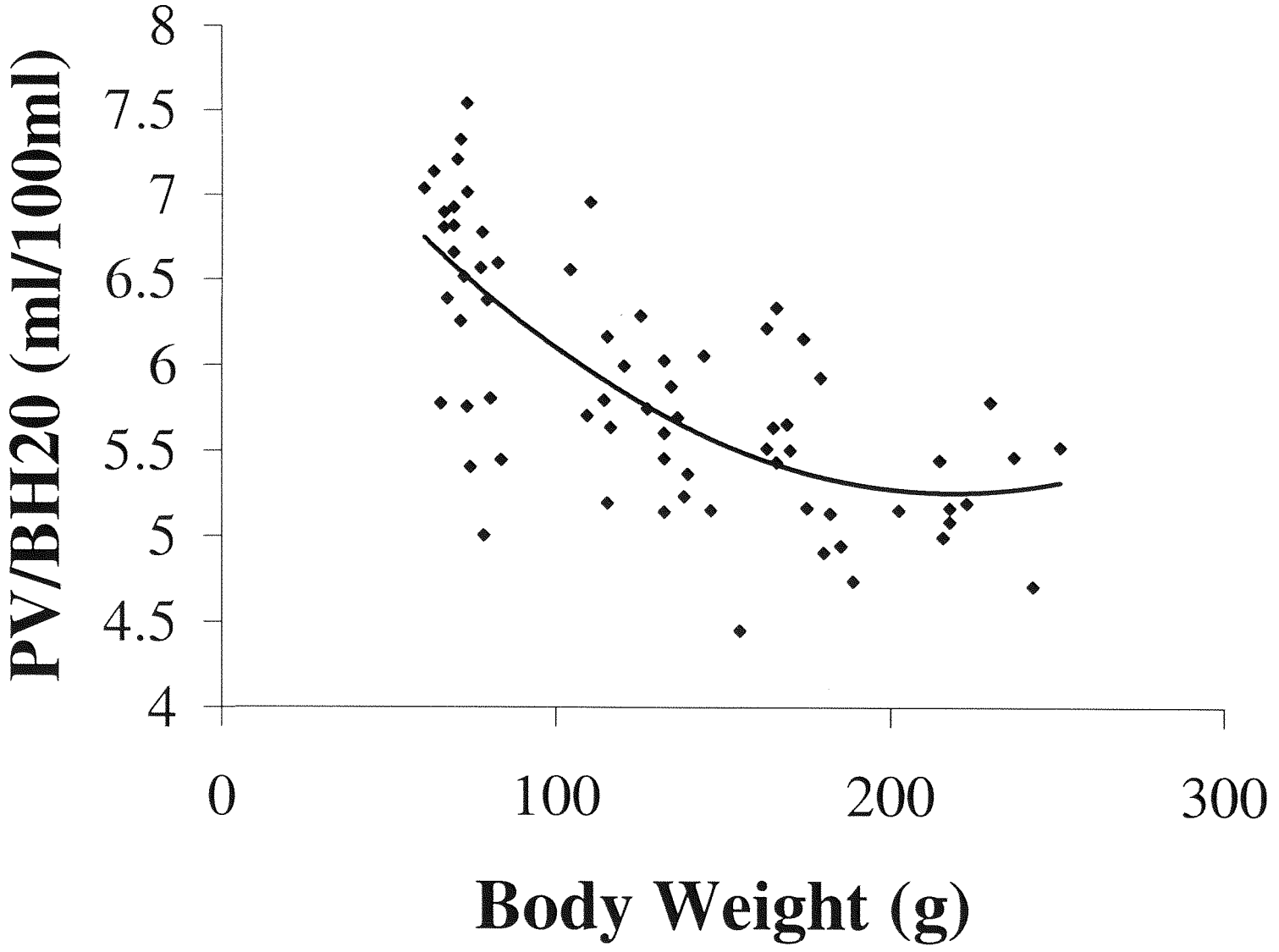

PV/BH20 (ml/100ml)
8
7.5
7
6.5
6
5.5
5
4.5
4
0
100
200
300
Body Weight (g)

Figure 5.9: Systolic blood pressure plotted as a dependent variable of PV/BH_20 in 9% casein exposed offspring.

Female Wistar rats were supplied either a 9% casein or an 18% casein diet throughout pregnancy. The offspring were used for determination of systolic blood pressure, plasma volume and body water content at 4, 6 and 8 weeks of age. Systolic blood pressure was examined in relation to PV/BH_20. The figure shows this relationship for 9% casein exposed animals. $r^2 = 0.457$, $P = 0.001$.

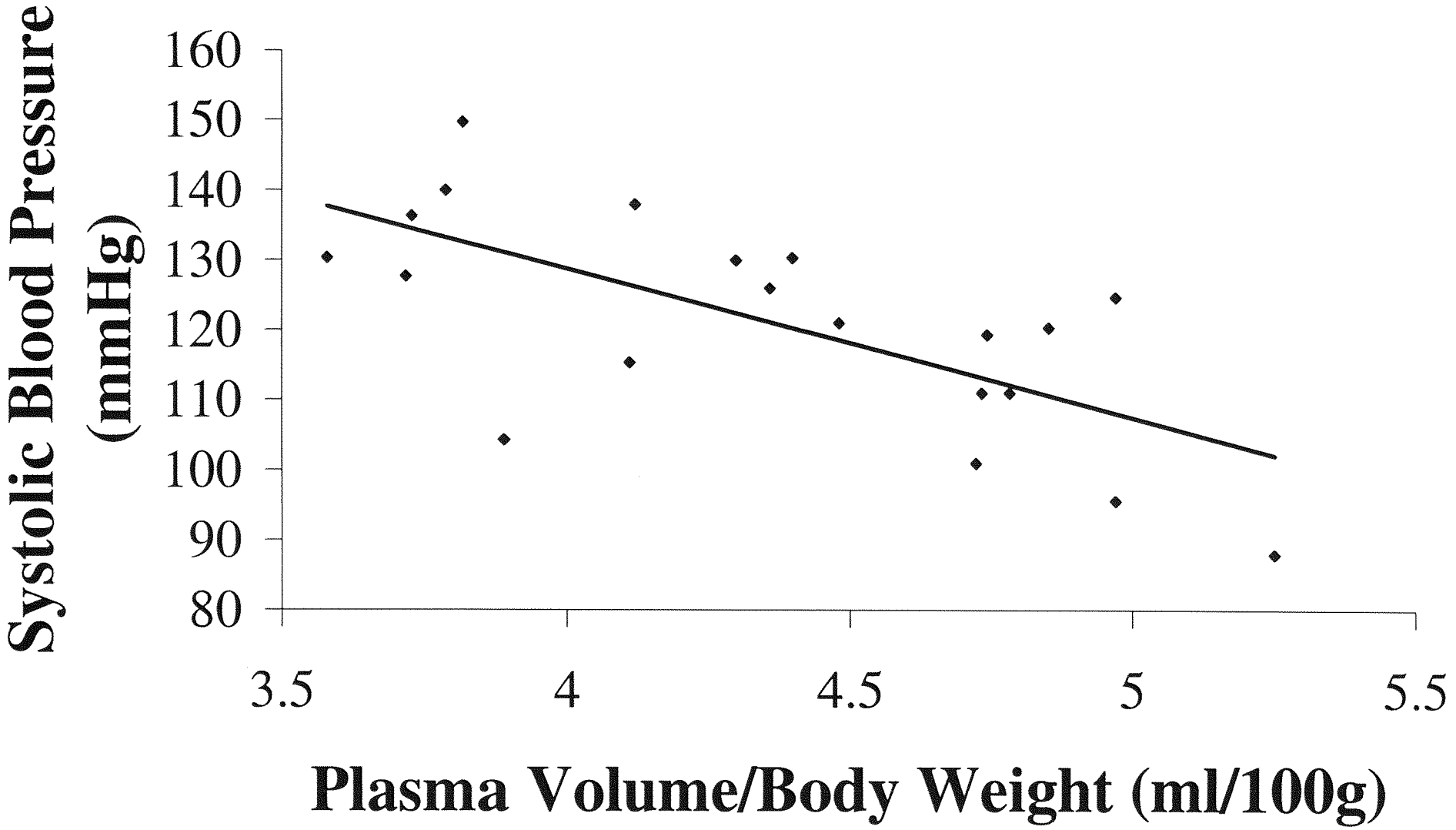
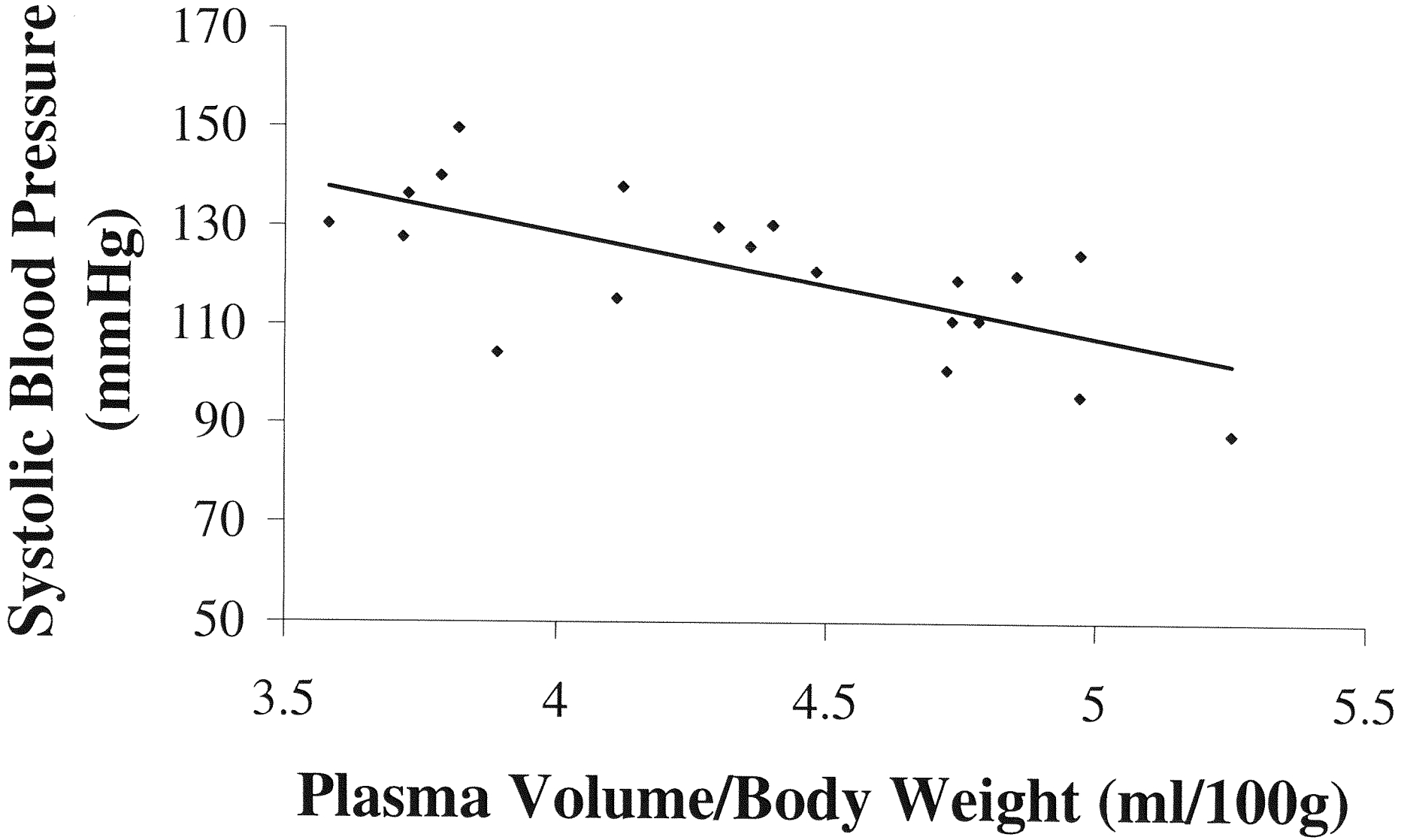

