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THE EARLY LIFE PROGRAMMING OF ADULT HYPERTENSION BY GLUCOCORTICOIDS

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ABSTRACT

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EARLY LIFE PROGRAMMING OF ADULT HYPERTENSION BY GLUCOCORTICOIDS

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Coronary heart disease remains the major cause of death in westernised societies of which hypertension is a major risk factor. As adults, the combination of a poor diet and physical inactivity leads to elevated systolic blood pressure but accounts for only a proportion of the variation in adult hypertension. The fetal and infant origins of adult disease hypothesis states that the propensity towards adult disease is programmed in utero by maternal nutritional and hormonal factors. The thesis presented examines the role of glucocorticoids in a model of hypertension programmed by maternal nutrition.

A low protein, isoenergetic diet (MLP) in the rat reduced maternal weight gain in late gestation and impaired the activity of placental 11β-hydroxysteroid dehydrogenase Type 2 at day 20 gestation, which changes in maternal hormonal status could not explain. MLP fetuses may therefore be exposed to a greater level of maternal glucocorticoid in late gestation. MLP pups at term were of low to normal birthweight yet exhibited degrees of disproportionate intrauterine growth that appeared to favour maintenance of substrate supply to the brain. Brain growth was maintained in proportion to growth of the body whereas growth of the liver, lungs and trunk was not.

MLP pups from term, into adulthood, demonstrated increased activities of glucocorticoid-inducible enzymes despite normal plasma corticosterone concentrations indicating a degree of hypersensitivity to glucocorticoid action. MLP pups were hypertensive relative to control rats from weaning age. Maternal adrenalectomy successfully ablated the hypertensive state of MLP-male rats which corticosterone, but not aldosterone, replacement to pregnant adrenalectomized rats restored. Hypertension in MLP-males is therefore likely to be glucocorticoid-dependent. Hypertension in MLP-female rats was either delayed or unaffected by maternal adrenalectomy, however, corticosterone replacement to MLP dams elevated the systolic blood pressure of MLP-female offspring.

Hypertension in MLP may be maintained into adult life by an interaction between glucocorticoids and angiotensin II -a principal mediator of glucocorticoid-dependent hypertension. Adrenalectomy of six week old MLP-male rats resulted in a significant decline of systolic blood pressure to control values, which corticosterone replacement negated. No effect of adrenalectomy on systolic blood pressure was observed in control rats. Adult MLP-females exhibited an exaggerated pressor response to intravenous angiotensin II injection at physiological concentrations relative to control female rats.

The data suggest that maternal undernutrition through maternal glucocorticoid influence, programmes hypertension in the resultant offspring in the rat. Increased sensitivity to glucocorticoid and angiotensin II action may partially maintain the hypertensive state. Undernutrition-induced fetal over-exposure to predominantly maternal glucocorticoids may contribute to mechanisms underpinning the incidence of adult hypertension associated with low birthweight.

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ABBREVIATIONS

11-HSD	11β-hydroxysteroid dehydrogenase
ACE	Angiotensin converting enzyme
ADP	Adenosine diphosphate
ADX	Adrenalectomy
AII	Angiotensin II
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
CORT	Corticosterone
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GC	Glucocorticoid
GR	Type II glucocorticoid receptor
HPLC	High Performance Liquid Chromotography
MADX	Maternal adrenalectomy
MLP	Maternal low protein diet (9% casein)
MR	Type I mineralocorticoid receptor
mRNA	Messenger ribosenucleic acid
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
NS	Not significant (P> 0.05)
RAS	Renin-angiotensin system
RU486	11β-(4-dimethylaminophenyl)-17β-hydroxy-17-(propynyl)-4,9-diene-3-one
SEM	Standard error of the mean

SBP Systolic blood pressure

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CHAPTER 1

INTRODUCTION

The founder of medicine, who developed a more rational concept of health and disease, is believed to be Hippocrates. Prior to his philosophy of *health* as a *state of equilibrium between man and his environment*, disease or sickness was thought to be divine in origin, ascribed to the Gods of the time. The abstract, Hippocratic concept of *disease* as a *disharmony between man and his environment* pervaded until late in the nineteenth century. Louis Pasteur and Robert Koch each designed experiments illustrating the specific causation of disease through introduction of specific micro-organisms into the body. That disease could be ascribed to a single factor apparently dispelled any Hippocratic notion of health.

However, working within Pasteur and Kochs' era, Claude Bernard and Charles Darwin made discoveries that married the two previous concepts of health and disease i.e. "Mans inclusive ability to resist infection by micro-organisms" *(specific causation of disease)*, and "the equilibrium of man with the environment" (*Hippocrates view of health*). Darwin was interested in the nature of evolutionary change. He hypothesised that individuals best adapted to the environment would have a better chance of survival. The probability of passing beneficial characteristics, what we now know as genes, into the next generation would therefore be increased. Bernard suggested that the survival of the fittest was a constant interplay between the internal environment (body) and external world. The best adapted individual was the one best able to maintain a constant *milieu interieur* in the face of environmental change. The dual hypotheses of inclusive fitness to the external environment and fixity of the internal environment provided a modern outlook on Hippocratic philosophy of "Mans sympathy with the environment".

Life therefore, is as much dependent upon the physiological and biochemical processes that enable growth and reproduction to take place, as on the control mechanisms that enable the fixity of the internal environment. Each controlled variable has a physiological range of accommodation which allows the organism to cope with environment change. If the range of accommodation is narrowed and subsequently challenged, or the control mechanisms fail, then the controlled physiological processes are disturbed and disease supervenes, as in hypertension. *Hyper* - in Greek meaning beyond, and *tension* - in mechanics meaning stretched or pulled when part of a system. In hypertension the cardiovascular system is stretched beyond its physiological range of accommodation, thus increasing the risk of disease.

LITERATURE REVIEW

1.1. THE CARDIOVASCULAR SYSTEM AND BLOOD PRESSURE

1.1.1. Blood pressure control and high blood pressure

Each cell in the body requires sufficient nutrients for either growth or maintenance and oxygen for oxidation of substrate, yielding energy to fuel these processes. The circulatory system transports these essential nutrients to the cells. The rapidity of nutrient delivery is due to pressure within the closed circulatory system of vertebrates. The pumping of the heart against the resistance of blood vessels generates pressure. Thus the pressure of the arterial circulation is a relationship between cardiac output and total peripheral resistance: ***arterial pressure = total peripheral resistance x cardiac output***

Many mechanisms tightly regulate mean arterial pressure in the short and long term (Levick 1991). The short term control of blood pressure is primarily achieved through neurogenic mechanisms which protect against acute variations in blood pressure. Chemoreceptors respond to variations in arterial P_{o2}, whereas baroreceptors respond to alterations in pressure. Increased complexity of arterial pressure control in the short term is achieved through the action of many hormones and local metabolites acting on vascular

smooth muscle causing either vasodilatation or constriction. Neurogenic mechanisms become desensitised to chronically elevated blood pressure and play a minor role in long term regulation (Sleight *et al* 1975). Long term control is thought to be primarily achieved by renal - body fluid mechanisms that are responsive to and maintain blood volume (Huang *et al* 1992). Blood volume is a function of the osmolality of extracellular fluid, with Na^+ being the major extracellular ion. Thus it is believed that the long term regulation of blood pressure is related to the control of Na^+ concentration (Huang *et al* 1992).

Defining elevated blood pressure is problematic. Pickering (1961) addressed this difficulty and described hypertension as a quantitative deviation from the mean as with "tall" or "overweight" (Pickering 1961). Such a definition may apply to animals also thus, hypertension, in a rat for instance, may be described as the pressure which is a clearly defined deviation from the mean blood pressure established in a given population of controls. Nevertheless, when in an individual blood pressure is elevated, the risk of cardiovascular disease is increased (Thom *et al* 1992), and thus categorisation of people as hypertensive or normotensive has a value for therapeutic decision-making. The US Joint National Committee for Detection, Evaluation and Treatment of High Blood Pressure recommends 'optimal' adult blood pressure be defined as SBP <120 mmHg and diastolic blood pressure <80 mmHg JNCV (1993). Blood pressure was further classified in adults over the age of 18 years as:

Classification of Blood Pressure

Category	Systolic BP (mmHg)	Diastolic BP (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

1.1.2. Forms of hypertension

The many factors that contribute to the regulation of blood pressure may be involved at some stage in the aetiology of primary or essential hypertension (hypertension of unknown origin) which accounts for up to 96% of the elevated blood pressure in humans (Pickering 1961). Hypertension is therefore described as being a multi-factorial disease. For blood pressure to remain elevated, it is likely that more than one factor is altered. In normotensives a perturbation of any component of cardiac output or peripheral resistance sets in motion a series of homeostatic responses that restore the pressure to its original level. In hypertensives either the homeostatic responses are altered, or the sustained elevation represents an operational fault in the central pressure regulatory centre.

Genetic hypertension

High blood pressure clusters in families (Ward 1990). Given that families tend to share a relatively similar environment the precise contribution of specific genes or the shared environment is difficult to determine. However, in a minority of cases hypertension can be ascribed to a single gene defect such as in the syndrome of Apparent Mineralocorticoid Excess (White *et al* 1997) or glucocorticoid-remediable aldosteronism (Lifton *et al* 1992). It may be that in the majority of cases of hypertension, an interaction between a genetic susceptibility together with predisposing environmental factors increase the susceptibility to hypertension. With the application of molecular biological techniques the strength of the contribution of specific genes to the rise in blood pressure may be evaluated together with the role of polymorphisms in candidate genes involved in blood pressure regulation.

Adult lifestyle factors and hypertension

Between-population studies of blood pressure enable the identification of environmental factors that may contribute to an increase in SBP. Dahl (1972) first observed the association between dietary salt intake and high blood pressure, followed by Glieberman (1973), who analysed data from 27 different populations and noted a positive relationship between dietary intake of salt and blood pressure. Although dietary intake of sodium and potassium may play a role in the pathogenesis of hypertension, the precise nature of the

interaction between dietary electrolytes and high blood pressure remains complex and controversial. Similarly obesity and hypertension co-aggregate (Tuck 1994). However, whether obesity precipitates hypertension or the two are merely markers for an underlying metabolic or hormonal abnormality is not known.

Certainly defects of insulin metabolism associate with obesity and hypertension and thus it has been suggested that changes in insulin metabolism may underlie the hypertensive state (Reaven & Hoffman 1987). The co-association of insulin resistance, hypertension, obesity, glucose intolerance and alterations in lipid metabolism, has been called 'Syndrome X', because no single factor can be attributed to the development of either obesity or hypertension in this syndrome (Reaven & Hoffman 1987). However, hypersecretion of the glucocorticoid cortisol, as occurs in Cushings syndrome, promotes both elevated SBP, central adiposity and insulin resistance. Consequently, Cushings patients are frequently diagnosed with hypertension and characteristics of the metabolic syndrome or "Syndrome X" (Ross & Linch 1982; Bujalska *et al* 1997). Physical inactivity is associated with elevated blood pressure and risk of CHD. Physical activity, either indirectly through effects on body weight regulation or direct effects on the sympathetic nervous system, insulin sensitivity, electrolyte balance, vascular structure or neural mechanisms reduces this risk (Kannel *et al* 1986). Additionally, excessive consumption of alcohol is believed to be causally related to hypertension (MacMahon 1987), perhaps through a direct pressor effect of alcohol itself or through an effect on the activity of 11-HSD, since alcohol inhibits 11-HSD *in vivo* (Stewart *et al* 1993).

1.1.3. Secondary hypertension

The above factors have an established involvement in the aetiology of essential hypertension. Some of the factors, may be remediable but, even following correction, hypertension may remain. Identifying the primary cause at a late stage of the process is clearly difficult given the complexity of the interactions. Two factors have been clearly identified for the promotion of primary hypertension and contributing to the maintenance of secondary hypertension: renal mechanisms and steroid hormones.

Renal mechanisms

Renal function determines fluid and electrolyte balance and exerts a major impact on the control of blood pressure in the long term. Patients with renal disease invariably develop secondary hypertension e.g. the occurrence of renal disease in hypertensives is approximately 4%, whereas up to 90% of patients with renal disease develop hypertension (Wilkinson 1994). Although the common endpoint of hypertension is an increase in total peripheral resistance (TPR), Guyton *et al* suggest that hypertension may develop in the face of a reduced TPR provided a kidney defect is present (Huang *et al* 1992). The evolution of hypertension of renal origin is associated with perturbations in sodium homeostasis (Hall & Granger 1994). Renal maintainance of blood volume is primarily through a cycle involving control of sodium concentration although other factors such as production of renal nitric oxide synthase may play a role. Increased plasma [Na^+] increases blood volume, leading to an increased renal perfusion pressure which stimulates pressure natriuresis to reduce blood volume and thus SBP (Hall & Granger 1994). Renal (Renin-angiotensin system) and cardiac (Atrial Natriuretic Peptide) hormones and other factors such as adrenomellin and endothelin also respond to changes in plasma [Na^+] and blood pressure (via baroreceptors), to readjust the sodium excretion appropriately (Hall & Granger 1994). Furthermore, decreased production of renal prostaglandins e.g. PGE_2, PGI_2 and 6-ketoPGE$_1$ which increase renal blood flow and sodium and potassium excretion are associated with hypertension (Quilley & McGiff 1994).

Steroid mechanisms

The major secretions of the adrenal cortex, mineralocorticoids and glucocorticoids, are integral to maintaining normal cardiovascular and metabolic homeostasis. The mineralocorticoids of the adrenal cortex are chiefly the steroids aldosterone and 18-hydroxycorticosterone (18OHB), both secreted from the zona glomerulosa (ZG) and deoxycorticosterone (DOC) and 18 hydroxy-deoxycorticosterone (18OH-DOC), which are secreted from the zona fasiculata (ZF). Mineralocorticoids, having a direct action on the kidney, primarily influence the pathogenesis of hypertension through effects on sodium

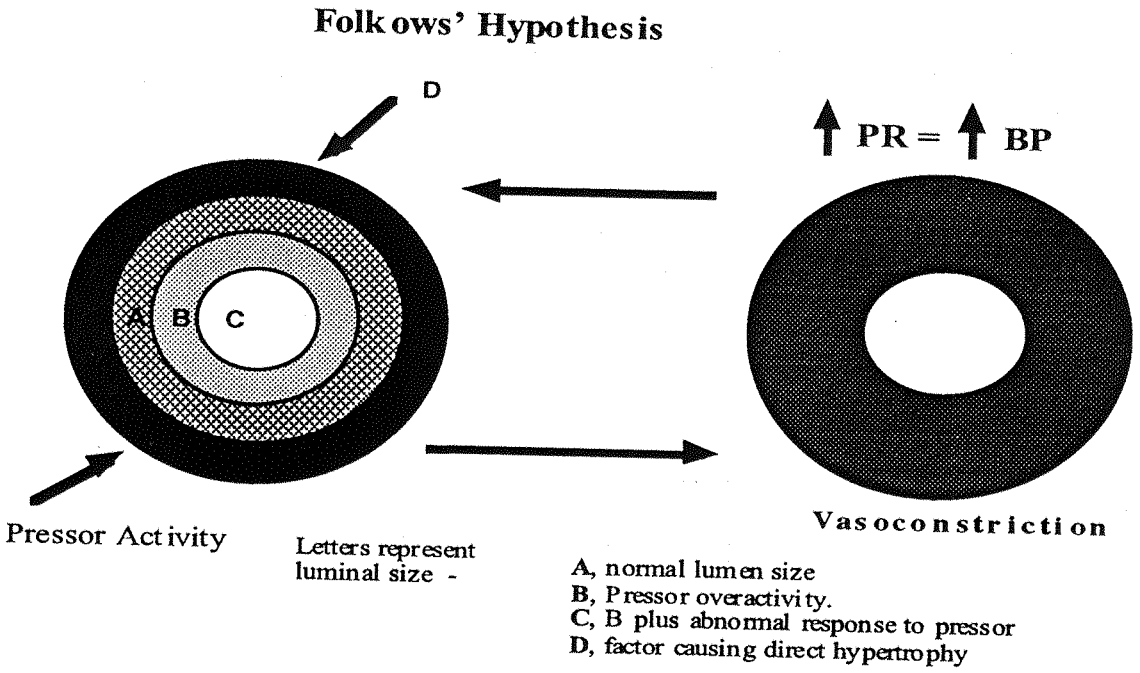
homeostasis (Mantero *et al* 1991). Defects in the ZG region, either inborn errors or adrenocortical tumours, promote primary aldosteronism (elevated plasma aldosterone), and hypertension (Mantero *et al* 1991). Hypersecretion of the glucocorticoid cortisol, from the ZF, as occurs in Cushing's syndrome, promotes elevated SBP. Consequently, patients with Cushing's frequently present with hypertension (Ross & Linch 1982). Glucocorticoids have both direct and indirect effects on blood pressure control as discussed later in this chapter.

A local excess of glucocorticoids may result from a defect in the activity of the enzyme 11β-hydroxysteroid dehydrogenase Type 2 (11-HSD2) (there are two isoforms 11-HSD2 and 11-HSD1 which differ in tissue distribution, characteristics, ontogeny and between species and are described in section 1.7). This enzyme metabolises bioactive cortisol to its inactive 11-oxo metabolite cortisone. Hypertension is a feature of AME, in which there is a reduced activity of 11-HSD2 (Stewart *et al* 1988). The high concentration of glucocorticoid freely stimulates the unprotected mineralocorticoid receptor (MR) leading to increased blood pressure. Similarly, a reduction in the activity of 11-HSD2 mediates elevated systolic blood pressure in people who excessively consume licorice, which contains glycyrrhizic acid and its product glycyrrhetinic acid, since both inhibit 11-HSD2 (Stewart *et al* 1987).

1.1.4. Maintenance of secondary hypertension

Whilst renal mechanisms and steroid hormones are clearly involved in the production of secondary hypertension, other additional factors may facilitate maintenance of the hypertensive state. Thus when a known primary cause of hypertension e.g. an adrenal adenoma has been removed, hypertension may still remain. Thus mechanisms secondary to the initial insult maintain the elevated SBP. Folkow has suggested a mechanism which has been widely accepted as the explanation (Folkow 1978). An elevation in SBP leads to a structural change in resistance vessels leading to a further elevation of blood pressure, a positive feedback cycle (Figure 1.1). The critical change was hypertrophy of vascular smooth muscle in response to chronically elevated pressure. The finding of a slow progressive rise of SBP in essential hypertension lends support to this view (Lever 1986).

Figure 1.1. **A diagrammatic representation of Folkows Hypothesis**



Controversy remains over the nature of the initial insult promoting vascular hypertrophy although, most agree, that it occurs early in development (Berry 1978; Folkow 1978; Lever & Harrap 1992). Short term over-activity of a trophin or mitogen coupled with an abnormal vascular response to that agent could initially raise blood pressure, initiating vessel hypertrophy. Both may be genetically or environmentally determined (Folkow 1978). Increased SBP in early cardiovascular experience and consequently the altered haemodynamic load that may follow was proposed by Berry (1978) to either constitute or amplify the initial stimulus.

The vascular action of angiotensin II, cortisol and insulin-like growth factors in early cardiovascular development may initiate changes in vascular cell structure and were suggested as initial stimuli in cardiovascular structural adaptation (Folkow 1978; Lever & Harrap 1992). SBP rises with age (Lever & Harrap 1992) and the degree of age-related increase may be partially determined by the genetic background but amplified by environmental factors acting early in life (Lever & Harrap 1992). The relative importance of each of these factors is uncertain, although recent evidence suggests that the early environment profoundly influences future metabolic competence, in terms of physiological control (Barker 1994).

Chronic elevation of SBP represents a major risk factor for CHD, the main cause of death in industrialised societies (Thom *et al* 1992). Projections for the future indicate an epidemic of CHD in developing countries (WHO 1991). The most important appraoch to the management of CHD is prevention. Prevention has primarily been directed towards changes in adult 'lifestyle' such as increasing physical activity and reducing obesity. However research conducted by Barker *et al* suggests that prevention of CHD in an individual may require consideration of factors which operate in the womb (Barker 1995).

1.2. THE FETAL AND INFANT ORIGINS OF CARDIOVASCULAR DISEASE

1.2.1. Epidemiological studies

Geographical studies of the incidence of CHD amongst different locations in England and Wales formed the basis of the 'early origin of adult disease hypothesis' (Barker 1994). Adult 'lifestyle' factors alone can not explain the difference in CHD between different areas of England and Wales, the highest rate of CHD being recorded in northern industrial towns and South Wales (Barker & Osmond 1986). Furthermore there is a close regional relationship between the incidence of CHD and infant mortality (Barker & Osmond 1986; Barker & Osmond 1987). Areas that had past high neonatal mortality are characterised by a higher proportion of mothers with poor physique and health, who give birth to lower birthweight babies (Barker & Osmond 1989). In addition, both stroke and CHD in adult life correlate with past maternal mortality (Barker & Osmond 1987). Adverse environmental conditions acting in early life which influence birthweight and neonatal and maternal mortality may, therefore, determine later risk of CHD.

To further pursue the hypothesis, cohorts of middle aged people were found, for whom detailed birth records were available. In Hertfordshire a collection of records (1911-30) detailed birthweight and weight at one year. Analyses of these data showed a striking inverse relationship between birthweight or weight at one year and the development of cardiovascular disease in later life (Barker *et al* 1989b). Lower weight at birth and at one year of age was

associated with a higher blood pressure in childhood and adult life and increased risk of cardiovascular mortality in men. The relationship was subsequently shown for women also (Law *et al* 1991; Osmond *et al* 1993). No measurements other than birthweight were recorded for the Hertfordshire cohort.

Birthweight is a relatively crude proxy for fetal growth, and length of gestation (not recorded in the Hertfordshire records) is obviously important in determining weight at birth. Therefore further cohorts (Preston (1935-43) and Sheffield (1907-23)) were studied for which length of gestation and specific body proportions had been recorded. The standardised mortality ratios for CHD in the Sheffield cohort were more strongly related to small head circumference and low ponderal index (weight/length^3) at birth than to birthweight alone (Barker *et al* 1992a). Importantly, these measurements were associated with small for gestational age babies rather than babies who were simply premature, as CHD was not related to gestation length. Thus from the Sheffield data it was inferred that adult CHD was related to a pattern of intrauterine growth leading to babies who were thin at birth with a small head circumference.

Data from the Preston cohort reaffirmed the inverse relationship between birthweight and adult CHD risk and also suggested that babies who were short at birth, with a reduced abdominal circumference in relation to head size, were as much at risk of CHD as the thin babies (Barker *et al* 1990). Furthermore there was also a positive relationship between placental weight and adult blood pressure (Barker *et al* 1990). Babies who were of low birthweight with a corresponding large placenta recorded the highest SBP. These results were subsequently duplicated in 4-year old children in a separate study (Law *et al* 1991). Elevated SBP is therefore more strongly related to disproportionate intrauterine growth than birthweight *per se*, and is evident from childhood into adult life.

The inverse association between birthweight and adult systolic and diastolic blood pressure was a consistent feature of all the data obtained from the Hertfordshire, Sheffield and Preston cohorts. Further data indicated relationships between birthweight and impaired glucose tolerance (Hales *et al* 1991), diabetes (Lithell *et al* 1996), plasma clotting factors (Barker *et al* 1992b) and serum cholesterol (Barker *et al* 1993b) which are all considered risk factors for CHD. The relationships found with adult disease are graded with birth weight and

more strongly graded with specific birth disproportions, and are observed, importantly, within the normal range of birthweight.

1.2.2. The early origin of adult hypertension

In all of the cohorts (Sheffield, Preston and Hertfordshire) the inverse relationship between systolic blood pressure in adult life and low birthweight was apparent (Barker *et al* 1990; Barker *et al* 1989b; Law *et al* 1993) The inverse relationship had been observed previously in a national sample in the UK (Wadsworth *et al* 1985) and a Swedish cohort (Gennser *et al* 1988) and has since been observed in over twenty studies (Law *et al* 1993) including many different populations and cultures (Forrester *et al* 1996; Launer *et al* 1993; Yajnik *et al* 1995). The nature of the association between low birthweight and adult systolic blood pressure in each study was graded, occurred within the normal birthweight range and was not related to gestation length. Blood pressure is known to 'track' growth during childhood and adulthood i.e. a rank order of blood pressure measurements is maintained in serial measurements of subjects, from an early age into adulthood (Law *et al* 1993; Lever & Harrap 1992; Whincup *et al* 1995). Hence, it is suggested that adult hypertension may be programmed in early life and become amplified later by environmental factors. For example, hypertension is most prominent among those who were of low birthweight and subsequently become obese as adults (Frankel *et al* 1996; Leon *et al* 1996).

The risk of adult disease and hypertension is related to early environment and in particular, factors that influence growth in early life leading to a disproportionate, low or normal birthweight baby at term. However, various factors that may confound the association between birthweight and adult disease should be addressed.

1.2.3. Confounding variables

The fetal origins of adult disease hypothesis has been criticised in the literature, predominantly in relation to the methodology used to determine the relationships seen with adult disease (Paneth & Susser 1995; Paneth 1996). Socio-economic disadvantage influences intrauterine growth and postnatal growth (Bartley *et al* 1994). Thus it has been suggested that the postnatal environment determines the association with adult disease. However,

associations between birthweight and adult disease are found for all social classes (Barker 1994), in varied populations such as Sweden (Gennser *et al* 1988), America (Yiu *et al* 1994), Jamaica (Forrester *et al* 1996) and India (Yajnik *et al* 1995) and in animal models (Langley & Jackson 1994). Migrant studies have been used to determine the relative contribution of environment. The relative risk for hypertension is carried from birth regardless of migration patterns in adult life (Osmond *et al* 1990). Furthermore the relationship between CHD and birth proportions appears to be specific, since in none of the studies has the relationship been observed with death from all non-cardiovascular causes (Barker 1994).

The association of high blood pressure with birthweight was not observed in adolescents (Matthes *et al* 1994). However adolescents undergo a hormonally induced pubertal growth spurt which may confound the results, since the age-related 'tracking' of blood pressure is also disturbed during adolescence (Lever & Harrap 1992). Twins are relatively smaller at birth than singletons. Consequently, it has been suggested that the incidence of CHD may, theoretically, be higher in adult twins. However no relationships between birthweight and adult cardiovascular disease have been noted in twins (Christensen *et al* 1995; Vagero & Leon 1994). Twins exhibit an early proportionate growth pattern. Thus growth of dizygotic and monozygotic twins is restricted early in gestation partly by intrauterine space and partly by placental factors, as opposed to the disproportionate growth retardation induced in singletons in late gestation where adequate intrauterine space is available. However, often one twin is born slightly lower in birthweight than the other twin, and thus the potential risk of hypertension and CHD in the smaller twin is of interest. One study proposing to counter the Barker hypothesis, analysed pre-term infants, infant nutrition and later blood pressure and found no relationship (Lucas & Morley 1994). Despite preterm infants being entirely different, in terms of early postnatal experience, to SGA infants, all of Barkers studies emphasize that the effects on the developing fetus are exerted *in utero* and only amplified postnatally.

1.3. REGULATION OF INTRAUTERINE GROWTH

1.3.1. Maternal nutrition

Birthweight is determined by genetic and environmental factors including maternal size and body composition, maternal nutrition, hormones and growth factors. The predominant influence on fetal growth is the maternal environment rather than the genotype of the fetus. Cross-breeding experiments between Shetland ponies and Shire horses (Walton & Hammond 1938) and the high correlation between birthweights of siblings attest to this. Furthermore birthweights of siblings with a common mother rather than father tend to be more highly correlated indicating the predominant influence of the maternal environment (Milner & Gluckman 1996). Thus in studies relating birthweight to adult disease, it is the factors that constitute the intrauterine environment and which influence fetal growth that are important.

The fetus in late gestation develops in an essentially constrained environment, as is suggested by the reduced birthweights of multiple pregnancies (Gluckman *et al* 1990). The fetal genome may determine the genetic potential for growth, but growth is potentially constrained by energy, nutrients and the quantity and quality of nutrients and oxygen that are delivered and made available to the fetus within the confines of the uterus (Gluckman *et al* 1990). Manipulation of the substrate supply i.e. maternal nutrition, can therefore have profound effects on the fetus (McCance & Widdowson 1974). The precise effects of maternal undernutrition on the fetus, however, may be modulated by many factors such as maternal metabolism, the placenta and the nature of the undernutrition itself i.e. the severity, duration and timing (Dobbing 1981; Widdowson & McCance 1963; Winick & Rosso 1974).

The earlier the undernutrition occurs, the more likely that permanent overall reductions in growth and form will be apparent (Widdowson & McCance 1963). McCance & Widdowson (1974) suggested this to be related to the particular stage in the growth process (McCance & Widdowson 1974). Early fetal growth, not being constrained by the maternal environment, is defined by genetically determined limits. The genetic potential can only be realised if the environment encountered is optimal. Nutrient restriction therefore precipitates an adaptive down-regulation of fetal growth in order to closely match demand with substrate

supply (McCance & Widdowson 1974). In early gestation, maternal undernutrition results in an adaptive reduction of fetal growth which is then able to be maintained in late gestation thus avoiding disproportion. Characteristically, babies undernourished in early development are symmetrically small (Barker 1995). These babies are, however, unable to catch-up in growth in postnatal life (Barker 1995; Widdowson 1971).

Mid-gestational undernutrition is thought to induce placental enlargement and reduce fetal growth (Pond *et al* 1991). The placenta, having a considerable reserve capacity for adaptive growth, exhibits compensatory hypertrophy in order to facilitate nutrient supply to the fetus (Pond *et al* 1991). In mid gestation the nutrient demands of the fetus per unit weight are relatively high, due to the large proportion of metabolically active organ tissue. The placenta therefore maintains a priority of growth in mid-gestation which the fetus may partially facilitate by mobilising endogenous stores following a nutritional insult (Owens *et al* 1994). The mid-gestationally undernourished fetus is therefore characteristically long and thin reflecting peripheral wasting (Barker 1995). Peripheral muscle loss may increase the susceptibility towards non-insulin dependent diabetes (NIDDM) given that muscle is the major site of insulin action (Hales *et al* 1991).

During late gestation in sheep, when fetal weight as a proportion of maternal weight is relatively high, undernutrition results in a prompt reduction in fetal growth (Harding & Johnston 1995). Blood supply is redistributed to organs that are essential for survival under conditions of intrauterine stress, such as the brain, heart and adrenal glands and to those organs that have completed a large proportion of their growth (Rudolph 1984). These adaptive responses produce a disproportionate pattern of fetal growth. The phenotype of the late gestational undernourished fetus is relatively shorter than would be expected from its head size (Barker 1994) reflecting, perhaps, the hierarchical nature of intrauterine growth in human fetuses i.e. maintenance of the brain > heart > adrenal > other peripheral organs such as the liver and lungs.

The long term impact of maternal undernutrition i.e. whether the organism is able to recover from the nutritional insult or not, may relate to the concept of cellular growth as proposed by Winick and Noble (Winick & Noble 1966). They suggest growth proceeds in three steps, a period of cellular hyperplasia early in development followed by a period of

hyperplasia and hypertrophy i.e. an increase in cell size, followed by a period of hypertrophy only (Winick & Noble 1966). Undernutrition is likely to exert a permanent effect, manifest as permanently reduced organ weights as a proportion of bodyweight, or an inability to display postnatal 'catch-up' growth, if encountered during periods of cellular hyperplasia. The actual number of cells may be reduced and if the undernutrition continues beyond the point at which cell division would normally end, then the insult is irreversible. If the insult occurs during periods of cellular hypertrophy then the actual size of existing cells may be reduced which is potentially reversible with refeeding (Winick & Noble 1966).

1.3.2. Maternal nutrition and later outcome

Human studies

Many studies have highlighted the importance of maternal nutrition in determining birth outcome and in relation to later cardiovascular anomalies. Thus an inverse relationship exists between maternal haemoglobin concentration and the systolic blood pressure of the subsequent children aged 10-12 years (Godfrey *et al* 1994). Moreover mothers with low haemoglobin had lower skinfold thickness', which were inversely related to placental weight (Godfrey *et al* 1991) and the child's systolic blood pressure (Godfrey *et al* 1994). Lower skinfold thicknesses and reduced haemoglobin are generally indicative of nutritional inadequacy. Mothers who demonstrated lower weight gains in pregnancy gave birth to children who were more susceptible to higher blood pressure (Godfrey *et al* 1994). However, reduced haemoglobin concentration is a crude marker for nutritional state. More accurate indices of maternal nutrition may be established through analysis of nutrient intake. A complex interaction between protein, carbohydrate, birthweight and adult SBP has been observed. In a cohort of 538 Southampton women, mothers consuming low intakes of dairy protein in late gestation gave birth to smaller babies (Godfrey *et al* 1996). However a high intake of carbohydrate in early gestation was also associated with lower birthweight. A follow-up study of 43-47 yr old men in Aberdeen indicated that higher SBP were recorded in

those men whose mothers consumed a low intake of protein coupled with a high intake of carbohydrate (Campbell *et al* 1996).

Dietary supplementation of Gambian women, who generally give birth to low birthweight babies relative to the UK, increased birthweight during the wet season, when the women would have been expected to be in negative energy balance without the supplement, but not during the dry season (Prentice *et al* 1987). It is suggested that a threshold may exist whereby supplementation is only effective when the mothers are at the limits of their ability to maintain a positive energy balance (Prentice *et al* 1987). In the Dutch Hunger Winter famine, which lasted seven months, babies who were exposed to the famine conditions in early gestation, and born after the famine ended, were of normal birthweight, but those that were exposed in mid-gestation were born of lower birthweight (Smith 1947).

Animal studies

The data in the human population finds much support in the literature from studies in animals of different species. The pig gives birth to multiple young who naturally vary considerably in size in relation to different positions within the uterine horns and the nature of the uterine blood supply. It was found that the most undernourished or 'runt' pig displayed large differences in body composition and postnatal growth compared to its littermates (Widdowson 1971). Restriction of dietary protein during gestation also has many effects on the resultant offspring. Reduced fetal growth, in association with increased placental size has been observed in pigs (Pond *et al* 1991) and rats (Langley & Jackson 1994; Levy & Jackson 1993). Studies in the rat illustrate how a maternal low protein diet influences aspects of physiology and cellular biochemistry in the adult offspring. The manifestations are primarily related to the effect of a late gestational nutritional insult i.e. on organs that exhibit a large proportion of growth in late gestation, such as the kidney, and the liver, whose growth is compromised to maintain growth of the brain in the face of a nutritional challenge. Hepatic function is altered in terms of glucose metabolism (Desai *et al* 1997; Desai *et al* 1995) and pancreatic function is altered in terms of insulin secretion (Swenne *et al* 1987). Pancreatic vascularisation is also reduced by a low protein diet (Dahri *et al* 1993) therefore influencing function (Dahri *et al* 1991; Snoeck *et al* 1990) that may lead to an insulin resistance syndrome

and NIDDM (Dahri *et al* 1993). The growth of the kidney is compromised by maternal protein restriction in terms of nephron complement (Zeman 1968) and function (Welham *et al* 1996).

In human populations, degrees of obesity are associated with low birthweight (Frankel *et al* 1996; Leon *et al* 1996) and are observed following a nutritional insult during gestation (Ravelli *et al* 1976; Smith 1947). Similarly, in animal studies, a maternal low protein diet, whilst reducing birthweight, leads to obesity (Anguita *et al* 1993; Ravelli *et al* 1976), degrees of glucose intolerance (Langley *et al* 1994) and an altered insulin secretory response to glucose (Pickard *et al* 1996) that then may predispose to obesity.

1.3.3. Hormonal regulation of growth

The major hormones that are classically involved in postnatal growth, such as growth hormone, have little influence in fetal life (Karlberg *et al* 1994). Fetal growth is essentially within a constrained environment (Gluckman *et al* 1990) and thus the major influence of hormones is to mediate the partitioning and utilisation of substrate by the fetus. Whilst fetal growth is enabled by substrate, the control is more complex than a direct relationship between substrate supply and demand. Reduced fetal and organ growth rates may be a response to maternal undernutrition but often reduced organ weights are noted prior to any obvious limitation on substrate supply, such as in early gestation when fetal demands are low (Pond *et al* 1991). The indirect effects of nutritional restriction on fetal endocrine status are therefore important in determining the relationship between undernutrition and fetal growth rates (Harding & Johnston 1995).

In early gestation fetal growth proceeds autonomously without obvious maternal constraint. During embryogenesis the somatomedins or insulin-like growth factors (IGF), acting in a paracrine/autocrine mode, influence cellular growth and differentiation (Han & Hill 1994). Glucocorticoids exert an influence on cellular differentiation (Meyer 1985) and in early gestation maternal glucocorticoids enter the fetal compartment (Murphy *et al* 1974), thus a role for glucocorticoids should be considered in early developmental events. In later gestation when the feto-placental unit has become well established, the placenta produces and secretes steroid hormones into the fetal compartment. Steroid hormones of maternal origin such as

cortisol are, however, largely prevented access to the fetus at this time due to placental metabolism (Murphy *et al* 1974). Thyroxine is able to permeate the placental barrier and appears essential to normal fetal development, especially that of the brain (Tanner 1989). The tissue specific action of thyroxine in fetal development is most likely permissive and in some cases, synergistic with glucocorticoid (Smith & Sabry 1983).