Systolic Blood Pressure (mmHg)
160
150
140
130
120
110
100
90
80
3.5
4
4.5
5
5.5
Plasma Volume/Body Weight (ml/100g)

Figure 5.10: Systolic blood pressure plotted as a dependent variable of PV/BH_20 in 18% casein exposed offspring.

Female Wistar rats were supplied either a 9% casein or an 18% casein diet throughout pregnancy. The offspring were used for determination of systolic blood pressure, plasma volume and body water content at 4, 6 and 8 weeks of age. Systolic blood pressure was examined in relation to PV/BH_20. The figure shows this relationship for 18% casein exposed animals. $r^2 = 0.103$, $P = 0.117$.


Systolic Blood Pressure (mmHg)
170
150
130
110
90
70
50
3.5
4
4.5
5
5.5
Plasma Volume/Body Weight (ml/100g)

5.4 Summary of results.

1. Both plasma volume and total blood volume were proportional to body weight in both control and low protein exposed rats.

2. Neither plasma volume nor total blood volume were modulated by maternal diet when expressed as absolute values or when corrected for body weight.

3. At 4 weeks of age, 9%Ca females had lower body water corrected for body weight than controls, whilst in 9%Ca males, it was elevated above control levels. In both 18%Ca females and 9%Ca males, the total body water declined with advancing age.

4. The proportion of body water comprising plasma volume (PV/BH_2O) declined rapidly in both dietary groups until a body weight of approximately 110g had been reached, at which point, this relationship tended towards an asymptote of around 4.59ml/100g body weight.

5. Systolic blood pressure increased with a decreasing PV/BH_2O in 9% casein exposed animals, but not in controls.

5.5 Discussion.

There are two primary associations between plasma volume and blood pressure. First, in untreated hypertensive individuals, plasma volume declines with increasing blood pressure (Danton *et al.* 1973). It is thought that venous constriction leads to intracapillary hypertension which favours the movement of fluid from the cardiovascular space to the interstitium thereby lowering the plasma volume. Secondly, an acute increase in plasma volume is associated with an elevation of blood pressure (Finnerty *et al.* 1970) which is depressed most effectively by the administration of diuretic drugs as opposed to direct vasodilators. This is particularly marked in individuals already displaying hypertension. Thus, hypertensive individuals not only

display disturbances of plasma volume relationships, but also have an enhanced responsiveness to changes in plasma volume.

In this study, plasma volume, body water content and blood pressure were examined in offspring of females fed a low protein diet during pregnancy. This was carried out in order to determine whether maternal diet might influence offspring plasma volume levels, and thus represent a possible mechanism by which blood pressure elevation is achieved in the low protein exposed group. It was found that low protein exposed animals demonstrated a decline in plasma volume corrected for body weight with increasing blood pressure. This relationship was not seen in controls and suggests that the hypertension seen in animals exposed to a mild maternal protein restriction reflects characteristics of essential hypertension (Danton *et al.* 1973) and may further implicate the kidneys in the development and progression of this disease.

Plasma volume and total blood volume were not modulated by maternal diet either as absolute values or when corrected for body weight. These data were in line with previously published values (Rosso & Streeter 1979; Fernandez *et al.* 1966). A fairly large degree of variation was seen in these data between 4 and 6 weeks of age and may be reflective of the fact that the growth curve of rats is at its steepest between these ages. Furthermore, it was found that the relationship between plasma volume and body water with increasing body weight is also changing rapidly between these ages (Figures 5.2 & 5.3). Thus, if any subtle differences did exist between dietary groups, both the rapid growth and the changing relationship between plasma volume and body water may well mask any effects of maternal diet.

The expression of plasma volume as a component of body water may highlight a possible reason why RBF, GFR and blood pressure are elevated from around 4 weeks of age in the SHR (Adams *et al.* 1989; Adams *et al.* 1990). In these animals, renal function is depressed relative to age matched Wistar-Kyoto controls until the blood pressure is elevated, after which time renal function is similar to that of controls (Harrap *et al.* 1986). The relationship of plasma volume corrected for body water, as a function of body weight, demonstrates that the proportion of body water comprising the plasma volume decreases rapidly until a body weight of around 110g is reached, after which,

there is little change in this relationship with increasing body weight. The plasma services the other body fluid compartments. Regulation of fluid and electrolyte homeostasis, and excretion of many breakdown products occurs via the action of the kidney on the plasma. As the proportion of body water comprising the plasma volume decreases, this effectively means that the volume of plasma available to the kidneys for the control of body fluid and electrolyte homeostasis also falls. It is not unreasonable to suggest that if the amount of fluid on which a system is able to exert control is diminished, then in order to maintain homeostasis, the work rate must be elevated. Thus, for the kidney to exert effective control of body fluid and electrolyte status, by acting on a volume of plasma that is diminishing relative to the total body fluid volume, there may well be changes in the factors which impinge upon the kidneys ability to do this. Furthermore, it was demonstrated that, at 4 weeks of age, but not at subsequent ages, the total body water (% body weight) of 9%Ca males was significantly greater than that of control males. This may suggest that the elevated blood pressure in the 9%Ca animals enables the regulation of total body water content and that this change may occur at approximately the same time as the renal functional changes seen in the SHR (Adams *et al.* 1989; Adams *et al.* 1990).

The ability of the kidneys to control fluid and electrolyte balance is enabled by the concerted action of a number of factors, both physical and hormonal. Variations in blood pressure are capable of substantially altering the levels of diuresis and natriuresis (referred to as pressure diuresis and pressure natriuresis respectively). Originally proposed by Guyton and colleagues (1972) the principle of pressure natriuresis and diuresis suggests that blood pressure is dominant in the day to day control of fluid and electrolyte balance. This was explained by examining the curves for sodium intake and sodium excretion against blood pressure. If sodium intake acutely exceeds sodium output, then fluid is retained within the body and the plasma volume is elevated. This increase in plasma volume results in an elevation of blood pressure which in turn leads to an increase in the excretion of sodium and water to a level exceeding that of intake. When plasma volume returns to normal, the blood pressure returns to its baseline level. Equally, if the level of sodium intake falls below output, then plasma volume declines and results in a depression of blood pressure which in turn allows the retention of fluid and electrolytes within the body. When plasma volume reaches its pre-set level, then

blood pressure returns to normal. Thus plasma volume is thought to be maintained at a predetermined point by the action of the blood pressure. It is thought (Guyton *et al.* 1972) that, in essential hypertension, the relationship between blood pressure and sodium intake/output is altered such that it requires a greater blood pressure to maintain body fluid and electrolyte balance.

Several hormones may also play a role in the renal control of body fluid and electrolyte balance. Sodium and water sparing hormones include angiotensin II, arginine vasopressin (AVP) and aldosterone. Of particular interest in relation to the rat model of hypertension employed in this thesis, is the potential role of angiotensin II. Langley-Evans and Jackson (1995) demonstrated that the hypertensive offspring of rats supplied a 9% casein diet during pregnancy demonstrated an elevated angiotensin converting enzyme (ACE) activity. Angiotensin converting enzyme is the enzyme which cleaves angiotensin I to form the vasocontrictive peptide angiotensin II (Oparil & Haber 1974a & 1974b). Treatment of low-protein exposed offspring with captopril, an ACE inhibitor, reduced their blood pressure to a level comparable with controls (Sherman & Langley-Evans 1998). The role of angiotensin II here, may actually be twofold. In addition to causing systemic vasoconstriction, angiotensin II also stimulates the release of aldosterone (Doi *et al.* 1984), a steroid hormone, whose action is to enhance sodium reabsorption from the kidney (Cannon 1977). Thus, an elevation of the concentration of angiotensin II may itself lead to an increase in blood pressure, both directly via its action on the vasculature, and indirectly by the sodium retaining action of aldosterone.

Previous chapters have shown that exposure to a maternal low protein diet during gestation leads to an impairment of nephrogenesis, resulting in animals with kidneys containing fewer glomeruli than controls. Renal filtration is maintained in the face of this nephron deficit, implying that the work rate of each individual nephron is higher than in control animals. Thus, these data highlight the possibility that blood pressure is elevated to enable the higher work rate of the nephrons. It may alternatively be the case that a reduced nephron deficit may result in an impaired ability of the kidney to respond to fluctuations in plasma and total body fluid volume. It was mentioned previously that the proportion of body fluid comprising the plasma diminishes rapidly between 4 and 6 weeks of age, during which time the blood pressure rises. It was suggested that in order

to exert control over the total body fluid by acting on an effectively diminishing pool of plasma, an elevation in renal work rate may be required. For animals which possess fewer nephrons, this increase in required work rate may be greater than that of control animals and hence, the rise in blood pressure with age is seen more clearly in this group. Thus, it may be the case that a nephron deficit alone is not necessarily sufficient to result in an elevation of blood pressure. However, when combined with the changing plasma volume relationships described above, the nephron complement may well be critical in determining whether an individual will become hypertensive or not.

An elevated ACE activity shown in previous work (Langley-Evans & Jackson 1994) may either be an additional factor in the dynamics of this system, or a response to the problem of an impaired ability to regulate fluid and electrolytes, resulting in the elevation of blood pressure seen in low protein exposed animals. Offspring of low protein fed mothers have been shown to be resistant to the hypertensive effects of a salt loading (Langley-Evans and Jackson 1996). This additionally points to an altered ability to balance fluid and electrolytes. If renal functional parameters, such as renal blood flow and glomerular filtration rate, are modulated differently within low protein exposed animals in response to a salt loading compared to controls, this may implicate the kidney as a causative agent in the aetiology of hypertension seen in these animals, i.e. if the kidneys are responsible for the elevated blood pressure in low protein exposed animals, then a further challenge to their function should identify the nature of the functional impairment.

Chapter 6.

Animals exposed to a low protein diet during gestation are resistant to the hypertensive effects of a salt load.

6.1 Introduction.

The experiments presented in earlier chapters of this thesis have demonstrated that exposure to a maternal low protein diet during gestation results in offspring with a reduced nephron complement and an elevated blood pressure. The filtration function of the kidneys is apparently unaffected, but in order to maintain a GFR similar to controls, it may be assumed that the single nephron GFR is raised.

The hypothesis proposed by Brenner and co-workers (1988) suggests that animals with a reduced nephron complement require an elevated blood pressure in order to maintain body sodium balance. This is derived from the hypothesis of pressure natriuresis proposed by Guyton and co-workers (1972). Guyton suggested that blood pressure is stable while sodium intake equals sodium excretion. If this balance is disturbed such that sodium intake is elevated above excretion, then blood pressure will rise. The elevation in blood pressure, it is proposed, results in a more rapid excretion of sodium thus reducing the body sodium content back to equilibrium levels. The reverse is also suggested, such that if body sodium falls then blood pressure falls, thus reducing the rate of sodium loss.

Guyton went on to suggest that individuals, who are prone to the development of hypertension, may have an altered response to a disturbance of the balance between sodium intake and excretion. He proposed that blood pressure rises as the ratio of sodium consumed to sodium excreted increases. Using a graph of this relationship, hypertension may be characterised in three ways. The curve may be shifted upwards, the gradient may be increased and/or, the gradient may be increased and the curve shifted upwards. All of these alterations would result in a higher blood pressure for any given level of sodium balance.

In the rat model of programmed hypertension it would be expected that, if the nephron deficit results in an elevation of blood pressure via the mechanism described above, as proposed by Brenner and colleagues (1988), then an increase in salt intake should further elevate the blood pressure. This has, however, already been shown not to be the case (Langley-Evans & Jackson 1996). A salt loading sufficient to elevate the blood pressures of control animals had no effect on those of low protein exposed animals. The proposed mechanism of natriuresis via the elevation of blood pressure, should increase the rate of sodium loss from the kidneys by elevation of the GFR. It may, therefore be the case that in this rat model, the GFR is elevated by means other than an increase in blood pressure, and hence delivery of blood to the kidney. The aim of the present chapter was to examine whether dietary "stressors" of renal function (salt-loading), might alter filtration function, in the absence of a systemic blood pressure change. In addition, the effect on blood pressure of feeding a 9% casein diet to offspring from weaning was also examined.

6.2 Protocol.

6.2.1 Study 1.

Six female Wistar rats weighing between 200g and 230g were mated and supplied either a 9% casein or an 18% casein diet throughout pregnancy (Materials and Methods 2.2). At parturition, animals were transferred to standard laboratory chow (SDS, Wincham Cheshire). Birthweights of offspring were measured. Offspring were weaned at 4 weeks of age, at which time the males were culled and the females retained until 19 weeks of age. Only females were used in this study because of the relative ease of cannulation of the urethra.

At 19 weeks of age, the animals were randomly allocated to 4 groups according to maternal diet, either 9% casein (9%Ca) or 18% casein (18%Ca), and those supplied saline (1.5% sodium chloride) in addition to normal drinking water (9%Ca-S; 18%Ca-S), or water (9%Ca-W; 18%Ca-W; Table 6.1). Standard laboratory chow (SDS, Wincham, Cheshire; Materials and Methods 2.2) was supplied *ad libitum*. A saline concentration of 1.5% NaCl was chosen in order to allow comparison with previous work (Langley-Evans & Jackson

1996). It was requested by the Home Office that saline given for any prolonged period of time, should be supplied along with normal drinking water. Systolic blood pressure was measured prior to imposition of the fluid protocol using the tail cuff method (Materials and Methods 2.5). The animals supplied water alone were untreated for 7 days. Those supplied water and 1.5%NaCl had their water removed on day 6 and were maintained solely on 1.5%NaCl for 24 hours. This procedure was performed in order that offspring of 18% casein fed females might achieve a significant elevation of blood pressure, in response to consumption of saline.

Systolic blood pressure was measured again after the 7 days. Saline and water consumption was measured gravimetrically and body weight was also measured prior to cannulation. After completion of the fluid regime the animals were anaesthetised and cannulated for the measurement of renal blood flow (RBF), glomerular filtration rate (GFR) and blood pressure which was continuously monitored by direct cannulation (Materials and Methods 2.6). After a post-surgery equilibration period of 30 minutes, 7 samples were taken at 15 minute intervals. At the end of the experiment the animals were terminally anaesthetised and the left kidney was removed and weighed.

6.2.2 Study 2.

Four virgin female Wistar rats weighing between 200g and 220g were mated and supplied either a 9% casein or an 18% casein diet during pregnancy. At parturition, females were transferred to standard laboratory chow. In order to determine whether exposure to a maternal dietary protein restriction during pregnancy may result in offspring whose blood pressure is "adapted" to cope with a low protein environment, offspring, at 4 weeks of age, were supplied either a 9% casein or an 18% casein diet. Systolic blood pressure was measured at 7 weeks of age using the tail cuff method (Chapter 2, section 2.3).

Table 6.1: Fluid protocol.

Experimental group.	n	Fluid regime.	Maternal diet.
18%Ca-W	9	Water	18% casein
18%Ca-S	6	Water + 1.5% NaCl	18% casein
9%Ca-W	6	Water	9% casein
9%Ca-S	6	Water + 1.5% NaCl	9% casein

Offspring of 9% casein (9%Ca) and 18% casein (18%Ca) exposed females were supplied either water (W) or a 1.5% NaCl (S; w/v) solution in addition to water for 7 days. Food intake was unrestricted. Blood pressure was measured before and after administration of the fluid protocol and animals were cannulated for the determination of GFR and RBF.

6.3 *Results.*

6.3.1 *Study 1.*

At parturition, birthweights of all pups were recorded (Figure 6.1). Birthweight was unaffected by maternal diet, although the male offspring of the 9%Ca group were heavier than their female littermates ($P<0.05$). Female animals were weaned at 4 weeks and retained until 19 weeks of age, at which time blood pressures were determined in each group. The animals were randomly assigned to one of the two fluid regimens (Table 6.1) and their blood pressures were again measured at the end of 7 days (Table 6.3).