The evidence to date suggests that fetal growth in late gestation is regulated by the IGFs (Gluckman 1995; Han & Hill 1994) and also by insulin (Fowden 1989). Insulin stimulates whole body tissue accretion and increases the availability of nutrients for cell growth and multiplication (Fowden 1989), whilst fetal IGFs determine the utilisation of nutrients at the cellular level and the partitioning of nutrients between the feto-placental unit in favour of the fetus (Gluckman 1995). Dissociating an auto/paracrine role for the IGFs from an endocrine role is, however, difficult.

Since fetal growth requires adequate substrate then the prime regulator of factors that influence fetal growth may be nutrient availability. Thus the action of IGFs is influenced by maternal undernutrition (Bauer *et al* 1995; Owens *et al* 1994). The density of tissue receptors for the IGFs, the IGF-binding proteins and the regulation of each by nutritional factors provide a further complexity through which the somatomedins influence fetal growth in late gestation (Han & Hill 1994). Maternal undernutrition in late gestation may change the hormonal environment from an anabolic to a catabolic state thus resulting in elevated concentrations of catabolic hormones such as glucocorticoid (Goland *et al* 1993) and reduced concentrations of IGF-1 (Bauer *et al* 1995; Lassarre *et al* 1991) and insulin (Fowden 1989).

There are many interactions between the nutritional and endocrine regulation of fetal growth. The physiological changes in the endocrine environment in response to maternal undernutrition and the resultant effects on fetal growth may be important in determining later outcome. The ability of early life events to permanently alter physiology, biochemistry and behaviour has been recognised for some time (Arai & Gorski 1968; Horn 1985) and has recently become known as 'programming' (Lucas 1991). The vulnerability of development to nutritionally-induced 'programming' of adult-onset pathophysiology such as hypertension and obesity represents a novel perspective on the development of chronic disease.

1.4. THE THEORY OF PROGRAMMING

1.4.1. Vulnerability in development and the concept of programming

Imprinting was a term coined by Konrad Lorenz. He observed how a gosling follows the first moving object it sees after hatching and then subsequently follows it thereafter, whether the object was its mother, a human, or a dog (Lorenz 1952). A developmentally sensitive period existed in which the image of the first moving object was 'imprinted'. For the young gosling this was a biological imperative, since the first moving object it saw was likely to be its mother. Imprinting therefore implies a physiological process and a notion of time. Widdowson & McCance (1975) stated that, "the whole developmental life of the animal may be profoundly altered by events during a very short period in it and thus have been termed the critical periods of development" (Widdowson & McCance 1975). A vulnerable period is therefore a defined window of time in an animals development where the physiological systems of the body are receptive or sensitive to particular set of stimuli.

Three concepts underpin the hypothesis of vulnerable periods in development:

1. Vulnerability occurs during growth of the organism.

The *adult* brain can survive and function normally after quite severe bouts of starvation whereas mild undernutrition during brain *development* is sufficient to produce marked changes in its physiology (Dobbing 1981).

2. Vulnerability depends on stage and velocity of growth.

Cells are more vulnerable during periods of relatively rapid growth. Programming of function/morphology is more likely to be permanent during periods of hyperplastic rather than hypertrophic growth (Winick & Noble 1966). In sheep a nutritional insult in late gestation impacts predominantly upon more rapidly growing fetuses. Those fetuses growing at a slower rate are able to maintain their rate of growth (Harding *et al* 1992).

3. Vulnerability implies a chronological aspect to development.

Genetic potential can only be fulfilled if the environment encountered is optimal (Widdowson & McCance 1975). Specific organs, tissues and even cells are especially susceptible to a particular stimulus at a given point in the development of that structure. The genetic background of an animal determines the timing and duration of the vulnerable period.

Timing of vulnerable periods may relate to the stage of growth i.e. hyperplastic *vs* hypertrophic, but also represents a period whereby physiological processes are able to respond to the surrounding environment, ultimately for the survival benefit of the fetus.

1.4.2. Programming stimuli

It is well established that outside the field of nutrition, many agents are able to permanently programme a set of homeostatic events. Hypothalamic organisation in the rat and secretion of gonadotrophins can be permanently switched from a female cyclical pattern to the male acyclical pattern by a single androgen injection at day 5 of postnatal life (Harris & Levine 1962). Rats subsequently develop as genetic females but do not ovulate or show normal female sexual behaviour (Barraclough 1961). A later injection (day 10-15) has no effect. Similarly, neonatal androgen exposure permanently alters hepatic androgen responsiveness (Gustafsson & Stenberg 1974) and properties of oestrogen-binding proteins (Sloop *et al* 1983). Exposure of rats to high levels of thyroxine during the first week of postnatal life permanently alters the subsequent function and ability of the hypothalamus to secrete thyroid stimulating hormone (Besa & Pascual-Leone 1984). Exposure to hormones, in particular steroid hormones, may therefore programme physiological functions and biochemical pathways at both the central (hypothalamic) and peripheral (hepatic) level. Aspects of neural function may also be programmed by events in early life. In kittens, during the first month of life brain neuronal connections are highly plastic, and the response properties of these neurones can be manipulated by altering the visual sensory input (Horn 1985). A similar protocol has no effect in adult cats.

Thus "programming", is the process whereby natural stimuli or environmental insults acting during sensitive periods of development may have lifelong or permanent consequences (Lucas 1991). Programming is a natural process facilitating survival of that organism, for example; sexual orientation, the following response of goslings and the persistence of an immunological memory (antibodies) after exposure to a single antigen (Abbas *et al* 1991). The relationship between cardiovascular anomalies in adult life and disproportionate intrauterine growth are not fully understood but are thought to represent part of a programming phenomenon that may be nutritional in origin (Barker 1994).

1.4.3. The programming of adult disease

Maternal nutrition and the hormonal environment may influence fetal growth and programme homeostatic functions and body structure in later life. Permanent alterations in the form and function of the body may be a consequence of programming at the cellular or subcellular level e.g. maternal protein restriction permanently reduces cell number and alters the size of many organs including the liver (Zeman & Stanborough 1969), brain (van Marthens & Shimomaye 1978), placenta (Hastings-Roberts & Zeman 1977) and kidney (Zeman 1968). This may be a later consequence of nutritional insults during periods of rapid cell growth i.e. during cell division (Winick & Noble 1966)

Alterations in enzyme activities that have been noted following maternal protein restriction (Desai *et al* 1995) may reflect programming of gene expression. Indeed a permanent elevation of mRNA for phosphoenolpyruvate carboxykinase (PEPCK) has been noted following maternal protein restriction (Desai *et al* 1997). A further example of enzymatic programming is temperature dependant sex determination in crocodiles, where at a specific temperature (33°C), a hypothalamic isomerase, that influences sex-specific organisational events in the hypothalamus and thus gonadotrophin secretion, is switched on resulting in all male offspring (Deeming & Ferguson 1989).

Whilst programming of gene expression is an attractive proposition, an alternative hypothesis may be through programming or clonal selection of specific cell lines such as occurs in the immune system (Abbas *et al* 1991). Undernutrition during specific periods may alter the clonal selection of cell types resulting in an altered proportion of cells within given organs that influences the later function of that organ (Lucas 1991). Maternal protein restriction alters hepatic enzyme activities that are specifically located to particular regions of the liver lobule (Desai *et al* 1995). Desai *et al* attribute the altered activities to differences in the proportion of cells in periportal and perivenous lobules of the liver leading to changes in hepatic function (Desai & Hales 1997).

For steroid hormones, programmed alterations in either receptor populations (Meaney *et al* 1988) or secretion (Besa & Pascual-Leone 1984) due to early life events may permanently programme the activity of steroid dependent enzymes. Indeed maternal protein deficiency in rats alters glucocorticoid receptor numbers in both the mother, which may be an

adrenal-dependant function, since maternal glucocorticoid levels may rise following protein restriction (Lunn & Austin 1983), and the fetus (Mulay *et al* 1982), which may thus have important consequences for the fetus and the range over which the fetal HPA responds in adult life. Changes in adult hormonal axes determined by prenatal nutritional insults may indirectly account for altered fetal physiology through effects upon all of the above programming mechanisms, or directly through hormonally induced mechanisms.

1.5. ANIMAL MODELS OF HYPERTENSION

Through the use of animal models in hypertension research, many preliminary insights into mechanisms that may potentially operate in hypertension have been discovered. Animal models carry many experimental advantages over human research. Experiments can be conducted that are invasive and could not be carried out in humans. Experimental control can be exerted so that single factors can be manipulated to elucidate the disturbed physiological processes in hypertension models. The hypertensive state can be monitored longitudinally over the animals lifetime or over generations within a relatively short time. Research using animal models allows the biochemical and physiological mechanisms involved in the control of blood pressure to be investigated. The evidence can then be used to design suitably focussed studies within the human population.

1.5.1. Genetic models of hypertension

A number of genetic models of hypertension are available as shown in Table 1.1. Perhaps the most widely used genetic model in hypertension research is the Spontaneously Hypertensive Rat (SHR). Okamoto and Aoki (1963) developed the SHR through selective in-bred matings of rats with the highest blood pressures (Okamoto & Aoki 1963). However, despite considerable success in elucidating mechanisms of physiological control in hypertension, use of genetically inbred strains of rats has yielded scant information on the genes involved. It is thought that eventually the candidate genes will be isolated and then investigated in human hypertension.

Table 1.1. Animal Models of Hypertension

Model of Hypertension	Type of Hypertension	F Generation	SBP (mmHg) above control
SHR	Genetic	20	50-80
DSS	Genetic	20	50-70
MHR	Genetic	70	30-50
LHR	Genetic	nd	40-50
NZHR	Genetic	20	20-30
SaHR	Genetic	nd	20-30
SHM	Genetic	nd	nd
Goldblatts 1	Renovascular	0	50
Goldblatts 2	Renovascular	0	50
SAC	Renovascular	0	40-50
RCP	Renovascular	0	30
DOCA	Steroid	0	*50
DEX	Steroid	0	*40-50
ARH	Steroid	0	50
MDEX	Steroid	1	10-30
IUGR	Nutritional	1	<10
MLP	Nutritional	1	10-20
Anaemic	Nutritional	1	10

SHR, spontaneously hypertensive rat; DSS, Dahl salt-sensitive; MHR, Milan hypertensive rat; LHR, Lyon hypertensive rat; NZHR, New Zealand hypertensive rat; SaHR, Sabre hypertensive rat; SHM, spontaneously hypertensive mouse; Goldblatts 1, 1 kidney-2 clip, 2, 2 kidney-1clip; SAC, suprarenal aortic coarctation; RCP, renal compression 'Page' hypertension; DOCA, deoxycorticosterone-acetate; DEX, dexamethasone induced; ARH, adrenal regeneration hypertension; MDEX, maternal dexamethasone exposure; IUGR, uterine arterial ligation induced intrauterine growth retardation; MLP, maternal low protein induced. Anaemic, maternal anaemia. SBP, systolic blood pressure (adult); * dependent upon dose; nd, no data available.Sources:*(Genetic, Renovascular & Steroid) - Textbook of Hypertension 1994. editor J.D.Swales(MDEX) - Benediktsson et al 1993; (IUGR) - Persson & Janson 1992; (MLP) Langley & Jackson 1994; (Anaemia) - Crowe et al 1995*

However, in the process of identifying a colony of rats which were susceptible to the development of high blood pressure, factors other than 'hypertensive genes' might also have been isolated. For example, the SHR is born of lower birthweight with a corresponding large placenta than Wistar-Kyoto (WKY) controls (Johnston 1995), an association identified for high SBP in the human population (Barker *et al* 1990). Furthermore the high blood pressure in the SHR can be modified experimentally by either embryo transfer (Azar *et al* 1991), or postnatal cross-fostering to WKY dams (Cierpal & McCarty 1987) indicating a maternal factor in the aetiology of *genetic* hypertension in the SHR.

1.5.2. Experimentally-induced hypertension

A number of animal models exist in which hypertension is produced through physiological manipulation (Table 1.1). The primary systems targeted by interventions are the kidney and adrenal gland. Among the models are renovascular-induced (Goldblatts, aortic coarctation) and steroid-induced (deoxycorticosterone-acetate, dexamethasone, adrenal regeneration). Renovascular hypertension is produced through clipping, with an adjustable silver clip, either one or both of the renal arteries, combined with/without unilateral nephrectomy, or through coarctation of the renal arteries. Hypertension is usually apparent a few days after the procedure (Wilkinson 1994). Renovascular models of hypertension lend insights into renal mechanisms involved in the control of blood pressure and maintenance of high blood pressure.

Steroid-induced hypertension is produced by excess administration of either mineralocorticoid (deoxycorticosterone-acetate) or glucocorticoid (dexamethasone) (Kenyon & Morton 1994). Through the use of these models the mechanisms by which either mineralocorticoids or glucocorticoids raise blood pressure have been partially elucidated. Therapeutic strategies can therefore be implemented in cases of primary/secondary hyperaldosteronism or hypercortisolism (Cushings syndrome). Experimentally-induced models of hypertension have been developed for use in adults and therefore fail to account of any early origin of hypertension. However, steroid-induced hypertension can be reproduced by dexamethasone injections throughout pregnancy in the rat (Benediktsson *et al* 1993), which also reduces the birthweight of the pups.

Ordinarily maternal steroids are metabolised by placental enzymes and glucocorticoids are no exception. The enzyme 11β-hydroxysteroid dehydrogenase Type 2 (11-HSD2) breaks down bioactive cortisol (corticosterone in the rat) to its inactive metabolite cortisone (11-dehydrocorticosterone) (Seckl 1997a). Dexamethasone, however, is only partially metabolised by placental 11-HSD2 and crosses the placenta into the fetal compartment. Fetal exposure to excess glucocorticoid permanently elevates SBP in the resultant offspring (Benediktsson *et al* 1993). Fetal exposure to excess *endogenous* maternal glucocorticoid can also be reproduced by carbenoxolone injections which inhibits placental 11-HSD2. Carbenoxolone injections, in doses that do not affect maternal SBP, reduce (20%) the birthweight and render the resultant offspring hypertensive (Lindsay *et al* 1996, Langley-Evans *et al* 1997b). This is a phenomenon that requires a product of the maternal adrenal gland, since carbenoxolone injections to adrenalectomized dams had no effect on offspring birthweight or blood pressure (Lindsay *et al* 1996).

The activity of placental 11-HSD2 is positively correlated with birthweight in rats (Benediktsson *et al* 1993) and humans (Stewart *et al* 1995) and negatively correlated with placental weight in rats (Benediktsson *et al* 1993). Furthermore the activity of placental 11-HSD2 in human placentas at term predicts birthweight (Benediktsson *et al* 1995). Thus Edwards *et al* contend that underlying the fetal origins of adult disease hypothesis is maternal glucocorticoid exposure, mediated by the variable activity of placental 11-HSD2 (Edwards *et al* 1993). However 11-HSD2 in Edward's model was not measured under favourable conditions (utilising first order kinetics) and therefore caution should be exercised in the interpretations of the correlations between 11-HSD2, birthweight and placental weight. However, neither the genetic, nor the experimental animal models of hypertension directly address the role for nutritional factors in the early origin of adult hypertension, as proposed by Barker (Barker *et al* 1993a; Barker & Osmond 1986).

1.5.3. Nutritional models of hypertension

In the SHR, hypertension can be modulated by nutritional means. Cross-fostering of SHR pups with WKY controls prevents the development of hypertension (McCarty & Fields-Okotcha 1994). The effect of cross-fostering is nutritionally-mediated rather than

through effects on maternal behaviour (McCarty & Lee 1996). Interestingly the milk of the SHR is characteristically low in protein (McCarty *et al* 1992) with an altered fatty acid profile (Mills *et al* 1990) relative to WKY.

Unilateral uterine arterial ligation throughout pregnancy in guinea-pigs produces litters with a split of pups between those that are growth retarded, from the ligated horn, and those that are of normal weight from the contralateral horn (Persson & Jansson 1992). The pups with most severe growth retardation, i.e. >47% reduction in birth weight relative to controls subsequently developed a mean arterial pressure (MAP) at weaning that was increased by 12%, compared to their normal sized litter mates (72.7 ±2.4mmHg *vs* 65.2 ±1.6 mmHg) (Persson & Jansson 1992). Thus a substantial nutritional deficit *in utero* elevated blood pressure in the resultant offspring. However, a severe surgical intervention was necessary to produce only a mild increase in MAP.

Manipulations of maternal nutrition have been shown to elevate SBP of the offspring in rats. Reduction of total food intake in pregnant rats to 30% food intake of controls produced lower birthweight pups that develop hypertension (SBP, 5-8mmHg above control values) from 30 weeks of age (Woodall *et al* 1996b). Similarly, rats rendered anaemic by feeding a low iron diet prior to and throughout pregnancy gave birth to lower birthweight offspring which develop hypertension (SBP, 10mmHg above control values) from 6 weeks of age (Crowe *et al* 1995). However, SBP was increased in the above interventions by only 5-10mmHg through feeding either a 70% reduction of food intake, which represents an almost pathological nutritional insult or by maternal anaemia which, whilst being a milder nutritional insult (rats were maintained on an adequate protein and energy diet) is only an indirect marker for nutritional state.

In contrast, a rat model of hypertension has been developed that involves mild maternal undernutrition and avoids any surgical interventions (Langley & Jackson 1994). Thus a range of maternal protein diets (60-120 g casein/kg) rendered the resulting offspring hypertensive, with SBP ranging from 15-22 mmHg above that of the 180 g casein/kg exposed controls (Langley & Jackson 1994). Indeed a restriction of dietary protein from 180 g casein/kg to 120g casein/kg, the recommended protein requirement of a pregnant rat (National Research Council 1978), elevated SBP in the offspring at 9 weeks of age by approximately

15mmHg. The diets were isoenergetic, therefore primarily reflecting protein restriction, and were replaced by laboratory chow at birth. The resultant offspring therefore differed only in prenatal nutritional experience.

The programming of hypertension in the maternal low protein exposed (MLP) offspring is apparent as early as 4 weeks of age, is independent of maternal blood pressure changes and is inversely related to maternal protein intake (Langley-Evans *et al* 1994). MLP offspring are born of lower birthweight with a corresponding larger placenta when measured at day 20 gestation (Langley & Jackson 1994), and have birth proportions similar to those identified as confering high risk of cardiovascular disease in human studies (Barker *et al* 1990). Interestingly, pharmacological blockade of glucocorticoid synthesis throughout the first two weeks of rat pregnancy prevents the development of hypertension in both male and female offspring from MLP rat dams (Langley-Evans 1997a). In addition, nutritionally-induced hypertension is associated with a reduction in the activity of placental 11-HSD2 in late gestation (Langley-Evans *et al* 1996c).

Since the barrier to maternal glucocorticoid is reduced by a maternal low protein diet, exposure to excess maternal glucocorticoid in gestation may therefore represent a common factor between nutritionally-induced adult hypertension and glucocorticoid-induced hypertension, as produced through either dexamethasone or carbenoxolone injections (Seckl 1997b). Injections of carbenoxolone to protein replete rat dams, either throughout or during specific periods of gestation, reduces the birthweight and elevates the SBP of the resultant offspring as effectively as maternal protein restriction (Langley-Evans 1997b).

1.6. THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS AND GLUCOCORTICOIDS

1.6.1. Glucocorticoid hormones

Glucocorticoids (GC) are a class of steroid hormone synthesised and secreted from the adrenal cortex and represent the end-product of hypothalamic-pituitary-adrenal (HPA) axis stimulation (Ballard 1979). GC bioactivity is exerted predominantly by cortisol

(humans), cortisol and corticosterone equally (guinea-pigs) and corticosterone solely (rats), dependent on species differences in the adrenocortical expression of 17-hydroxylase (Orth *et al* 1992). Corticosterone/cortisol is predominantly synthesised and secreted from the zona fasciculata of the adrenal cortex (*see* Figure 1.2). In terms of their synthesis, structure and mechanism of action GC share many similarities with other steroid hormones including, in mammalian systems, mineralocorticoids, oestrogens, progestogens and androgens. Figure 1.2 illustrates the synthetic pathways involved in steroid biosynthesis.

Gluco-corticoids are so-called because they are products of the adrenal ***cor***tex and influence ***gluco***se metabolism. Many metabolic functions are attributed to GC and, in fact, virtually every cell in the body bears a GC receptor through which their actions are almost exclusively exerted (Burnstein & Cidlowski 1989), indicating the multiplicity of GC influenced functions.

1.6.2. The functions of glucocorticoids

Glucocorticoids have numerous functions in peripheral tissues and generally mobilise energy substrates and increase plasma glucose concentration. Thus GC stimulate hepatic gluconeogenesis and import of gluconeogenic substrates such as amino acids into the liver (Dallman *et al* 1989). Associated increases in the activity of gluconeogenic enzymes and mobilisation of amino acids from muscle facilitate gluconeogenesis. During starvation the increased utilisation of fatty acids as energy substrates, rather than glucose, promotes a 'glucose sparing' effect. In muscle, GC can induce protein catabolism and inhibit protein synthesis (Dallman *et al* 1993). The action of insulin, which promotes peripheral glucose uptake, is generally antagonised (Dallman *et al* 1989). The diurnal variations in HPA axis function to maintain the constant provision of energy from endogenous sources, in the face of variations in food intake and thus external nutrient supply (Dallman *et al* 1993). During 'stress' (as defined as any stimulus that may threaten or challenge the internal environment) glucocorticoid actions are directed towards suppressing the defence mechanisms (immune and inflammatory systems) that are activated. This prevents these systems from threatening the homeostatic environment (Munck *et al* 1984).

Figure 1.2. **Steriod hormone biosynthesis in the human adrenal cortex**

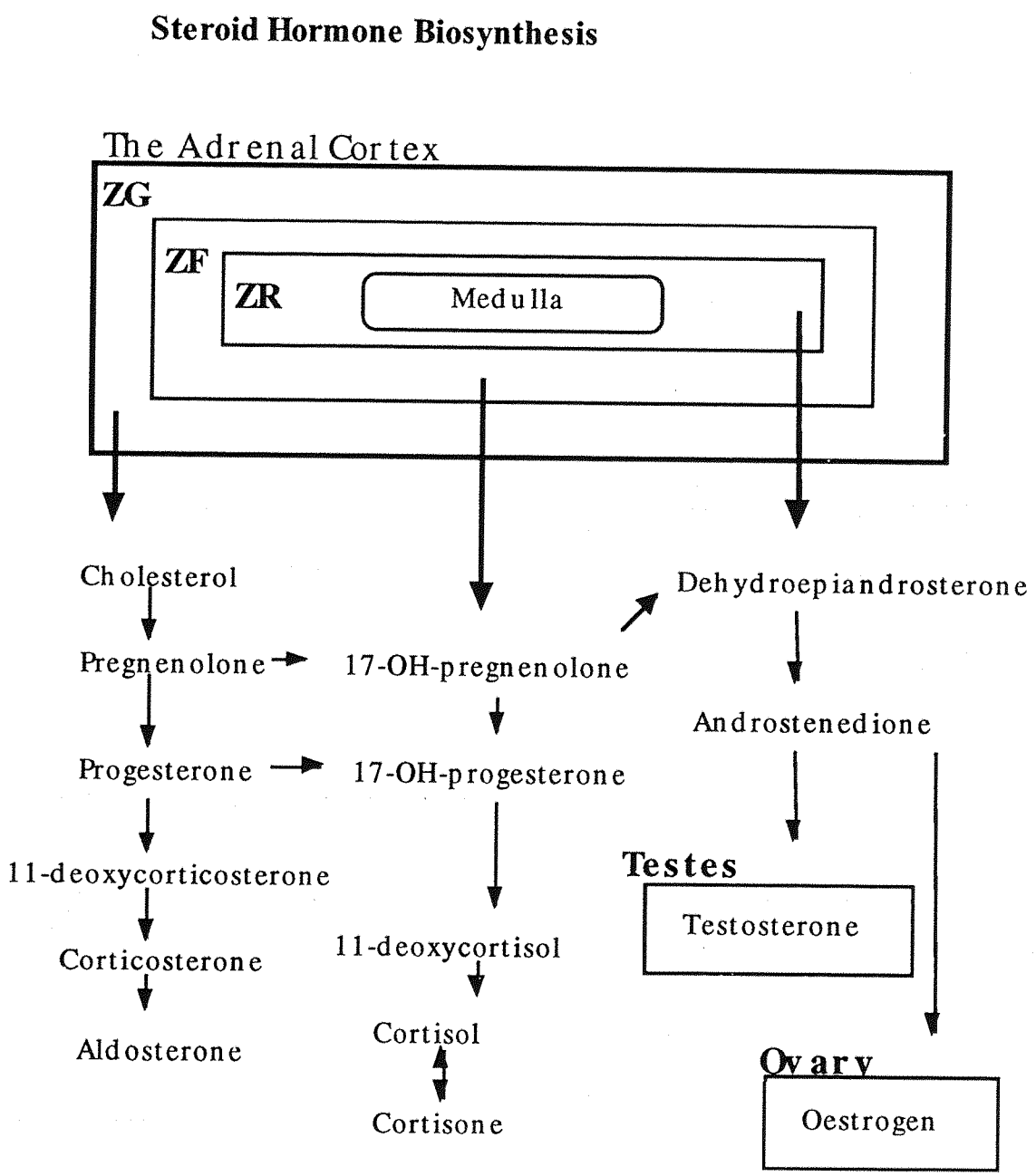


Figure 1.2.
A schematic representation of the human adrenal cortex and steroid biosynthesis. Adapted from Orth *et al* (1992).
ZG, zona glomerusa; ZF, zona fasciculata; ZR, zona reticularis; Medulla, adrenal medulla

During development GC, in addition to potentiation of the fetal response to stress i.e. in terms of catecholamine and angiotensin II action, promote the maturation and differentiation of many tissues. In the brain, GC potentiate the development and differentiation of adrenergic and neural systems, and have potent stimulatory effects on glial cells (McEwen *et al* 1986). In high concentrations GC inhibit brain growth and cytodifferentiation. This may, however, be time and dose dependent. Neonatal high dose GC suppresses neuronal differentiation (Meyer 1985), density and axonal sprouting (McEwen *et al* 1986), whilst adrenalectomy induced permanent cellular hypertrophy (Devenport 1979). During the neonatal period the immature rat brain is relatively unresponsive to stress. Sapolsky and Meaney have termed this period the "Stress non-responsive period" (Sapolsky & Meaney 1986). Thus either high or low GC concentrations, which may have deleterious consequences upon neuronal maturation are avoided by adaptive changes in GC receptor concentrations which operate at this time to maintain a low and relatively stable GC concentration (Sapolsky & Meaney 1986).

Maturation of the fetal HPA axis exhibits distinct species differences. The late gestational effects of GC upon the fetus have been best characterised in the fetal sheep. Thus during late gestation in the sheep, a marked increase in fetal adrenal GC secretion occurs which stimulates the onset of parturition (Liggins 1969) and prepares the fetus for an *ex utero* existence. GC induce accelerated maturation of fetal tissues, especially the lung, where the increased production of surfactant and antioxidant enzymes protect against an oxygen rich environment. The increase in fetal plasma GC in late gestation is of fetal origin since the passage of bioactive GC from mother to fetus in humans is prevented in late gestation by the activity of 11-HSD2 (Beitins *et al* 1979). Furthermore, adrenalectomized rat dams exhibit normal plasma GC concentrations in late gestation (Chatelain *et al* 1980) which correlate with the number of live fetuses being carried (Dupouy *et al* 1975). The overactivity of the fetal HPA in late gestation following maternal adrenalectomy maybe a response to inhibit the high maternal ACTH secretion. The concommitant increase in the fetal concentration of ACTH and corticosterone may therefore have important developmental consequences given the effects of high GC upon the fetus (Reinisch *et al* 1978). Prevention of maternal bioactive GC flux across the placenta in late gestation allows increased fetal ACTH production and thus

increased corticosterone secretion, and increased autonomy of the fetal HPA axis in preparation for an external existence (Bourdouresque *et al* 1988; van Dijk & Challis 1989).

1.6.3. The Hypothalamic-Pituitary-Adrenal axis

The functions of GC during stress only describes their predominant physiological effects after adrenocorticotrophic hormone (ACTH) stimulation. ACTH secretion not only rises significantly after stress but also on a circadian basis regardless of stressful stimuli (Orth *et al* 1992). During the physically active period (day in humans, night in rodents) ACTH and consequently GC levels may rise 2-3 fold above basal concentrations and then fall thereafter to basal levels (Martin *et al* 1978). The production of ACTH and secretion from the anterior pituitary is under the dual control of corticotrophic hormone (CRH) and arginine vasopressin (Orth *et al* 1992) (*see* Figure 1.3).

Stress (metabolic; signalled via glossopharyngeal and vagal nerves to the brainstem, or emotional; from corticolimbic pathways) or influences from the circadian pacemaker (suprachiasmatic nuclei in ventral hypothalamus) initiate the secretion of CRH from the parvocellular region of the paraventricular nucleus (PVN) into the hypophysial-portal circulation (see Figure 1.3). CRH then, alone or together with the co-secretagogues arginine-vasopressin (AVP) and to some extent oxytocin and encaphelins, stimulates the production and secretion of pro-opiomelanocortin (POMC) derived peptides, including ACTH (Orth *et al* 1992). ACTH then enters the systemic circulation and promotes the synthesis and secretion of GC from the adrenal cortex.

Prolonged exposure to a chronic GC excess has potentially deleterious consequences including; muscle atrophy, insulin insensitivity, diabetes, reduced immune competence, hypertension and arterial disease (Munck *et al* 1984). A biological imperative therefore is the ability to effectively 'turn off' activation of the HPA axis. Thus negative feedback loops exist at the level of the pituitary (short-loop), the hypothalamus (long-loop) (Widmaier 1992) and the hippocampus (Sapolsky *et al* 1986) where inhibitory neurons project into the PVN, reducing CRH and consequently ACTH secretion (Figure 1.3). The feedback sensitivity and responsiveness of the HPA axis exhibits a distinct diurnal variation (Dallman *et al* 1978).

Figure 1.3. **A schema illustrating the Hypothalamic-Pituitary-Adrenal (HPA) axis**

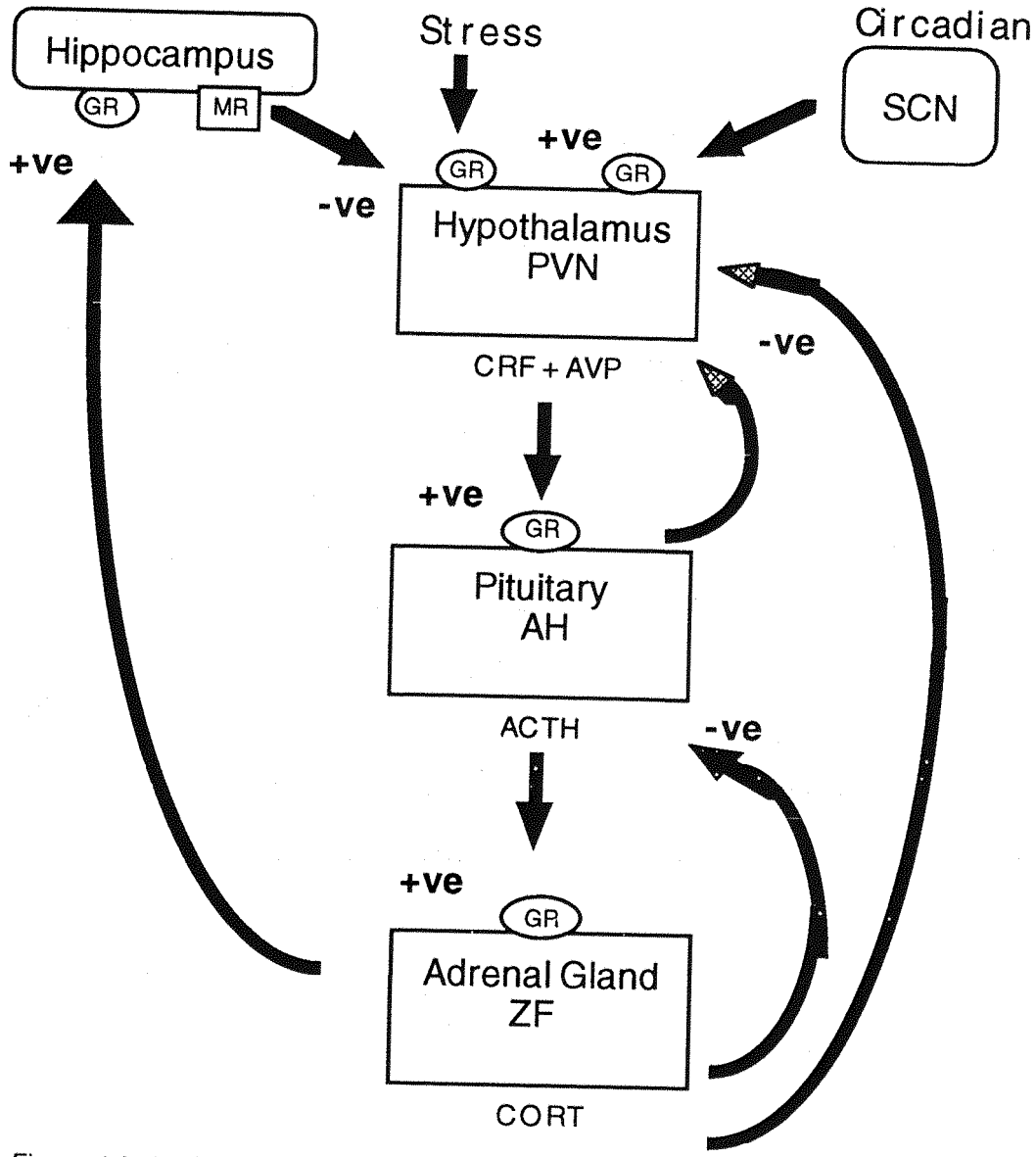


Figure 1.3. A schematic representation of the Hypothalamic-Pituitary-Adrenal (HPA) axis. SCN, suprachiasmatic nucleus; PVN, paraventricular nucleusCRF, corticotrophin releasing factor; AVP, arginine vasopressin; GR, glucocorticoid receptor; MR, mineralocorticoid receptorAH, adenohypophysis; ZF, zona fasiculata; CORT, corticosterone

Sensitivity to stress may therefore be low during peak corticosterone secretion and high during the diurnal trough. Sensitivity to negative feedback is highest during peak glucocorticoid secretion. The rhythms operate to integrate, in association with insulin secretion, an optimal metabolic state throughout periods of feeding and fasting (Dallman *et al* 1993; Devenport *et al* 1989).

1.6.4. The cellular action of glucocorticoids

In common with other steroids, GC exert their effects through binding to cytoplasmic/intranuclear receptors and initiating gene transcription (Figure 1.4) (Burnstein & Cidlowski 1989). The resultant synthesised protein then mediates the action of GC. The highly lipophilic steroid freely crosses membranes and binds to a soluble, cytosolic/nuclear, unoccupied receptor. The ensuing dissociation of heat shock proteins and conformational change in the tertiary structure of the protein 'activates' the receptor i.e. unmasking the DNA binding site, which then dimerises and binds to specific DNA sequences or hormone responsive elements. The hormone-receptor complex, once bound to DNA, alters GC-specific transcription factors, such as AP1, that induce either up or down regulation of gene expression dependent upon the particular transcription factor bound to DNA (Burnstein & Cidlowski 1989). The relative distribution of GC receptors between the cytosolic and nuclear compartments has recently been proposed to influence GC action and tissue sensitivity to GC (Jenson *et al* 1996) which may be of significance in terms of GC programming of adult disease in the present rat model since binding to GR is increased in MLP (Langley-Evans *et al* 1996c).