Fluid consumption was similar in both 18% and 9% casein exposed groups regardless of fluid regimen (Table 6.2). In both dietary groups, those animals supplied water and 1.5%NaCl consumed approximately twice the volume of fluid ($P<0.05$) consumed by those supplied just water. Calculating sodium intake from fluid consumption showed that, during the first 6 days, both 18% and 9% casein exposed animals supplied saline consumed around 2 times the amount of sodium (approximately 226mg/day & 214mg/day respectively) as that taken in by animals provided with water only (approximately 90mg/day), whose only intake of sodium was through the chow diet. On the 7th day, the 18%Ca animals consumed 3 times the sodium intake of controls (approximately 267mg/day), while the 9%Ca group took in 4 times the sodium intake of controls (approximately 397mg/day).

Consumption of 1.5% NaCl over the 7 day study elicited a rise in the blood pressures of 18%Ca animals to a level comparable to that of the 9%Ca animals provided water only ($P<0.05$). The blood pressure of the 9%Ca animals supplied 1.5% NaCl however, remained unchanged, as did that of the 18%Ca and 9%Ca animals supplied water (Table 6.3).

The glomerular filtration rate (GFR) and renal blood flow (RBF) were measured by the infusion of ^{14}C labelled inulin and ^{3}H labelled para-amino hippuric acid (PAH). There were no differences in GFR due to maternal diet (Table 6.4). The GFR of the 18%Ca animals appeared to be slightly elevated by consumption of 1.5%NaCl (NS). Measurement of RBF was hampered by problems associated with the ^{3}H-PAH label. Some results were obtained

however and, although the low numbers prevented useful statistical analysis, they did suggest that baseline RBF in 9%Ca animals was lower than that of controls (data from animals supplied water only), and that exposure to 1.5%NaCl appeared to increase the RBF of 9%Ca offspring (NS; Table 6.4). A necessary part of the measurement of GFR and RBF was to precisely determine the volume of urine produced during each 15 minute period in between blood sampling. These urine volumes were analysed and shown not to be influenced by maternal diet or with saline consumption (Table 6.4).

Body weight was unaffected by maternal diet (Table 6.5). Although animals were randomly allocated to each fluid regimen, the 9%Ca-W animals were significantly lighter than those of the 9%Ca-S group both before ($P<0.05$) and after ($P<0.05$) the one week experimental period (Table 6.5). The kidneys of 9%Ca-S animals were heavier ($P<0.044$) than those of 9%Ca-W animals, but this may well have been due to the unforeseen difference in size between the two groups. Furthermore, the kidney weights, when corrected for body weight were similar between the 9%Ca-S and 9%Ca-W animals, suggesting that the difference in kidney weight *per se* between these two groups was due to the initial difference in size. The kidneys of the 9%Ca-W animals were lighter than those of 18%Ca-W controls ($P<0.05$), but this relationship disappeared when kidney weight was corrected for body weight. It is interesting to note, however, that within the two groups of 18%Ca control animals, the kidney weights of those supplied salt were heavier than in those of animals supplied water, both in absolute terms ($P<0.05$) and when corrected for body weight ($P<0.05$; Table 6.5).

6.3.2 Study 2.

At parturition, offspring exposed to a 9% casein diet *in utero* were significantly lighter than controls ($P<0.05$; Figure 6.2). At 7 weeks of age, in both males and females, the systolic blood pressures of low protein exposed offspring were significantly higher than those of control animals ($P<0.05$; Table 6.6). A two-way ANOVA showed that the diet supplied from weaning did not influence the effect of maternal diet on offspring blood pressure ($P=0.687$).

Figure 6.1: Birthweights of offspring from 9% casein and 18% casein fed dams.

Female Wistar rats were supplied either a 9% casein or an 18% casein experimental diet throughout pregnancy. At parturition birthweights were measured. Values for birthweight are expressed as means ± SEM. * represents a significant difference ($P<0.05$) between males and females within the same dietary group. In the 18% casein group, for males - n=16, females – n=15; 9% casein group, males n=14, females n=12.

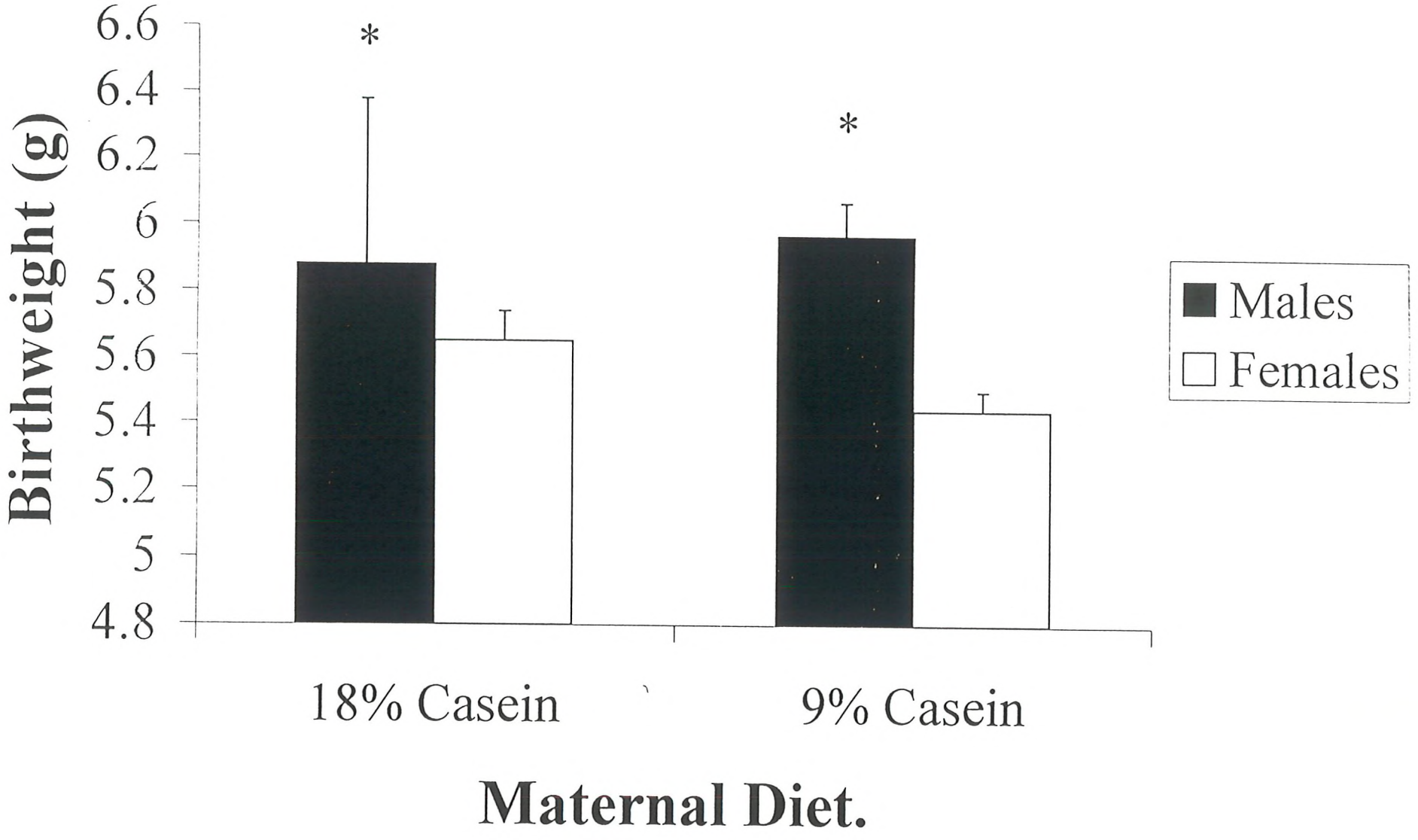

Birthweight (g)
6.6
6.4
6.2
6
5.8
5.6
5.4
5.2
5
4.8
*
*
Males
Females
18% Casein
9% Casein
Maternal Diet.

Table 6.2: Fluid consumption in rats provided with water and saline.

Group.	n	1.5%NaCl consumed by day 6 (ml/day).	1.5%NaCl consumed from day 6 - day 7 (ml).	Water consumed (ml/day).
18%Ca-W	9	NA	NA	33±2
18%Ca-S	6	23±5	30±3	37±4
9%Ca-W	6	NA	NA	29±4
9%Ca-S	6	21±4	52±13	27±3

Female offspring of rats supplied either a 9% casein or an 18% casein experimental diet during pregnancy were left undisturbed until 19 weeks of age. The animals had their blood pressures determined and were then randomly assigned to one of two fluid protocols (Table 5.1), either water only or water plus 1.5%NaCl (w/v). Food intake was unrestricted. At the end of 6 days, animals provided water plus 1.5%NaCl had their water removed for 24 hours. Fluid consumption was measured every day. Values are expressed as means ± SEM.

Table 6.3: Systolic blood pressures of offspring at 19 weeks of age before and after the administration of the fluid protocol (Table 5.1).

	Systolic Blood Pressure (mmHg).			
Dietary group.	**n**	**Before fluid regimen.**	**n**	**After fluid regimen.**
18%Ca-W	9	120±5	9	114±7
18%Ca-S	5	126±6	6	139±6*
9%Ca-W	6	143±8*†	6	140±7*
9%Ca-S	5	133±8	6	123±9

Female offspring from rats supplied either a 9% casein or an 18% casein experimental diet during pregnancy had their systolic blood pressures measured at 19 weeks of age using the tail cuff method (Materials and Methods 2.5). The animals were then randomly assigned to one of two fluid protocols (Table 5.1). At the end of 6 days, animals on water plus 1.5%NaCl had their water removed for 24 hours. Systolic blood pressure was again measured at the end of this 7 day period. Values are expressed as means ± SEM. * represents a significant difference relative to 18%Ca-W animals ($P<0.05$). † represents a significant difference relative to 18%Ca-S animals.

Table 6.4: Glomerular filtration rate (GFR), renal blood flow (RBF) and urine production in offspring of 9% casein and 18% casein exposed dams supplied either water alone, or water plus 1.5%NaCl from 19 weeks of age.

	n	GFR (ml/min).	n	RBF (ml/min).	n	Urine (µl/min).
18%Ca-W	9	2.39±0.39	4	6.70±1.15	9	60±7
18%Ca-S	6	2.65±0.08	4	6.01±0.79	6	62±16
9%Ca-W	6	2.42±0.15	4	4.49±0.45	6	62±9
9%Ca-S	6	2.52±0.27	3	6.59±1.38	6	87±14

Female offspring from rats supplied either a 9% casein or an 18% casein experimental diet during pregnancy were left until 19 weeks of age. The animals had their blood pressures determined and were then randomly assigned to one of two fluid protocols (Table 5.1). Glomerular filtration rate (GFR) and renal blood flow (RBF) were measured in anaesthetised animals (Chapter 2, section 2.6). Values for GFR, RBF and urine production over the cannulation period are expressed as means ± SEM.

Table 6.5: Body weights, kidney weights and kidney weight to body weight ratios of offspring of 9% casein and 18% casein exposed dams supplied either water alone, or water plus 1.5%NaCl from 19 weeks of age.

	n	Body weight before fluid (g)	Body weight after fluid (g)	Kidney weight (g)	KW/BW %
18%Ca-W	9	278±4	284±6	0.90±0.03	0.32±0.01
18%Ca-S	6	284±9	282±6	0.98±0.02*	0.35±0.01*
9%Ca-W	6	267±5	272±7	0.84±0.01*	0.31±0.01
9%Ca-S	6	284±3‡	290±4‡	0.94±0.03‡	0.32±0.01

Female offspring from rats supplied either a 9% casein or an 18% casein experimental diet during pregnancy were left until 19 weeks of age. The animals had their blood pressures determined and were then randomly assigned to one of two fluid protocols (Table 5.1). At the end of 6 days, animals on water plus 1.5%NaCl had their water removed for 24 hours. Prior to administration of the fluid protocol, body weights were determined. After completion of the fluid protocol, animals were cannulated for the measurement of RBF and GFR and at the end of the cannulation procedure, the left kidney was removed and weighed from each animal. Body weights and kidney weights are expressed as means ± SEM. * represents a significant difference compared to 18%Ca-W animals ($P<0.05$) and ‡ represents a significant difference compared to 9%Ca-W animals ($P<0.05$).

Figure 6.2: Birthweights of offspring from females supplied either an 18% casein or a 9% casein diet throughout pregnancy.

For 18%Ca males, n=10; females, n=9. For 9%Ca males, n=9; females, n=8. Birthweights are shown as means ± SEM. * represents a significant different compared with sex matched controls ($P<0.05$).

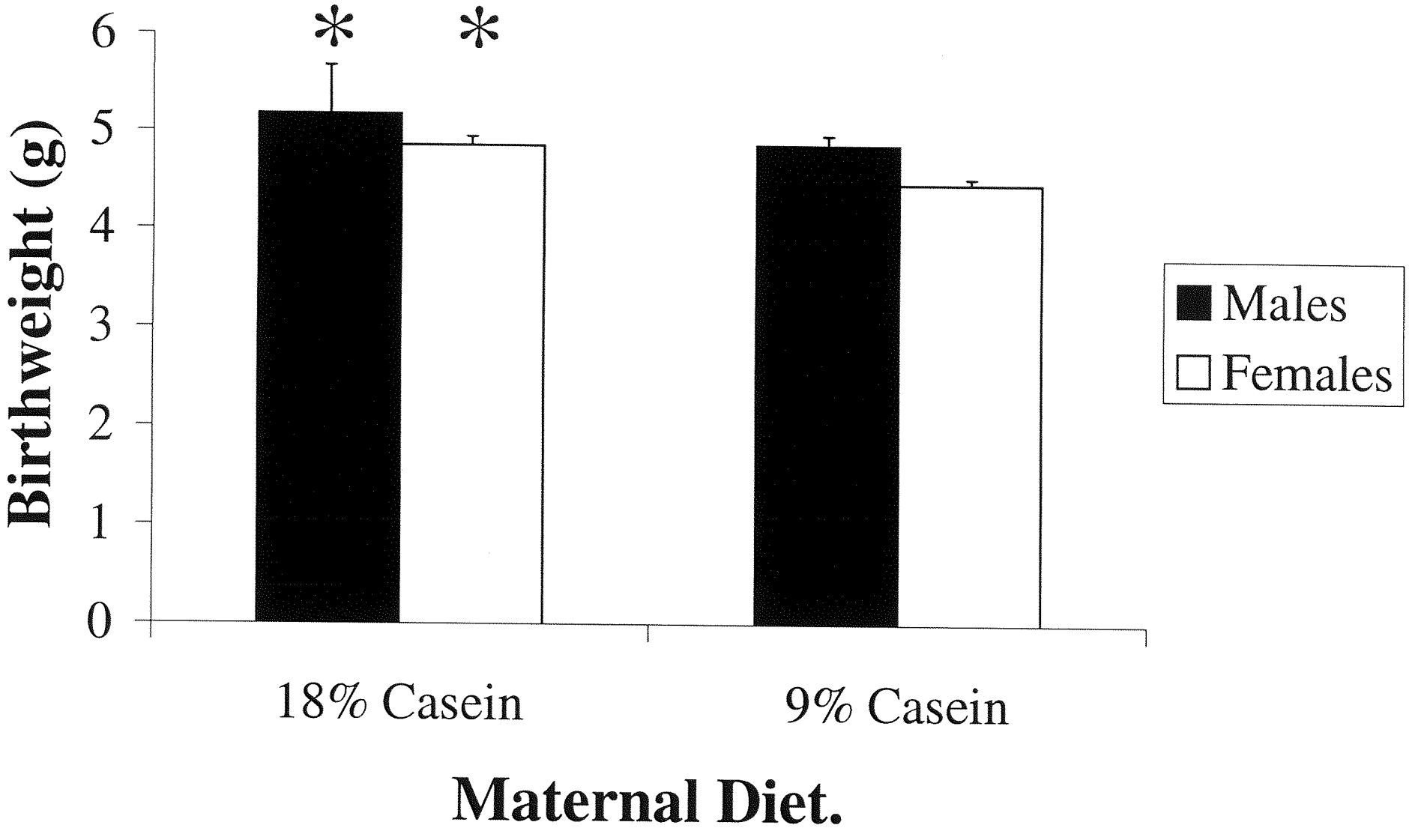

Birthweight (g)
6
5
4
3
2
1
0
*
*
Males
Females
18% Casein
9% Casein
Maternal Diet.

Table 6.6: Systolic blood pressure of offspring at 7 weeks of age.

	Systolic blood pressure (mmHg).			
Diet.	**n**	**Males.**	**n**	**Females.**
18%Ca - 18%Ca	5	107 ± 5	3	109 ± 12
18%Ca - 9%Ca	5	96 ± 2	6	122 ± 15
9%Ca - 18%Ca	3	127 ± 15*†	3	136 ± 15*
9%Ca - 9%Ca	3	125 ± 12*†	4	135 ± 24*

Female Wistar rats were supplied either a 9% casein (9%Ca) or an 18% casein (18%Ca) diet throughout gestation. At 4 weeks of age, offspring were supplied either a 9% casein or an 18% casein diet. At 7 weeks of age the systolic blood pressures of offspring were measured. Values are shown as means ± SEM. * represents a significant difference relative to 18%Ca-18%Ca controls ($P<0.05$). † represents a significant difference relative to 18%Ca-9%Ca animals ($P<0.05$). Two-way ANOVA showed no influence of diet supplied at weaning on the hypertensive effect of a maternal 9% casein diet ($P=0.687$).

6.1.4 Summary of results.

1. Fluid consumption was unaffected by maternal diet.

2. Consumption of salt caused the blood pressures of control animals to be elevated but had no effect on those of low protein exposed animals.

3. The GFR, RBF and urine production were all comparable between different dietary groups and were not modulated by consumption of salt, although the RBF of low protein exposed animals tended to be lower than in controls.

4. In 18%Ca control animals, consumption of salt appeared to result in an increase in kidney weight, both in absolute terms and when corrected for body weight.

5. In study 2, offspring exposed to a maternal 9% casein diet *in utero* were smaller than control offspring at birth.

6. The blood pressures of 7 week old rats were modulated by maternal diet supplied during pregnancy, but not the diet supplied at 4 weeks of age.

6.1.5 Discussion.

Birthweight demonstrated a variable response to a maternal 9% casein diet. One study showed birthweight to be reduced by a maternal 9% casein diet whilst the other, in line with data described in previous chapters, highlighted no differences between dietary groups. Little significance should be attributed to findings of birthweight as an individual measure of fetal growth in this model partly because the maternal dietary protein restriction is slight compared to that used by previous workers who used a 4% casein diet as their low protein group (Zeman 1968; Merlet-Bernichou *et al.* 1994; Hastings-Roberts & Zeman 1977) and partly because previous work has shown that the trajectories of the fetal body weight curves of low protein exposed and control animals with advancing gestation follow different paths, but intersect at around full-term (Langley-Evans *et al.* 1996c). Low protein exposed fetuses were shown to accelerate

growth rate in early gestation compared with control animals. This rate of growth then declined such that by birth, offspring body weights were comparable. The model used in these studies is in fact a model of low-normal birthweight, produced through complex variations in fetal growth throughout mid-late gestation.