1.6.5. Glucocorticoid receptors

There are two functionally distinct glucocorticoid receptor types which were elucidated using synthetic steroid ligands (Reul & deKloet 1985). One has a high affinity for corticosterone but also binds aldosterone and so was called the mineralocorticoid (MR) or Type I receptor. The MR is located in mineralocorticoid target areas such as the colon, kidney and parotid gland (McEwen *et al* 1986). The second glucocorticoid receptor has a

Figure 1.4. **The cellular action of steroid hormones**

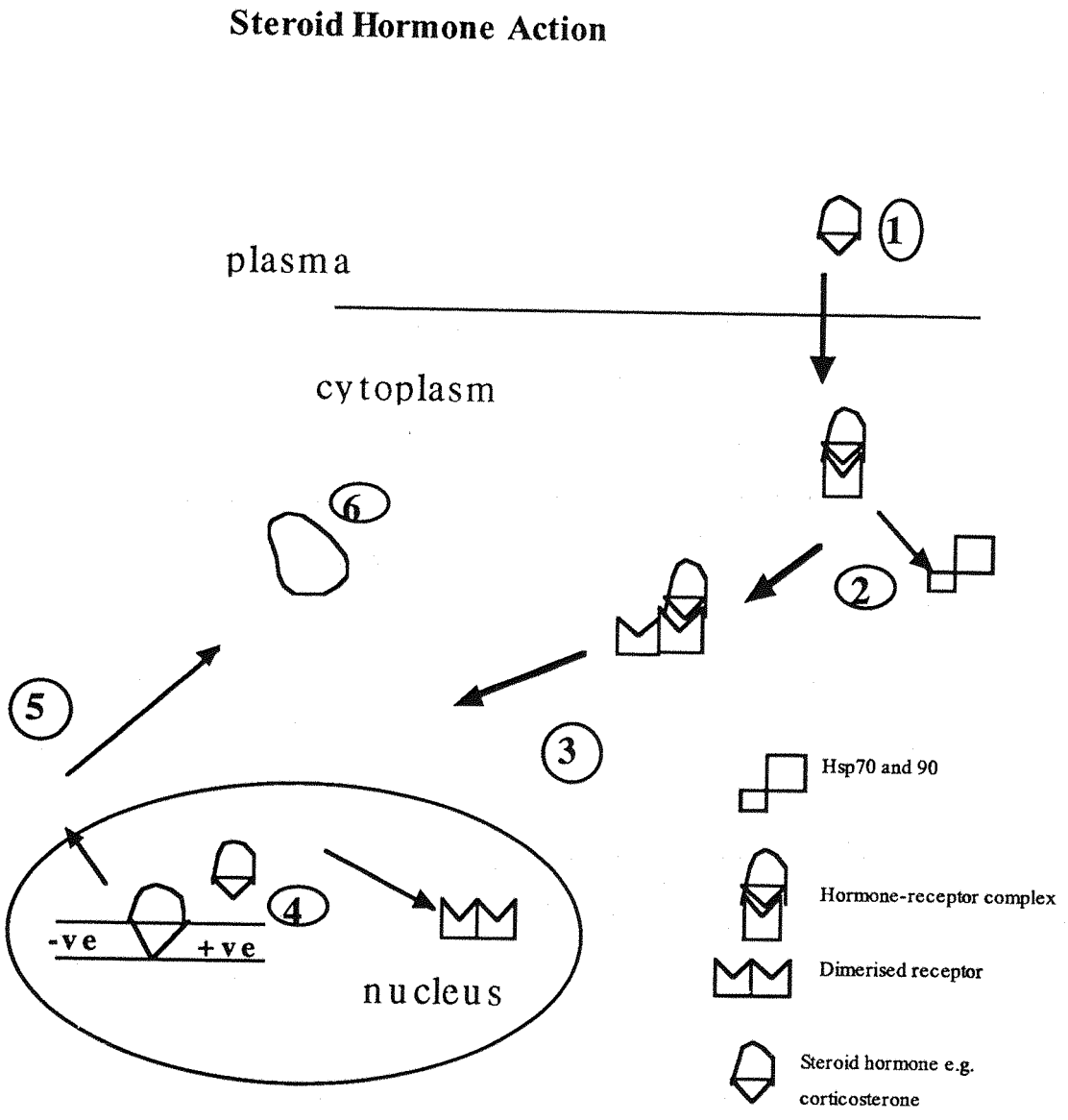


Figure 1.4.
A schematic representation of steroid hormone action. Free steroid (1) crosses the plasma membrane, binds to its receptor causing heat shock proteins 70 and 90 to dissociate (2), thus allowing the now dimerised hormone-receptor complex to cross nuclear membrane (3) and initiate transcription, (4). The resultant mRNA is translated (5) thus forming a new protein that effects the response (6) of the hormone. Steroid binding to DNA can either positively (+ve) or negatively (-ve) regulate transcription

widespread central distribution, being found in both glial cells and neurones (Meyer & McEwen 1982). It has an equal affinity for corticosterone and aldosterone yet only binds aldosterone at high concentrations and so was called the glucocorticoid or Type II (GR) receptor. The highest central densities of GR are found in the hypothalamus (Reul & deKloet 1985), but GR are present throughout the peripheral tissues (Burnstein & Cidlowski 1989). In addition, splice variants of GR have been isolated in the human that differ only at their carboxyl terminal. hGRα is believed to influence target tissue patterns of gene expression and hGRβ is thought to inhibit the activity of hGRα (Oakley *et al* 1996).

The existence of distinct corticosterone receptors with differing affinities reflects the dual metabolic actions of corticosterone (Devenport *et al* 1989). At basal concentrations up to 90% of the higher affinity MR, located in non-classical mineralocorticoid target regions e.g. the hippocampus, are occupied by steroid (Reul *et al* 1987b), providing a tonic influence on hippocampal related functions such as mood or cognition. As GC concentrations rise to mobilise fuel reserves when the gut is most empty (i.e. prior to dark-onset in rats), MR become saturated and thus the lower affinity GR become increasingly occupied (Reul *et al* 1987b). Occupation of GR promotes a catabolic metabolic response, releasing fuel substrates, which prevails until the ingestion of food (Devenport *et al* 1989).

Following food ingestion, the catabolic effects of GR stimulation gradually remit and the MR become increasingly occupied promoting energy storage (Devenport *et al* 1989). At basal levels only 10-15% of GR are occupied and consequently GR are extremely sensitive to daily fluctuations in corticosterone concentration. During stress and peak glucocorticoid secretion, occupation of GR rises to 70-80%. GR therefore mediates the glucocorticoid response to stress and negative feedback within the HPA (Reul *et al* 1987b). However, a role for MR in potentiating GR mediated negative feedback has recently been proposed that involves potentiation of GC negative feedback during peroids of high GC concentrations, such as occur during the diurnal peak in GC secretion (Bradbury *et al* 1994).

1.6.6. Ontogeny of glucocorticoid receptors

During development the GR population shows a distinct ontogenic and tissue specific pattern of expression that reflects the differing tissue specific GC functions at a given timepoint e.g. lung receptor numbers are high in late gestation when glucocorticoid induces surfactant production (Liggins 1969). Central GR mRNA has been observed in mid-gestation in rodents and concentrations increase towards late gestation to approximate adult levels (Brown *et al* 1996; Kitraki *et al* 1996; McEwen *et al* 1986). After birth, levels initially decrease, producing a stress unresponsive period (Sapolsky & Meaney 1986). GR expression then progressively increases to adult levels around the third postnatal week of life (Meaney *et al* 1993). In adult, and perhaps fetal, life GC auto-regulate the GR population (deKloet & Reul 1987). Thus adrenalectomy produces a marked up-regulation of receptors (McEwen *et al* 1974) and stress down-regulates GR (Sapolsky *et al* 1984).

MR are present in fetal rat brain (Rosenfeld *et al* 1988) and in contrast to GR, densities at postnatal day 2-4 are similar to levels observed in the adult (Meaney *et al* 1993). The MR population is relatively stable, only being regulated through endocrine status due to factors such as ageing (Seckl & Olsson 1995). MR appear unresponsive to the circulating corticosterone concentrations. Up to 80% are proposed to be bound by steroid at basal corticosterone concentrations (however this may be tissue specific dependent upon level of co-expression with 11-HSD2) and occupation was not depleted following maximal suppression of corticosterone by dexamethasone (Reul *et al* 1987a). During intrauterine development in the mouse, central MR are not co-localised with 11-HSD2 (Brown *et al* 1996) and thus may be bound by the higher circulating concentrations of corticosterone. This may have important consequences with respect to GC action *in utero*, regarding the rat model used in the present studies, since circulating corticosterone concentrations rise in the rat in late gestation (Chatelain *et al* 1980) and GC stimulation of central GR promotes increases in SBP (van den Berg 1990). It is not known whether the corticosterone measured in these early studies reflects bioactive corticosterone.

Whilst steroid hormones predominantly exert effects through nuclear receptors, recent evidence suggests that membrane steroid receptors may exist. The rapidity of effect i.e. changes in ion permeability or neurotransmitters release in response to steroid stimulation is

too fast to involve protein synthesis (Chen *et al* 1991; McEwen 1991; Orchinik *et al* 1991). GC may modulate neuronal action potentials through membrane receptors (Chen *et al* 1991). This effect may be brought about by altering the intracellular concentration of calcium ions, or by a synergistic interaction between genomic and non-genomic effects i.e. the action of steroids at the cell surface might be able to trigger changes in gene expression indirectly (McEwen 1991).

1.6.7. Modulation of tissue glucocorticoid action

Corticosterone binding globulin

GC circulate at up to 100 fold greater concentrations than aldosterone, yet the MR remains selective for aldosterone as its principal agonist in aldosterone target sites, despite having a similar affinity for corticosterone (Krozowski & Funder 1983). Initially, corticosterone binding globulin (CBG) was thought to confer the specificity. CBG binds approximately 90% of circulating plasma corticosterone (Smith & Hammond 1991), but not aldosterone. Once bound, corticosterone cannot cross plasma membranes, therefore restricting its biological activity. CBG therefore probably acts as a circulating reservoir for corticosterone, enabling flexibility in the stress response.

The levels of CBG change over development in the rat. CBG is present at approximately 50% of maternal levels during fetal life, reduces dramatically in the peripartum period and rises again after 1-2 weeks, in concert with rising corticosterone levels, up to mature adult levels at postnatal week 6 (Smith & Hammond 1991). Hepatic CBG synthesis is stimulated by oestrogen and in general females tend to have higher circulating concentrations (Smith & Hammond 1991). However where concentrations of extravascular CBG are very low, such as in the adult rat brain (McEwen *et al* 1986) or during the neonatal period (Meaney *et al* 1993), the MR is able to remain selective for aldosterone. Thus CBG is unlikely to confer specificity upon the MR.

11β-hydroxysteroid dehydrogenase (11-HSD)

As previously stated, MR has equal binding affinity for corticosterone as aldosterone yet in mineralocorticoid target sites such as the colon and kidney aldosterone exclusively

binds to MR despite the higher circulating concentration of corticosterone. Thus the MR in classical mineralocorticoid target sites is said to be 'protected' from the action of corticosterone. The protection of MR is conferred at the pre-receptor level. 11-HSD, which co-localises with the MR in classical MR target sites (Edwards *et al* 1988), protects MR through conversion of corticosterone (B) to dehydrocorticosterone (A), which does not bind MR (Figure 1.5) (Funder *et al* 1988). In Apparent Mineralocorticoid excess (AME), a congenital deficiency of 11-HSD, hypertension, hypokalaemia and suppression of plasma aldosterone and renin activity occur (Stewart *et al* 1988) which represent manifestations of cortisol binding to MR. 11-HSD is also expressed in the ovine liver, which is not a mineralocorticoid target site (Yang & Yu 994) and the high K*m* of hepatic 11-HSD for GC suggested this isoform was genetically distinct from the kidney isoform. Two separate 11-HSD isoforms have thus been demonstrated, one predominantly expressed in the ovine liver (11-HSD1) and the other in the mineralocorticoid target sites as mentioned previously (11-HSD2).

1.7. THE 11β-HYDROXYSTEROID DEHYDROGENASE COMPLEX

1.7.1. Gene and kinetics

The two isoforms of 11-HSD, 11-HSD1 and 11-HSD2 are the product of two separate genes and differ in tissue localisation and physiological function. 11-HSD1 has an apparent molecular weight of 34K and in tissue homogenates *in vitro* is bi-directional expressing co-factor preference for NADPH (reductase) (predominantly?) and NADP (dehydrogenase activity) (Seckl 1997a). The *K*m for 11-HSD1 has a μmolar affinity for its substrate (cortisol/corticosterone) as oppose to the much higher affinity 11-HSD2 isoform which has a *K*m for substrate in the nmolar range (50nM) and shows exclusively NAD-dependent dehydrogenase activity (Brown *et al* 1993) (Figure 1.5).

1.7.2. Tissue localisation and physiological role of 11β-hydroxysteroid dehydrogenase

11-HSD1 shows a widespread distribution but is predominantly concentrated in the liver (Monder & White 1993). The physiological role of 11-HSD1 is thought to be in

regulating ligand access to GR and thus to modulate glucocorticoid hormone action (Whorwood *et al* 1994). In the liver the regeneration of active GC may facilitate metabolic effects including antagonising insulin action and promoting gluconeogenesis. Bioactive GC has been proposed to increase 11-HSD1 activity and thus would further increase GC action within hepatic tissue. In contrast 11-HSD2 is only present in high densities in mineralocorticoid target sites (colon, salivary glands, kidney). To initial surprise and confusion, the MR has an equal affinity for corticosterone and aldosterone *in vivo* yet is able to exclusively bind aldosterone (Edwards *et al* 1988). It was established that the physiological role of 11-HSD2, which co-localises with the MR, was to 'protect' MR in kidney, colon from the much higher circulating concentrations of glucoccorticoid (Edwards *et al* 1988). The higher affinity of 11-HSD2 for substrate enables it to fulfil this role more adequately than 11-HSD1. Whorwood *et al* have shown that MR which is not co-localised with 11-HSD2 is likely to be unprotected against GC binding (Whorwood *et al* 1994). During pregnancy, the fetal placenta predominantly expresses an identical isoform to renal 11-HSD2 as has been determined in the mouse (Brown *et al* 1993) and other species such as the sheep (Yang 1995) and human (Seckl 1997)a. 11-HSD1 has been noted in ovine maternal decidua (Yang 1995). The 11-HSD complex in placental tissue is proposed to regulate the transport of maternal glucocorticoid into the fetal compartment (Murphy *et al* 1974). Thus in early gestation when fetal GC concentrations are low then decidual 11-HSD1 may facilitate fetal GC exposure whilst in late gestation when the fetal HPA axis is maturing bioactive GC from the maternal circulation may be metabolised by 11-HSD2 in fetal membranes allowing independence of the fetal HPA (Burton & Waddell 1994; Pepe *et al* 1990; Stewart *et al* 1995)

1.7.3. Regulation of 11β-hydroxysteroid dehydrogenase

11-HSD is regulated in a species, tissue and sex-specific fashion which is consistent with the presence of two distinct isoforms of 11-HSD. In rats 11-HSD1 expression is stimulated by glucocorticoids in the brain (hippocampus), liver (Seckl 1997a) and fat (Bujalska *et al* 1997).

Figure 1.5. **The action of 11β-hydroxysteroid dehydrogenase**

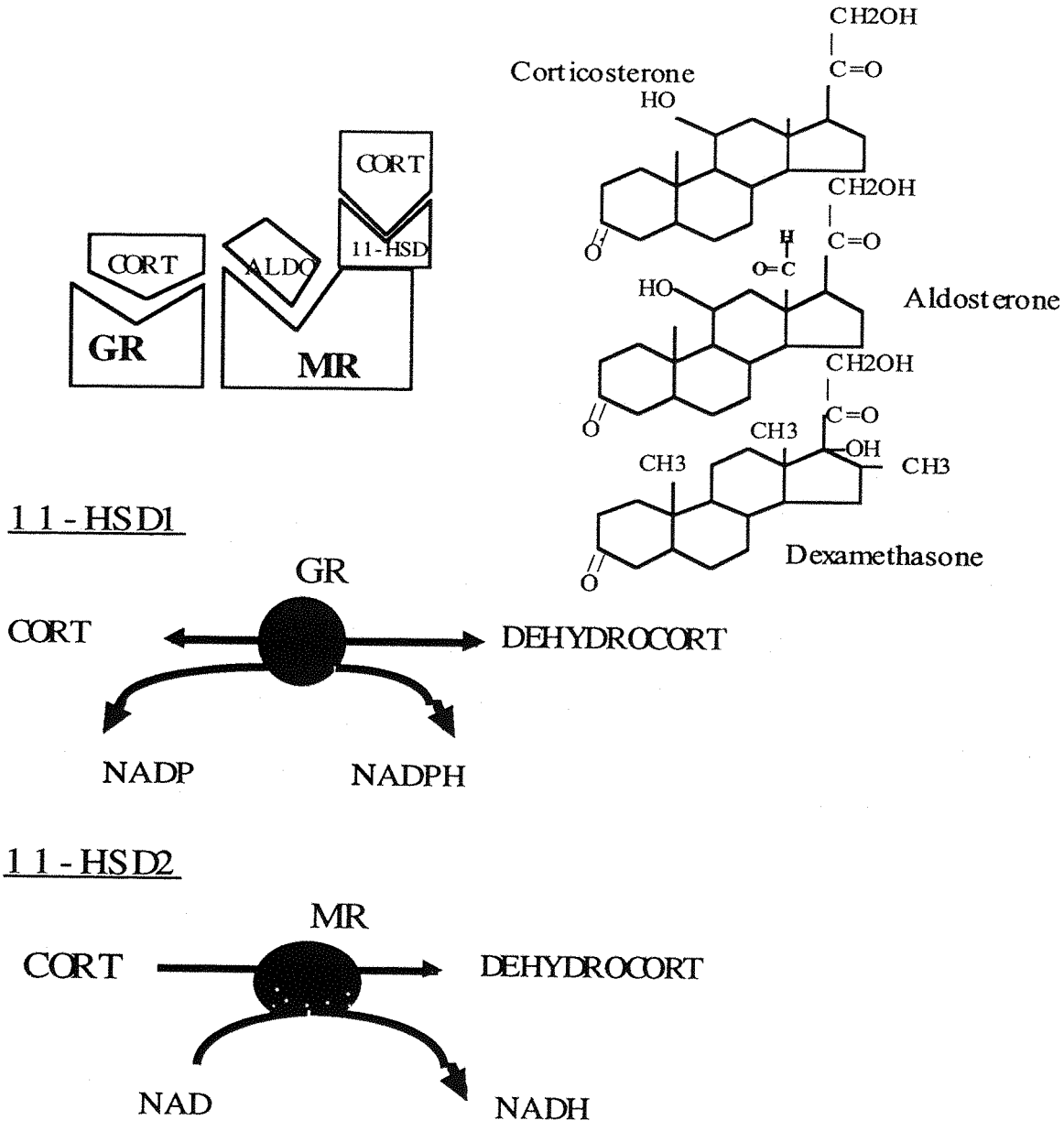


Figure 1.5. A schema illustrating the action of 11 beta-hydroxysteroid dehydrogenase (11-HSD). **ALDO**, aldosterone; **CORT**, corticosterone; **Dehydrocort**, 11-dehydrocorticosterone; **GR**, glucocorticoid receptor; **MR**, mineralocorticoid receptor

11-HSD1, predominant location in adult liver, lung, pituitary, cerebellum

11-HSD2, predominant location in adult colon, kidney, (placenta)

In addition growth hormone, thyroid hormones and insulin have been reported to regulate 11-HSD1 in peripheral tissues (Hammami & Siiteri 1991; Low *et al* 1993; Whorwood *et al* 1993). In terms of the sex-specific regulation of 11-HSD1 then following gonadectomy in rats, the activity of 11-HSD1 decreased in male liver, yet increased in female liver, an effect which was reversible with oestrogen replacement (Low *et al* 1993). In contrast, increased activity was noted in both male and female kidney following gonadectomy with oestrogen replacement (Low *et al* 1993) indicating the tissue specific regulation of 11-HSD1. The explanation for sex-specific differences in 11-HSD1 activity may be explained by differences in pituitary growth hormone secretion (Low *et al* 1994).

The sex steroids also regulate the activity of 11-HSD2, as identified by work in the baboon placenta (Baggia *et al* 1990). Whether the sex steroids regulate 11-HSD2 in the rat placenta is unknown. Oestrogen is proposed to increase the activity of placental 11-HSD2 whilst progesterone is postulated to decrease its activity, thus providing fetal autonomy in late gestation, in preparation for an *ex utero* environment (Baggia *et al* 1990). Progesterone inhibits the activity of 11-HSD2 in human kidney homogenates (Stewart *et al* 1995). In addition, corticosterone, cortisone, 11-dehydrocorticosterone and thyroid hormone are proposed to regulate 11-HSD2 (Stewart *et al* 1995). However these studies were conducted on kidney homogenates in unphysiological conditions and consequently the precise characteristics of the regulation of 11-HSD2 *in vivo* as yet are unclear.

1.7.4. Ontogeny of 11β-hydroxysteroid dehydrogenase

Glucocorticoids have potent effects on neural and glial cell differentiation and in high concentrations can inhibit brain growth (Devenport & Devenport 1983; Meyer 1985). Free glucocorticoid levels in the first few weeks of life approximate adult levels since CBG concentrations are low and brain MR approximate adult concentrations. GC may thus have potent developmental effects acting through MR, if unprotected (Seckl *et al* 1993). 11-HSD1 activity has been demonstrated in primate, mouse and rat brain (Seckl *et al* 1993). In rat hippocampus it has been demonstrated that 11-HSD1 activity was moderately high at birth, subsequently declining to a nadir around postnatal day 10 and then gradually increasing to

reach adult activity values (Moisan *et al* 1992). Thus, around the neonatal period, 11-HSD1 may influence glucocorticoid hormone action within the brain in the rodent. In contrast whilst the expression of 11-HSD1 in the liver was found to be similarly very high at birth, expression did not decline but further increased towards adulthood (Krozowski *et al* 1990). However it should be noted that whilst it is likely that both activity and expression of 11-HSD1 were being measured in the latter two studies the actual molecular characteristics had yet to be determined which yielded two genetically distinct isoforms, 11-HSD1 and 11-HSD2.

The expression of 11-HSD2 is high in fetal brain from day 11.5 in the mouse (Brown *et al* 1996). However, from late-gestation (d15) the widespread expression of 11-HSD2 declines such that at term it is only detectable in specific regions, such as the thalamus (Brown *et al* 1996). High concentrations of mRNA for GR are also observed in mid-gestation rat brain (Brown *et al* 1996; Kitraki *et al* 1996). Therefore in the developing, early rat brain the high concentrations of 11-HSD2 may regulate GC access to GR. In later gestation, the declining expression of 11-HSD2 may allow GC stimulated developmental events to occur, that is if declining expression translates into a decline in 11-HSD2 protein and activity (Seckl 1997a).

1.8. GLUCOCORTICOID PROGRAMMING OF ADULT HYPERTENSION

There are many features of intrauterine glucocorticoid excess that suggest fetal *overexposure* to maternal GC may underlie the fetal and infant origins of adult disease. GC influence many developmental processes in the fetus and modulate blood pressure in adult life and, perhaps, in the fetus (Tangalakis *et al* 1992). Glucocorticoids induce both symmetrical (Katz *et al* 1990) and disproportionate (Mosier *et al* 1982) intrauterine growth retardation (IUGR), depending on timing, length of exposure and administered dose. IUGR is also associated with high CRF levels (Goland *et al* 1993). SHR rats rendered diabetic through neonatal streptozotocin treatment produce offspring of low birthweight, and the degree of hypertension exhibited by the resultant offspring is inversely related to birthweight (Iwase *et*

al 1995). Interestingly, streptozotocin-treated diabetic rats have high circulating corticosterone levels and the activity of placental 11-HSD2 in these rats is maintained throughout pregnancy at a higher level than controls, perhaps to protect against the deleterious effects of high maternal corticosterone on the fetus (Heller *et al* 1988). Whether placental 11-HSD2 activity declines following maternal adrenalectomy and correlates positively with birthweight in streptozotocin-treated rats, as in untreated rats (Benediktsson *et al* 1993), is not clear at present. However maintenance of an elevated placental 11-HSD2 *throughout* gestation and the altered hormonal environment produced by streptozotocin treatment, may mask such correlations with birthweight in this experimental model.

Experimentally induced fetal exposure to excess maternal bioactive GC, either through glucocorticoid injections to pregnant rats or inhibition of placental 11-HSD2 reduces birthweight and elevates adult SBP (Benediktsson *et al* 1993; Lindsay *et al* 1996). Similar associations with birthweight, 11-HSD2 activity and blood pressure are observed when rat dams are fed a low protein diet (Langley & Jackson 1994; Langley-Evans 1997a; Langley-Evans 1997b). The mechanism for the down-regulation of placental 11-HSD2 following protein restriction is unclear. 11-HSD2 activity is positively related to birthweight in rats and humans suggesting that the placental enzyme has a role in the regulation of fetal growth (Benediktsson *et al* 1993; Benediktsson *et al* 1995; Stewart *et al* 1995).

The mechanisms through which intrauterine GC excess programme later hypertension are unknown. In the adult, GC interact with the sympathetic nervous system and renin-angiotensin system (RAS) in the regulation of blood pressure (Walker & Williams 1992). Increased pressor responsiveness to glucocorticoid has been observed in individuals with essential hypertension (Walker *et al* 1996). Thus GC increase vascular reactivity to vasoconstrictors such as angiotensin II (AII) and noradrenaline (Grünfeld & Eloy 1987; Whitworth *et al* 1995). Increased vascular reactivity may be mediated through alterations in membrane potential, since GC increase the transmembrane transport of Na+ and Ca^{2+} (Kornel *et al* 1995) or increased receptor densities. The potent vasoconstriction elicited by AII is potentiated by GC in the SHR through an up-regulation of AII (AT-1) receptors (Provencher *et al* 1995). Moreover GC further interact with the renin-angiotensin system through

induction of hepatic angiotensinogen transcription and increasing the activity of angiotensin converting enzyme (Mendelsohn *et al* 1982).

An interaction between GC, the sympathetic nervous system and the RAS may exist *in utero* and thus may irreversibly alter organ and/or vascular structure, predisposing the individual to later hypertension. Cortisol infusions in fetal sheep directly raise SBP through effects on the RAS (Tangalakis *et al* 1992) and furthermore influence renal function (Hill *et al* 1988) and sensitivity to adrenergic stimuli in later life (Bian *et al* 1992). AII regulates renal development (Tufro-McReddie *et al* 1995) and alterations in the activity of the RAS may influence renal development and later function. A programmed deficit in renal nephrons has been proposed to underlie the fetal origins of adult disease (Mackenzie & Brenner 1995).

Since GC effects are largely mediated through GR, then prenatal stress (high GC levels), which is known to programme later increased hippocampal GR density (Meaney *et al* 1993) may influence the regulation of SBP. In specific regions of the fetal brain where the high densities of MR are unprotected, and in mid-late gestation when the activity of 11-HSD2 declines (Brown *et al* 1996), glucocorticoid stimulation of MR will promote increases in SBP (Gomez Sanchez 1995; van den Berg *et al* 1990). Furthermore increased central GC exposure may influence the development of cardiovascular control areas i.e. metabolism of amino acid neurotransmitters, sympathetic activity, resulting in a higher 'setting' of SBP (Talman *et al* 1984). In the periphery, transient elevations in SBP and increased vasoconstrictor activity as a result of GC excess may influence the development of blood vessels (Berry 1978) and initiate cardiovascular responses leading to structural hypertrophy and permanent hypertension (Folkow 1978).

Thus increased bioactive glucocorticoid exposure *in utero,* enabled through a reduction in the activity of placental 11-HSD2, secondary to a maternal low protein diet, may influence a plethora of functions integral to cardiovascular development and control. The evidence to date suggests that maternal glucocorticoid exposure may programme vital functions and structures within the fetus that predispose, or increase the propensity of the individual to adult disease and especially hypertension.

AIMS OF THE THESIS

Epidemiological data in human populations around the world has highlighted a role for maternal nutrition in the fetal origin of adult hypertension. In order for the mechanisms underlying this association to be investigated an animal model of nutritionally-induced hypertension has been developed.

The aim of the present thesis is to explore the hypothesis of a role for maternal glucocorticoids, secondary to maternal undernutrition, in the programming and development of hypertension of early origin.

Firstly, the relationship between adult cardiovascular disease and intrauterine growth, although clearly defined in epidemiological studies has not been considered in animals. Initial experiments will investigate the effects of prenatal protein restriction in rats upon fetal, placental and neonatal growth. Undernutrition and its effects upon maternal growth and energy balance will additionally be evaluated. Fetal growth responses to a low protein diet will be considered in terms of systolic blood pressure in later life.

Since prenatal exposure to maternal glucocorticoids has been shown to programme later hypertension in rats (Benediktsson *et al* 1993). The role of glucocorticoids as the hormonal mediators of nutritionally-induced hypertension will be determined through:

1. Assessment of placental 11-HSD2 activity and measurements of fetal and maternal glucocorticoid. This will, albeit indirectly, test the hypothesis that nutritional downregulation of 11-HSD2 may increase bioactive GC transfer from mother to fetus.

2. Assay of biochemical markers of glucocorticoid action in fetal and neonatal tissues.

3. Ablation of maternal glucocorticoid production in order to assess the glucocorticoid-dependence of the hypertensive state in the offspring

4. Measurements of indirect HPA axis function in the offspring and;

5. Adrenalectomy of the offspring in postnatal life to assess the role of glucocorticoids in maintaining the hypertensive state.

CHAPTER 2

METHODOLOGY

2.1. CHEMICALS

Standard chemicals and reagents were purchased from Sigma (Poole, UK). All radiochemicals were purchased from Amersham (Little Chalfont, UK), unless otherwise stated in the text.

2.2. ANIMALS

All experimental animal work was carried out under licence from the Home Office and in accordance with the Home Office Animals Act (1986). Rats of the Wistar strain bred in the University of Southampton animal facility were used in all experiments. The animals were housed in either wire mesh or perspex cages at an ambient temperature of 24°C on a 12hr light/dark cycle. All rats were offered food *ad libitum* either standard non-purified laboratory chow diet (CRMX, Special Diet Services, Cambridge, UK) or a semi-synthetic control (180g/kg casein) or low protein (90g/kg casein) diet. Dietary composition of synthetic diets and chow are shown in Appendix 1 and 2 respectively. The semi-synthetic diets were balanced in terms of energy, fibre (cellulose), fat (corn oil), minerals, vitamins and were supplemented with D,L-methionine to avoid sulphur deficiency. The shortfall in energy from protein in the low-protein diet (50% reduction) was balanced by an increased availability of energy from starch (14% increase) and sucrose (14% increase).

2.2.1. Nutritional regimen for generation of animals

In initial experiments, virgin female rats weighing between 200-250g were habituated to the semi-synthetic diets for 14d prior to mating and maintained on the diet throughout the mating period (1-7d) and gestation (21-22d). Where possible only 1-2 males were used for

mating to limit paternal genetic variability in studies. However, between studies over a period of 3 years, different males were used but were derived from a homogenous strain therefore limiting genetic variability. The maternal weight range selected was the weight at which female rats are reproductively mature and have almost achieved full growth. For all experimental protocols at least n=6 dams/dietary group were mated generating approximately n=5/6 litters/dietary group from which the n for postnatal experimental groups were derived. If offspring were harvested at different timepoints then a greater number of rat dams were mated. Recent experiments (Langley-Evans *et al* 1996f) have determined no effect of the pre-mating habituation period on any metabolic parameter in the offspring including blood pressure and consequently all animals were maintained on the semi-synthetic diet only during pregnancy (as stated in text). Day 0 of gestation was determined by the presence of a vaginal plug on the floor of the cage. Immediately upon giving birth the diet was replaced with standard chow diet (CRMX, Special Diet Services, Cambridge, UK; Appendix 2) and litters culled to 8 pups per litter (4 male, 4 female). The offspring differed, therefore, only in their prenatal dietary experience and the period (1-3d) of maternal postnatal recovery from the diet. At 3-4 wks old the pups were weaned onto the chow diet.

2.3. ADMINISTRATION OF HORMONES AND ANTIHORMONES

2.3.1. Corticosterone and Aldosterone

Corticosterone was administered to the rats by subcutaneous injection (0.05-0.1ml volume) at a dosage of between 10-20mg/kg bodyweight/day, suspended in arachis oil or polyethylene glycol. Aldosterone was given by the same route at a dosage of 100µg/kg bodyweight/day suspended in polyethylene glycol. Control animals received an equivalent volume of vehicle, by the same route.

2.3.2. Mifepristone (RU486)

RU486 was administered subcutaneously suspended in polyethylene glycol (0.05ml) vehicle at a dosage of 30mg/kg/day. Injections were given daily at 9.00am and 5.00pm. Control animals received an equivalent volume of vehicle only, by the same route.

2.4. TISSUE COLLECTION

2.4.1. Fetuses and Neonates

Pregnant rat dams were rapidly killed by decapitation and the uterus excised. All day 14 gestation fetuses were either killed by decapitation or snap frozen in liquid nitrogen whilst all 20 day old fetuses were killed by concussion. All fetal membranes and maternal tissues were carefully removed to allow accurate measurements of body length and weight of both placentas and fetuses. The day 20 fetuses were killed in a random order and dissected to obtain whole brain, lung and liver tissue which was frozen immediately in liquid nitrogen and stored at -70°C for up to a maximum of 3 months until required for biochemical analysis. Term neonates were killed and dissected as above.

2.4.2. Visceral organs

Rats of weanling age or older were either decapitated or killed by cervical dislocation. Liver, kidneys, heart, spleen, adrenals and lung were rapidly excised and weighed. The brain was removed and stored on ice for dissection. Blood was collected into heparinised tubes either through decapitation of the animal within 30 seconds of removal from its cage or by cardiac puncture under terminal pentobarbitone anaesthesia. Blood tubes were immediately shaken and placed on ice prior to separation of the plasma component. All tissues collected were frozen immediately in liquid nitrogen and stored at -70°C for up to 3 months until required for biochemical analysis.

2.4.3. Brain dissection

The cerebellum was immediately obtained by making a vertical cut at the back of the hindbrain and cortical tissue removed. For removal of the hippocampus, the cerebral cortex was initially sliced laterally away from the midline and eased clear to reveal the hippocampus and related limbic structures. Sufficient hypothalamic tissue was obtained by cutting through the optic chiasma and then 0.5cm proximal to the brain stem. The resulting block of tissue was cleared of cortical tissue. The pituitary was not obtained as in small animals it was difficult to identify and remove with precision. All regions obtained were rapidly placed in liquid nitrogen and stored at -70°C for up to 3 months until required for biochemical analysis.

2.4.4. Preparation of blood

Blood samples were removed from the heparinised collection tubes into microfuge tubes and centrifuged for 5 mins to separate red blood cells from the plasma fraction. The supernatant plasma was then aspirated and stored at -70°C.

2.4.5. Bilateral adrenalectomy

Rats were anaesthetised with either pentobarbitol *i.p.* at a dosage of 72mg/kg bodyweight, or diazepam *i.p.* (10mg/kg bodyweight) with the neuroleptic fentanyl fluanisone *i.m.* (5mg/kg bodyweight). The righting response was lost within 5 minutes, and all rats were identified as being under deep anaesthesia when they failed to respond to pinching of the sole of the foot. Two dorsal incisions were made with minimal damage to the abdominal capsule. The adrenal glands, with associated fat pads, were located and carefully withdrawn from the animal. The capsule was sutured and the skin was held together with surgical clips and sutures. The animals were then placed in a room maintained at 27°C with free access to chow, water and saline (0.9% NaCl). Animals recovered within 2-5 hrs after surgery. The mortality rate after surgery was established to be approximately 1-3%.

2.5. ARTERIAL AND VENOUS CANNULATION

Rats were anaesthetised with 72mg/kg bodyweight sodium pentobarbital *i.p.* The arterial and venous cannulation and infusions of angiotensin II were based on the method of Saglikes *et al* (Saglikes *et al* 1985). Once anaesthetised, the ventral skin of the neck was removed. Fatty, muscular and membranous tissue were cleared and a tracheotomy performed. The right carotid artery was located, cleared to allow access, and clamped with two wire arterial clamps placed 1cm apart. Prior to cannulation, the cannulae and pressure transducing dome were checked for leaks or air bubbles. A small incision was made, into which a heparinised, saline filled cannula (0.4mm/0.8mm, Portex, UK) was inserted and secured with sterile surgical braid. The arterial cannula was attached to a saline filled, circular plastic dome containing a distensible membrane. The dome was attached to a pressure transducer which, through a pressure amplifier recorded pressure changes which were transcribed onto a pre-calibrated (110mmHg-160mmHg) chart recorder. The arterial clamp distal to the head was then removed to allow blood flow into the cannula. It is accepted that physiological measurements under anaesthesia may not necessarily reflect *in vivo* responses since pentobarbitone acts as mild cardiovascular depressor, however the technology was not available at the time of experiments to measure the SBP of rats in free living conditions. Thus the animals were left for 30mins following anaesthetic administration after which a stable, direct blood pressure reading could be determined. All measurements were made by the same operator.