At 19 weeks of age, animals were randomly assigned to a fluid protocol. By chance, the 9% casein exposed groups assigned to a salt regimen were significantly heavier than those supplied just water. This did not appear to have any bearing on the results subsequently obtained in these animals. Within the 18%Ca control groups, the kidney weight, both in absolute terms and when corrected for body weight appeared to be affected by consumption of salt. This was not seen in the 9%Ca groups and may reflect some mechanistic alteration in the sodium handling activity of the kidney in these animals. The fact that the kidneys of 18%Ca animals supplied salt apparently became heavier than their water supplied counterparts, suggests a degree of intrarenal fluid retention. It is unclear as to why this might be the case, but it may represent a reason why the blood pressure of these animals was elevated. If, in 18%Ca animals, consumption of salt leads to fluid retention at the baseline level of blood pressure, then an elevation in blood pressure may be required to remove this excess fluid.

The data show that animals placed on similar fluid protocols consumed equivalent volumes of fluid. However, it is clear that animals supplied 1.5%NaCl consumed approximately twice the volume of fluid of animals supplied water only, which is consistent with previous work (Langley-Evans & Jackson 1996a). This reflects the problematic nature of studies examining the effect of a salt loading. In the wild state, animals and individuals will consume both salt and water at a level determined by individual choice. Thus, supply of 1.5% saline alone does not reflect the conditions of the organism's environment. Under experimental conditions, it is clearly necessary to minimise the number of variables within the study. By supplying both salt and water however, it is difficult to determine whether any effects which are seen, are a consequence of an elevated salt intake, or merely an elevated fluid intake. An increase in salt intake is likely to result in an increase in the overall fluid intake since body fluid levels of sodium are tightly regulated (Ganong 1991). It is unclear as to the degree to which fluid moves in and out of individual compartments (i.e. extracellular,

intravascular) in response to alterations of fluid and electrolyte intake. There is resistance to movement of fluid into all compartments. For fluid to move into the intravascular compartment there are at least two potential motive forces; an increase in the osmotic potential of the blood plasma, and the capacity of the extracellular space. Therefore, if the blood plasma becomes hyperosmotic compared to the extracellular fluid, then water will move from the extracellular fluid into the intravascular compartment. If the extracellular space is reduced, or the volume of fluid within it increases, then again there is a drive to move fluid from this, into the intravascular space. However, there are also forces resistant to the movement of fluid from compartment to compartment. For example, the intravascular volume (i.e. the capacity of the cardiovascular system to contain fluid) is limited. This may also become altered by the actions of some hormones (angiotensin II, vasopressin, prostaglandins) and other molecules (nitric oxide). Thus, by contraction or relaxation of blood vessels, the volume of the intravascular space is modulated and therefore, the relative forces acting on the movement of fluid between compartments may also be varied. There are undoubtedly numerous other factors which may affect this. The consideration of all data regarding salt loading must therefore take into account the fact that alterations in these relationships, which are not well understood, may result under differing circumstances.

The previous chapter demonstrated that, at 4 weeks of age, animals exposed to a maternal 9% casein diet possess fewer glomeruli and thus fewer nephrons than controls (Chapter 3). It was also shown in Chapter 4, that the rate of nephrogenesis declined towards the end of gestation. The implication was that the production of cortical nephrons was more at risk from a maternal dietary protein restriction than juxtamedullary nephrons, since juxtamedullary nephrons are formed first (Abrahamson 1991). The juxtamedullary nephrons, with their long loops of Henle, provide the major sodium resorbtive capacity of the kidney and therefore the means by which water retention is enabled. The data from the previous chapters, not only suggest a reduction in the nephron complement of 9% casein exposed animals, but also that the proportion of cortical nephrons to juxtamedullary nephrons is lower. Should this be the case, then it may be that kidneys of animals from a low protein fed mother have a greater sodium resorbtive ability than controls. This might be examined using the slow infusion of ^{22}Na in low protein exposed and control animals maintained on either a low, normal or high

salt intake. Determination of the rate of excretion of ^{22}Na should highlight variations in the resorbtive capacity of the kidneys. If low protein exposed animals do have a greater sodium resorbtive capacity than controls, then it is not unreasonable to suggest that blood pressure may be elevated in order to prevent excessive sodium retention.

Data obtained for blood pressure in this study reflected that observed in a previous study by Langley-Evans and co-workers (Langley-Evans et al. 1996a). The blood pressures of 18%Ca animals rose in response to salt consumption, whilst there was no effect of salt intake on the blood pressure of 9% casein exposed animals. It should be pointed out that in the present study, sole consumption of a 1.5% NaCl solution without additional access to water was required in order to achieve an elevation in the blood pressure of 18%Ca animals. Systolic blood pressure was measured at day 6 when both saline and water were supplied, and no elevation in blood pressure was found in the 18% casein exposed group. The study was designed to examine the effects of a salt loading which elicited an increase in blood pressure in control animals, on low protein exposed offspring, thus it was necessary to withdraw water for one day. It would appear from the data that the 9% casein exposed animals were resistant to the hypertensive effects of a salt loading, whilst the 18%Ca animals were not, although the possibility that a longer exposure to salt loading, or a more severe salt loading, might result in an elevation in the blood pressures of the 9%Ca animals, cannot be ruled out. The principle of pressure natriuresis (Guyton *et al.* 1972) states that blood pressure is increased in order to excrete excess sodium, enabling a return to balance between sodium intake and sodium excretion. The offspring of 9% casein exposed dams, by virtue of their reduced nephron complement may already be at the upper limit of blood pressure achievable for increase of sodium excretion, whilst 18%Ca animals at a lower basal level of blood pressure have a greater range available.

It is interesting to note that, although there are numerous studies linking salt consumption and elevated blood pressure (Meneely 1967; Friedman *et al.* 1990; Reid & Laragh 1965), other studies have shown little or no effect on blood pressure of an elevated salt intake (Smith *et al.* 1988; Intersalt Co-operative Research Group 1988). However, it has been shown that a high salt intake may reduce the survival of rats, despite a lack of salt-induced blood pressure elevation. Tobian and Hanlon (1990)

examined the effect of a high salt diet on the survival of Dahl salt resistant rats (Dahl-R rats). After 8 weeks of feeding a high salt diet, only 47% of animals survived, and all had died after 15 weeks of salt feeding. All animals fed a low sodium diet survived beyond this point. Post-mortem examination revealed that most of the animals fed a high salt diet had died from cerebrovascular disease with brain infarction, i.e. stroke. The feeding of a high salt diet in this study had not caused an elevation in blood pressure. It has further been shown that many Japanese people with a high salt intake, showed a greater susceptibility to stroke (Sasaki 1962). This suggests that in individuals and animals that show resistance to the hypertensive effects of a salt loading, there is a potentially lethal sensitivity to salt consumption, which perhaps would not become manifest were the blood pressure response to be present. It is also extremely interesting that within the Japanese population there is also a tendency for individuals to possess fewer nephrons and to be prone to the development of hypertension (Takashi *et al.* 1957). As mentioned above, it has been shown in previous chapters that offspring of females exposed to a 9% casein diet, during pregnancy, possess fewer nephrons than control animals and consistently show an elevated blood pressure. It may be, that this rat model of hypertension closely approximates the hypertensive condition of certain human populations.

Renal blood flow, as in the previous chapter, was not altered by maternal protein restriction. Salt loading did not modify blood flow in the kidneys. The GFR was measured in order to determine whether the lack of a blood pressure response to a salt load was accommodated for by an increase in the filtration capacity of the kidneys. The GFR was apparently unaltered by either maternal diet (in keeping with the results from Chapter 4) or salt consumption. In 9% casein exposed animals, a lower nephron complement suggests that the single nephron GFR (i.e. the volume of fluid filtered at each glomerulus) is elevated. This raises an interesting question regarding the consequences of an artificially induced depression of renal perfusion pressure (via hydraulic control of blood flow to the kidney) i.e. what happens to sodium homeostasis in offspring of 9% casein fed females if perfusion pressure is lowered? As was mentioned above, the kidney weight of 18%Ca controls apparently increased in response to salt loading. This was not seen in 9%Ca animals. This may be due to the fact that the blood pressure is already elevated to a level which prevents this apparent

renal fluid retention. Thus, by artificially lowering the blood pressure of 9%Ca animals, would their kidneys gain weight when exposed to a salt load?

Study 2 examined the effect on blood pressure of supplying either a 9% casein or an 18% casein diet to offspring of 9% casein and 18% casein fed mothers, from 4 weeks of age, in order to assess whether the hypertensive effect of a maternal low protein diet might be modulated by exposure to a similar diet postnatally. This addresses the idea of the thrifty phenotype hypothesis proposed by Hales and Barker (1992). This hypothesis suggests that the fetus may adapt to conditions *in utero* which reflect those of the outside world. Thus, it was suggested that maternal undernutrition during pregnancy would be communicated to the fetus in some way and the fetus would then develop in such a way as to be better able to cope, in a nutritionally deprived environment, postnatally. There was shown to be no effect on blood pressure of feeding either of the synthetic diets after weaning. It therefore may be suggested that exposure to a maternal 9% casein diet during gestation does not result in the fetus accommodating its blood pressure to a potentially nutritionally hostile environment, or if it does, the level of blood pressure does not maintain a direct relationship with postnatal protein consumption. This does not preclude the possibility that the hypertensive effect of a maternal dietary protein restriction during pregnancy, occurs as a result of other changes which are themselves, adaptations to deal with an environment in which food is scarce.

In conclusion, 9% casein exposed animals are insensitive to the hypertensive effects of an elevation of salt consumption. Salt excretion is not enhanced by an increase in the glomerular filtration rate. It may be the case that the kidneys of the 9% casein exposed offspring are already working at a level sufficient to cope with the increase in salt intake, without the necessity of altering parameters such as blood pressure and GFR. Another aspect of the principle of pressure natriuresis is that an expansion of plasma volume is regarded as the cause of the rise in blood pressure, which enables sodium excretion to be increased. The previous chapter indicated that plasma volume fell with increasing blood pressure in low protein exposed offspring only, suggesting, along with the present data, that the mechanism of fluid and electrolyte homeostasis may be impaired in these animals.

Chapter 7.

General discussion.

Epidemiology is the study of the association between the exposure to a range of environmental factors and disease outcomes amongst populations. From these associations, individual factors, or a combination of factors may be identified which pose a risk of developing a disease. Association does not of itself prove causality, but might imply possible causal factors. In order that specific causal factors and their modes of operation in inducing disease might be identified, more detailed exploration at other levels of organisation is required.

The techniques which are used in molecular biology have enabled detailed exploration of ways in which mechanisms might operate at the molecular level to change the behaviour of cells. However, each possible change in the behaviour of a cell must be accommodated within the homeostatic mechanisms which maintain the normal function of tissues and organs. In order to elucidate the causal mechanism or mechanisms at the molecular level, and to direct appropriate molecular and cellular studies, an understanding of relationships at the level of the whole body can be invaluable. Thus the work in this thesis has been carried out to explore which factors might be of importance in integrating the response to maternal dietary protein restriction, at the whole body level. The objective has been to draw a link between epidemiological observations, which indicate a relationship between fetal growth impairment and adult cardiovascular disease, and possible cellular and sub-cellular processes and mechanisms through which blood pressure might be programmed.

All of the studies carried out in this thesis employed the supply of a synthetic 9% casein diet to rats during pregnancy in order to impose a maternal dietary protein restriction during this period. Control animals were fed a synthetic 18% casein diet during pregnancy. In examining changes in fetal growth and development, particularly in response to a maternal dietary intervention, it is necessary for a number of factors to be considered. These include the nature of the exposure, i.e. does the dietary protein content fall within the normal range consumed during pregnancy?, the effects on the

pregnant female of such an intervention, the periods during development when sensitivity to a maternal dietary protein restriction may be high, and the nature of the outcome in the offspring.

The nature of the maternal dietary protein restriction was chosen to reflect the lower limit of protein intake considered normal and sufficient to support a successful pregnancy (Clarke *et al.* 1978). This level of protein restriction may also reflect a level of nutrition in human mothers not considered to be undernourished.

Developmental events occur according to a genetically determined time-scale, for example, the kidney develops from around day 11 of gestation to 10 days postnatally (Tuffro-McReddie *et al.* 1993). Particular developmental processes may be susceptible to the programming effects of exposure to a maternal 9% casein diet. Offspring blood pressure, late gestation nephrogenesis and renal function were therefore examined in animals exposed to a maternal 9% casein diet during discrete weeks of pregnancy.

The results obtained in this thesis reflect the view that exposure to a maternal 9% casein diet reduces the metabolic competence of the offspring (Jackson 1996). Clearly, a relative nephron deficit, which has been shown to result from exposure to a maternal 9% casein diet (chapter 3), pre-determines that the "reserve capacity" of the kidneys of these animals is diminished with respect to that of controls. Thus, if nephron loss occurs with ageing, the point at which the reserve capacity is lost will be sooner in life in those animals exposed to a maternal dietary protein restriction than in controls (which may never lose their reserve capacity).

Determination of the glomerular complement between day 20 of gestation and full term was employed in order to assess the effects of a maternal 9% casein diet during discrete weeks of pregnancy and throughout gestation, on nephrogenesis. It was found that exposure to a maternal low protein diet during the second week only, the third week only or throughout gestation impaired the late gestation formation of new nephrons. A reduction in nephron number has similarly been shown to result from exposure to maternal diets of 5% and 6% protein (Merlet-Bernichou *et al.* 1994; Zeman 1968). Further, other organs have been shown to be affected in this way. The pancreas of

animals exposed to a maternal 8% casein diet possesses fewer β-cells (Snoek *et al.* 1990). Thus, it is clear that reduction of the protein component in the maternal diet during pregnancy has a substantial detrimental effect on the development and morphology of the offspring.

Data from human studies have similarly shown a relationship between fetal growth impairment and renal development. Kidneys of babies which are small for gestational age have been shown to grow more slowly *in utero* than those in babies of appropriate size for gestational age (Konje *et al.* 1996) and they possess fewer nephrons at birth (Hinchliffe *et al.* 1992). It would be useful in future to examine these same parameters in association with maternal nutritional status. Also, it would be interesting to examine the growth of human fetal kidneys using the ultrasound method described by Konje and co-workers (1997) and follow up the children in postnatal life and examine their renal function and blood pressure.

The mechanisms by which a maternal dietary protein restriction during pregnancy may impair fetal growth include nutrient restriction to the fetus and programming of the fetus by maternal hormones. Protein enables the construction of new cells. Thus, an obvious pathway by which the structural alterations described above may come about is if, by lowering the maternal protein supply, the physical amount of protein available for the production of new cells within the fetus is reduced below that which is demanded. Maternal plasma amino acid concentrations have been shown to be depressed by the consumption of a low protein diet during pregnancy (Reusens *et al.* 1995), suggesting that this may be the case. In addition, the expansion of maternal plasma volume with advancing pregnancy has been shown to be impaired by consumption of a 9% casein diet (Welham *et al.* 1998). Maternal undernutrition also reduces uterine blood flow (Rosso & Kava 1980) and this, in turn, is associated with fetal growth impairment (Croall *et al.* 1978). This, it is suggested, may occur by restricting the supply of nutrients to the placenta and thus, the fetus.

An alternative explanation for the mechanism by which maternal dietary protein restriction impairs fetal growth and programmes blood pressure, involves the action of maternal glucocorticoids. Fetal exposure to glucocorticoids results in offspring with

elevated blood pressures (Celsi *et al.* 1998; Benediktsson *et al.* 1993). In the placenta, the enzyme 11-β hydroxysteroid dehydrogenase (11-β HSD) protects the fetus from exposure to maternal glucocorticoids. The activity of this enzyme in the placentas of low protein exposed offspring is lower than in controls (Langley-Evans *et al.* 1996d) and therefore may allow the passage of maternal glucocorticoids to the fetus in this group. Manipulation of maternal glucocorticoid production has suggested that this hormone may play a causative role in the development of low protein induced hypertension (Langley-Evans *et al.* 1996f; Gardner *et al.* 1998). Furthermore, kidney development has been demonstrated to be sensitive to glucocorticoid exposure (Celsi *et al.* 1998). Administration of dexamethasone (a glucocorticoid analogue not metabolised by 11-β HSD) to pregnant rats resulted in offspring with a significant nephron deficit and elevated blood pressure. Exposure to maternal glucocorticoids, therefore, may be critical in the programming of hypertension in the offspring of low protein fed dams, and there is some evidence to suggest that this may occur through the action of glucocorticoids on the developing kidney.

The association between a relative nephron deficit and elevated blood pressure is well established (Brenner *et al.* 1988; Mackenzie & Brenner 1995). It was proposed that kidneys possessing fewer nephrons might require an elevated blood pressure in order to maintain sodium homeostasis. The higher blood pressure is thought to lead to further nephron loss due to an excessive workload, and therefore a further increase in blood pressure in order to continue to maintain sodium homeostasis. Studies in spontaneously hypertensive rats (SHR) demonstrated that prior to the blood pressure elevation (at around 4 weeks of age), renal blood flow (RBF) and glomerular filtration rate (GFR) are significantly lower than in Wistar-Kyoto control rats (Harrap & Doyle 1986). The onset of the blood pressure elevation in the SHR results in the "normalisation" of RBF and GFR. Thus, although measurements in adult SHR's may not highlight differences in the RBF and GFR compared with that of controls, it appears that they are maintained at control levels by the elevated blood pressure.

There was apparently no difference in the RBF or GFR of animals exposed to a maternal 9% casein diet during gestation, but their blood pressures were significantly higher than those of controls, possibly suggesting the presence of a similar mechanism

to that which occurs in SHR's. It would be useful in the future to examine the ability of the kidneys of low protein exposed animals to excrete sodium under different pressures by artificially controlling renal perfusion pressure. This may clarify whether the relative nephron deficit of animals exposed to a maternal 9% casein diet *in utero* physically alters the sodium excreting capabilities of their kidneys.

In humans diagnosed hypertensive and receiving no antihypertensive treatment, it has been shown that the plasma volume declines with increasing blood pressure (Dustan *et al.* 1973). A similar relationship was found in the offspring of rats exposed to a maternal low protein diet (Chapter 5). This may be a function of the fact that plasma volume is unaffected by exposure to a maternal dietary protein restriction during pregnancy, whilst blood pressure is. Thus, since blood pressure is elevated and continues to rise to some degree with age, this relationship may merely be a numerical one with little biological significance. However, the apparent insensitivity of blood pressure to a salt loading in offspring of 9% casein exposed animals (Chapter 6; Langley-Evans & Jackson 1996) suggests that control of sodium homeostasis is more tightly regulated at a higher blood pressure. This may mean that, in addition, control of plasma volume is equally tight in these animals and perhaps more so than in controls. Therefore, the lack of an association between plasma volume and blood pressure in control animals may be reflective of less rigid control than that seen in low protein exposed offspring.