The right femoral vein was cannulated using a similar procedure. The ventral skin of the right hind leg was removed and fat and muscular tissue teased aside to reveal the right femoral vein and artery. The vein and artery were separated and a section of femoral vein was isolated. A heparinised cannula (0.4mm/0.8mm, Porters, UK) was inserted and the proximal clamp removed. A heparinised syringe was attached to the cannula and a small amount of blood was drawn into the syringe to eliminate air bubbles. A rectal thermometer was inserted and the rat was maintained at 37.5°C with the aid of an overhead lamp. Through the cannula doses of angiotensin II (AII) (1, 5, 10, 20 and 40ng bolus injections in 0.1ml NaCl at 37°C) or vehicle (0.1ml NaCl at 37°C) were administered in random order. The exact protocol was

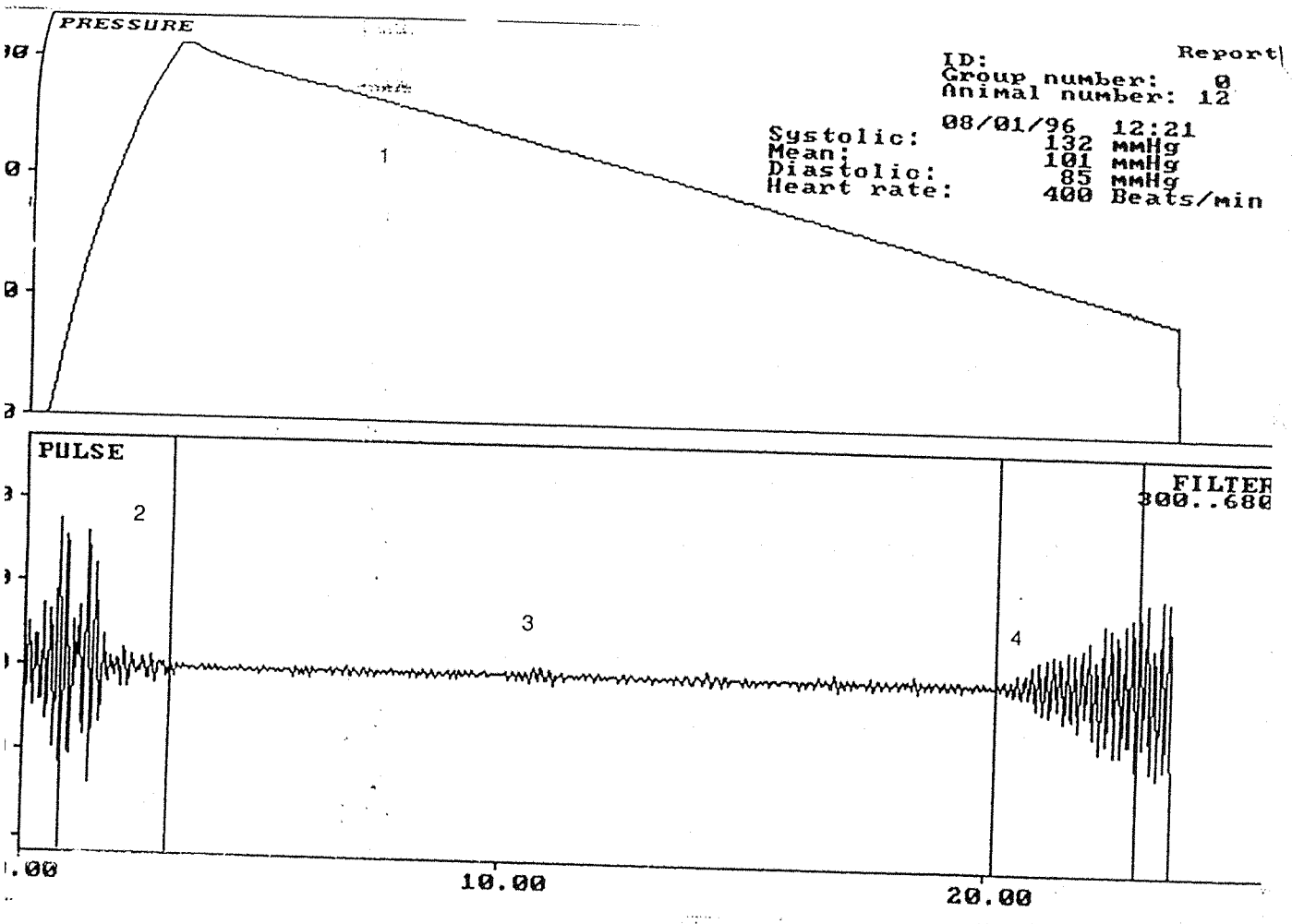
developed in 7 chow-fed rats (Appendix 4). Intravenous doses of AII ranging between 1-80ng were injected to characterise the dose response curve. Figure A1 (Appendix 4) illustrates that the vascular response to AII injection reached a maximum (asymptotic plateau) at a bolus dose of AII greater than 40ng. Doses of AII at or below 40ng were therefore used. Figure A2 (Appendix 4) characterises the recovery rate after AII infusion at any given dose and demonstrates that by 3mins blood pressure had returned to baseline values. The vascular response was therefore not followed beyond this point. However, an interval of at least 5mins was maintained between injections to allow for any vascular overcompensation and consequent oscillation of pressure. The variation in maximal vascular response between animals was 8.1%, 9.3%, 9.3%, 10.5%, 8.9% and 17.4% for doses of 1.25, 2.5, 5, 10, 20 and 50ng AII respectively. The individual variation in maximal vascular response at a given dose was 6.5%, 9.2%, 5.8%, 6.7% and 5.2% for doses of 1.25, 2.5, 5, 10 and 20ng AII respectively.

2.6. MEASUREMENT OF SYSTOLIC BLOOD PRESSURE

Blood pressure was measured non-invasively in consious rats. Prior to measurement rats were habituated for two hours in the test laboratory maintained at 27°C. This temperature avoids heat stress which, in rats, occurs at 30°C and above. Tail vein pulses can be detected at 27°C but are undetectable below 27°C using the indirect tail-cuff method (Model 229 Blood Pressure Monitor; Linton Instrumentation, Diss, Norfoik, UK; Figure 2.1). The tail-cuff method has been validated against direct methods used to determine SBP in conscious, unrestrained rats (Pfeffer *et al* 1971).

Rats were initially placed into an appropriately sized perspex restraint tube and allowed to settle. Preliminary studies indicated that a settling period of 4-5 minutes was optimum for reproducible recording of blood pressure (Appendix 5, Figure A1). An appropriately sized tail cuff was placed around the tail and inflated to 300mmHg. Pulses were recorded during deflation at a rate of 3mmHg/sec. Measurements (*see* Figure 2.1) were made in either triplicate or quadruplicate and the average values for systolic blood pressure and heart rate were recorded. Preliminary studies indicated that pre-training the animals to the apparatus did not significantly affect the measurement of blood pressure (Appendix 5, Figure

Figure 2.1. **A typical blood pressure trace using tail-cuff sphygmomanometry**



Blood pressure was determined following the methodology described in section 2.5.

Key:

1. The cuff deflation curve (300-0 mmHg at a rate of decrease of 3mmHg / minute)
2. Detectable tail pulses
3. Blockade of blood flow
4. The onset of blood flow, equivalent to systolic blood pressure

A2). Intra and inter-assay variations were determined through 6 consecutive readings of 6 animals and through twice daily readings of 6 animals over a 3-day period. Intra and inter-assay variations of 4.7% and 7.9% respectively were recorded. An IITC model 229 blood pressure monitor linked to a computer software package reduced observer bias and the operator (of which there was only one doing all measurements) was blinded to the dietary origin of the animals.

2.7. BIOCHEMICAL ASSAYS

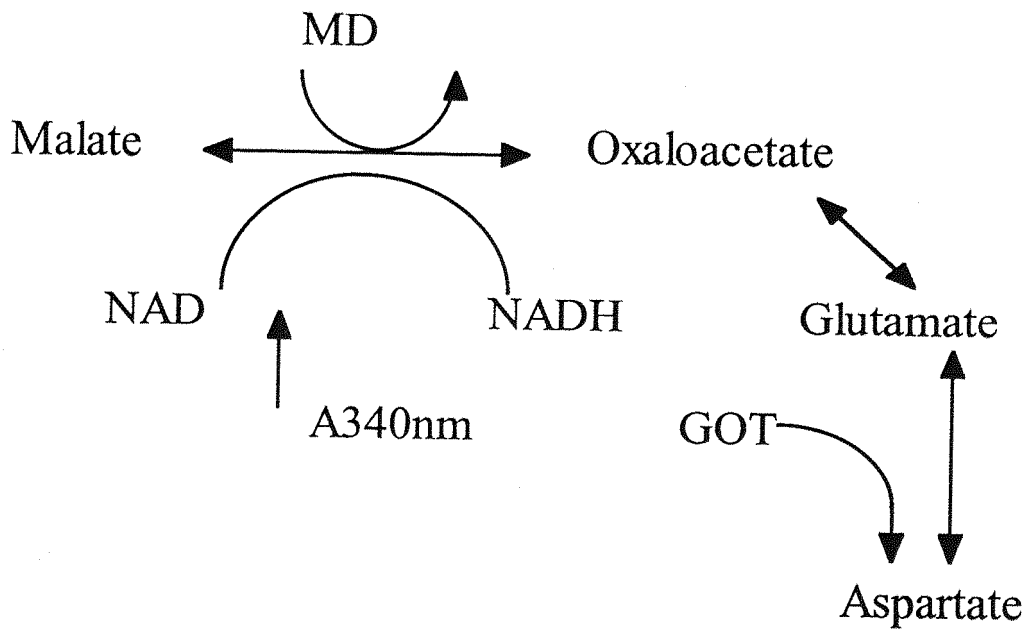
2.7.1. Malate dehydrogenase (MD)

MD (EC 1.1.1.37) was assayed using the method of Langley and York (Langley & York 1990). Brain samples were homogenised in 10 volumes of homogenising buffer (Appendix 3) and stored on ice. 15µl of homogenate was then incubated with 1ml reaction buffer (Appendix 3) for 10 mins at room temperature. The reaction was terminated by the addition of 40µl 10M NaOH. The activity was measured in the direction of malate oxidation and read against a protein free blank at A340nm to determine the reduced state of NAD (*see* Figure 2.2). Enzymic activity was linear at protein concentrations between 50-300 µg protein at pH 9.5. The intra-assay variation was assessed by measuring 6 repeats of the same sample and was 2.7%.

2.7.2. Glycerol phosphate Dehydrogenase (GPDH)

GPDH (EC 1.1.1.8) is a glucocorticoid-sensitive enzyme (Kitraki *et al* 1996) which was assayed following the method of Langley and York (Langley & York 1990). Brain samples were homogenised in 10 volumes homogenising buffer (Appendix 3) and subsequently centrifuged at 20 000g for 40 mins at 4°C. 100µl of supernatant was incubated with 0.8ml reaction buffer (Appendix 3) at 37°C. The reaction was started by adding 100µl of dihydroxyacetone phosphate (8.33µmoles). The decrease in absorbance at 340nm was measured over one minute (*see* Figure 2.3). Beyond one minute, activity was at Vmax and thus measurement would not reflect first order kinetics. Analysis of enzyme activity against

Figure 2.2. **A schema of the malate dehydrogenase (MD) reaction**



Malate dehydrogenase is a glucocorticoid-insensitive reversible enzyme. It is located both on the inner mitochondrial membrane, where it converts malate to oxaloacetate which in combination with acetyl coenzyme A enters the tricarboxylic acid cycle (TCA) as citrate, and within the cytoplasm where its role is the exact reverse, i.e. to reduce oxaloacetate to malate.

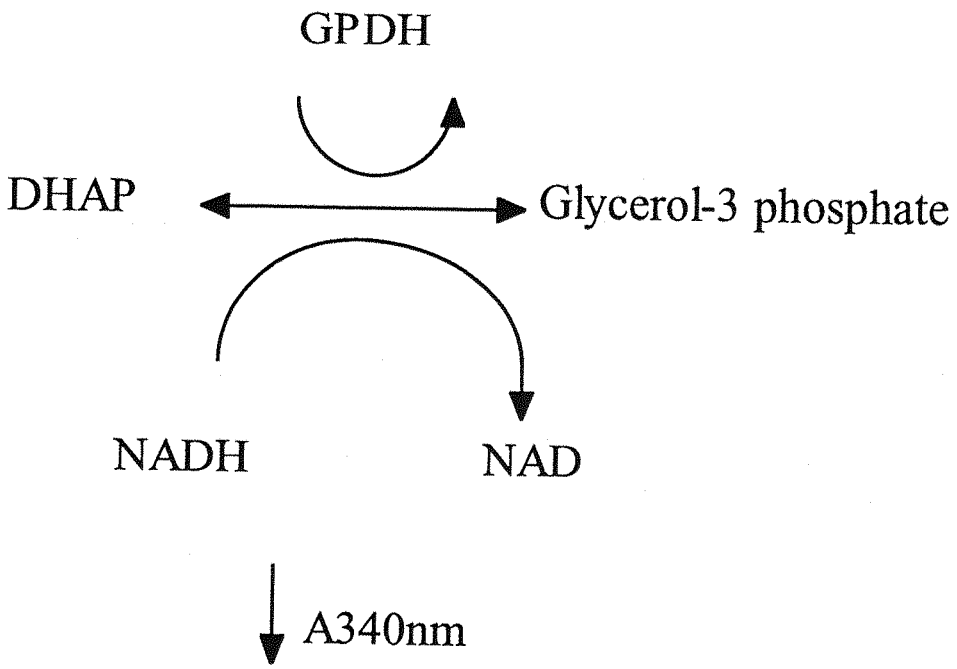
Key:

GOT, glutamate-oxaloacetate transaminase

MD, malate dehydrogenase

A340, absorbance is read at a wavelength of 340nm

Figure 2.3. **A schema of the glycerol-3 phosphate dehydrogenase (GPDH) reaction**



Glycerol phosphate dehydrogenase is a glucocorticoid-sensitive enzyme, predominantly located within the central nervous system (CNS) in oligodendrocytes (Kitraki *et al* 1996). It is located within cytoplasm where it mediates the conversion of dihydroxyacetone phosphate to glycerol-3 phosphate, therefore providing a precursor of phospholipids for incorporation into glial cell membranes.

Key:
DHAP, dihydroxyacetone phosphate
GPDH, Glycerol phosphate dehydrogenase
A340, the decrease in absorbance is read at a wavelength of 340nm

protein concentration revealed a linear relationship between 50-750 μg protein at pH 7.15. An intra-assay variation of 2% was recorded from measurement of 8 repeats of the same sample.

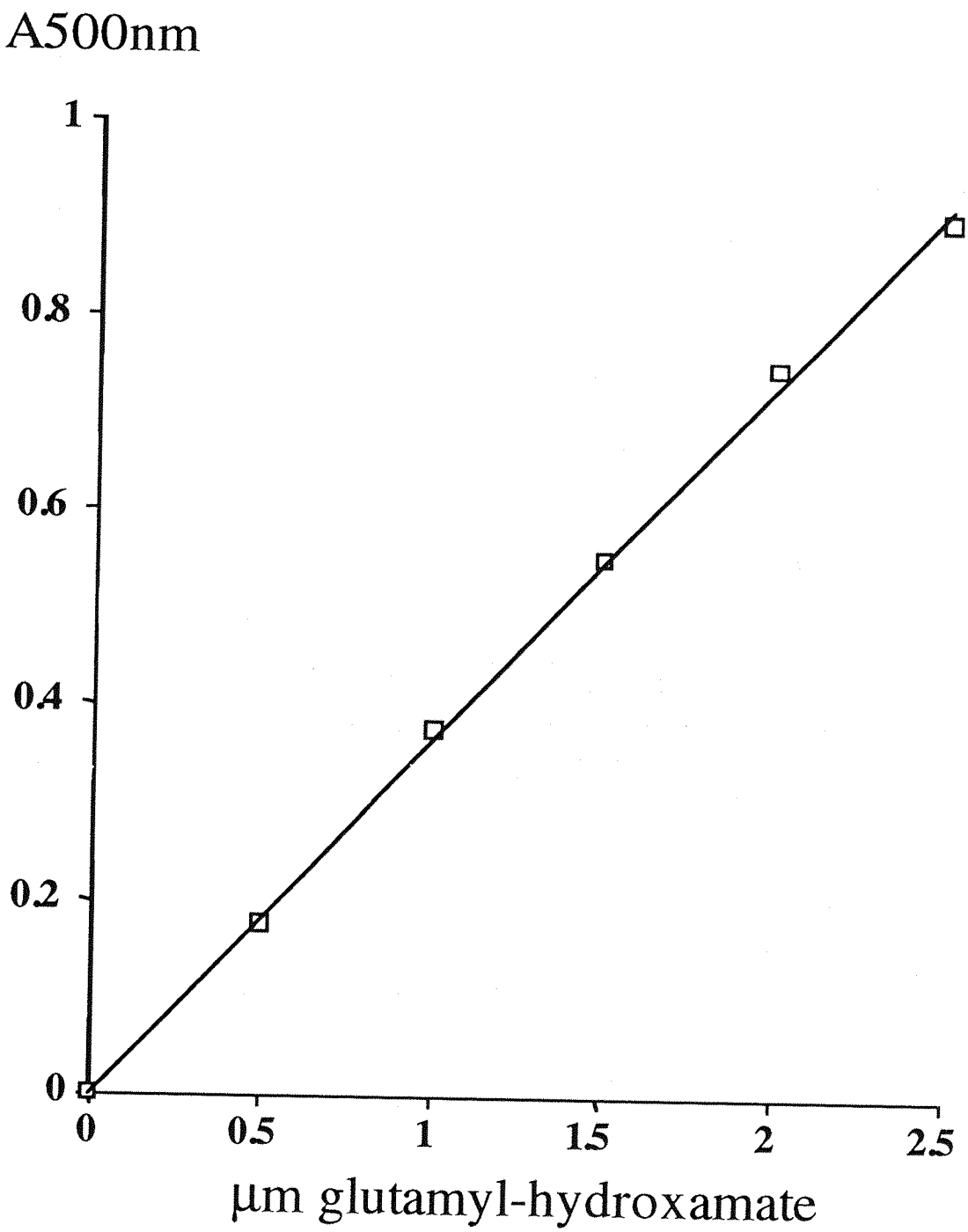
2.7.3. Glutamine Synthetase (GS)

GS (EC 6.3.1.2) is a glucocorticoid-sensitive enzyme (Patel *et al* 1983) which was assayed following the method of Langley and York (Langley & York 1990). Brain samples were homogenised in 10 volumes homogenising buffer (Appendix 3) and sonicated six times at 26kHz for 10 seconds with a 5 second interval. The samples were then centrifuged at 3000rpm for 5 mins. 100μl sample protein was incubated with 0.9 ml reaction mixture at pH 8.0 (Appendix 3) for 20 mins at 37 °C. The reaction was terminated by the addition of 1.5mls colour reagent (Appendix 3). Absorbance at 500nm was measured and the quantity of product formed was determined from a standard curve of 0-2 μmoles glutamyl-hydroxamic acid (Figure 2.4). The intra-assay coefficient of variation for this assay was determined as 1.8% by measuring 6 repeated readings of the same sample. Figure 2.5 outlines the GS enzymic reaction that determines the quantity of product formed.

2.7.4. Tyrosine aminotransferase (TAT)

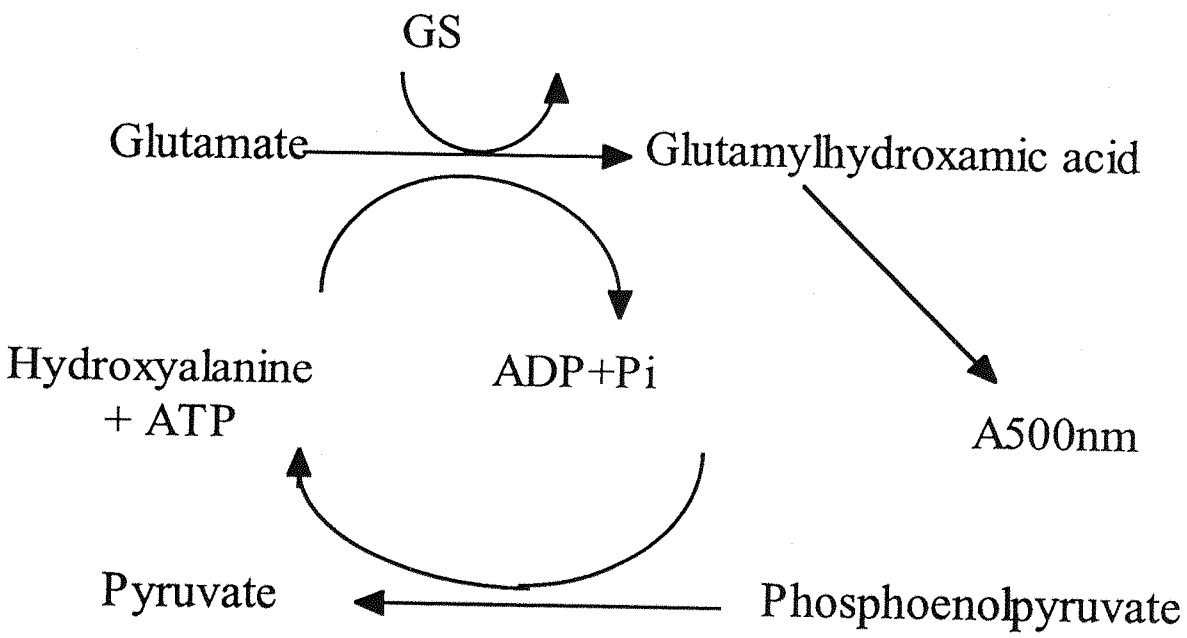
TAT (EC 2.6.1.5.) is a glucocorticoid-sensitive enzyme (Shargill *et al* 1983) which was assayed following the method of Shargill *et al* (Shargill *et al* 1983). The liver cytosol was prepared by homogenisation in 20 volumes buffer (Appendix 3) and centrifuged at 105 000g for 1hr at 4°C. 25μl sample (75-175μg protein) was added to 200μl reagent (Appendix 3) and incubated for 15min at 37°C. 20μl 10M KOH was added and samples further incubated for 30min at 37°C. The reaction was terminated with the addition of 1ml ice-cold water. Samples were read at 331nm against a zero-time blank. Data are expressed as moles p-hydroxybenzaldehyde formed/min/mg protein (*see* Figure 2.6). The intra-assay variation for this assay was established to be 3.2% by measuring 8 repeats of the same sample.

Figure 2.4. **A standard curve of A500nm against μmoles glutamylhydroxamic acid for the determination of glutamine synthetase activity.**



Data points reflect the mean of duplicate readings.

Figure 2.5. **A schema of the glutamine synthetase (GS) reaction**

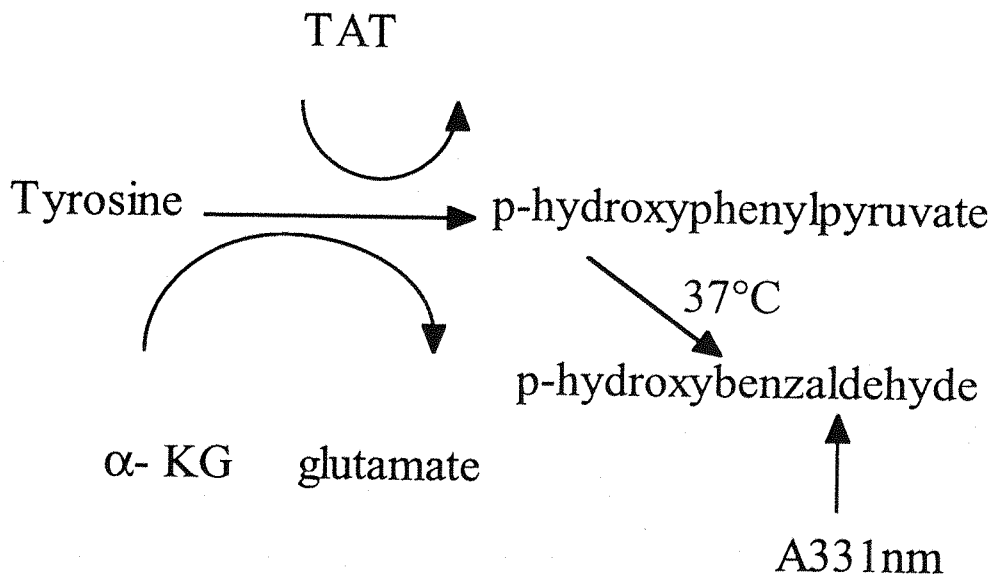


Glutamine synthetase is a glucocorticoid-sensitive enzyme which, within the CNS, is predominantly localised in astrocytes mitochondria, where it plays an important role in the processing of amino acid neurotransmitters (Patel *et al* 1983). Thus after glutamate release it is taken up into astroglia and converted to glutamine by GS. The glutamine is then hydrolysed to glutamate and stored in the nerve terminals.

Key:

GS, Glutamine synthetase
PK, Pyruvate kinase
A500, the absorbance is read at a wavelength of 500nm and activity determined against a standard curve of 0-2 µmoles glutamylhydroxamic acid

Figure 2.6. **A schema of the tyrosine aminotransferase (TAT) reaction**



Tyrosine aminotransferase is a glucocorticoid-sensitive enzyme (Shargill *et al* 1983). It is located within the cytoplasm where it functions as the rate limiting enzyme in tyrosine catabolism.

Key:

KOH, potassium hydroxide
TAT, tyrosine aminotransferase
α-KG, alpha-ketoglutarate
A331, the increase in absorbance is read at a wavelength of 331nm

2.7.5. Pyruvate Kinase (PK)

PK (EC 2.7.1.40.) was assayed by the method of Langley and York (Langley & York 1990). Tissues were homogenised in 10 volumes homogenising buffer (Appendix 3), and centrifuged at 25 000g for 20 mins at 4°C. 100 µl supernatant (50-250 g protein) was incubated with 800 µl reaction mixture (Appendix 3). The reaction began with the addition of 100µl 1mM phosphoenolpyruvate. The decrease in absorbance was followed at 340nm for one minute (*see* Figure 2.7). Intra-assay variation was established as 2.1% for 9 replicates.

2.7.6. 11β hydroxysteroid dehydrogenase (11HSD2)

Tissue 11HSD2 activity was assayed according to the method of Benediktsson *et al* (Benediktsson *et al* 1993). Briefly, fresh placenta (including both maternal decidua and fetal placenta) was excised and immediately washed in Krebs-Ringer buffer (KRB, pH7.4) (Appendix 3) to remove excess blood. The washed placenta was homogenised in 10 volumes KRB and centrifuged at 2000 rpm for 1 min to remove large tissue fragments. Conditions for the assay were optimised using homogenate containing 500µg/ml protein was added to 2.5µCi ^{3}H-corticosterone (12.5 nmoles/L) in KRB, 2g/l glucose, 2g/l BSA and 200µM NAD in a total reaction volume of 250µl. After 10 min incubation at 37°C the reaction was terminated with 2.5ml ice-cold ethyl acetate. At 10 min the initial velocity of enzymic reaction had reached Vmax (*see Figure 2.8*) and therefore data should be treated with caution. Tubes were centrifuged at 2200 rpm for 10 min and the organic phase was aspirated and dried under nitrogen. Samples were reconstituted in a mixed dehydrocorticosterone/corticosterone solution (100µl methanol) and steroids separated using high performance liquid chromatography (HPLC), using an isocratic Gilson system with a reverse-phase C_{18} column utilising a 65% methanol:35% water (v/v) running buffer. A UV detector at 240nm linked to Gilson software determined peaks of activity and fractions were collected into scintillation vials and radioactivity (disintegrations per minute) measured in a liquid scintillation counter. Variation between 8 repeats of the same sample within this assay was established to be 3.5%. Utilising the present methodology the proportions of B metabolised to other reduced metabolites could not be determined. Typical % conversions for placental 11-HSD2 were

between 20-35% at day 20 gestation. The assay was validated in placental tissue for incubation time (Figure 2.8).[1]

2.7.7. Glucose assay

Plasma glucose was determined according to the method of Langley *et al* (Langley *et al* 1994). Plasma was initially deproteinised by dilution (v/v) with 10% trichloroacetic acid, vortexed and spun in a microcentrifuge for 5 mins. The resulting supernatant was then diluted further with an equal volume of sodium buffer pH 7.0, to restore the pH to within the range needed for glucose oxidase to operate efficiently (pH 6.5-7.5). 100µl of glucose reagent (Appendix 3) was added to 40µl of diluted plasma in a microplate and incubated for 5 mins at 37°C in the dark. Glucose concentration was determined on a spectrophotometer at 620nm against a glucose standard curve (0-0.5 g/l). Inter and intra-assay variations of 4% and 9% were recorded respectively for this assay.

2.8. DETERMINATION OF PROTEIN

All specific enzyme activities were expressed as nmoles converted/min/mg protein. The protein concentration of each sample was determined by using the bicinchoninic (BCA) method (Smith *et al* 1985). In one of the enzyme assays (GS) the buffer interfered with the copper based protein assay (Smith *et al* 1985) and in this case protein concentration was determined using the method of Lowry *et al* (Lowry *et al* 1951).

2.8.1. Bicinchoninic acid method

A standard protein stock (1mg/ml bovine serum albumin (BSA) made up in 0.1M NaOH) was prepared and diluted to give a range from 0.2mg/ml to 1mg/ml. 10 µl of standard

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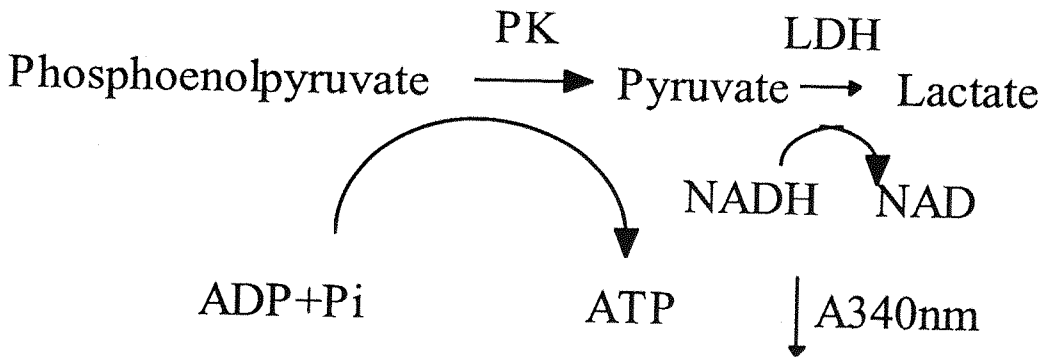
[1] It is clear that under the conditions of the assay placental 11-HSD2 activity at 10min was not measured under conditions of substrate excess. Therefore this data must be treated with caution. However any differences between dietary groups for placental 11-HSD2 activity are therefore likely to be underestimated.

Figure 2.7. **A schema of the pyruvate kinase (PK) reaction**



Pyruvate kinase is a glucocorticoid-insensitive irreversible enzyme. It mediates a key regulatory step in the glycolytic pathway, the transfer of phosphate from phosphoenolpyruvate to ADP forming pyruvate and ATP.

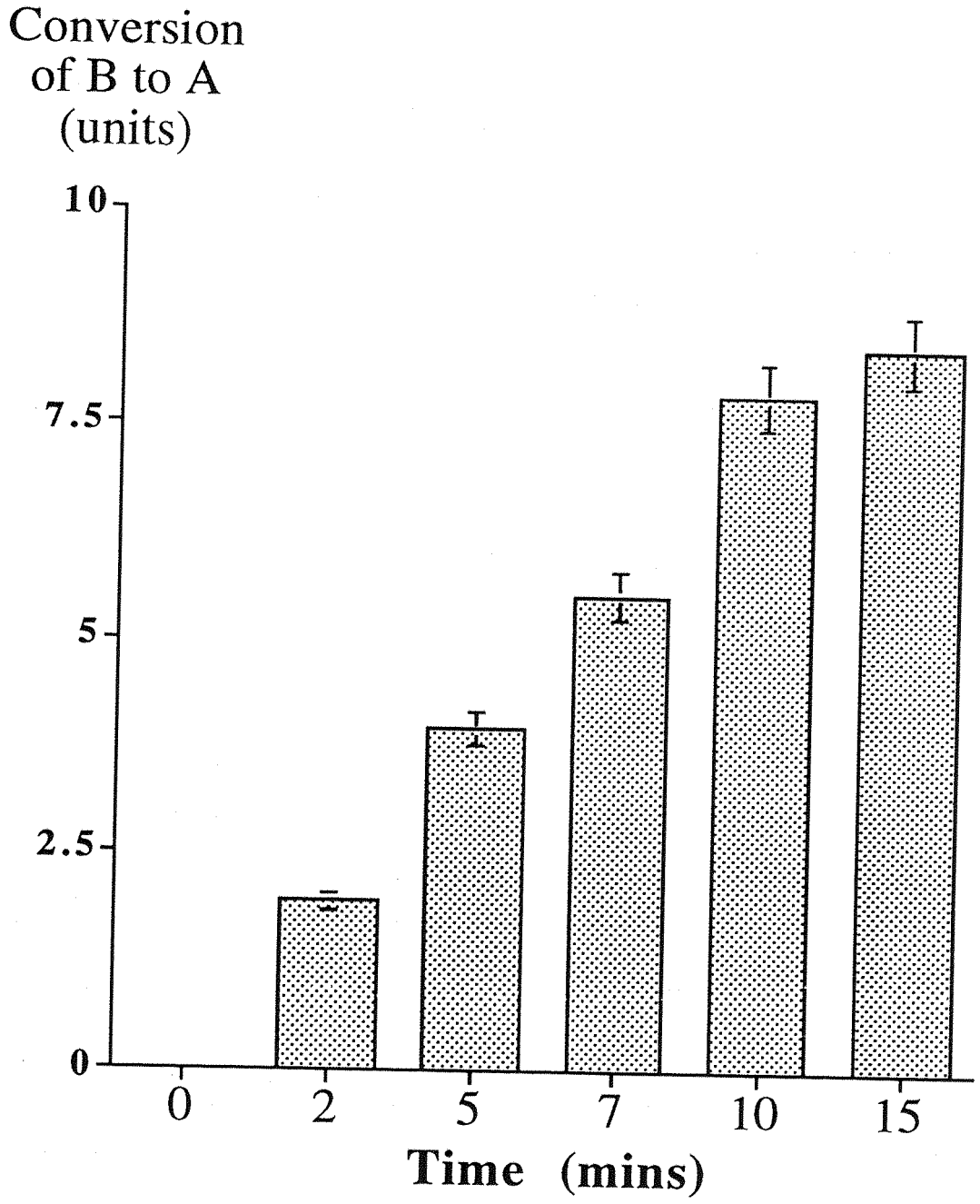
Key:

PK, pyruvate kinase

LDH, lactate dehydrogenase

A340, the decrease in absorbance is read at a wavelength of 340nm

Figure 2.8. **Assay of 11-HSD2 against incubation time at 37°C.**



The percentage conversion of corticosterone (B) to 11-dehydrocorticosterone (A) was established on duplicate samples of placental homogenates. The conversion was linear upto 10 mins incubation and consequently the assay was run for a 10 minute period thus reflecting enzyme activity in conditions of substrate excess i.e. first order kinetics. Units of activity are presented as pmoles A formed/mg protein/10mins.

or sample were assayed in duplicate. 200 µl of bicinchoninic solution (Appendix 3) was added to each sample and incubated for 30mins at 37°C. The absorbance was then measured at 550nm on a spectrophotometer. Intra and inter-assay variations of 4.4% and 5.5% were recorded respectively.

2.8.2. Method of Lowry

1ml of working reagent (Appendix 3) was added to 20 µl of sample or BSA standard (0-50 µg) and vortexed. After 10 minutes incubation at room temperature, 0.1ml Folin and Ciocalteu's phenol reagent (1:3 dilution) was added and tubes were immediately vortexed again. They were then incubated at 37°C for 10 minutes and absorbance at 750nm was determined.

2.9. HORMONE ANALYSES

Steroid hormones were analysed using radioimmunoassay (RIA) techniques with tritium (^{3}H) as the radioactive tracer, and a dextran-coated charcoal suspension (DCC) as the precipitation factor. Insulin and adrenocorticotrophic hormone utilised a double antibody radioimmunoassay protocol that employed ^{125}I as the radioactive tracer. Hormones (corticosterone) measured repeatedly within this laboratory were validated in our own hands. Hormones measured on only one occasion (ACTH, insulin, progesterone, 17ß-oestradiol, androstendione) were not validated in-house and thus the manufacturers validations were accepted. Appendix 3 lists all reagents used in the RIA including; standards, dilution buffers, dextran coated charcoal suspension and radio-active tracers.

2.9.1. Corticosterone

Corticosterone was assayed in rat plasma by competitive binding radioimmunoassay techniques following the method of Langley and York (Langley & York 1990). The corticosterone antibody was provided as a vial of lyophilised powder. To obtain the working solution of antibody 50mls of 0.05M Tris-HCl buffer, pH 8.0 containing 0.1% BSA and

0.1% sodium azide were added to the vial of lyophilised powder. Plasma from rats was diluted in ethanol to achieve a final concentration within the range of the standard curve (1:24, 1:12, or 1:6 (v/v)). The dilution varied depending upon the time of the day when the plasma was collected. The mixture was vortexed for 1min and centrifuged for 10mins at 3000rpm. The supernatant (containing sample hormone) was decanted and subsequently used within the assay.

Ethanolic plasma extracts were assayed in triplicate. Briefly, 0.1ml unknown plasma sample or standard (0-2ng corticosterone) and 0.5ml diluted antiserum were vortexed and incubated for 30 min at room temperature. The 0 ng corticosterone standard represented the "zero" binding tube as no corticosterone could bind to antiserum. In addition triplicate tubes containing either plasma and buffer (i.e. no antiserum representing "Blank" tubes) or buffer only were prepared to account for non-specific binding within tubes and total radioactivity respectively. After 30 min, 0.2ml of diluted ^{3}H-corticosterone was added to all tubes. . All tubes were then vortexed and incubated at 37°C for 1h. The tubes were then cooled for 15 min at 4°C. 0.2ml dextran coated charcoal was rapidly added to each tube (excluding tubes designated as total radioactivity) to separate bound and free steroid, vortexed for 30 sec and incubated for 10 min in ice cold water. The total volume in each tube was made upto 1ml with buffer. All tubes were then centrifuged at 2000g for 15 min at 4°C. Finally 0.6ml of the resultant supernatant was taken from each tube and placed into a scintillation vial with 4ml liquid scintillation cocktail (Appendix 3). The radioactivity present within tubes was then determined on a scintillation counter. The above system has been found to be sensitive to 30pg/tube with <0.3% cross reactivity with progesterone, cortisol and other sex steroids (data from Sigma datasheet).

Radioactivity (from ^{3}H-corticosterone) was inversely proportional to the concentration of unlabelled corticosterone and plasma concentrations were determined as follows: The average measured radioactivity (disintegrations per minute, dpm) of triplicate tubes designated non-specific binding (Blank tubes) was subtracted from the averaged measured radioactivity in all tubes containing either sample or standard, yielding the radioactivity due to binding of ^{3}H-corticosterone alone. This value was then divided by the proportion of unbound steroid (i.e. average counts for 0ng corticosterone standard (zero) tube

minus average counts for blank tubes). The sample plasma corticosterone concentration was determined from a curve of standard corticosterone concentration against the %bound (B)/ unbound (Bo) of standards (0-2ng/ml corticosterone). The intra-assay variation for this assay was 12% as determined in our own laboratory.

2.9.2. 17β Estradiol

17β Estradiol was assayed in ethanolic plasma extracts using an antibody raised against 17β estradiol in whole rabbit antiserum (Sigma). The antiserum was provided as a vial of pre-diluted lyophilised powder. To obtain the working solution 50mls of 0.05M Tris-HCl buffer, pH 8.0 containing 0.1% gelatin, 0.1M NaCl and 0.1% sodium azide was added to one vial of lyophilised powder. Plasma was diluted 1:5 in ethanol. The mixture was vortexed hard for 1min and centrifuged for 10min at 3000rpm. The supernatant was decanted and subsequently used within the assay.

0.1ml sample or standard and 0.5ml diluted antiserum (except BLANK & TOTAL) were added to glass tubes, vortexed and incubated for 30 min at room temperature. 0.2ml of diluted ^{3}H- 17β estradiol was then added to all tubes, vortexed and incubated at 37°C for 1h. The tubes were then cooled for 15 min at 4°C. 0.2ml DCC was rapidly added to each tube (except TOTAL), vortexed for 30 sec and incubated for 10 min in ice cold water. All tubes were then centrifuged at 2000g for 15 min at 4°C. Finally 0.6ml of the supernatant was taken from each tube and placed into a scintillation vial with 4ml liquid scintillation cocktail (Appendix 3) to determine the amount of radioactivity present.

The above system has been found to be sensitive to 5pg/tube with <0.1% cross reactivity with other sex steroids (Sigma datasheet). Plasma 17β estradiol concentration was determined by extrapolation from a curve of standard 17β estradiol concentration against the %bound (B) / %unbound (Bo).

2.9.3. Androstenedione

Androstenedione was assayed in ethanolic plasma extracts using an antibody raised against androstenedione in whole rabbit antiserum (Sigma). The antiserum was provided as a vial of pre-diluted lyophilised powder. To obtain the working solution 50mls of 0.05M Tris-

HCl buffer, pH 8.0 containing 0.1% gelatin, 0.1M NaCl and 0.1% sodium azide was added to one vial of lyophilised powder. Plasma was diluted 1:5 in ethanol. The mixture was vortexed hard for 1min and centrifuged for 10min at 3000rpm. The supernatant was decanted and subsequently used within the assay.

0.1ml sample or standard and 0.5ml diluted antiserum (except BLANK & TOTAL) were added to glass tubes, vortexed and incubated for 30 min at room temperature. 0.2ml of diluted ^{3}H- androstenedione was then added to all tubes, vortexed and incubated at 37°C for 1h. The tubes were then cooled for 15 min at 4°C. 0.2ml DCC was rapidly added to each tube (except TOTAL), vortexed for 30 sec and incubated for 10 min in ice cold water. All tubes were then centrifuged at 2000g for 15 min at 4°C. Finally 0.6ml of the supernatant was taken from each tube and placed into a scintillation vial with 4mls liquid scintillation cocktail (Appendix 3) to determine the amount of radioactivity present.

The above system has been found to be sensitive to 10pg/tube with <2% cross reactivity with other sex steroids (Sigma datasheet). Plasma androstenedione concentration was determined by extrapolation from a curve of standard androstenedione concentration against the %bound (B) / %unbound (Bo).

2.9.4. Progesterone

Progesterone was assayed in ethanolic/ether plasma extracts using an antibody raised against progesterone in whole rabbit antiserum (Sigma). The antiserum was provided as a vial of pre-diluted lyophilised powder. To obtain the working solution 50ml of 0.05M Tris-HCl buffer, pH 8.0 containing 0.1% gelatin, 0.1M NaCl and 0.1% sodium azide was added to one vial of lyophilised powder. 100µl plasma was diluted 1:1 in ethanol, vortexed and 2mls petroleum ether added. The mixture was vortexed hard for 1min and the supernatant removed by freezing the organic (aqueous) layer in dry ice and placed in glass tubes. The tubes were then dried down overnight in a fume cupboard.

0.5ml diluted antiserum (except BLANK & TOTAL) was added to glass tubes, vortexed and incubated for 30 min at room temperature. 0.2ml of diluted ^{3}H- progesterone was then added to all tubes, vortexed and incubated at 37°C for 1h. The tubes were then cooled for 15 min at 4°C. 0.2ml DCC was rapidly added to each tube (except TOTAL),

vortexed for 30 sec and incubated for 10 min in ice cold water. All tubes were then centrifuged at 2000g for 15 min at 4°C. Finally 0.6ml of the supernatant was taken from each tube and placed into a scintillation vial with 4ml liquid scintillation cocktail (Appendix 3) to determine the amount of radioactivity present.

The above system has been found to be sensitive to 5pg/tube. The antibody shows little (<1%) cross-reactivity with progesterone metabolites and with other steroids (Sigma datasheet). Plasma progesterone concentration was determined by extrapolation from a curve of standard progesterone concentration against the %bound (B) / %unbound (Bo).

2.9.5. Insulin

Insulin was assayed using a double-antibody binding radioimmunoassay kit (Diagnostic Systems Labs, Texas, USA). 100µl standard (0 - 12ng/ml) or sample, 100µl insulin antiserum (except BLANK & TOTAL) and 100µl ^{125}I were added to glass tubes, vortexed and incubated at 4°C for 16 hrs. 1ml precipitating reagent was then added to all tubes (except TOTAL), vortexed and tubes incubated for a further 15min at room temperature. All tubes were then centrifuged at 3000rpm for 20min and the supernatant aspirated off. The resultant pellet was then counted on a gamma counter to determine the amount of radioactivity present.

The above system has been found to be sensitive to 5pg/tube with <0.01% cross reactivity with other peptides (glucagon, somatostatin, pancreatic polypeptide and insulin-like growth factor-1). Plasma insulin concentration was determined by extrapolation from a curve of standard insulin concentration against the log transformation of %bound (B) / %unbound (Bo). Plasma insulin was determined on only one occasion and therefore the validations for the assay were accepted according to the manufacturers specifications. The kit has an inter and intra-assay variation of 8% and 6% respectively as determined by Diagnostic Systems Laboratories.

2.9.6. Adrenocorticotrophic hormone (ACTH)

ACTH was measured using a commercial competitive binding RIA kit (Penninsula Laboratories, Belmont, California, USA) within one month of purchase. All buffers and reagents used in this assay are described in Appendix 3. Plasma was diluted 1:1 (v/v) in Buffer A and centrifuged at 6000g for 20 min at 4°C. A SEP-COLUMN (Pennisula) was equilibrated by washing with Buffer B (1ml) once followed by Buffer A (3.0ml) three times. The plasma solution was loaded onto the pre-treated C_{18} SEP-COLUMN and washed with Buffer A (3.0ml) twice and the peptide eluted with Buffer B (3.0ml) once. The final eluant was collected into a polypropylene tube and evaporated to dryness in a rotary evaporator.

Prior to assay, the residue was dissolved in 250µl RIA buffer, vortexed and centrifuged at 6 000g for 20 min at 4°C. RIA buffer was diluted to 200ml with distilled water. Standard peptide was reconstituted in 1ml of buffer and anti-peptide serum in 13ml buffer. A range of ACTH concentrations between 1pg/tube - 128pg/tube was prepared with RIA buffer. 100µl of standard or sample and 100µl of anti-peptide serum was added to tubes, vortexed and incubated for 16-24h at 4°C. The ^{125}I-peptide was reconstituted with 1.0ml RIA buffer and mixed. 100µl of ^{125}I-peptide tracer was added to each tube, vortexed and incubated for a further 16-24h at 4°C. 100µl Goat Anti-Rabbit IgG GARGG and 100µl Normal Rabbit Serum NRS was added to each tube and contents vortexed and incubated for 90mins at room temperature. 500µl RIA buffer was then added to each tube and contents vortexed. All tubes were then centrifuged for 20 min at 3 000rpm, 4°C. The resulting supernatant was aspirated off and the amount of radioactivity present was counted on a gamma counter.

The above system has a sensitivity of 13pg/tube and shows no cross-reactivity with either ACTH 7-38 (Human), α-MSH, LH-RH or β-endorphin (Peninsula datasheet). Plasma ACTH concentration was calculated by extrapolation from the curve of standard ACTH concentration against the log transformation of %bound (B) / %unbound (Bo).

2.10. STATISTICAL ANALYSIS

Data are expressed as means ±SEM unless otherwise stated. Results were initially compared by analysis of variance (ANOVA) followed by suitable post hoc statistical methods (Bonferroni on Statview for Macintosh) for individual comparisons where differences were indicated. Differences were accepted as statistically significant at $P < 0.05$.

CHAPTER 3

THE ACUTE AND CHRONIC METABOLIC AND PHYSIOLOGICAL CONSEQUENCES OF MATERNAL PROTEIN RESTRICTION

3.1. INTRODUCTION

Pregnancy, second only to lactation, represents the most pronounced anabolic process experienced by any healthy, adult female animal. In order to fuel the demanding growth process, a constant supply of substrate is required. Maternal food intake thereby forms the basis of the nutrition that the fetus requires in order to grow. Manipulating the maternal nutritional supply has the potential to disrupt the intrauterine growth process (Robinson *et al* 1994), whether severe, such as 70% energy restriction (Woodall *et al* 1996a) or subtle, such as slight deficits in protein content (Langley & Jackson 1994).

In terms of reproductive outcome the pregnant rat is said to require a diet containing a minimum of 12% protein (National Research Council 1978). In many studies of maternal undernutrition in the rat, only extremes of dietary protein intake, either high (240-300 g/kg) (Hastings-Roberts & Zeman 1977; Zeman & Stanborough 1969) or low (0.7%) (Pond *et al* 1991) or even protein free (Hastings-Roberts & Zeman 1977) have been used, which do not represent the habitual intake of the rat. The diet used in the present studies (9% protein) therefore reflects only a marginal deficit (25%) in protein and in terms of reproductive success is not limiting (Langley & Jackson 1994).

Recent epidemiological evidence suggests a relationship between maternal undernutrition, compromised intrauterine growth and adult disease (Barker *et al* 1993a). Babies born within the normal birthweight range but disproportionate i.e. relatively long and thin or short in relation to head circumference are more disposed to cardiovascular disease (Barker 1994). This introduces a more subtle outcome by which to assess the adequacy of maternal nutrition. The concept that alterations to adult homeostatic systems reflect a

metabolic memory of prenatal nutrition is supported by animal studies (Dahri *et al* 1991; Desai *et al* 1995; Langley & Jackson 1994; Swenne *et al* 1987). A hormonal (glucocorticoid) mechanism has also been forwarded to explain the 'Barker Hypothesis' (Edwards *et al* 1993). This model however exhibits features common to nutritionally-induced hypertension (Langley-Evans *et al* 1996d) and thus a similarity may exist between the two apparently distinct models.

The following chapter characterises the acute effects of a low protein diet upon maternal and fetal growth patterns and explores the more chronic consequences of the diet in terms of blood pressure and glucocorticoid axis control.

3.2. PROTOCOL

The animals (n=12 dams/dietary group, unless otherwise stated in text) were fed diets containing either 18% casein (control) or 9% casein (maternal low protein; MLP) during pregnancy as described in *Methods*. Diets were substituted for standard laboratory chow at birth such that offspring differed only in their prenatal dietary experience. Enzymes and hormones were assayed according to previously described methodology, as presented in the relevant sections of *Methods*.

SECTION 1

THE EFFECT OF DIETARY PROTEIN RESTRICTION UPON MATERNAL FOOD INTAKE, GROWTH AND HORMONE STATUS

3.3.1. RESULTS

3.3.1.1. Maternal growth and food intake during gestation

Initially the animals were habituated to the semi-synthetic diets for 14 days prior to mating. During this prepregnancy period the animals from both dietary groups achieved a steady increase in weight and, after the two week period, animals on the control (18% casein) diet had gained 37 ±3g, n=11, compared to 38 ±3g, n=11 for animals on the maternal low protein (9% casein; hereafter referred to as MLP; Maternal Low Protein) diet (P= NS). At the time of mating, the average bodyweight of animals from each dietary group were similar (266 ±2g *vs* 264 ±3g for control and MLP groups respectively). The mating period (1-7d) did not inflence the gradual weight gain, so that at conception (d0 gestation) the animals from each dietary group weighed 279 ±10g *vs* 278 ±8g (NS) for control and MLP groups respectively. During pregnancy (Table 3.1) the weight gain of both dietary groups was significantly higher in week 3 than in week 2 or week 1 ($P< 0.05$). In week 3, control rats gained significantly more weight than MLP rats (P= 0.01).

Weight gain of pregnant rats fed laboratory chow at the Southampton University animal facility was found to be typically less than either the 18% or 9% casein groups in the first two weeks of pregnancy. In the second week this difference was statistically significant (P= 0.02) relative to 18% casein control rats. Food intake was not measured in chow-fed rats and thus decreased food intake could explain the decreased weight gain relative to rats fed the semi-synthetic diet. The synthetic diet is more energy dense (cal/g) than rat chow containing 10% fat as opposed to 2.9% fat in laboratory chow (see Appendices 1 and 2). The relatively lower weight gain of chow-fed animals may therefore reflect dietary differences. Similar to the

rats fed the semi-synthetic diet, weight gain of rats fed standard chow was significantly increased in week 3 relative to weeks 1 and 2 (P= 0.0003).

During the first week of pregnancy the average food intake of both controls and MLP fed animals increased relative to the food intake during the habituation period (Table 3.1), although this was not statistically significant. For the remainder of pregnancy the MLP rats maintained a steady intake of diet that did not differ significantly between week 1, 2 or 3. Rats fed control diet increased food intake significantly in mid-gestation (P= 0.01) when compared to day 0 and to week 1 (Table 3.1).

3.3.1.2. Maternal growth and food intake during lactation

Following delivery of the litters all dams were significantly heavier than at conception (P <0.05). During early lactation, despite similar food intakes in the two dietary groups (Table 3.2), MLP dams exhibited a greater gain in body weight than controls (P >0.05). Thus, by the time of weaning (3-4 wks post partum), the slight deficit in weight gain exhibited by MLP dams during gestation, was effectively compensated for (weight gain from postnatal d 1-21: control 89 ±6g; MLP 93 ±10g, P= NS).

3.3.1.3. Lactational performance of dams fed either a low protein or control diet during gestation

Pup weight gain represents a simple proxy for lactational performance, since all nutrition obtained by the pups during the first two weeks of lactation is from the dam's milk. During the first week of lactation the weight gain of low protein exposed pups was markedly reduced compared to controls (P <0.05) (Table 3.2). Weight gain was subsequently greater (NS) in the low protein group during week 2. MLP dams sequestered more of the total food intake than did control dams during early lactation as reflected in the relative efficiency of food utilisation for weight gain (*see Figure 3.1 legend)* between the two groups (Figure 3.1).

Table 3.1. **Maternal weight gain and food intake of rats fed either a control, low protein (MLP) or standard chow diet throughout gestation**

Weight Gain and Food Intake during Pregnancy (g/d)							
Weight Gain				**Gestation**			
Group	**Day 0**	**Week 1**	**n**	**Week 2**	**n**	**Week 3**	**n**
Control	-	5.4± 0.4	11	6.5± 0.5	10	10.7± 0.5 §	8
MLP	-	5.0± 0.4	11	5.7± 0.5	12	7.9± 0.9 *	8
Chow	-	4.3 ±0.8	7	5.0 ±0.3	7	9.3 ±1.2 *	7
Food Intake							
Control	25.2± 1.2	27.8± 1.5	9	32.5± 1.5	9	28.2± 1.5	9
MLP	24.6± 1.7	26.8± 0.5	11	30.2± 0.4	11	25.8± 0.4	11

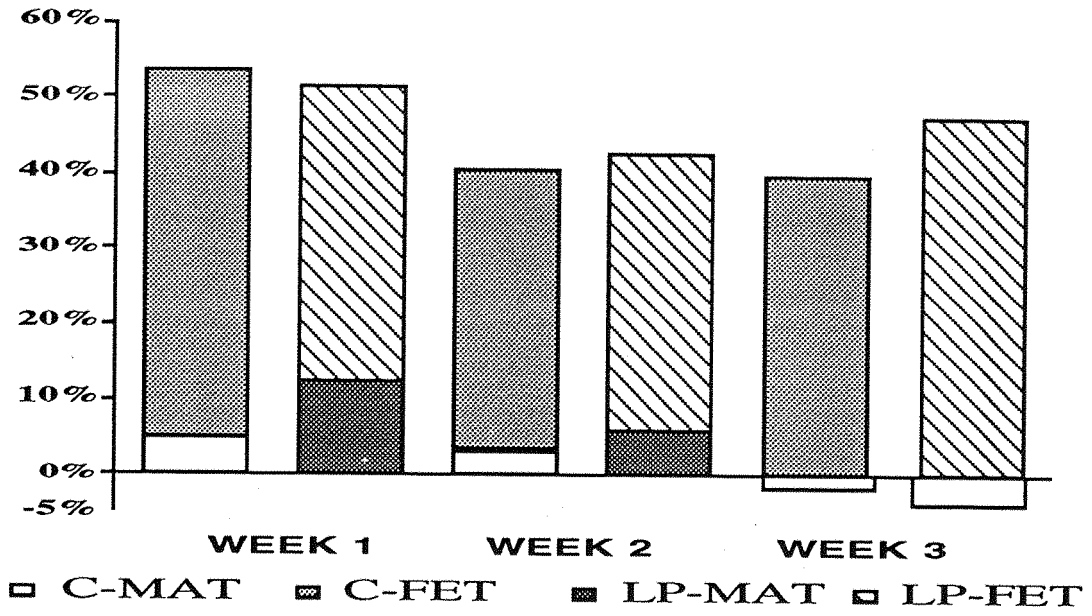
All data are given as Mean± SEM. Dams (n=8-12/dietary group) were housed in wire mesh cages on a 12 hr light:dark cycle at a temperature of 24°C. Animals were habituated to the experimental diets (Control, 18% casein; MLP, 9% casein) or chow (18.8% protein) for 2 wks prior to mating. Date of conception was determined by the presence of a plug on the cage floor and designated day 0 of gestation. Weight gain (g/d) and food intake (g/d) were determined during the first, second and third trimesters (Weeks 1, 2 and 3 respectively; day 21-22 = term). Two-way ANOVA indicated a significant effect of diet ($F= 4.96$, $P= 0.009$) and time ($F= 36.3$, $P< 0.0001$) on gestational weight gain. Food intakes were similar between groups for all weeks. * indicates significant difference to weeks 1 and 2 ($P<0.05$), § indicates significant difference to weeks 1 and 2 and to MLP week 3 ($P<0.05$) .

Table 3.2. **Weight gain and food intake of dams and pups during lactation exposed to either a low protein or control diet during gestation**

Weight Gain and Food Intake during Lactation (g/d)				
Maternal food intake				
	Week 1	**Week 2**	**Week 3**	***n litters***
Control	26.3± 3.2	46.5± 2.4 §	37.2± 1.6 *	4
MLP	26.0± 3.7	45.7± 3.3 §	29.6± 2.8 *	4
Maternal weight gain				
Control	1.33± 1.2	1.45± 1.0	-0.3± 0.6	4
MLP	3.2± 1.8	2.3± 0.3	-1.7± 0.7	4
Pup weight gain (g/d/pup)				
Control	1.28± 0.06	1.55± 0.07 *	1.51± 0.10 *	4
MLP	1.02± 0.05 ¶	1.73± 0.09 *	1.58± 0.10 *	4

All data are given as Mean± SEM. Dams (n=4/dietary group) were generated as outlined in methods. Refer to Table 3.1 for details. Litters were culled to 10 pups/litter (5male/5female) at birth to increase the data available for analysis. Data were analysed by two way ANOVA and subsequent Students t-test where differences were indicated. For pup weight gain there was a significant effect of age ($F= 16.5$, $P= 0.001$) and a trend towards an interaction between age and diet ($F= 3.1$, $P=0.06$). * indicates a significant difference to week 1 ($P<0.05$), § indicates a significant difference to weeks 1 and 3 ($P< 0.05$), ¶ indicates a significant difference to Control rats ($P<0.05$).

Figure 3.1. **The % efficiency of food utilisation for growth (food intake/total growth, [g/g]) achieved by dam & pup**



Offspring from rat dams (n=4 dams/group) were generated as outlined in *Methods*. Litters were culled to 10 pups/litter (5male/5female) at birth. Food intake (g/d) and weight gain (g/d) of dams and weight gain (g/d/pup) of pups were determined during the first, second and third weeks of lactation (Weeks 1, 2 and 3 respectively). Bars represent the total energy efficiencies (combining proportions contributed by dam and pup) as calculated below. Data reflect the Mean± SEM efficiencys of dams and pooled male and female data for pups (n=10pups/litter).

Total energy efficiencies were calculated as: $\frac{\text{Total weight gain of dam plus litter}}{\text{Total food intake/day}} \times 100$

The proportion energy (%) directed towards either fetal (A) or maternal (B) weight gain was then calculated from the total efficiency by:

$$A = \frac{\text{weight gain of litter}}{\text{total energy efficiency}} \times 100 \qquad B = \frac{\text{weight gain of dam}}{\text{total energy efficiency}} \times 100$$

In week 3 of lactation the dams from each dietary group were losing weight despite increases in weight of their litters, therefore B is negative and has not been shown on the Figure.

KEY: C-MAT - Control maternal efficiency **C-FET** - Control fetal efficiency

LP-MAT - Low protein maternal efficiency **LP-FET** - Low protein fetal efficiency

3.3.1.4. The effect of a control or low protein diet upon maternal and fetal hormone concentrations

Maternal diet and/or stage of gestation had no effect upon maternal plasma insulin, 17β-estradiol, androstenedione or glucose concentrations (Table 3.3). Plasma concentrations of corticosterone and glucose in fetuses at d20 gestation were all significantly lower ($P< 0.01$) than maternal concentrations. There was no effect of maternal diet upon the fetal concentrations of corticosterone or glucose.

Maternal plasma corticosterone concentrations were significantly higher at d20 gestation than at d14 gestation in both dietary groups. At both mid (d14) and late (d20) gestation MLP dams had modestly elevated (NS) corticosterone concentrations relative to controls (d14, 26% greater; d20, 6% greater) (Table 3.3). There was a significant influence of diet and stage of gestation ($F= 5.36$, $P= 0.03$), upon maternal plasma progesterone concentrations. MLP dams had elevated plasma progesterone concentrations at d20 relative to d14. The late gestational elevation in plasma progesterone concentrations was not apparent in control animals.

3.3.1.5. The activity of 11β-hydroxysteroid dehydrogenase (type II) at d14 and d20 gestation in placentas from rats fed either a control or low protein diet

The activity of 11β-hydroxysteroid dehydrogenase (11-HSD2) was lower in the placentas of MLP dams at both d14 and d20 relative to control rats. However when expressed as total activity per placenta, activities in MLP remained lower at day 20 but were greater than control activities at day 14 ($P<0.01$). Two way analysis of variance for diet and stage of gestation indicated a significant effect of diet ($F= 6.12$, $P= 0.01$) and stage of gestation ($F= 200$, $P< 0.0001$) (Table 3.4). Both fetal and placental masses were significantly higher in the MLP group at d14 gestation ($P< 0.05$) and relative to fetal mass the placental mass was markedly increased. At day 20 gestation the mass of fetus and the mass of placenta of MLP exposed rats were significantly ($P< 0.05$) reduced compared to controls and there was no difference in fetal mass relative to placental weight (Table 3.4).

Table 3.3. **Maternal and fetal hormone and glucose concentrations during gestation in rats exposed to either a control or low protein diet during pregnancy.**

	Maternal				Fetal	
	Pregnancy day 14		Pregnancy day 20		day 20	
Hormones (ng/ml)	**Control**	**MLP**	**Control**	**MLP**	**Control**	**MLP**
Insulin	2.1± 0.1	2.0± 0.1	2.1± 0.2	1.8± 0.1	-	-
Corticosterone	19.5± 3.3	24.6± 1.3	26.9± 2.4§	28.6± 2.2§	17.1± 1.5†	17.1± 1.1†
17β-estradiol	7.5± 0.7	7.1± 0.9	4.6± 1	8.5± 1.2	-	-
Progesterone	12.1± 1.8	10.7± 1.7	7.0± 1.2	12.2± 1.1*	-	-
Androstenedione	3.7± 0.5	4.3± 0.6	3.2± 0.4	4.4± 0.5	-	-
Glucose (mM)	4.6± 0.2	4.8± 0.2	4.3± 0.3	4.5± 0.2	1.3± 0.5†	1.8± 0.6†

Values are presented as Mean± SEM for n=6 dams/group. Data were analysed by two-way ANOVA for diet and age. Animals were generated as described in Methods. At day 14 a proportion of the rats (n=6/group) were decapitated within 1min of removal from the cage, and maternal blood collected. The remainder (n=6/group) proceeded to day 20 gestation and maternal and fetal blood collected. Maternal blood was collected in heparinised tubes, whilst fetal blood was drawn into capillary tubes and ejected into a heparinised container. It was necessary to pool blood from 3-4 fetuses within each of the 6 litters to obtain a sample that was sufficiently large for assay. This was only possible at day 20 gestation. Plasma was centrifuged and stored for up to 3 months at -80°C for analysis. *indicates a significant difference to controls at day 20 ($P<0.05$), † indicates a significant difference to maternal plasma hormone concentrations ($P<0.01$), § indicates a significant difference to plasma hormone concentrations at d14 ($P<0.05$).

Table 3.4. **The activity of 11β-hydroxysteroid dehydrogenase (11-HSD) in placental homogenates from rats exposed to either a control or low protein diet at d14 and d20 gestation**

Parameter	Pregnancy day 14		Pregnancy day 20	
	Control	MLP	Control	MLP
11-HSD activity (*units*)[2]	29.6 ±2.4	24.3 ±2.2*	6.54 ±0.29	5.39 ±0.19*
Total activity/placenta (*units*)	272 ±35	461 ±44*	360 ±20	287 ±10*
Fetal mass (mg)	131 ±15	167 ±40	3258 ±77	2940 ±40*
Placental mass (mg)	179± 7	290± 26*	566± 9	524± 8*
Placenta:Body mass	1.36 ±5	1.74 ±4*	0.17 ±0.004	0.17 ±0.003
Total protein / placenta (mg)	8.8 ±0.7	20.9 ±2.0*	55.4 ±2.0	53.9 ±1.5

Values are presented as Means± SEM for n= 19-27 offspring for each dietary group from a total of 24 rats that were mated and fed a diet containing either 18% (Control, n=12) or 9% (MLP, n=12) casein from d0 of gestation. At day 14 a proportion of the rats (n=6/group) were sacrificed and fetuses and placentas excised. The remainder (n=6/group) proceeded to day 20 gestation, were sacrificed and organs removed. From each rat dam within each group, the first three placentas from the ovarian end of right horn were removed in a random order, and assayed for 11-HSD2 activity following the method of Benediktsson *et al* (Benediktsson *et al* 1993) and described in section 2.7.6. of *Methods*. Protein was determined by the method of Smith *et al* (1985) and presented as total protein (mgs) per placenta. Two-way analysis of variance (ANOVA) indicated a significant effect of diet ($F= 6.12$, $P= 0.01$) and stage of gestation ($F= 200$, $P<0.0001$). Where differences were indicated a suitable post-hoc test was applied (Fishers' PLSD). *units* - activity expressed as pmoles corticosterone converted/mg reaction protein/ 10 mins. * indicates a significant difference to control rats $P<0.05$.

[2] The activity of 11-HSD2 in d14 placentas are much higher than in validation experiments. This is due to validations being done in d20 placentas. Therefore data at d14 may not reflect true activities. The difference in activity may in fact be an underestimate of the true difference.

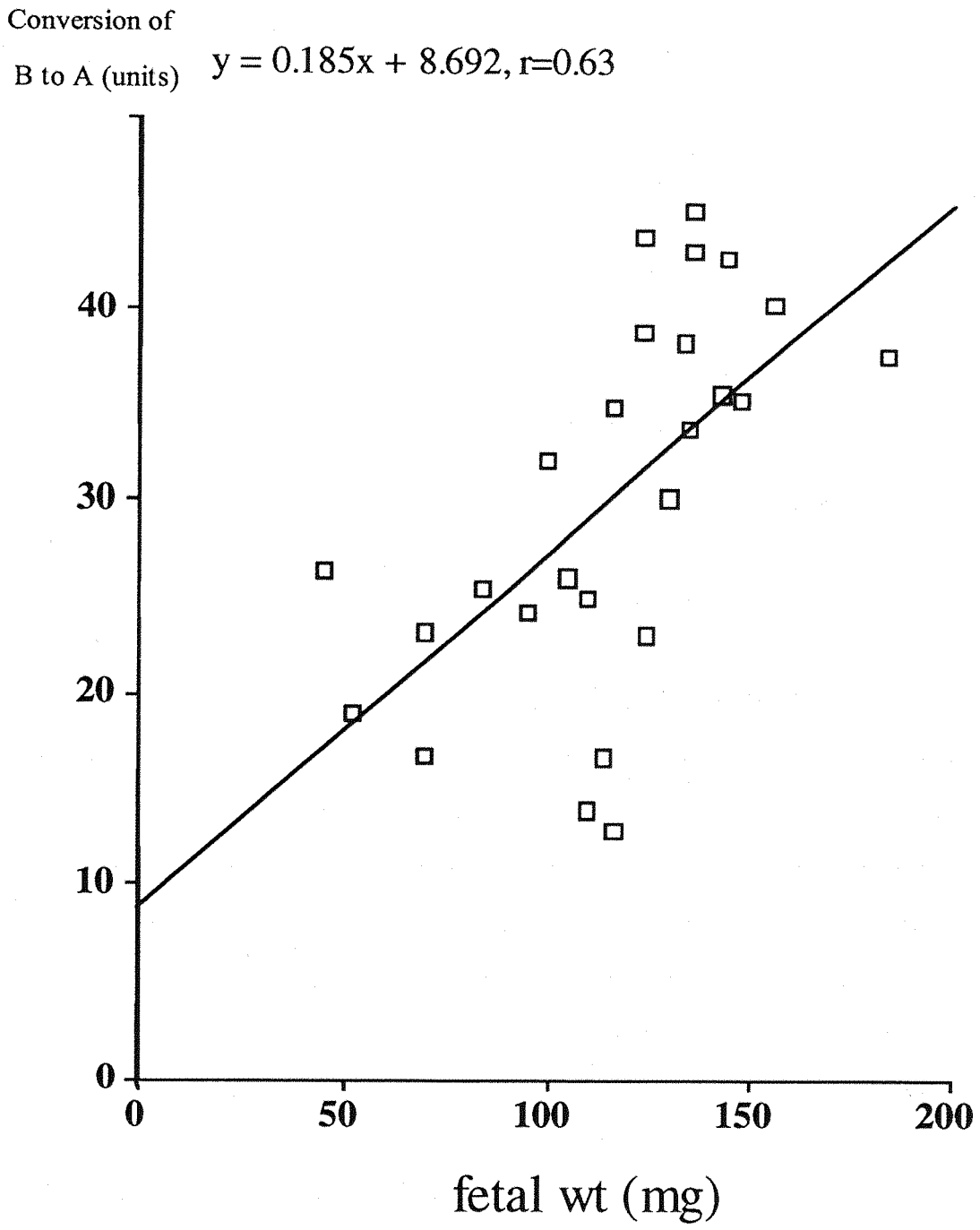
At both stages of gestation and in both dietary groups fetal weight increased as placental weight increased ($r= 0.39$, $P< 0.05$). At d14 ($r=0.63$) and d20 ($r=0.52$) there was a positive relationship between 11-HSD2 activity and fetal weight in rats fed control diet but not in MLP. The relationship at d14 is shown in Figure 3.2.

3.3.1.6. DISCUSSION

Food intake was similar between dietary groups at each stage of gestation. Whereas in late gestation dams fed the control diet had a significant increase in bodyweight, the MLP dams failed to exhibit a similar increase in weight. The concentrations of insulin, 17-β estradiol, corticosterone, progesterone and androstenedione were unaffected by the dietary regimen in mid-gestation, but progesterone concentration was higher in MLP than in controls in late gestation. Protein restriction did not alter the maternal plasma concentration of glucose or the fetal concentrations of corticosterone or glucose. The activity of 11β-hydroxysteroid dehydrogenase type II (11-HSD2) was reduced in placental homogenates of MLP at d20, yet increased when expressed per placenta at d14 gestation.

In order to satisfy the demands of pregnancy in terms of reproductive capability the pregnant rat dam requires a minimum of 12% dietary protein content (National Research Council 1978). The MLP diet used in this study reflects a mild protein restriction (25%) and does not compromise reproductive success in MLP, given that litter sizes were similar between dietary groups (*section 3.3.2.2.*). In early gestation maternal weight gain was similar in the two groups. Towards the end of the second week and certainly by the third the effects of MLP were manifest as decreased maternal weight gain relative to controls. This observation is commonly found when rat dams are fed a low protein diet (Desai *et al* 1996; Zeman 1967). Thus, in late gestation, the control rats were gaining upwards of 2g per day more than MLP animals. The greater weight gain exhibited by control rats relative to MLP cannot be explained by an increase in food intake since food intakes were not significantly different between the dietary group. It appears that the impact of the MLP diet upon the dam is primarily restricted to late gestation. It has been suggested that during early gestation, the dam 'prepares' for the increased demand of the late gestational fetal growth spurt by depositing nutrient reserves

Figure 3.2. **The relationship between 11-HSD2 activity and fetal weight in control rats at d 14 gestation.**



Data are 11-HSD2 activities in control rats (n=26 from 6 litters) at day 14 gestation. Refer to Table 3.4 for details. 11-HSD2 activity was assayed following the method of Benediktsson *et al* (Benediktsson *et al* 1993) and described in section 2.7.6. of *Methods*. Units are presented as pmoles A formed/mg protein/10 mins

(amino acids and fat) (Naismith 1969; Naismith & Morgan 1976) which are then subsequently utilised, either in late gestation, or during lactation. Whether the MLP diet interrupts this anabolic process and therefore compromises the ability of the dam to sustain fetal demands adequately in late gestation cannot be determined from the present results.

Mayel-Afshar & Grimble observed protein synthesis to continue in the face of a low protein diet in early gestation (Mayel-Afshar & Grimble 1983). Maternal protein catabolism in late gestation, however, occurred earlier and more rapidly when a low protein diet was fed. The apparent failure of MLP to match the weight gain of controls in late gestation is, therefore, primarily due to the actual deficit in protein *per se* at a time when demands are increased, thus forcing MLP dams to mobilise their own bodily stores to a greater extent than controls. This is perhaps reflected in the reduced weight gain of MLP relative to controls after the products of conception have been removed (*section 3.3.1.2.*).

Whether the nutritional challenge presented to MLP, reflected in reduced late gestational weight gain relative to controls, is sufficiently severe to compromise delivery of nutrients to the fetuses is not known. More severe nutritional interventions (6% protein) than the current model reduce placental glucose transport (Rosso 1977) and amino acid transport (Malandro *et al* 1996) to rat fetuses, perhaps mediated by changes in their respective transporter systems (Malandro *et al* 1996). In an experimental ovine model of intrauterine growth retardation the placental flux of leucine was reduced by over 50% (Ross *et al* 1996). Furthermore there is evidence to suggest that nutrient restriction during pregnancy in the rat reduces blood flow to the placenta, mediated perhaps by a reduced cardiac output (Rosso & Kava 1980) or attenuated plasma volume expansion (Rosso & Streeter 1979). This would compound any changes to the physical transfer of nutrients across the placental barrier.

The effect of nutritional restriction during gestation on maternal food intake may relate to the severity of the restriction. Severe restriction of the protein component of the diet results in a decrease in food intake (Nelson & Evans 1953; Zeman 1967), perhaps due to the excess energy provided in the form of carbohydrate when on a isocaloric, low protein diet. Moderate dietary restriction can lead to an increase in food intake, possibly in an attempt to increase the availability of a limiting nutrient (Widdowson & McCance 1975). The MLP

dams in this study ate less than controls but this was not statistically significant. In mid-gestation, a time when the fetal growth rate is significantly increasing (Langley-Evans *et al* 1996b) the control rats increased their food intake, presumably to supply the demands of the growing fetuses. No such effect was observed in MLP animals thus contributing further to the decreased weight gain observed in MLP over late gestation. The principal dietary inadequacy in the present model is of protein. Overall food intake and thus energy intake between the two dietary groups over the course of gestation is not vastly different, and the protein content of the MLP diet is reduced by 50% whereas the carbohydrate proportion is only increased by 14% (*Appendix 1*).

Unfortunately from an initial total of n=6/group for measurement of lactational weight gain two dams from each dietary group resorbed their pregnancies and thus data for maternal weight gain during lactation shows increased variation and therefore must be interpreted with caution. However, combined with the food efficiency data for both dam and pup there is an indication of the postnatal response from MLP diet to a protein replete diet. During early lactation there were no differences in food intake between each dietary group. Food intake in both groups increased markedly in the second week of gestation, a time when pup growth was greatest. In late lactation (week 3) food intake in MLP was lower than controls despite the MLP pups growing at a faster rate. This may reflect either an increased metabolic efficiency with which MLP pups handle substrate, as occurs in the genetically obese Zucker rat (Langley 1990) or be due to differences in milk intake or quality (Rose & McCarty 1994). Weight gain of the dams during early lactation was greater in MLP than controls. Given the limited weight gain experienced by MLP dams during gestation, when measured post-partum (*section 3.3.1.2.*), then an increased partitioning of substrate during early lactation towards their own demands may reflect recovery from the effects of the MLP diet.