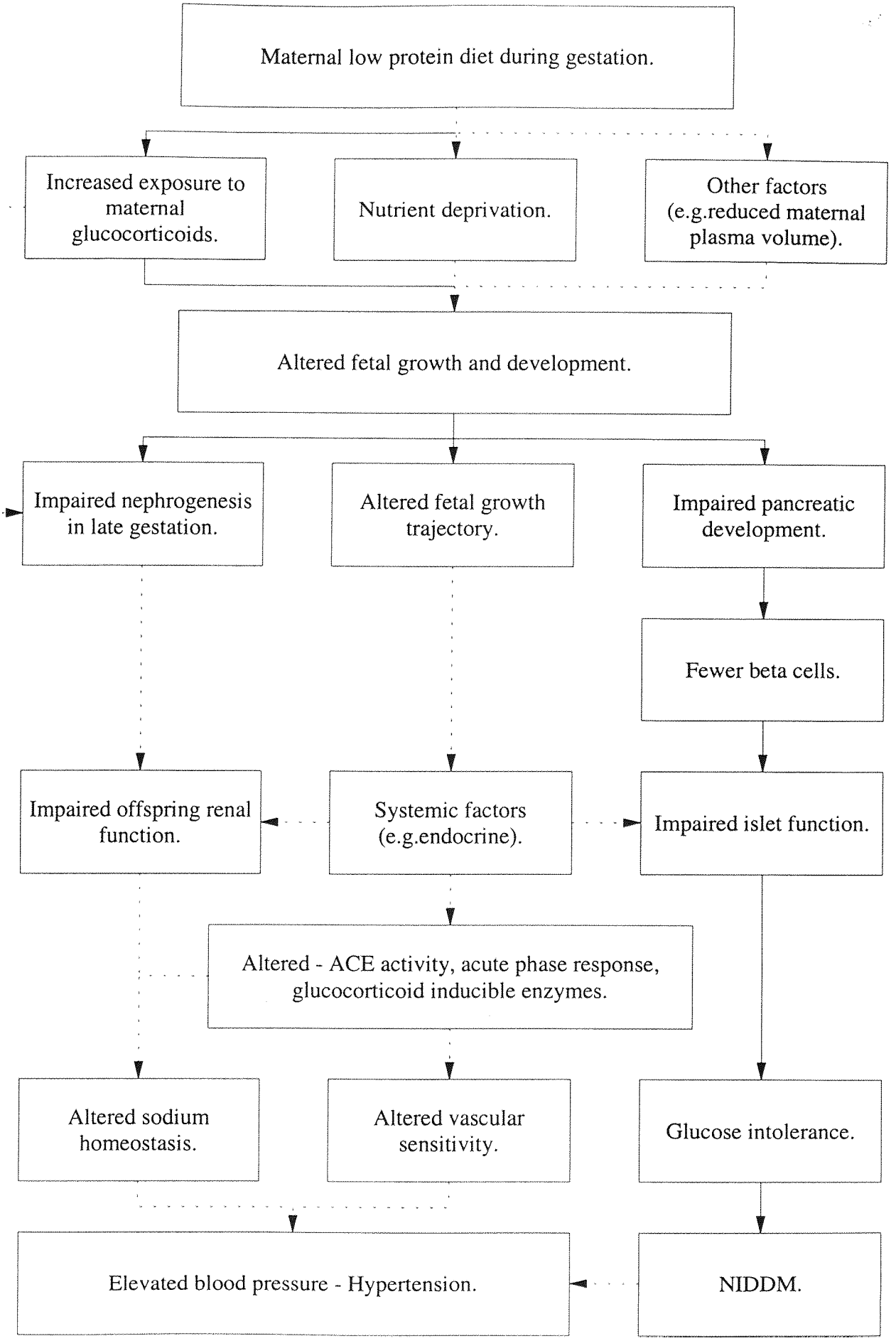
The working hypothesis for the mechanisms of fetal programming of adult diseases employed during, and adjusted as a result of, work in this thesis is outlined in Figure 7.1. In the figure, it is postulated that a maternal low protein diet might affect the fetus in one or all of three ways: via increased exposure to maternal glucocorticoids, a deprivation of nutrients, or other factors yet to be discovered. Their effect is to alter the fetal growth trajectory and specifically to impair pancreatic development and late gestation nephrogenesis. These changes may result in impairment of function (renal, pancreatic and hormonal) which subsequently may predispose the individual or animal to the development of elevated blood pressure and non-insulin dependent diabetes (NIDDM).

Further work in this field should encompass a range of disciplines. It has already been mentioned that human fetal morphometric analysis, particularly in association with maternal nutrition and subsequent examination of children postnatally, would provide useful information regarding impairment of renal growth *in utero* and it's effect on postnatal renal physiology and blood pressure control. In the rat, and indeed other animal models, renal development might be examined in more detail in order to more clearly understand the mechanisms by which a maternal dietary protein restriction impacts upon renal development. Embryological studies examining the role of maternal glucocorticoids and various growth factors may highlight potential specific targets of intrauterine programming stimuli. Physiological studies of particular importance would, as mentioned previously, examine the sodium excretory capacity of kidneys from low protein exposed offspring.

Postnatal factors which are of importance in blood pressure control not covered by this thesis include, in particular, the renin-angiotensin system which has been shown in a number of studies to be disturbed in offspring of rats fed a 9% casein diet during pregnancy (Langley-Evans & Jackson 1995; Langley-Evans *et al.* 1996). Further work might usefully examine the developmental changes which could result in alterations of expression of the angiotensin converting enzyme (ACE) and the vascular sensitivity to angiotensin II. This has been demonstrated to be increased in low protein exposed animals (Gardner *et al.*. 1998) and may be modulated by exposure to maternal glucocorticoids (Langley-Evans 1997).

Figure 7.1: An outline of the factors associated with the programming of hypertension and non-insulin dependent diabetes (NIDDM) by maternal dietary protein restriction during rat pregnancy.

The figure shows the potential effects of a maternal low protein diet, supplied during pregnancy. Solid lines represent associations for which there is direct evidence and dotted lines represent those which are postulated and qualified by indirect evidence.


Maternal low protein diet during gestation.
Increased exposure to maternal glucocorticoids.
Nutrient deprivation.
Other factors (e.g.reduced maternal plasma volume).
Altered fetal growth and development.
Impaired nephrogenesis in late gestation.
Altered fetal growth trajectory.
Impaired pancreatic development.
Fewer beta cells.
Impaired offspring renal function.
Systemic factors (e.g.endocrine).
Impaired islet function.
Altered - ACE activity, acute phase response, glucocorticoid inducible enzymes.
Altered sodium homeostasis.
Altered vascular sensitivity.
Glucose intolerance.
Elevated blood pressure - Hypertension.
NIDDM.

In conclusion, maternal dietary protein restriction impairs late gestation nephrogenesis and results in offspring with a reduced nephron complement. There is some evidence to suggest that this relative nephron deficit may impair renal function in postnatal life and that this, in turn, may be responsible, at least in part, for the elevation of blood pressure seen in animals exposed to a maternal 9% casein diet during gestation. Further work is needed to clarify whether indeed this is the case, and if so, to elucidate the mechanisms by which a maternal 9% casein diet supplied during pregnancy impairs renal development.

Appendix.

Acid Alcohol.

Acid alcohol was prepared for renal histological analysis as follows (Carltons Histological Technique 1967):

Haematoxylin and Eosin Stain.

To 70% alcohol was added hydrochloric acid at a concentration of 1%.

Staining with haematoxylin and eosin produces the following results:

Tissue.	**Colour after staining.**
Nuclei.	Blue to blue black.
Karyosomes.	Dark blue.
Cartilage.	Pink or light blue to dark blue (depends on the haematoxylin used).
Calcium and calcified bone.	Purplish blue.
Basophil cytoplasm (plasma cells and osteoblasts).	Purple.
Red blood cells, plasmosomes, eosinophil granules, paneth cell granules, zymogen granules, keratin.	Bright orange red.
Cytoplasm.	Shades of pink.
Muscle fibres, thyroid colloid, thick elastic fibres, decalcified bone matrix.	Deep pink.
Collagen and osteoid tissue.	Light pink.

Bradford Reagent.

Bradford reagent for the determination of tissue protein content (section 2.8) was prepared as follows:

200mg of Coomassie Blue (Sigma, UK) was dissolved in 100ml ethanol (Sigma, UK). To this was added 200ml phosphoric acid (Sigma, UK) and the solution was made up to 2.5L with distilled water. The reagent was stored in a dark place for 1 week prior to use. The reagent was used within 2 months.

Tris-HCl Buffer.

Tris-HCl buffer (pH 7.4) was used as a medium for homogenisation of tissue samples for protein and DNA analysis (sections 2.8 and 2.9) and was prepared as follows:

Tris buffer was mixed to a concentration of 10mM with $MgCl_2$ to a concentration of 1mM and 0.02% TX-100 (v/v) and made up to a final volume of 1L with distilled water.

DPA Reagent.

The DPA reagent for use in the DNA assay (section 2.9) was prepared as follows:

To 40g of diphenylamine was added 0.1ml of paraldehyde. This was made up to a litre with glacial acetic acid. All chemicals were obtained from Sigma (Poole, UK).

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