Partitioning of nutrients towards the mother, however, may be at the expense of the pup since the MLP pups gained significantly less weight than controls in early lactation (*section 3.3.1.2.*). Although the dams were changed from the low protein diet to laboratory chow at birth, metabolic adjustment to the higher protein diet might not have been instantaneous. The possibility exists that delayed adjustment to a protein replete diet in MLP during lactation influences future blood pressure cannot be assessed in the present

results. The effects of a protein replete diet in gestation followed by a protein deficient diet during lactation have not been determined using the present model. Certainly the ability to demonstrate postnatal catch-up growth is not seen in pups who are fed a low protein diet during lactation (Desai *et al* 1996). This is not surprising given that the relative demands on the dam are considerably higher during lactation. Programming of altered cholesterol and triacylglycerol metabolism has also been shown to be dependent upon either prenatal or postnatal undernutrition (Lucas *et al* 1996).

Within a defined range the body is able to adapt to changes in protein supply (Danielson & Jackson 1992). The process of adaptation to protein undernutrition is directed by hormones and the ability of dietary factors to influence hormone concentrations in the rat have been described (Edozien *et al* 1978). Pregnancy itself represents a period of dramatic hormonal change in the maternal environment in mammals. Clearly, maternal nutrient restriction may be expected to alter the maternal hormonal environment, and many studies have demonstrated this phenomenon (Dwyer & Stickland 1992; Gluckman 1995; Harding & Johnston 1995). In the present study, however, the maternal plasma concentrations of insulin, 17β-estradiol and androstenedione were similar between dietary groups at mid (d14) and late (d20) gestation and were within the range previously described for rats (Dwyer & Stickland 1992, Martin *et al* 1978, Lunn & Austin 1983). However, such snapshot measurements of hormonal concentrations do not yield sufficient evidence for rates of hormonal flux and only limited conclusions can be drawn from the current results regarding nutrient-hormone interactions. For example, the measurement of plasma insulin was made without an overnight fast and thus no valid conclusion can be drawn from the insulin data. Due to time and financial constraints a further, more meticulous investigation (i.e. multiple measurements throughout gestation) was not carried out and this represents an area for further research.

The plasma concentration of progesterone in MLP rats was greater at d20 gestation than at d14. Towards late gestation progesterone is observed to rise (Garland *et al* 1987). MLP diet abolished the fall in plasma progesterone seen in the control group. The reason for the apparently unchanged progesterone concentrations in controls is not known but may simply reflect a phase-shift in progesterone secretion in MLP, as occurs for steroid hormones

in rats on a food restricted regimen (Oliveira *et al* 1993). Thus the peak of progesterone secretion that ordinarily occurs in late gestation may have been missed in controls due to sampling at only two timepoints in gestation. Maternal plasma corticosterone concentration in MLP rats was slightly greater than concentrations observed in control rats at d14 (25%) and d20 (6%) gestation. The energy intakes between dietary groups were not significantly different and maternal plasma corticosterone concentration is related to total energy restriction rather than protein (Lunn & Austin 1983). However feeding either very low protein diets (0.005 protein:energy ratio) (Coward *et al* 1977) or a balanced reduction (40%) in total feed intake (Dwyer & Stickland 1992) elevates maternal plasma corticosterone.

Perhaps the single most important result, regarding the effect of the MLP diet upon the dam, is the observation of lower placental 11-HSD2 at day 20. The decrease in 11-HSD2 activity does not merely reflect a reduction in placental size, as it may appear from the data, since activities were lower when expressed per mg placental weight (Control; 0.64 ±0.03, MLP; 0.54 ±0.02 pmoles converted/per mg placenta/10 mins, P=0.02). Previous work has noted, impaired placental 11-HSD2 activity (33% decrease) in day 20 placentas (Langley-Evans *et al* 1996e)[3]. Methodological errors as previously highlighted may account for the difference in 11-HSD2 activity between this thesis and the activity recorde by Langley-Evans *et al* 1996e). Lower activity of placental 11-HSD2 implies the potential for increased transfer of maternal corticosterone into the fetal compartment. However, given the efficacy of placental 11-HSD2, a reduction in activity of 18-33% may well be accommodated by the enzyme (Stewart *et al* 1995) and when expressed per total placenta (which reflects *in vivo* physiology) the activity of 11-HSD2 at day 14 (but not day 20) was actually increased. The increase in total 11-HSD2 at day 14, as a consequence of a large placental weight in MLP relative to controls may facilitate the greater weight gain exhibited by MLP in early gestation, since GC retard intrauterine growth (Reinisch *et al* 1978).

MLP-induced hypertension is remediable by pharmacological adrenalectomy (PHADX) with metyrapone and corticosterone replacement successfully restores the hypertensive state in females (Langley-Evans 1997a). The activity of 11-HSD2 following

[3] These studies were flawed on methodological grounds. However the difference in 11-HSD2 activity between dietary groups (33%) may be an underestimate of the true difference

PHADX has not been measured. However the effect of diet upon placental 11-HSD2 is unlikely to be direct but rather a product of the maternal adrenal acts as an intermediate in the regulation of placental 11-HSD2. To test this hypothesis the activity of 11-HSD2 could be measured following maternal adrenalectomy. Furthermore, PHADX may cause increased fetal ACTH which has been proposed to inhibit placental 11-HSD2. The data presented offer no suggestion as to the mechanism of lower placental 11-HSD2 activity. Since the activity of 11-HSD2 is lower at day 20 even when expressed per placenta, then a physiological effect of low protein upon the late gestational activity of 11-HSD2 might be assumed. A specific role for maternal GC excess in the programming of elevated SBP is inferred. An effect of MLP upon the activity of 11-HSD1 cannot be excluded from the present results since maternal decidual tissue was incorporated within the reaction volume. 11-HSD1 activity has been demonstrated in rat placenta (Burton & Waddell 1995) and an increase in this activity at day 20 may have a similar effect on the fetal environment as a diminution of 11-HSD2 activity. Thus future analyses of the oxidative capacity of 11-HSD1 in maternal decidua following MLP diet may be important. Without direct measurements of placental hormone flux (i.e. through radioisotope measurements), however, the extent of MLP fetal exposure to excess maternal GC is unknown. The present data did not exhibit a negative correlation between placental weight and 11-HSD2 activity as has been observed by Benediktsson *et al* (1993). The disproportionate growth of MLP i.e. their increased growth rate to day 20 and their heavy yet thin and long phenotype at day 20 relative to controls may mask such a correlation in this group but the reason for a lack of a correlation in controls is not known.

The mechanism linking the MLP diet with lower placental 11-HSD2 at d20 gestation is not clear. 11-HSD2 has been reported to be regulated by GC, oestrogen, thyroid hormone, progesterone and growth hormone (Baggia *et al* 1990; Low *et al* 1993; Monder & Shackleton 1984; Stewart *et al* 1995; Whorwood *et al* 1994). Many of these earlier studies were performed in mice, as well as rats, and the regulation of 11-HSD2 differs between the two species (C.Whorwood - *personal communication*). Furthermore, earlier work failed to distinguish between the two isoforms of 11-HSD, which are now known to be genetically distinct (Brown *et al* 1993). Thus, at present, data regarding the specific regulation of rat placental 11-HSD2 is scarce. The hormonal profile established for MLP in the present study

offers no definitive answer to explain the association between maternal diet and reduced placental 11-HSD2 activity. The elevated concentration of progesterone in MLP relative to controls in late gestation could, theoretically, explain a reduction in 11-HSD2 since progesterone in high concentrations inhibits 11-HSD2 activity (Stewart *et al* 1995). However whether the low progesterone identified in control rats at day 20 is robust needs to be established. Consideration of placental 11-HSD2 mRNA expression at d14 suggests that a down-regulation occurs, at the level of transcription. Furthermore, decidual 11-HSD1 mRNA may perhaps be increased in late gestation, thus further increasing the fetal glucocorticoid exposure (S.C. Langley-Evans & C. Whorwood - *personal communication*). Despite lower placental 11-HSD2 at day 20 there was no evidence of an increase in corticosterone concentration in fetal plasma at this time. Plasma could not be obtained at day 14 due to the small size of fetuses. Perhaps a more sensitive marker of corticosterone action, such as GPDH mRNA (Schlatter *et al* 1990), may offer a more robust marker of the fetal glucocorticoid environment.

The present results offer a common factor between two apparently diverse models of hypertension. Edwards, Seckl and colleagues have established a rat model of hypertension that is dependant upon maternal glucocorticoids for the genesis of the hypertensive state (Edwards *et al* 1996). In a series of studies where placental 11-HSD2 has either been bypassed (using dexamethasone-a synthetic glucocorticoid and weak substrate for 11-HSD2) or inhibited (using carbenoxolone), to increase the endogenous flux of maternal glucocorticoid to the fetus, lower birthweight and hypertension follow (Seckl 1997b).

Prenatal treatment with glucocorticoids has been shown to retard intrauterine growth (Katz *et al* 1990; Reinisch *et al* 1978). Placental 11-HSD2 therefore appears to play an important role in the regulation of intrauterine growth, protecting the fetus from the potentially deleterious effects of excess maternal bioactive glucocorticoid exposure. Stewart *et al* (1995) suggest that the role of placental 11-HSD2 is to maintain fetal HPA axis autonomy in late gestation since the fetus displays 11-HSD2 activity, which will therefore modulate any fetal glucocorticoid excess produced through a lowering of placental 11-HSD2 activity. Activity correlates positively with fetal weight in rats (Benediktsson *et al* 1993) and humans (Stewart *et al* 1995). In humans, the activity of placental 11-HSD2 in an *ex vivo* system

correlates closely with, and is able to predict, birthweight (Benediktsson *et al* 1995). Low birthweight infants, therefore, that are of smaller size than would be predicted by placental weight will theoretically have been exposed to a higher level of maternal glucocorticoid. Thus, intrauterine glucocorticoid exposure is proposed by Edwards *et al* (Edwards *et al* 1993) to underscore the fetal origins of adult hypertension hypothesis (Barker 1995).

However there are methodological drawbacks to both dexamethasone (i.e. increasing maternal SBP) and carbenoxolone (i.e. inhibits 11-HSD1 in addition to 11-HSD2) treatment during pregnancy in the rat which may preclude a specific effect of maternal glucocorticoid being inferred. Indeed dexamethasone is a weak substrate for 11-HSD2 (Best *et al* 1997). Moreover, the negative correlation of 11-HSD2 activity with placental weight that Benediktsson *et al* (1993) report will be cancelled out when total activity per placenta is related to fetal weight. From the outset Barker and colleagues, however, suggested nutritional factors as being important (Barker & Osmond 1986) and have since collected evidence to support this notion (Campbell *et al* 1996; Forrester *et al* 1996; Godfrey *et al* 1996). The present results provide an early mechanism, i.e. lower placental 11-HSD2 activity, whereby nutritional factors (MLP diet) may influence the development of hypertension through the action of hormonal factors.

SECTION 2

THE EFFECT OF FEEDING A MATERNAL LOW PROTEIN DIET UPON THE PRE AND POSTNATAL DEVELOPMENT OF THE OFFSPRING

3.3.2. RESULTS

3.3.2.1. Fetal mass at day 20 gestation

Overall litter size was unaltered between dietary groups (Table 3.5). Relative to controls, MLP fetuses were larger, in terms of total body mass (P= 0.001) and longer, in terms of nose-anal length (mm) (P= 0.0001) at day 20 gestation. Similarly the brain, liver,

lung and placenta were all heavier in MLP relative to control fetuses (Table 3.5). Only the latter did not achieve statistical significance ($P< 0.05$). When the mass of each organs was expressed relative to body weight evidence of disproportion emerged. Both the placenta and brain were significantly smaller relative to body weight in the MLP group compared to control fetuses ($P< 0.05$). Similarly, the relative liver size was reduced although this did not reach statistical significance. The Lee index (body mass:length^3), which provides a measure of body proportionality (Bernadis & Patterson 1968), indicated that the fetuses of dams fed the low protein diet were longer and/or thinner in proportion to body weight than control fetuses (Table 3.5).

3.3.2.2. Body and organ masses of term neonates

Litter sizes and body weight were similar in the two dietary groups follwing delivery (Table 3.6). The protein restriction regimen impacted predominantly upon the liver, which was both smaller in absolute weight ($P= 0.04$) and in relative terms ($P= 0.0006$) compared to the pups of dams fed the control diet. Interestingly the nose-anal length of MLP pups was significantly shorter at term ($P= 0.04$) compared to control animals, the opposite to what was observed in the fetuses at d20. Analysis of body proportion (Lee index) suggested that the MLP pups were relatively shorter and/or heavier for their length ($P= 0.0001$) compared to control animals (Table 3.6).

3.3.2.3. Fetal growth in late gestation from protein restricted and control dams

Measuring individual body and organ weights can yield information about absolute differences between two groups at a given point in time but does not give an indication about the nature of the protein restriction in terms of intrauterine growth dynamics. Therefore, by measuring the increase in organ weights from d20 to term, expressed as a percentage of the weight at d20, an indication is given as to how the effects of maternal protein restriction manifest at the organ level in late gestation fetuses. As Figure 3.3 illustrates the control fetuses doubled in weight over the last two days of gestation. Protein restriction attenuated this late gestation increase in body weight by 25%. Both liver and lung growth of MLP fetuses in late gestation were severely reduced compared to controls (14% *vs* 53% for

Table 3.5. **The organ masses and relative body proportions of day 20 rat fetuses exposed to either a maternal control or low protein diet**

	Maternal Diet		
	Control	**MLP**	***P***
Litter size	14 ±1	13 ±1	NS
Fetal body weight (g)	3.01 ±0.07	3.09 ±0.04	0.001
Placental weight (mg)	547 ±9	558 ±10	NS
Brain weight (mg)	109 ±3	122 ±2	0.0003
Liver weight (mg)	122 ±6	152 ±4	0.002
Lung weight (mg)	78 ±4	105 ±2	0.0001
Nose-anal length (mm)	34 ±0.4	40 ±0.8	0.0001
Placenta:Bodyweight (%)	18.8 ±0.3	18.4 ±0.4	0.04
Brain:Bodyweight (%)	4.49 ±0.15	3.76 ±0.09	0.0003
Liver:Bodyweight (%)	4.99 ±0.17	4.60 ±0.15	NS
Lung:Bodyweight (%)	3.11±0.10	3.19 ±0.06	NS
Lee Index (Bodyweight:Length^3)	0.075 ±0.003	0.052 ±0.001	0.0001

All data are presented as Means ± SEM for n= 21-55 offspring (Control) or n= 29-52 offspring (MLP) from a total of 5 dams from each dietary group. Dams were terminated at day 20 gestation and fetuses and organs excised. Animals were generated as outlined in *Methods*. NS = not significant.

increase in liver weight at term relative to day 20, 24% *vs* 56% for lungs. {MLP *vs* control rats respectively}). In contrast the brains of MLP fetuses maintained 84% of the growth achieved by the control rats (Figure 3.3). The severe reduction in truncal growth from d20 to term (Figure 3.3) in MLP fetuses compared to controls (26% *vs* 54%, MLP *vs* controls) may, in part, explain the observed reciprocal change in the Lee index over this period (*see Tables 3.5 and 3.6).*

3.3.2.4. Postnatal growth of offspring from protein restricted and control dams

Growth was measured from birth to 40 weeks of age in male and female offspring. Following weaning, there was no difference in postnatal growth between the two dietary groups (Figure 3.4). As expected, males from each dietary group were significantly heavier than females from 5-6 weeks of age.

3.3.2.5. Body and organ masses at weaning age

At weaning age (3-4 wks) MLP pups were significantly heavier than control pups (P= 0.03) (Table 3.7). MLP had significantly larger livers (P= 0.004) and hearts (P= 0.01). However, when expressed as a percentage of body mass these differences were no longer seen. Absolute wet weights of brain, lung and kidney were similar in the two dietary groups although as a percentage of body mass, both the lung and kidney were significantly smaller in MLP rats than in control animals (P< 0.01) (Table 3.7).

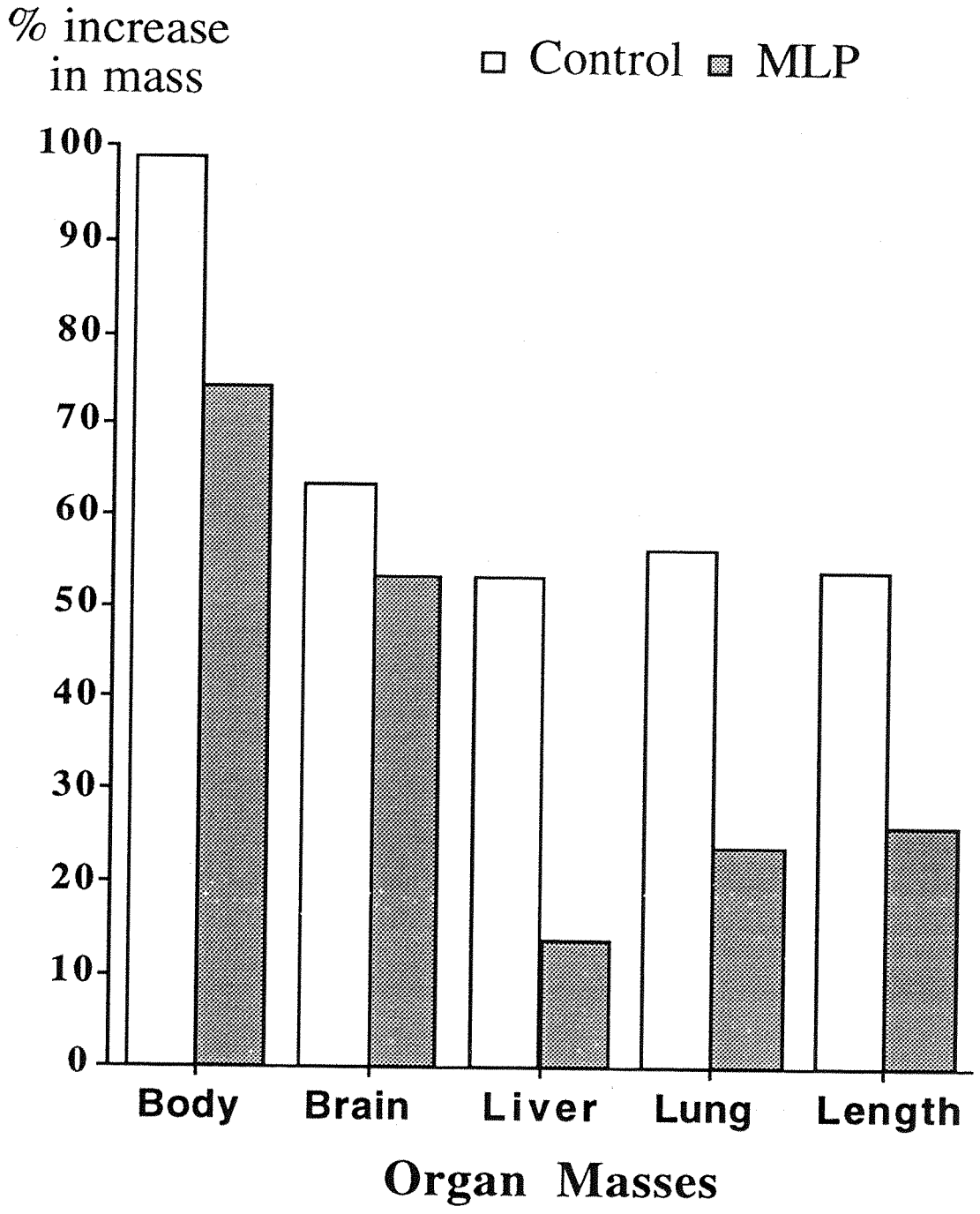
The greater body weight exhibited by the MLP rats at weaning was peculiar to females (a 13% increase, P= 0.007) (Table 3.9) rather than males (an 8% increase, NS) (Table 3.8). The greater absolute wet weight of hearts, observed in MLP animals, was only apparent in males (P= 0.05), but when analysed relative to bodyweight, only females had significantly smaller hearts (P= 0.05) (Table 3.9). Similarly, the previously observed decrease in the kidney:body mass ratio in low protein exposed animals relative to controls appeared to be specific to females, *P*= 0.01 (Table 3.9). In contrast to the kidney, the relative differences in lung proportions were only apparent in males (Table 3.8), probably due to the fact that in females the absolute lung weights were significantly heavier than controls (P= 0.04).

Table 3.6. **The organ masses and relative body proportions of term rat neonates exposed to a maternal control or low protein diet**

	Maternal Diet		
	Control	**MLP**	***P***
Litter size	13± 1	14±1	NS
Body weight (g)	5.49± 0.08	5.75± 0.10	NS
Brain weight (mg)	178± 5	187± 4	NS
Liver weight (mg)	187± 5	173± 5	0.04
Lung weight (mg)	122± 4	130± 3	NS
Nose-anal length (mm)	52± 0.4	50±0.5	0.04
Brain:Bodyweight (%)	3.28± 0.10	3.27± 0.09	NS
Liver:Bodyweight (%)	3.45± 0.08	3.02± 0.08	0.0006
Lung:Bodyweight (%)	2.24± 0.05	2.22± 0.05	NS
Gestation length (d)	22.9± 0.1	22.8± 0.1	NS
Lee Index (Bodyweight:Length^3)	0.036± 0.0007	0.046± 0.001	0.0001

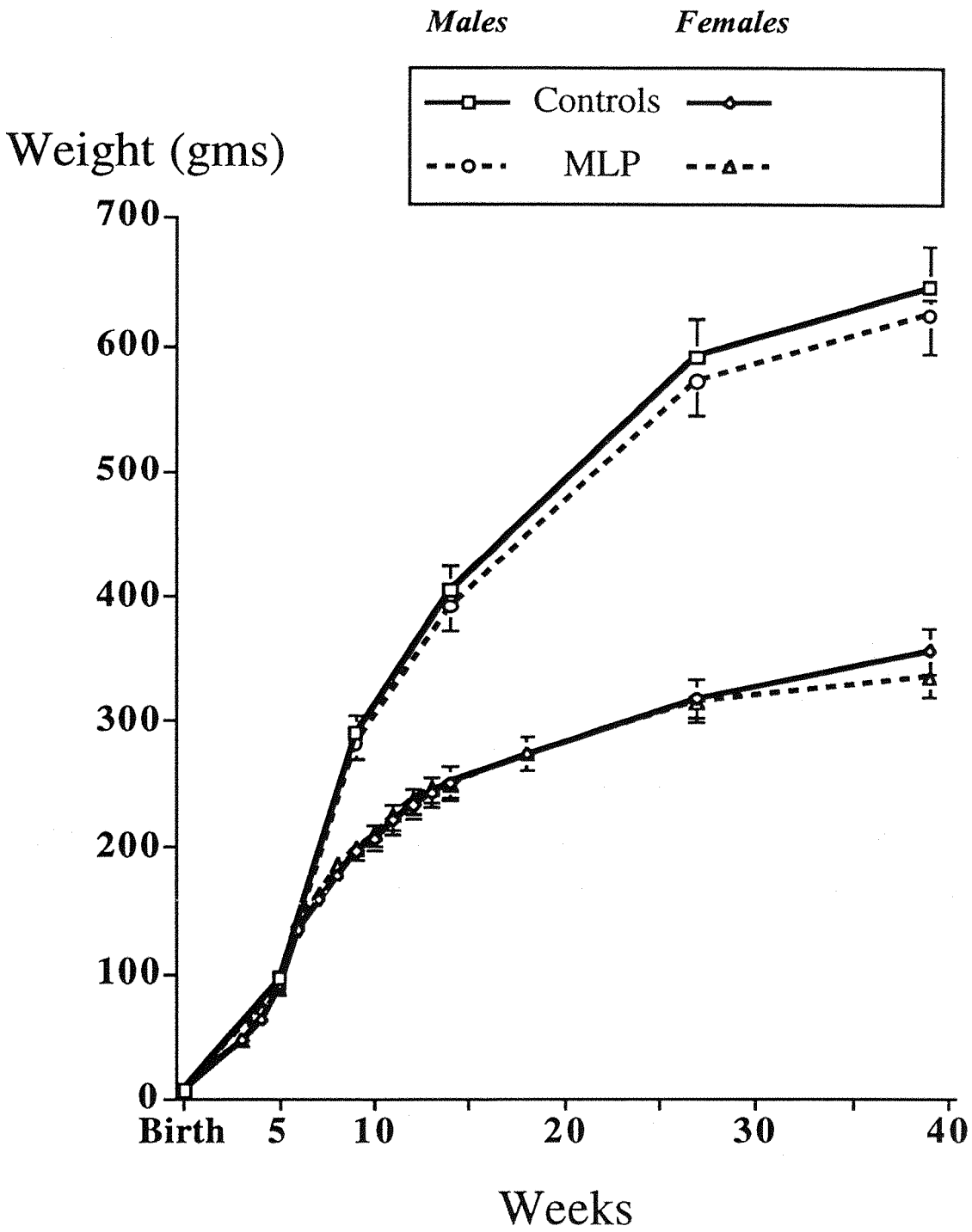
All data are presented as Means± SEM for *n*= 30-53 offspring (Control) or *n*= 38-65 offspring (MLP) from a total of 5 litters from each dietary group. Litters were killed within 12hrs of birth. Animals were generated as outlined in *Methods*. NS = not significant.

Figure 3.3. **The percentage increase in mass and length between day 20 and full term in rat fetuses exposed to either a control or low protein maternal diet**



Values are expressed as the mean percentage of the fetal mass/length as determined at day 20 gestation. A total of 20 rat dams were fed either 18% casein (control, n=10) or 9% casein (MLP, n=10) for two weeks prior to mating and throughout gestation. At day 20 gestation 5 dams from each dietary group were sacrificed and fetal body/organ masses recorded. The remaining pregnancies proceeded to term. Offspring were sacrificed within 12 hrs of the dam giving birth and organ weights determined.

Figure 3.4. **The postnatal growth of offspring from maternally protein restricted (MLP) or control fed dams from birth to adulthood**



Data are Mean± SEM. For all values other than birth n= 9-16 offspring/dietary group for males and females from a total of 6 litters per dietary group. At birth n= 53 offspring (control) or n= 65 offspring (MLP) for combined male and female data from a total of between 10-12 litters/dietary group. Offspring from either dietary group were generated as outlined in *Methods*. At 4 weeks of age all litters were weaned onto laboratory chow diet. Animals were then weighed at regular intervals up to 40 weeks of age.

Table 3.7. **The organ masses and relative body proportions of weanling rat pups (*male and female*) exposed to either a control or low protein maternal diet**

	Maternal Diet		
	Control	MLP	*P*
Body weight (g)	85.5± 2.5	93.6± 2.8	0.03
Brain weight (g)	1.30± 0.18	1.32± 0.28	NS
Liver weight (g)	4.07±0.06	4.61± 0.14	0.004
Lung weight (g)	0.84± 0.02	0.88± 0.02	NS
Heart weight (g)	0.54± 0.01	0.59± 0.01	0.01
Kidney weight (g)	0.48± 0.01	0.48± 0.01	NS
Brain:Bodyweight (%)	1.46± 0.03	1.41± 0.03	NS
Liver:Bodyweight (%)	4.70± 0.06	4.76± 0.04	NS
Lung:Bodyweight (%)	0.99± 0.02	0.92± 0.01	0.01
Heart:Bodyweight (%)	0.62± 0.01	0.61± 0.01	NS
Kidney:Bodyweight(%)	0.53± 0.009	0.50± 0.007	0.008

All data are presented as Means± SEM for *n*= 35-51 offspring (Control) or *n*= 38-53 offspring (MLP) from a total of 6 dams fed on each diet. All litters were culled down to 8 pups/litter (4 male/4 female). Offspring were weaned at 4 wks of age and sacrificed within 48hrs. Animals were generated as outlined in *Methods.* The Lee Index was not measured on weanling animals. NS = not significant.

Table 3.8. **The organ masses and relative body proportions of weanling rat pups (*male only*) exposed to either a control or low protein maternal diet**

	Maternal Diet		
	Control	**MLP**	***P***
Body weight (g)	93.6± 4.2	101.5± 5.9	NS
Brain weight (g)	1.35± 0.21	1.39± 0.20	NS
Liver weight (g)	4.35± 0.17	4.89± 0.26	NS
Lung weight (g)	0.93± 0.04	0.90± 0.05	NS
Heart weight (g)	0.55± 0.02	0.62± 0.03	0.05
Kidney weight (g)	0.52± 0.02	0.51± 0.02	NS
Brain:Bodyweight (%)	1.40± 0.04	1.38± 0.06	NS
Liver:Bodyweight (%)	4.69± 0.09	4.84± 0.06	NS
Lung:Bodyweight (%)	1.00± 0.03	0.89± 0.03	0.03
Heart:Bodyweight (%)	0.59± 0.01	0.62± 0.01	NS
Kidney:Bodyweight(%)	0.53± 0.01	0.50± 0.01	NS

All data are presented as Means± SEM for *n*= 17-20 offspring (Control) or *n*= 16-20 offspring (MLP) from a total of 6 dams fed on each diet. Offspring were weaned at 4 wks of age and sacrificed within 48hrs. All litters were culled down to 8 pups/litter (4 male/4 female). Animals were generated as outlined in *Methods*. NS = not significant.

Table 3.9. **The organ masses and relative body proportions of weanling rat pups (*female only*) exposed to either a control or low protein maternal diet**

	Maternal Diet		
	Control	**MLP**	***P***
Body weight (g)	81.6± 3.2	93.4± 2.7	0.007
Brain weight (g)	1.25± 0.02	1.28± 0.04	NS
Liver weight (g)	3.99± 0.17	4.38± 0.13	NS
Lung weight (g)	0.78± 0.03	0.86± .003	0.04
Heart weight (g)	0.53± 0.02	0.56± 0.02	NS
Kidney weight (g)	0.44± 0.01	0.47± 0.01	NS
Brain:Bodyweight (%)	1.52± 0.05	1.42± 0.03	NS
Liver:Bodyweight (%)	4.85± 0.07	4.69± 0.07	NS
Lung:Bodyweight (%)	0.97± 0.02	0.93± 0.02	NS
	0.65± 0.01	0.61± 0.01	0.05
Kidney:Bodyweight(%)	0.54± 0.01	0.50± 0.007	0.01

All data are presented as Means± SEM for *n*= 17-24 offspring (Control) or *n*= 23-25 offspring (MLP) from a total of 6 dams fed on each diet. Offspring were weaned at 4 wks of age and sacrificed within 48hrs. All litters were culled down to 8 pups/litter (4 male/4 female). Animals were generated as outlined in *Methods*. NS = not significant.

3.3.2.6. DISCUSSION

In the present section intrauterine and postnatal growth of rats exposed to either MLP or control diet was examined. The intrauterine growth rate of MLP rats was greater than control rats up to day 20 gestation. From d20 to term, the growth rate of MLP rats slowed, falling behind that of control rats. The faltering somatic growth of MLP relative to control rats in late gestation was accompanied by disproportionate growth of specific organs relative to body weight. Thus, the body proportions of a pup at term exposed to MLP *in utero* was markedly different to a pup exposed to control diet. From birth to weaning a degree of catch-up growth was exhibited by MLP. However the growth of both the lungs and kidneys in MLP was reduced as a proportion of body weight. Postnatal growth from weaning into adulthood was similar between dietary groups.

Nutritional restriction during periods when the demands for specific nutrients are increased, such as during growth, imposes a metabolic stress on the developing organism. The manifestations of the metabolic stress may specifically relate to the nature of the nutrient restriction i.e. the length of restriction, severity, timing and degree of energy restriction (Harding & Johnston 1995; McCance & Widdowson 1974; Widdowson & McCance 1963). Other factors such as maternal height and the plane of maternal nutrition upon entering pregnancy which will necessarily influence maternal weight and body composition at the time of conception and may influence how an early nutritional restriction impacts upon the fetus (Mellor 1983; Robinson *et al* 1994).

The present data indicate that fetal growth in MLP rats was enhanced, despite a 50% reduction in the protein content of the diet, up to day 20 relative to control rats. Fetuses were of greater bodyweight and longer than control animals. Other data obtained within this laboratory has also demonstrated a greater bodyweight in MLP relative to controls from as early as day 14, persisting up to day 20, but thereafter declining such that at term MLP pups are of low to normal birthweight (Langley-Evans *et al* 1996b). The placenta, which closely correlates with fetal size in the present model, is also greater in size at day 14 gestation in MLP animals relative to controls (Table 3.4). (Langley-Evans *et al* 1996b). Undernutrition in early gestation induces compensatory growth of the placenta in sheep (McCrabb *et al* 1991; Robinson *et al* 1994) and rats (Langley-Evans *et al* 1996f) and mothers diagnosed as having

maternal anaemia, a marker of nutritional status, exhibit increased placental size (Godfrey *et al* 1991). Increased placental size during mid-late gestation may facilitate the supply of amino acids and other energy substrates necessary for fetal growth (Owens *et al* 1987), perhaps enabling the greater early growth rate in MLP relative to control rats. Furthermore, increased total placental 11-HSD2 activity (Table 3.4) during early gestation may further facilitate the more rapid rate of growth of MLP in early gestation. The stimulus for early placental compensatory growth is not known but may perhaps depend on the relative distribution of cells between the inner cell mass, which develops into the fetus, and the trophectoderm, which forms the placenta. Early (d1-4) protein restriction results in a greater migration of cells towards the trophectoderm rather then the inner cell mass, and at d14 these animals exhibit greater placental mass (T. Fleming - *personal communication*).

At day 20 gestation the placenta as a proportion of fetal bodyweight was significantly smaller and fetuses larger in MLP animals than controls. This appears at variance with the data presented in Table 3.4 where no difference in placental weight was observed and fetuses were of lesser weight than protein replete controls. Whilst the difference in the data may represent study to study variation this is unlikely. However, when an analysis which includes data from a large sample size is conducted, the effects of litter size upon birthweight are reduced and a more representative picture emerges (Langley-Evans *et al* 1996b) as shown in Table 3.5. The combination of a small placenta and large fetus is interesting and may suggest a more efficient feto-placental transfer of nutrients, or increased handling efficiency of substrate in MLP. Nutritional status influences the insulin-like growth factors, IGF-I and -II (Owens *et al* 1994) and their binding protein concentrations (Straus *et al* 1991). Both fetal IGF-I and -II correlate positively with fetal pO_2 and plasma glucose concentration (Owens *et al* 1994) and IGF-I with placental weight and birthweight (Osorio *et al* 1996). IGF-I and -II are synthesised in, released from and abundantly expressed in placental tissue (Han & Hill 1994). Thus nutritional modulation of IGFs may influence the local control of placenta development/function, placental substrate supply to the fetus and consequently fetal and placental growth (Owens *et al* 1994).

At day 20 gestation the Lee index (bodyweight:length^3), a measure of body proportionality (Bernadis & Patterson 1968), suggests that MLP fetuses are longer than

expected by body mass measurement. This resembles the phenotype of babies postulated to have undergone mid-late gestational undernutrition, that are more susceptible to adult hypertension and non-insulin-dependent diabetes (NIDDM) (Barker 1994). Considered alongside the data at term it is apparent that MLP fetuses continue along a disproportionate growth curve that reflects a late gestational fetal undernutrition (Harding & Johnston 1995). Thus MLP initially exhibit a greater increase in weight relative to controls until late gestation when the demands of fetal growth upon the dam increase. Growth rate of MLP then rapidly slows presumably to match substrate availability. Nutrient restriction slows the growth of more rapidly growing ovine fetuses whereas previously slow growing fetuses maintain their growth trajectory (Harding *et al* 1992). Undernutrition in late gestation presents an obvious physiological challenge given that nutrient demands are higher but, together with an earlier nutritional insult, may further compromise fetal development. In early pregnancy dams deposit nutrient reserves (protein and fat) in preparation for increased later demands (Naismith 1969; Naismith & Morgan 1976).

At term, MLP pups were shorter than controls both in absolute terms and relative to body mass, although no difference in birthweight was apparent. However birthweight alone is a crude indicator of the quality of fetal growth. Despite no difference in birthweight, MLP had significantly smaller livers as a proportion of body weight than controls. These specific growth disproportions, not highlighted by a simple measurement of birthweight, are more related to adult disease than birthweight alone (Barker 1995; Hales & Barker 1992). A cross-sectional analysis of body and organ weights at day 20 and at term may provide an index of organ growth trajectory over late gestation and indicates how the nutritional restriction may impact upon fetal growth. It is clear from *Figure 3.3.* that whilst growth of the body is attenuated (15-25%) in MLP relative to control rats, which double in weight over the last two days of gestation, the growth of the brain appears relatively maintained in late gestation. This appear likely to be at the expense of lung, liver and truncal growth, emphasising the hierarchy with which a prenatal stress impacts upon the developing fetus. A certain proportion of difference in wet weight may be attributed to differences in water content since dry weight was not recorded in the present studies. At term the brain:liver ratio, which is indicative of a redistribution of nutrients, is higher in MLP relative to controls (1.08 *vs* 0.95 respectively).

However this effect appears confined to late gestation since the brain:liver ratio is reduced in MLP relative to controls at day 20.

Reduced growth of visceral organs and peripheral tissue perhaps reflects a redistribution of the energy substrates to maintain the brain in the face of a challenging environment (Rosso 1990). Furthermore, if the nutritional restriction is sufficiently severe, fuel molecules (amino acids, glucose) may be mobilised by the fetus itself, contributing to peripheral wasting (Harding & Johnston 1995). The data presented lend support to this hypothesis. A redistribution of energy substrates, favouring maintenance of the brain, requires alterations in blood flow (Rudolph 1984). Altered haemodynamics and blood pressure during development have the potential to transiently affect the function or structure of vascular smooth muscle (VSM) leading to morphological changes in arterial vessels during later life (Berry 1978). Blood pressure is known to track growth from an early age suggesting that early factors are involved in the regulation of blood pressure (Law *et al* 1993; Lever & Harrap 1992). Altered circulatory dynamics and consequently vascular blood vessel structure may initiate VSM hypertrophy. A positive feedback cycle is entered which will promote further increases in blood pressure (Folkow 1978). Such a self-perpetuating mechanism may explain why hypertension can remain after the primary cause e.g. a renal clip, has been removed. Thus, in the present model, the primary cause may be altered maternal nutrition leading to necessary adjustments in haemodynamics during fetal life. The positive feedback mechanism is then operable in postnatal life contributing to the generation of elevated blood pressure in MLP.

Jackson (Jackson 1996) proposes a general model of growth and development that suggests the growth of any tissue or organ consists of an increasing functional capacity. A tissue/organ ordinarily operates well within its maximum functional capacity, allowing an operational reserve. Compromised development of an organ reduces its maximum capacity and therefore reserve. Under conditions where the functional demand of that particular organ increases i.e. for the liver -nutritional excess, then the maximum capacity is reached earlier and a metabolic stress is imposed. This model complements the 'thrifty phenotype hypothesis' proposed by Hales & Barker (Hales & Barker 1992). This hypothesis suggests that the fetus adapts to intrauterine undernutrition by adopting a thrifty metabolism. Such adaptations,

especially in the pancreas and liver, may predispose the individual in later life and under conditions of nutritional excess to disease processes. Hales and colleagues have gathered considerable evidence to support this hypothesis (Desai *et al* 1995; Desai & Hales 1997; Hales *et al* 1991; Ozanne *et al* 1996). In the present results, impaired late gestational growth of the liver (14% *vs* 53% for increase in liver weight at term relative to day 20), as a result of maternal undernutrition, may subsequently alter its functional capacity in adult life, predisposing the individual to metabolic abnormalities associated with liver function, such as NIDDM (Hales & Barker 1992). MLP rats demonstrate an insulin resistance syndrome as determined by a glucose tolerance test (Langley *et al* 1994; Pickard *et al* 1996), insulin resistance being a major feature of NIDDM.

Growth from birth to weaning of MLP-female rats was greater than in control rats, an effect that is consistently reproduced in our hands (Langley & Jackson 1994). It cannot be determined from the present studies what constitutes the extra weight in MLP-female rats. Further analysis of body composition may reveal how the extra weight is manifest, although animal (Anguita *et al* 1993) and human (Ravelli *et al* 1976) studies suggest that increased postnatal deposition of fat occurs as a result of intrauterine malnutrition. At weaning (3-4 weeks) only the lungs and kidneys of MLP rats as a proportion of body weight were significantly small relative to control rats. This suggests the effect of the MLP diet is predominantly exerted in late gestation, since both the lungs (Liggins 1969) and kidneys (Zeman 1968) experience a significant proportion of their growth in late gestation. Moreover, the maturation of the lung appears related to the maturational state and integrity of the kidney. Thus lung growth will proceed normally only in the presence of an intact, uncompromised kidney (Peters *et al* 1991) which suggests that a renally derived humoral factor may influence lung growth. Relative to bodyweight the hearts of MLP-female rats but not MLP-male rats were smaller than controls. The reason for this observation is not clear but may potentially contribute to the sexually dimorphic response to maternal adrenalectomy noted later in this thesis.

Prenatal protein restriction has been demonstrated to impair kidney development (decreased glomeruli and nephrons) and function in a series of studies by Zeman and colleagues (Hall & Zeman 1968; Zeman 1967; Zeman 1968; Zeman & Stanborough 1969).

These results have been replicated using the MLP diet. Rats exposed to MLP had decreased nephron number (S.J.M. Welham - *unpublished observations*) and altered functional characteristics (decreased renal blood flow) (Welham *et al* 1996). In humans intrauterine growth retardation, as defined by birthweights below the 10th centile, is associated with deficits in nephron number (Hinchcliffe *et al* 1992). A programmed deficit in nephron number as a result of poor maternal nutrition may, for example, impair the ability of the kidney to excrete sodium resulting in a gradual elevation of systolic blood pressure i.e. 'pressure natriuresis' (Huang *et al* 1992). This is proposed by Brenner and colleagues (Mackenzie & Brenner 1995) to underpin the epidemiological observations made by Barker and co-workers. Attenuated growth of the kidney in late gestation, leading to a functionally compromised organ in adult life, may thus contribute to the development of high blood pressure in MLP animals in later life. This could, perhaps, be experimentally tested through unilateral nephrectomy of MLP and observing the *rate of increase* of SBP of MLP relative to controls.

SECTION 3

THE EFFECT OF A MATERNAL LOW PROTEIN DIET UPON THE SYSTOLIC BLOOD PRESSURE AND GLUCOCORTICOID MARKER ENZYMES AND AXIS OF THE OFFSPRING

3.3.3. RESULTS

3.3.3.1. Systolic blood pressure and heart rate

Systolic blood pressure (SBP) was determined in 3-15 week old, male and female offspring of dams fed control or MLP diets in pregnancy. At all ages measured, MLP offspring had significantly ($P<0.05$) elevated SBP compared to controls (Figure 3.5). At each age the SBP was between 10-20mmHg higher in MLP relative to controls. Blood pressure in both groups of animals varied as a function of time with pressures being significantly lower at age 3-4 weeks than at any other age measured ($P= 0.0001$). Maximal SBP was attained in both groups of rats between 6 and 10 weeks of age (Figure 3.5).

Figure 3.6 illustrates the heart rates of the animals from each dietary group. At both 3-4 weeks of age and 5-6 weeks of age the MLP animals had moderately lower heart rates than control animals, under the conditions of the blood pressure measurement protocol. Due to the degree of variation between animals this effect was non-significant. The heart rates of adult rats (10-15 wks) were similar between dietary groups. In both groups of animals heart rate varied as a function of age, the rate was higher at weaning age than at any other age measured and thereafter declined gradually (Figure 3.6).

3.3.3.2. Diurnal effects on blood pressure

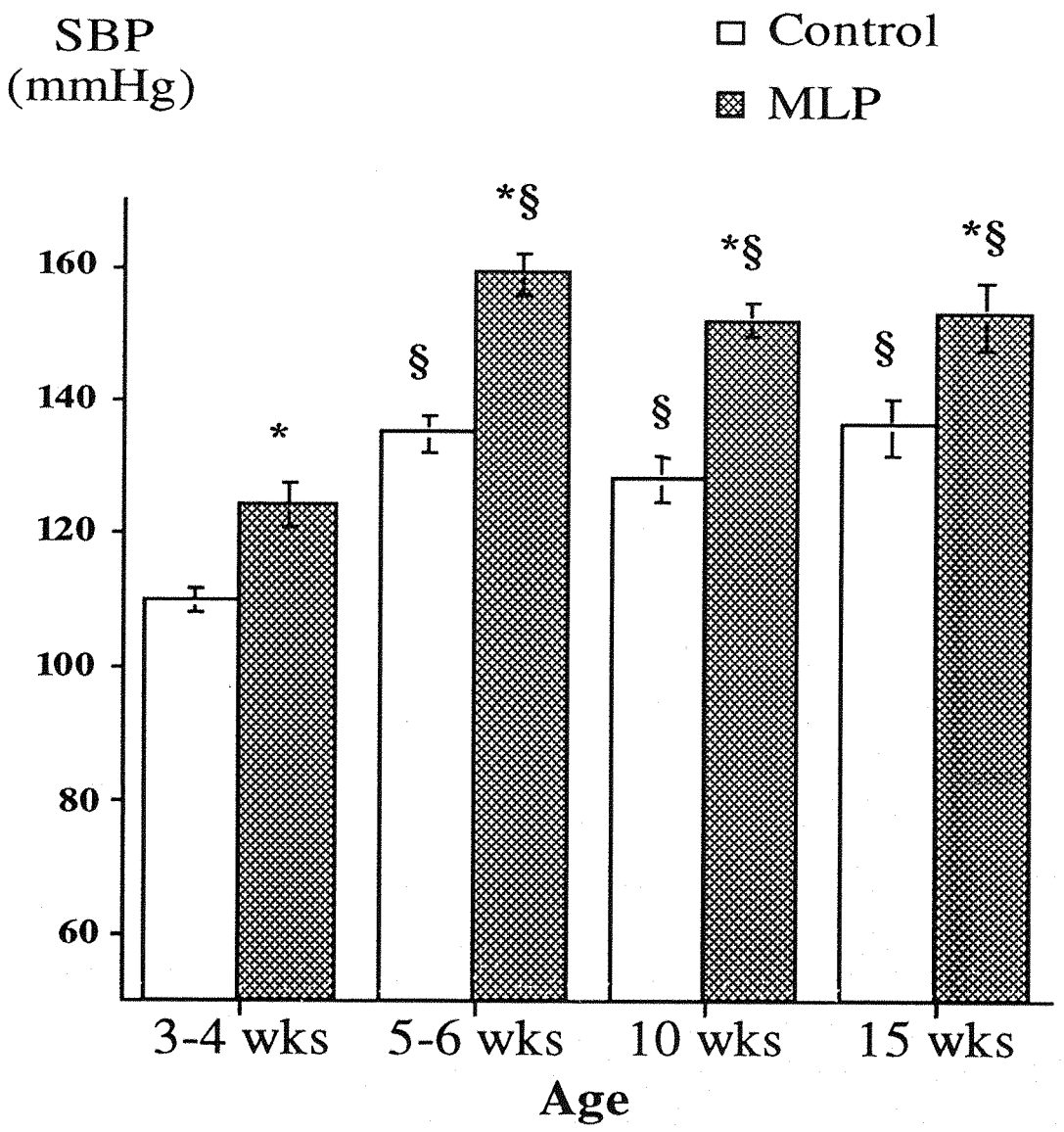
Analysis of the diurnal variation in blood pressure revealed no significant effect of time of day upon blood pressure (Table 3.11). Consistent with previous findings (*Figure 3.5*) the MLP offspring demonstrated elevated blood pressure, throughout the diurnal cycle, compared with control animals ($P< 0.05$).

3.3.3.3. DISCUSSION

The present results represent a unique observation, in that, by prenatal nutritional restriction the future control of blood pressure in the resultant offspring has been modified. Male and female rats from MLP dams exhibit elevations in systolic blood pressure (SBP) in the order of 10-20mmHg above controls from as early as 3-4 weeks of age. The nutritionally programmed hypertensive state appears permanent, given that the SBP is higher in MLP rats relative to controls in adulthood (10 & 15 weeks of age as presented) and has been measured up to 44 weeks of age (Langley-Evans & Jackson 1995). The elevated SBP was not associated with an elevation in heart rate, which was in fact lower in group MLP relative to controls in measurements taken up to 6 weeks of age.

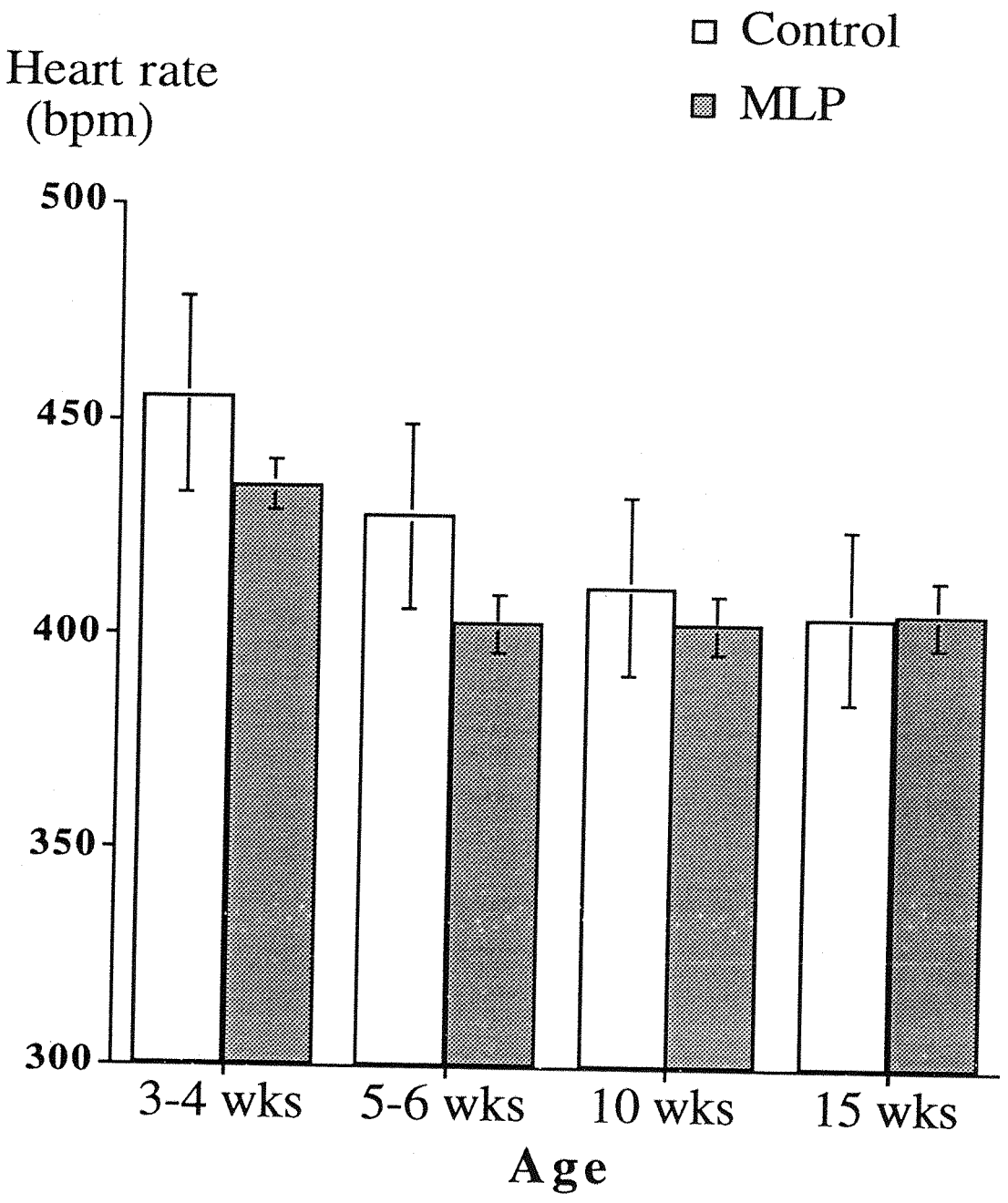
Elevated adult systolic blood pressure in the offspring of rats from nutritionally restricted dams has been reproduced consistently in this laboratory (Langley & Jackson 1994; Langley-Evans & Jackson 1995; Langley-Evans *et al* 1994; Langley-Evans *et al* 1996f) and by other workers (Crowe *et al* 1995; Isherwood-Peel *et al* 1997; Persson & Jansson 1992; Woodall *et al* 1996b). In a prospective human study, it was found that both birthweight and placental mass were inversely related to the intake of carbohydrate in early pregnancy and protein in late pregnancy (Godfrey *et al* 1996), reflecting the animal studies. Moreover, a follow-up design human study further indicated the importance of an interaction between low protein and high carbohydrate in programming the future blood pressure of the offspring. Thus Campbell *et al* (Campbell *et al* 1996) found that if maternal dietary intake of protein was < 50g/day then a high carbohydrate intake was associated with a higher SBP in the resultant adult (40yrs of age) offspring.

Figure 3.5. **The systolic blood pressure (mmHg) of prenatally protein restricted and control rats at four different ages**



All data are given as Mean± SEM. Offspring were generated as outlined in section 2.2. The data are the combined results of male and female recordings and n= 5-45 offspring (control) and 5-34 offspring (MLP) from a total of 6 litters/dietary group at each age. Measurement of systolic blood pressures were made using the tail cuff method by a single operator as outlined in Methods. Statistical analyses were done using paired and unpaired Students t-test. At all ages studied the low protein exposed animals had significantly higher systolic blood pressure than control animals (P= 0.001). Blood pressure also varied against time, with pressures being significantly higher at weaning age than at any other age measured (P< 0.05). * Significantly different to control animals, § significantly different to pressures recorded at age 3-4 weeks for each dietary group (P<0.05).

Figure 3.6. **The heart rate (beats/min) of rats exposed to either a control or low protein maternal diet at four different ages**



All data are given as Mean± SEM. Offspring were generated as outlined in *Methods*. The data are the combined results of male and female recordings and n= 5-45 offspring (Control) and 5-34 offspring (MLP) from a total of 6 litters/dietary group at each age studied. Measurement of systolic blood pressures were made using the tail cuff method as outlined in Methods. Two way ANOVA suggested an interaction between diet and age on HR ($F=1.50$, $P= 0.06$). HR was lower in MLP upto 6 weeks of age but thereafter was not significantly different to controls.

Table 3.10. **The diurnal variation in blood pressure (mmHg) of rats exposed to either a control or low protein maternal diet**

Time	Control	MLP
09.00am	101± 2.9	122± 4.8 *
15.00pm	104± 5.9	121± 3.9 *
21.00pm	112± 3.5	125± 2.6 *
03.00am	98± 8.3	129± 6.4 *

Data are Mean± SEM for n= 6 offspring (18% casein, control) and n= 5 offspring (9% casein, MLP) from a total of 6 litters/dietary group. The animals were generated as outlined in Methods and SBP measured as previously described (*see* Figure 3.5 for details). Analysis of variance (ANOVA) indicated a significant effect of diet. * indicates a significant difference to control rats ($P< 0.05$).

It is becoming increasingly clear from studies in our own and other laboratories that rather than the deficit in protein *per se,* programming the hypertensive state (despite protein intake being negatively correlated with SBP in our model (Langley & Jackson 1994)), it is the overall balance and interactions of nutrients that is important in the programming of cardiovascular control. Thus, in studies that have reproduced nutritionally-induced hypertension by dietary means (Langley & Jackson 1994, Isherwood-Peel *et al* 1997) rather than surgical (Persson & Jansson 1992) the shortfall in energy from protein has been compensated for by an increase in energy from carbohydrate in the form of starch and sucrose. If the energy deficit is made up with glucose alone, hypertension is only observed in the offspring if the diet is fed throughout gestation, lactation and up to d70 (Petry *et al* 1997) rather than through gestation only (Lucas *et al* 1996). However, the diet used in these studies is not only different to our own diet in the source of carbohydrate but also in methionine content and source of fat (soybean oil *vs* corn oil) (Desai *et al* 1995). Interestingly, soybean oil contains 7-fold more linolenic (18:3) acid than corn oil and variations in maternal fatty acid intake are known to modulate the SBP of the offspring (Langley-Evans *et al* 1996a). If an adequate (18% casein) protein diet is coupled with coconut oil as the fat source (which has a low linolenic acid content) rather than corn oil as is normally used (*Appendix 1*) the resultant pups are of reduced birthweight and develop hypertension as adults (Langley-Evans 1996). It is therefore clear that whilst nutritional programming of hypertension is a demonstrable phenomenon, i.e. through either a low protein or high saturated fat intake, it is the overall balance and interactions between nutrients that are important, rather than specific nutrient deficits being the active programming agent.

It has been suggested that the elevation in SBP observed within this model is more a reponse to a stressed environment during the tail-cuff procedure rather than an habitual state (van den Berg *et al* 1994). During the stress response blood pressure is known to rise, together with an increased heart rate. It has clearly been demonstrated that the heart rate of MLP is not elevated relative to controls and in fact is lower at weaning age. Also, through direct measurement of SBP via a carotid cannula in anaethetised animals the same difference in SBP between dietary groups is observed (*section 5.6.2.*) without any change

in heart rate. The observation of a lower heart rate in MLP appears anomalous given that the activity of the sympathetic nervous system (SNS) of MLP is enhanced (i.e. increased plasma concentrations of noradrenaline) (Langley-Evans - *unpublished observations*). Phillips & Barker (1997) also correlate an increasing pulse rate with declining birth weight and suggest increased SNS activity may contribute towards elevated SBP. However heart rate is regulated by a multitude of factors and is a poor proxy for estimates of sympathetic activity. Analyses of noradrenaline turnover may provide a more accurate index of smpathetic activity in MLP relative to controls.

SBP is the product of a relationship between cardiac output and total peripheral resistance (TPR). Assuming a constant TPR, an elevation of 10-20mmHg, as is seen in our model, would require an increased cardiac output. Cardiac output is determined by the product of heart rate and stroke volume. Heart rate is either similar, or reduced in MLP, relative to controls and thus for a change in cardiac output to occur would require an increase in stroke volume in the order of 10-20%. Given that the relative size of the hearts of MLP are no different to, or even smaller than controls, it seems unlikely that a such a change in stroke volume occurs. On present evidence such a prediction cannot be made with confidence and some future measurement of cardiac output or left ventricular mass is essential.

To achieve an elevation in SBP in the face of a either a constant or perhaps slightly increased cardiac output requires an elevation in the total peripheral resistance (TPR). The mechanisms that may raise TPR and consequently SBP are numerous and maybe thought of as either short-term mechanisms restoring SBP to a 'set point' or more longer-term adaptive responses. Upon deviations in SBP the central nervous system (CNS), catecholamines, renin-angiotensin system (RAS), prostaglandins, adrenal corticoids and the nitric oxide generating system (NOS) are activated which together, in the short term, restore the pressure back to normal values. The majority of these systems, excluding the CNS and NOS, have been demonstrated to be up-regulated/enhanced in the MLP model of hypertension (Langley & Jackson 1994; Langley-Evans *et al* 1996c; Langley-Evans & Jackson 1995; Sherman *et al* 1996). Information about the cardiovascular system is transmitted to the CNS via baroreceptors

which act as pressure transducers. An increase in SBP increases the neuronal firing rate from the baroreceptor which in turn, via the central cardiovascular centre, leads to a decreased sympathetic outflow and reduced TPR resulting in a reduced SBP (Levick 1991). The baroreceptor threshold increases within 15mins of exposure to the new SBP level, adapting to the new higher pressure. This system therefore provides the brain with information about changes in blood pressure rather than the current SBP (Levick 1991). The baroreceptors therefore have no particular role in the long term control of SBP. It is unlikely that changes in baroreceptor function are a primary cause of hypertension (Levick 1991) but rather as a secondary consequence. Elevated activity of the SNS may contribute to the hypertensive state given that MLP exhibit elevated levels of plasma catecholamines (Langley-Evans - *unpublished observations*). The role of nitric oxide nor of arginine have not been evaluated in MLP to date.

Glucocorticoids, produced from the zona fasciculata of the adrenal cortex, modulate blood pressure as part of the stress response and, if over-produced, may lead to hypertension, as in Cushings syndrome (Ross & Linch 1982). Recent evidence suggests that a mild peripheral hypersensitivity to glucocorticoid (GC) action may explain a proportion of cases diagnosed as essential hypertension (Walker *et al* 1996). In MLP exposed rats the hypothalamic-pituitary-adrenal (HPA) axis demonstrates a degree of hypersensitivity in both peripheral and central tissues. The activities of enzymes that are stimulated by GC are permanently increased despite similar plasma corticosterone concentrations to control animals (Langley-Evans *et al* 1996c) (also *section 3.3.3.5. & 3.3.3.6.*). Furthermore MLP demonstrate 3-fold increased binding of corticosterone to vascular GR relative to controls and have more receptors in the hippocampus. This may provide the mechanism for increased peripheral and central sensitivity to GC action (Langley-Evans *et al* 1996c).

The evidence to date, from both animal and human studies, now clearly implicates a role for glucocorticoids in essential hypertension and in the pathogenesis of hypertension of fetal origin. Patients with 'Cushingoid' features frequently demonstrate a marked hypertensive state (Ross & Linch 1982), and a feature of patients with essential hypertension is increased peripheral sensitivity to GC (Walker *et al* 1996). The renin-

angiotensin system (RAS) system is also modulated by GC. GC induce the expression of the angiotensin II (AII) precursor molecule angiotensinogen predominantly from the liver and increase the activity of peripheral ACE (Mendelsohn *et al* 1982). Interestingly the elevated SBP of MLP is reduced to values observed in controls following ACE inhibition with captopril (Langley-Evans & Jackson 1995). The physiological action of AII is potentiated by GC in the SHR via increases in AII receptor number (Provencher *et al* 1995). Furthermore, the production of vasodilatory prostaglandins of renal origin, known to be important in kidney function and modulating RAS activity, are inhibited by GC (Walker *et al* 1992). The action of the sympathetic nervous system and catecholamines are potentiated through the permissive action of GC (Walker *et al* 1992) and transepithelial cation transport is increased to aid volume expansion, mediated through Type II GR receptors (Kornel *et al* 1993a). Thus, in general, GC upregulate vasoconstrictor mechanisms such as angiotensin II and noradrenalin, whilst inhibiting vasodilatatory mechanisms such as prostaglandins and nitric oxide (NO) (Saruta 1996).

Interestingly, and perhaps providing a mechanism whereby maternal diet modulates future systolic blood pressure, the cardiovascular actions of glucocorticoids in the peripheral vascular system (Tangalakis *et al* 1992) and kidney (Hill *et al* 1988) also operate *in utero*. Intrauterine GC may modulate the utilisation of substrate by organs and acquisition of substrate from the maternal circulation during periods of prenatal stress. Thus a prenatal stress imposed by maternal protein restriction, could theoretically produce changes in the fetal GC environment *in utero*. Increased GC action at an inappropriate stage of development may subtly alter the intrauterine homeostatic environment. This may result in altered development of both the cardiovascular system and organ (kidney, brain) structure which, during postnatal development, lead the organism towards a hypertensive state.

SECTION 4

THE EFFECT OF A FEEDING A MATERNAL LOW PROTEIN DIET UPON GC-INDUCIBLE MARKER ENZYMES AND CIRCULATING ACTH AND CORTICOSTERONE CONCENTRATIONS

3.3.4.1. The effect of a control or maternal low protein diet on central glucocorticoid marker enzymes in pre-term fetuses and term neonates

The activities of glycerol 3-phosphate dehydrogenase (GPDH) and glutamine synthetase (GS) were assessed as indicators of glucocorticoid activity. Both are glucocorticoid inducible activities (GPDH, (Kitraki *et al* 1995); GS, (Patel *et al* 1983)). The activities of malate dehydrogenase (MD) and pyruvate kinase (PK) were also assessed as glucocorticoid insensitive control marker enzymes (Langley & York 1990). The activities of GPDH, GS, MD and PK in whole brain and total protein content of whole brain were unaltered by prenatal dietary experience in day 20 rat fetuses (Table 3.12). Similarly, at term, there was no difference in central activities of GS, MD or PK between MLP and control pups (Table 3.12). However, the activity of GPDH, a glucocorticoid-inducible glial cell enzyme, was almost 3-fold (260%) greater in MLP rats than the activity in control animals. Importantly, the higher activity was present despite a lack of diet-induced changes in either brain protein concentration or in the activities of steroid-insensitive enzymes (MD and PK).

3.3.4.2. Central glucocorticoid marker enzymes in specific brain regions of weanlings

Since the brain was of sufficient size to enable dissection of specific GC target areas in weanling animals, the activities of the GC-sensitive enzymes were assayed in hypothalamus, hippocampus and cerebellum of 4 week old weanling animals (Table 3.13). Whole brain GC-sensitive enzyme activity was not measured in weanling animals to avoid the dilution of enzyme activity by areas known to exhibit weak

Table 3.11. **The activities of glucocorticoid marker enzymes in whole brains of day 20 fetuses and term neonates exposed to either a control or low protein maternal diet**

Age..	Day 20 fetuses		Term neonates	
Maternal Diet.	**Control**	**MLP**	**Control**	**MLP**
GPDH *units*	2.7 ±0.4	2.5 ±0.3	1.8 ±0.3	6.5 ±0.4*
GS *units*	119.2 ±4.5	112.7 ±3.9	117.5 ±6.3	112.8 ±4.1
PK *units*	425 ±9	387 ±16	337 ±15	373 ±37
MD *units*	25.3 ±0.6	24.2 ±4.6	37.1 ±1.3	38.9 ±1.2
Protein *mg/g tissue*	70.2 ±3.7	88.0	70.2 ±3.7	68.3 ±1.6

All data are presented as mean± SEM for n= 7-10 offspring (Control) or 8-10 offspring (MLP) of male and female data combined. A total of 20 rats were habituated for two weeks to either a control protein diet (Control) or maternal low protein (MLP) diet and then mated. Conception was determined through the presence of a mucus plug on the floor of the cage. At day 20 a proportion of the dams (n=5 per dietary group) were sacrificed and fetal tissues excised. The remainder proceeded to term. The newborns were sacrificed within 12hrs of birth and tissues obtained.

GPDH, glycerol 3-phosphate dehydrogenase; GS, glutamine synthetase; PK, pyruvate kinase; MD, malate dehydrogenase. *Units*, enzyme activity expressed as nmoles product formed/min/mg protein.* indicates a significant difference to control rats ($P < 0.0001$)

Table 3.12. **The activities of glucocorticoid marker enzymes in brain regions of weanling rats exposed to either a control or low protein maternal diet**

	Maternal diet		
Enzymes / Regions	**Control**	**MLP**	***P***
GPDH *units...*			
Hypothalamus	43.6 ±2.4	52.9 ±2.6	0.006
Hippocampus	25.8 ±1.6	31.8 ±2.5	0.09
Cerebellum	14.8 ±1.3	21.0 ±0.9	0.001
GS *units...*			
Hypothalamus	186 ±4	236 ±8	0.001
Hippocampus	152 ±6	186 ±13	0.03
Cerebellum	184 ±8	242 ±12	0.0008
MD *units...*			
Hypothalamus	26.9 ±1.0	27.1 ±1.8	NS
Hippocampus	22.9 ±3.9	18.2 ±1.1	NS
Cerebellum	32.8 ±1.1	25.9 ±0.8	0.0001
Protein (mg/g tissue)			
Hypothalamus	133 ±9	150 ±3	NS
Hippocampus	134 ±9	139 ±12	NS
Cerebellum	130 ±11	155 ±15	NS

All data are presented as mean± SEM for n= 5-10 offspring (Control) or 9-10 offspring (9% casein, MLP) of male and female data combined from a total of 5 litters/group. See Table 3.12 for details. GPDH, glycerol 3-phosphate dehydrogenase; GS, glutamine synthetase; PK, pyruvate kinase; MD, malate dehydrogenase. *Units*, enzyme activity expressed as nmoles product formed/min/mg protein.

GC sensitivity. No significant differences in protein concentration were observed in any region. This is reflective of the greater *n* in the present study as opposed to the data presented in Table 3.10. Measurement of MD was considered a satisfactory control and thus no further measurements of PK activity were made. As in day 20 fetuses and term neonates, prenatal dietary experience had no effect upon the activity of MD in the hypothalamus or hippocampus, although activity in the cerebellum was significantly lower in MLP animals than in controls (P= 0.0001). In contrast, the activity of GS was significantly greater in hypothalamus (27% greater), hippocampus (22%) and cerebellum (30%) of MLP rats, relative to controls. Similarly the activity of GPDH in specific brain regions (hypothalamus and cerebellum only) was significantly higher in MLP rats than in controls (P<0.01). Thus in the hypothalamus, hippocampus and cerebellum GPDH activities, relative to controls, were 21%, 23% and 42% greater respectively (Table 3.13).

3.3.4.3. Central and peripheral glucocorticoid marker enzymes in adult rats

To determine whether enzyme activities were permanently programmed by the the MLP diet, enzymes were assayed in adult rats at 10 weeks of age (Table 3.14). Due to a limitation of rat numbers during this study enzyme activities could only be measured in specific regions and not throughout all regions. Since activities of GPDH were highest in the cerebellum in weanling rats the activity of MD, the enzyme control, was measured in this region to differentiate between a GC-specific effect and a general up-regulation of all enzyme systems in GC target areas. The activity of central GPDH in MLP rats was greater in both the cerebellum (13% greater, *P* =0.05) and hippocampus (12%, *P*= 0.09) relative to controls. No diet-related alterations to protein concentration in either of these brain regions was observed and similarly no change in the activity of hypothalamic MD, between dietary groups, was noted (Table 3.14). Hypothalamic protein concentration was significantly lower (P= 0.03) in MLP rats relative to control rats. The activity of hepatic TAT, a GC-sensitive enzyme, was significantly greater (55%) in the MLP rats, although hepatic GS was unaltered by prenatal dietary experience (Table 3.14). Hepatic protein concentration and MD activity were unaffected by exposure to a maternal low protein diet.

Table 3.13. **The activities of central and hepatic glucocorticoid marker enzymes in adult rats exposed to either a control or low protein maternal diet**

	Maternal diet		
Enzymes / Regions	**Control**	**MLP**	***P***
GPDH *units...*			
Hippocampus	34.1 ±0.7	38.2± 1.6	0.09
Cerebellum	48.7 ±2.0	55.1 ±2.0	0.05
GS *units...*			
Liver	146 ±8.3	145 ±5.4	NS
TAT *units...*			
Liver	4.9 ±0.1	7.6 ±0.4	0.01
MD *units...*			
Liver	51.0 ±6.3	47.5 ±5.0	NS
Protein (mg/g tissue)			
Hypothalamus	181.0 ±4.0	166.6 ±3.8	0.03
	85.0 ±1.0	88.0 ±3.0	NS
Cerebellum	83.3 ±3.0	79.0 ±2.0	NS
Liver	147 ±16	108 ±23	NS

All data are mean± SEM for n= 6 offspring (Control) or n= 5 offspring (MLP) for male rats. Offspring from a total of 5 litters/group were generated as outlined in *Methods* and sacrificed at 10 weeks of age and whole brain removed for immediate dissection of regions. GPDH, glycerol 3-phosphate dehydrogenase; GS, glutamine synthetase; TAT, tyrosine aminotransferase; MD, malate dehydrogenase. *Units*, enzyme activity expressed as nmoles product formed/min/mg protein.

3.3.4.4. Discussion of enzyme data

The enzymes glycerol 3-phosphate dehydrogenase (GPDH), glutamine synthetase (GS), and tyrosine aminotransferase (TAT) were assayed as an index of glucocorticoid (GC) activity in the brains and liver of control and MLP rats. Central GPDH (Kitraki *et al* 1995; Masters *et al* 1994) and GS (Patel *et al* 1983) and hepatic TAT (Shargill *et al* 1983) are enzymes sensitive to variations in plasma GC concentrations. In order to determine whether prenatal dietary experience exerts a GC-specific effect on enzyme activities, the activities of the GC-insensitive enzymes malate dehydrogenase (MD) and pyruvate kinase (PK) were also assayed together with tissue protein contents, as previously described (Langley 1990).

In fetal whole brain tissue, intrauterine dietary experience did not influence the activities of the GC-inducible enzymes. In contrast, at term, the activity of GPDH in MLP was increased three fold relative to controls. Moreover, within specific central GC target areas *(hippocampus, hypothalamus, cerebellum)* slightly elevated GPDH activity in MLP relative to controls was maintained into adult life. However the elevation of GPDH activity was somewhat attenuated relative to the increase seen in neonatal brains suggesting, perhaps, that the programming effects of GC are diminished with age. When measured at weaning (GS) and in adult life (TAT), the activities of central GS and hepatic TAT were also greater than the activities in control animals. A specific GC-inducible effect on enzyme activities is inferred given that no increase in the specific activities of the control enzymes (MD and PK) or protein content in MLP relative to control animals was observed at any stage (except in hypothalamic protein content in 10wk old animals).

That a maternal low protein diet may elevate activities of GC-specific enzymes, an effect that is apparently lifelong, is both interesting and potentially important. The obvious implication is that the maternal low protein diet renders the subsequent offspring subject to increased GC hormone action. Since the effects of glucocorticoids are almost exclusively mediated by the type II (GR) receptor (Meyer 1985) and that GC autoregulate their receptor population (Svec 1985) the former appears a more likely

explanation. This hypothesis is bolstered by the fact that the MLP group exhibit elevated GR receptor populations in vascular and brain tissue (Langley-Evans *et al* 1996c), (in central tissues GC may up-regulate their receptor population as a negative feedback mechanism in contrast to regulation in the periphery) and that circulating corticosterone concentrations were similar between dietary groups.

Early life stress has been previously demonstrated to permanently programme GC receptor populations. The mild stress of neonatal handling increases the density of central GC receptors (Meaney *et al* 1988). The mechanism by which GC receptor populations are permanently altered by early environmental events is unknown but may relate to increased second messenger generation (cAMP) through the action of serotonin (Meaney *et al* 1993). Elevations in the activity of GPDH may reflect an adaptive response to *in utero* stress since the increased generation of NAD^+ would facilitate continued glycolysis and the resulting product formed, glycerol 3-phosphate, could eventually be incorporated into the citric acid cycle for energy production (Schlatter *et al* 1990).

Despite a lack of effect of MLP on the activities of fetal GC-inducible enzymes in the present study, the hypothesis of an increased intrauterine GC signal programming the elevations in later enzyme activities remains. Both GPDH and, in particular GS, are present in fetal brains but only become functionally active in later life. GPDH activity in the rat is maximal around postnatal days 20-40, coinciding with the most active period of myelination (Kitraki *et al* 1995) reflecting the role of GPDH in providing glycerol-3-phosphate for the biosynthesis of phospholipids (Meyer 1985). Similarly, the activity of GS is dependent upon the maturity of the brain, being very low in neonatal rat brain (Patel *et al* 1983) but increasing substantially between postnatal days 14-20 (Rao *et al* 1987). This may explain why GS activity is increased in MLP relative to controls in weanling as oppose to neonatal rat brains. The demonstration of increased GPDH activity within the first few days of life after exposure to a low protein diet may therefore suggest a premature induction of the GPDH gene by GC. This may be the result of an *in utero* overexposure since a latency period of 2-3 days exists between accumulation of GPDH mRNA (indicative of GC induction and a sensitive marker for the

stress response (Masters *et al* 1994)) and an increase in the specific activity of GPDH (Schlatter *et al* 1990). GPDH activity has a long half-life and consequently declines slowly over time (Schlatter *et al* 1990), the signal for increased GPDH activity at birth may therefore occur *in utero*. Interestingly, MLP diet fed throughout the third week of pregnancy only elevates GPDH in fetal rat brains (Langley-Evans - *personal communication*) suggesting premature induction of GPDH by elevated GC.

Both GS and GPDH are tissue specific, being located in glial cells and the brain of the fetal rat at day 20 predominantly consists of neurones (Meyer 1985). Since whole brain homogenates were used in the assay the reported activities could have been lowered by the relatively large ratio of neuron:glial cells. Alternatively, the elevated activities of GPDH and GS in the brains of MLP exposed animals relative to controls may simply represent a higher density of glial cells. There were no significant elevations in DNA concentrations in the brains of MLP exposed neonatal and weanling animals relative to control animals and this, therefore, would argue against an increase in glial cell number. Histological examination and analysis of a GC-inducible neuronal marker enzyme, such as tyrosine hydroxylase (Tank & Weiner 1995), may give a more detailed indication of intrauterine GC action in fetal and neonatal rat brain.

Prenatal dietary experience had no effect on the activity of hepatic GS in contrast to central GS. The regulation of GS by GC appears to be tissue specific. In the developing brain the primary function of GS is the metabolism of the amino acid neurotransmitter, glutamate (Patel *et al* 1983), in the periphery, GS provides nitrogen from glutamine for the synthesis of purines, pyrimidines and consequently anabolic processes. Consequently hepatic GS is more sensitive to the action of growth hormone than GC (Wong *et al* 1980). Alternatively, the tissue specificity of gene specific responses to GC action may relate to tissue-specific differences in the concentration of transcription factors such as AP-1.

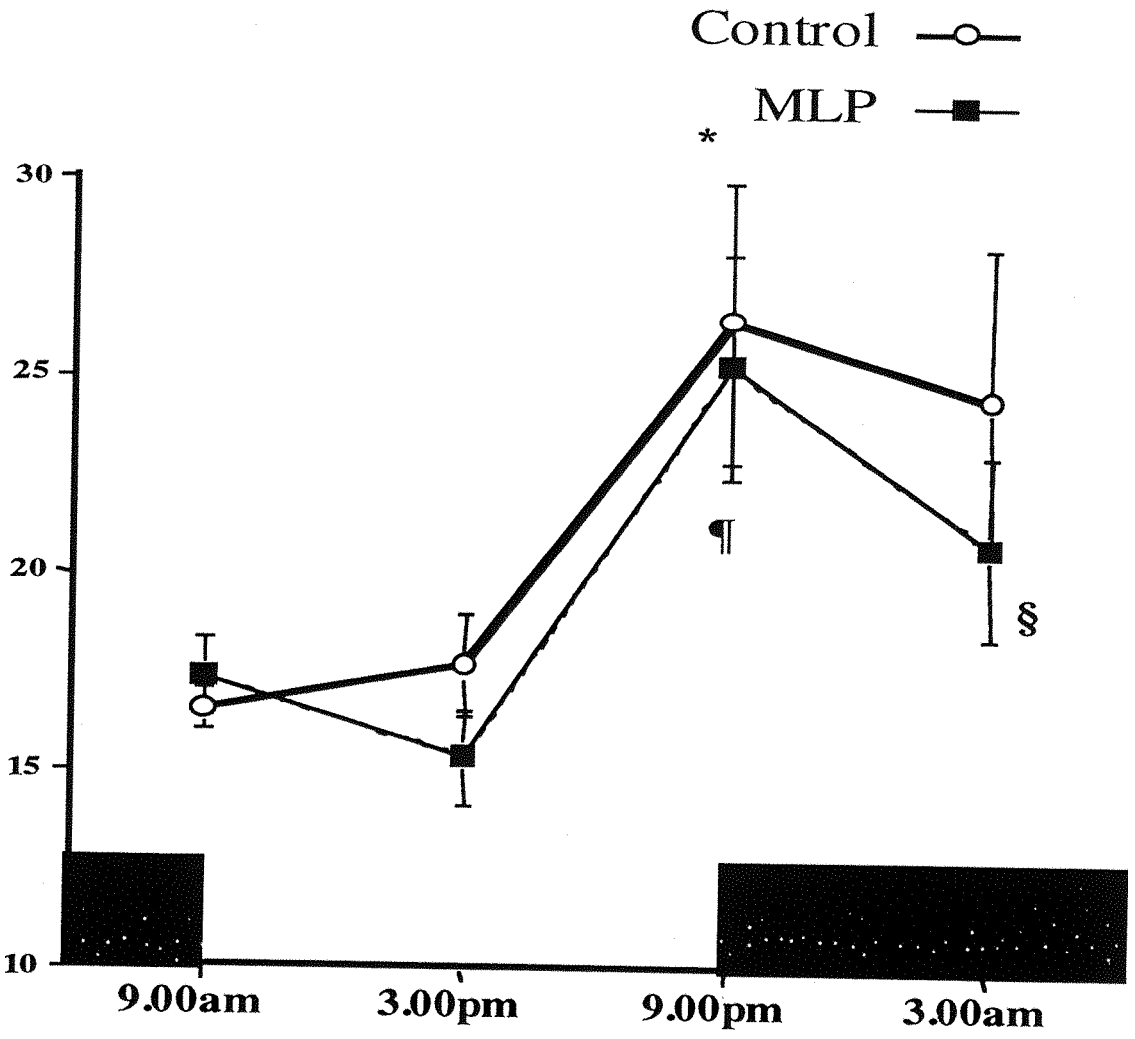
Desai *et al* (Desai *et al* 1995) have suggested that prenatal protein restriction alters the metabolic activity of the liver in favour of glucose production rather than utilisation. It is proposed that prenatal undernutrition influences the differentiation of hepatic cells during fetal development, resulting in a contraction of the perivenous population and an

expansion of the periportal population (Desai & Hales 1997). The altered liver biochemistry may then contribute to poor glucose handling leading to insulin resistance (Ozanne *et al* 1996). The activity of GS, being predominantly localised in perivenous cells, would therefore be expected to decrease. In our experiments, no change in the activity of hepatic GS as a result of prenatal protein restriction was observed. This may reflect the different carbohydrate composition of the two diets. Whereas our semi-synthetic diet contains a slight increase in both starch and glucose to offset the protein-energy deficit (Appendix 1), only glucose makes up the deficit in the diet used by the Cambridge group (Desai *et al* 1995). Alternatively the differences may reflect the fact that GS in the present study was measured in hepatic homogenates which may have masked the specific effect of a contracted perivenous cell population in a manner analagous to the difference between measuring GPDH in whole brain and specific brain regions.

3.3.4.5. The diurnal variation of plasma adrenocorticotrophic (ACTH) hormone and corticosterone concentrations

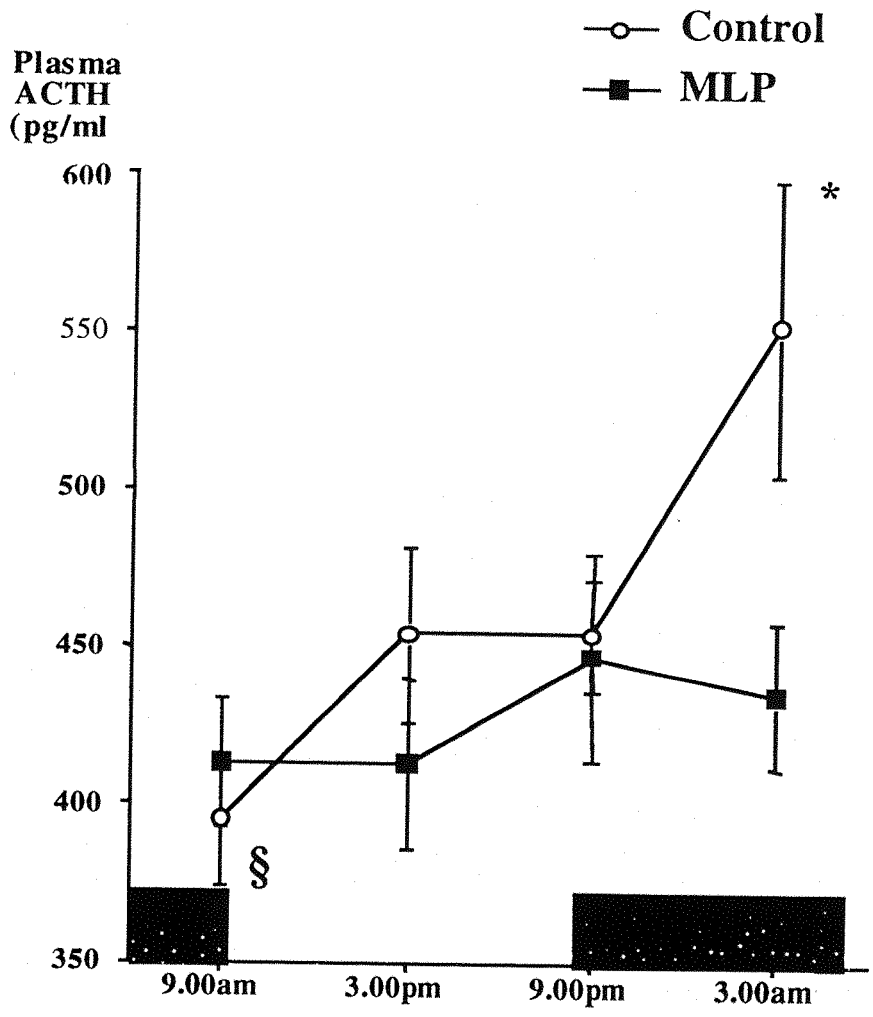
Corticosterone concentrations exhibited a distinct circadian variation, that corresponded with the lights on, lights off schedule, in both groups of animals (Figure 3.7). Concentrations were significantly higher at 9.00pm than at either 3.00pm or 9.00am in both dietary groups ($P< 0.05$). The plasma concentration of corticosterone was also significantly higher at 3.00pm than at 3.00am ($P= 0.03$) in the MLP group. In control animals, the plasma concentration of ACTH also showed a marked diurnal variation (Figure 3.8). Thus, plasma ACTH concentration in these animals was significantly lower at 9.00am (395 pg/ml) than at either 9.00am or 3.00am (552 pg/ml) ($P= 0.02$). In contrast, and exhibiting an apparent dissociation from plasma corticosterone concentrations, ACTH in the MLP group varied only between 413 pg/ml (9.00pm and 3.00pm) and 435 pg/ml (9.00pm) over the whole period studied. Thus at 3.00am ACTH concentrations were significantly higher in control animals than in the MLP group ($P= 0.01$). Two-way analysis of variance suggested a tendency for an interaction between maternal diet and time of day ($P= 0.09$). Area under the curve analysis revealed that the 24hr secretion of ACTH by the MLP group was reduced by 23% compared to control animals ($P<0.05$).

Figure 3.7. **The diurnal variation of plasma corticosterone concentration in rats exposed to either a control or low protein maternal diet**



Plasma corticosterone levels were assessed at four different time points (3.00am, 9.00am, 3.00pm and 9.00pm) in the 24hr cycle in 4wk old weanling female rats that had been exposed to either a control or maternal low protein (MLP) diet. Animals were decapitated within 1 minute of removal from their cages and plasma obtained. Corticosterone was assayed by radioimmunoassay as outlined in Methods. All data are presented as Mean ±SEM for 5-6 offspring per group from a total of 5 litters/group. Two way analysis of variance (ANOVA) indicated a significant effect of time of day (P = 0.0005) on plasma corticosterone concentration. In both dietary groups concentrations were higher at 9.00pm than at 3.00pm (18% Casein *P= 0.04, 9% Casein ¶P= 0.01) or 9.00am (P= 0.03 for both groups). In addition the low protein exposed group exhibited a higher corticosterone concentration at 3.00am than at 9.00am (§P= 0.04). Black boxes denote time at which lights go off.

Figure 3.8. **The diurnal variation of plasma adrenocorticotrophic hormone in rats exposed to either a control or low protein maternal diet**



Plasma adrenocorticotrophin (ACTH) concentrations were assessed as described in Figure 3.7. All data are presented as Mean ± SEM for 4-6 offspring per dietary group from a total of 5 litters/group. Two way analysis of variance (ANOVA) indicated a significant effect of time of day ($F= 3.38$, $P= 0.02$) and maternal diet ($F= 3.45$, $P= 0.01$) on plasma ACTH concentrations. For the control rats, ACTH concentrations were significantly lower at 9.00am than at 3.00am or 9.00am (§$P= 0.02$). The ACTH concentration was significantly higher at 3.00am in the control protein group than in the low protein exposed animals (*$P= 0.01$). Area under the curve analysis indicated the secretion of ACTH in the 9% casein group was reduced by 23% compared to protein replete animals ($P<0.05$). Black boxes denote the time at which lights go off.

3.3.4.6. Discussion of hormone data

The hypothalamic-pituitary-adrenal (HPA) axis in all species is characterised by a clearly defined circadian rhythm. Diurnal profiles of plasma ACTH and corticosterone concentrations were determined to examine whether prenatal protein restriction influences the fundamental characteristics of the HPA axis.

The data illustrate a clearly defined circadian variation in plasma corticosterone in the rat. The cycle follows the lights on/lights off schedule and thus the onset of feeding behaviour in the nocturnally active rat. The present results are in agreement with other studies in the rat (Carroll *et al* 1975; Martin *et al* 1978). Corticosterone levels were significantly higher at 9.00pm than at either 9.00am or 3.00pm in both dietary groups. Similarly ACTH, the major secretagogue for corticosterone, displayed a distinct diurnal variation in control animals. In these animals plasma ACTH was significantly lower at 9.00am than at either 9.00pm or 3.00am as has been reported elsewhere (Atkinson & Waddell 1995). In contrast, no variation in the plasma concentration of ACTH was observed in the MLP group. The maintenance of 'normal' plasma corticosterone despite an increased central sensitivity (as reflected by enzyme data) may reflect a reduced pituitary production of ACTH concommitant with an increased adrenal sensitivity to ACTH. A reduced output of ACTH in the face of normal corticosterone concentrations may be determined through dexamethasone infusion and measuring the concommitant ACTH output.

The mechanism for the blunting of the diurnal surge in ACTH as occurs in MLP is unclear. Mild stress (handling) in early life leads to a suppression of the adult HPA axis (Meaney *et al* 1988). Increased GR populations in the hippocampus, as a consequence of neonatal handling, are believed to underlie this effect (Meaney *et al* 1992). Thus, increased GR numbers in the hippocampus increase the central sensitivity to corticosterone feedback. This, therefore, enhances the action of inhibitory neurones projecting from the hippocampus into the paraventricular region of the hypothalamus, and so reducing the CRF surge and blunting the diurnal surge in ACTH (Seckl & Olsson

1995). In support of this hypothesis, hippocampal lesions or reduced hippocampal GR receptors are associated with an exaggerated ACTH response to stress (Seckl & Olsson 1995). An analogous scenario may be operating in the MLP model of hypertension, since significantly elevated (41%) corticosterone binding to hippocampal GR receptors has been noted (Langley-Evans *et al* 1996c). Alternatively, since only four recordings were made throughout the 24hr cycle, the peak in ACTH in MLP may have been missed or MLP demonstrate altered sine curve profiles relative to controls.

It is apparent that there is a dissociation between plasma ACTH and corticosterone concentrations in MLP rats. This phenomenon is readily observed in other animal models. Thus the diurnal corticosterone rhythm persists either after pre-treatment of rats with dexamethasone to inhibit ACTH output (Dallman *et al* 1978), or following hypophysectomy (Meier 1976) which will abolish ACTH secretion. Furthermore, hypophysectomised rats with ACTH implants, delivering a constant level of ACTH, still demonstrate a clear, rhythmic, secretion of corticosterone (Ottenweller & Meier 1982). In dogs, the rise in cortisol that necessarily follows haemorrhage is elicited before a rise in ACTH occurs (Wood *et al* 1982). It has been observed that the rat adrenal gland exhibits a diurnal sensitivity to ACTH that is dissociable from the rhythm in ACTH secretion. Adrenal sensitivity to ACTH was found to be approximately 2-3 fold higher in the evening (lights off) than in the morning (lights on) (Dallman *et al* 1978). The changes in adrenal sensitivity are not related to rhythms in the metabolic clearance of either ACTH or corticosterone (Kaneko *et al* 1981). Ottenweller & Meier (1982) surmised that superimposed upon the central circadian pacemaker located in the suprachiasmatic nucleus was an extrapituitary factor that may control the sensitivity of the adrenal to ACTH and thus couple the secretion of corticosterone from the adrenal gland to the secretion of ACTH from the pituitary.

The extrapituitary factor that drives the adrenal sensitivity to ACTH stimulation is believed to be neural in origin. Spinal cord transection at T-7, disrupting adrenal innervation, abolishes the diurnal corticosterone rhythm whereas transection at L-1, which will not directly impact upon adrenal innervation, does not (Ottenweller & Meier 1982). Splanchnic nerve transection, therefore severely restricting adrenal sympathetic

innervation, blunts the diurnal surge in corticosterone by 50% yet has no effect on basal corticosterone secretion (Dijkstra *et al* 1996). Thus sympathetic innervation of the adrenal gland potentiates ACTH stimulation, resulting in an enhanced corticosterone response to ACTH in the evening, which may thus maintain the characteristic diurnal peak of plasma corticosterone in the face of a blunted diurnal ACTH peak.

A generally low secretion pattern of ACTH and corticosterone in adult MLP suggests that excess exposure to maternal corticosterone during mid-late gestation, a critical time in the development of the fetal HPA (Bourdouresque *et al* 1988), alters the *in utero* development of the fetal brain leading to altered hormonal secretion in adult life. This may be interpreted as deleterious in this animal model given that these animals are hypertensive. The significance of the present observations is that maternal undernutrition has programmed aspects of the fetal HPA axis leading to dysfunction in adult life. Elevations in the activity of glucocorticoid sensitive enzymes reflects an increased sensitivity to glucocorticoid action. Whilst increased enzyme activity and an altered secretion profile of ACTH may not be causally related to increased systolic blood pressure in MLP, they provide useful biochemical markers of HPA axis activity.

3.4. GENERAL DISCUSSION

Hypertension is a major risk factor for coronary heart disease (CHD). CHD is the major cause of death in westernised societies (Thom *et al* 1992). Whilst it is accepted that adult 'lifestyle' factors i.e. low physical activity, obesity, saturated fat, salt and alcohol intake can lead to elevated blood pressure (Ward 1990), these factors only partially determine cardiovascular risk. Barker and colleagues have proposed that factors operating in early life may determine individual susceptibility towards hypertension and thus CHD (Barker *et al* 1989a). Birthweight is determined by both genetic and non-genetic factors. There is a high correlation between birthweights of siblings, which follows the mother rather than the father, indicating the importance of a maternal factor (Milner & Gluckman 1996). The overriding single determinant of birthweight is the maternal physical environment rather than maternal genes. Transfer of a shetland embryo to a shire horse uterus will result in a foal of greater birthweight than would a shetland foal from a shetland mare uterus (Walton & Hammond 1938). Growth is an anabolic process requiring nutrients and thus maternal undernutrition can potentially influence the intrauterine growth process. Classically it was thought that mild undernutrition had little effect on the fetus, as evidenced by the Dutch Hunger Winter Famine, where birthweight was reduced only when maternal caloric intake fell below 1500 kcal/day (Smith 1947). However, the essence of the fetal origins of adult disease hypothesis is that subtle degrees of undernutrition leading to an asymmetric baby at term, but a baby that is within the normal birthweight range, may have long term consequences for the health of that individual (Barker 1995).

The feeding of the MLP diet to pregnant rats did not reduce reproductive performance since litter sizes and birthweights were similar between dietary groups. Maternal weight gain in late gestation was, however, restricted and this may have influenced the late gestational and early lactational pattern of fetal growth. The MLP rats, although of low to normal birth weight, exhibited degrees of disproportionality at term, reflecting reduced peripheral (liver, lung) organ growth *in utero*. Whilst brain growth continued over late gestation in proportion to the increase in body size, growth of the

trunk, lungs and liver lagged behind. *The mechanisms that co-ordinate the redistribution of fuel substrates away from the periphery favouring the brain in response to undernutrition may be critical in the programming of cardiovascular disease.*

In response to a deficiency of specific nutrients within the maternal diet the fetus adapts by mobilising endogenous stores and redistributing blood flow (Rudolph 1984). These adaptations made to accomodate the stress are likely hormone based. Both insulin, being a major anabolic hormone (Fowden 1989), and GC are important in mediating prenatal energy budgets and growth (Dallman *et al* 1993; Seckl 1994) and modulate the control of the cardiovascular system (Hill *et al* 1988; Tangalakis *et al* 1992). Edwards *et al* first postulated that maternal glucocorticoid hormones might be the key programming agents in the fetal origin of adult disease (Edwards *et al* 1993). In early prenatal development, maternal glucocorticoids are important at facilitating fetal blood pressure control prior to the fetus having acquired an adequate capability to autoregulate its cardiovascular system (Tangalakis *et al* 1992). Thus under conditions of metabolic stress, that the MLP diet may impose in late gestation, fetal blood pressure may increase in order to facilitate aquisition of substrates from the placenta, and to redistribute blood flow and consequently nutrients, aided by an increased flux of maternal GC .

GC have many recognised actions in the cardiovasculature which result in elevated blood pressure and often hypertension (Ross & Linch 1982; Walker & Williams 1992; Whitworth *et al* 1995). In the sheep glucocorticoids infused *in utero* raise blood pressure (Hill *et al* 1988) by mechanisms relating to peripheral reactivity and potentiation of the pressor effect of angiotensin II (Tangalakis *et al* 1992) and sympathetic activity (Walker *et al* 1992). Tissue specific alterations to fetal GC receptor populations (through which GC actions are mediated) may therefore have the potential to initiate rises in blood pressure and redistribute blood flow to the brain, through effects on peripheral vascular reactivity. Young adult MLP exhibit 3-fold increased vascular GR binding relative to controls which may, if present in fetal life, faciliate such a mechanism (Langley-Evans *et al* 1996c). Intrauterine haemostatic and hormonal alterations may provide the initial stimulus necessary to render the individual susceptible to later elevated blood pressure even aften the initial stimulus has been removed. Berry suggested that "...changes induced

in (blood) vessels by high pressure or flow are adaptive... but these changes may predispose to degenerative disease" and that "blood vessel structure is determined in later life by haemodynamic stress during growth" (Berry 1978). Thus factors operating *in utero* may initiate haemodynamic and structural vascular changes that lead to primary hypertension (Lever 1986). Since the rate of increase of adult SBP is associated with pressure in childhood (Lever & Harrap 1992) then elevated pressure in early life may precipitate and facilitate mechanisms, i.e. smooth muscle hypertrophy that maintain secondary hypertension (Folkow 1978). The hypertensive state of MLP is clearly initiated by factors operating in early life and a secondary mechanism may therefore maintain the elevated blood pressure.

GC hormones are therefore important in determining vasoactive hormone activity *in utero* and in adult life which may then in turn influence the genesis of the hypertensive state in MLP. The following chapter(s) investigate the hypothesis that GC hormone activity *in utero* is important in establishing primary hypertension (*Chapter* 4) and that postnatal GC influence is essential for the mainenance of the hypertensive state (*Chapter 5*).

CHAPTER 4

THE INFLUENCE OF THE MATERNAL ADRENAL AND ITS PRODUCTS ON HYPERTENSION PROGRAMMED BY MATERNAL PROTEIN RESTRICTION

4.1. INTRODUCTION

Maternal glucocorticoids are postulated by Edwards *et al* (Edwards *et al* 1993) to underpin the fetal origins of adult disease hypothesis (Barker 1995). Maternal corticosterone is ordinarily metabolised in the placenta to its inactive, labile form 11-dehydrocorticosterone (Murphy *et al* 1974) by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11-HSD2). 11-HSD2 displays a wide variation in activity and, in the rat (Benediktsson *et al* 1993) and human (Benediktsson *et al* 1995; Stewart *et al* 1995) correlates positively with birth weight, indicating a role for this enzyme in the regulation of fetal growth and development. Either inhibition of 11-HSD2 by carbenoxolone (Langley-Evans 1997b; Lindsay *et al* 1996) or bypassing 11-HSD2 activity with dexamethasone (Benediktsson *et al* 1993) lowers birthweight and renders the resultant offspring hypertensive. Maternal adrenalectomy abolishes the hypertensive action of carbenoxolone (Lindsay *et al* 1996) indicating a maternal steroid-dependent effect.

Protein restriction similarly lowers the activity of placental 11-HSD2 (Langley-Evans *et al* 1996d). The resultant offspring display disproportionate patterns of fetal growth leading to a full-term neonate of low-normal birthweight. The MLP offspring exhibit elevated activities of GC-inducible enzymes and altered adrenocorticotrophic hormone secretion indicating a widespread disturbance of hypothalamic-pituitary-adrenal axis function. The MLP offspring subsequently develop hypertension from an early age (*see Chapter* 3). Fetal exposure to maternal glucocorticoids, secondary to undernutrition, may therefore lead to hypertension in later life.

The use of dexamethasone and carbenoxolone in pregnant rats to elucidate a role for maternal glucocorticoid has methodological drawbacks as discussed in Chapter 3. However actual blockade of maternal glucocorticoid influence throughout pregnancy presents specific problems. Metyrapone (an 11β-hydroxylase inhibitor) reduces both maternal and fetal corticosterone levels (Baram & Schultz 1990) and therefore cannot be used in late gestation since corticosteroids are essential for late gestational lung maturation (Liggins 1969). Later use would thus severely impact upon fetal viability. Moreover, the channelling of metabolites into other steroid pathways following 11β-hydroxylase inhibition and the hypertension that may follow precludes a specific glucocorticoid effect being determined. RU-486, a Type II GC antagonist, also acts as an anti-progestogen, thereby restricting use in pregnancy (Baulieu 1988). A specific Type II (GC) receptor antagonist is not yet available.

In order to address the influence of the maternal adrenal upon fetal programming of adult hypertension in MLP, a number of approaches are left. Using steady state radioisotope techniques the placental flux of corticosterone into the fetal compartment during gestation may be assayed. Whilst this approach is direct it presents a number of difficulties. For example, radioisotope measurements in mother and fetus would necessarily have to be made under anaesthesia which may influence the maternal physiological environment. Furthermore a number of preliminary experiments would be required to determine the optimal dose of radiolabelled corticosterone, the optimal time between administration and sampling and the recovery of labelled steroids from the maternal and fetal plasma. Such an experiment may also be confounded by conversion of A to B in fetal tissues and possible nutritional effects upon fetal 11-HSD1 and 11-HSD2. Thus, surgical adrenalectomy prior to pregnancy represents the only realistic means of selectively removing all maternal corticosteroid influence (without influencing ambulatory blood pressure) throughout gestation and thus addressing whether maternal glucocorticoid excess programmes nutritionally-induced hypertension in the resultant offspring.

4.2. PROTOCOL

12 rats were bilaterally MADX as outlined in section 2.4.5. and maintained on 0.9% saline, drinking water and standard chow diet *ad libitum*. After a 1 month recuperation period

the rats were mated and offspring generated as outlined in section 2.2.1. Rat dams were sacrificed at weaning and blood samples taken for corticosterone analysis to determine completeness of ADX. Blood pressures were determined on the offspring of both dietary groups using the indirect tail-cuff method (*section* 2.6.) at 6 and 10 weeks of age alongside age matched offspring from adrenal-intact rat dams. Unfortunately data were not available for intact males at 6 and 10 weeks of age and therefore data for MADX-males at these ages have been removed. All animals were sacrificed by decapitation following the final blood pressure measurement, and blood and tissue samples obtained.

SECTION 1

THE EFFECT OF MATERNAL ADRENALECTOMY ON LOW PROTEIN-INDUCED HYPERTENSION

4.3. RESULTS

4.3.1. Maternal weight gain after MADX

MADX rat dams were mated as described in Methods approximately 1 month after surgery and following conception were allocated to receive either control (18% casein) (n=6) or low protein diet (9% casein; MLP) diet (n=6) throughout pregnancy. All MADX dams conceived successfully. Of the 12 rats that were mated 2 rats (1/dietary group) failed to go to term. The average weight of MLP dams on day 0 gestation was approximately 35g heavier than the control group (262 ±11g *vs* 227 ±15g). Since data for weights at surgery and food intake were not available, the weight gain of each dietary group cannot be compared prior to mating. Control animals gained on average 6g/day throughout pregnancy, mostly during the last week of pregnancy (Table 4.1). Thus, control dams gained relatively more weight in week 3 than either week 1 (P= 0.03) or week 2 (P= 0.07). In contrast MLP did not exhibit an increase in late gestational weight gain relative to week 1 or 2 (Table 4.1). Thus controls dams gained approximately 2.5g/d more weight than MLP dams during Week 3.

Table 4.1. **The weight gain of MADX rats fed either a control or low protein diet during pregnancy**

	Gestational Weight Gain (g/d)				
	Week 1	**Week 2**	**Week 3**	**Average**	**n**
Control	4.88 ±0.6	5.44 ±0.3	9.04 ±1.7*	6.03 ±0.6	5
MLP	4.93 ±0.7	5.46 ±0.5	6.56 ±1.8	4.98 ±0.7	5

All values are presented as mean ±SEM. Rats were bilaterally adrenalectomized (ADX) under sodium pentobarbitone anaesthesia two months prior to mating. They had free access to drinking water, 0.9% saline to maintain fluid balance and laboratory chow diet. Upon conception, detected by the appearance of a plug on the floor of the cage and consequently designated d 0 of pregnancy, rats were transferred to a semi-synthetic diet containing either 18% (control) or 9% (low protein; MLP) casein as protein source. The dams on the control diet gained more weight in week 3 than in week 1 (*P= 0.03) or week 2 (P=0.07). MLP dams failed to exhibit a similar magnitude of weight gain in the final week relative to controls. Thus, by week 3 MLP dams were gaining approximately 2.5g/day less weight than the control group. *n* = number of dams in each group

4.3.2. Reproductive success after maternal ADX

Maternal adrenalectomy (MADX) did not affect overall litter size (Table 4.2), although perinatal mortality was higher in both dietary groups compared to intact controls (data not shown). Average birth mortality in the control group was 10% with total neonatal mortality reaching 25%. Protein restriction increased both birth and neonatal mortality by at least two-fold (Table 4.2) predominantly due to the dams eating their litters. The absence of a maternal adrenal did not appear to affect birth weight in males or females of the control group compared to adrenal-intact animals (*comparison to birth weights in Table 3.6*). Birth weight was significantly reduced in both male and female MLP animals compared to protein replete controls. Females from the MLP group appeared especially vulnerable to the growth retarding effect of MADX coupled with protein restriction (7% *vs* 14% smaller for MLP males and females respectively) (Table 4.2).

4.3.3. The systolic blood pressure of 6 weeks old female rats after maternal ADX

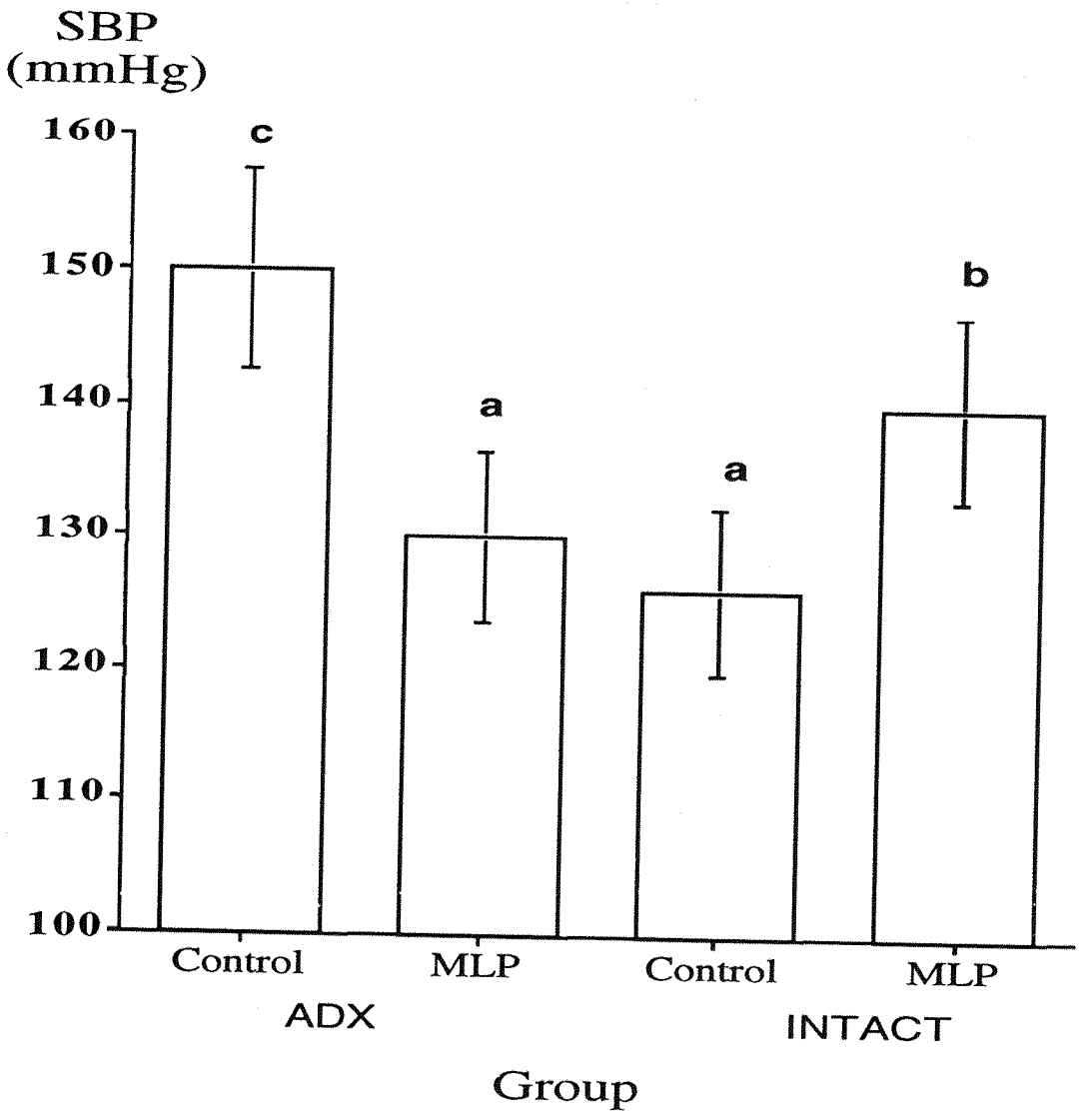
Systolic blood pressures were measured using the tail-cuff method as previously described. All pressures obtained in females were compared to age matched offspring of adrenal-intact rats fed control or MLP diets, to determine the effect of MADX (Figure 4.1). There were no equivalent data for intact males at the age studied and thus the effect of MADX could not be determined in male animals and data have not been presented. MADX significantly decreased blood pressure in MLP females compared to age matched females from adrenal-intact MLP dams (P= 0.03). SBP of MLP females were significantly lower than in the female offspring from control MADX rats (P= 0.002) (Figure 4.1). Females of the control MADX group had significantly increased blood pressure compared to age matched female offspring from control adrenal-intact rat dams (P= 0.002) (Figure 4.1).

Table 4.2. **Reproductive success of MADX rats fed either a control or low protein diet during pregnancy**

	Dietary Group	
	Control	**MLP**
Litter size	10± 0.8	11± 1.7
Birthweight (g)...		
Females	5.52± 0.06	4.75± 0.19§
Males	5.67± 0.04 *	5.30± 0.15 *
Mortality...		
Born dead (%)	10	25
Survival (%)	75	50

All values given are mean ±SEM. See Table 4.1 for details. Litter size was calculated as the mean of 5 litters born (control) and 3 litters born (MLP). The percentage born dead was calculated as those that were dead upon first inspection within each litter as a proportion of the total litter size. The survival rate was calculated as the ratio of the pups that survived to weaning age per litter to the total number that were born (alive and dead) in that litter, expressed as a percentage. Neonatal mortality was higher and survival rate lower in the low protein exposed group, although litter size was unaffected. Two way ANOVA indicated a significant effect of sex ($F= 12.73$, $P= 0.0006$), diet ($F= 33.68$, $P< 0.0001$) and an interaction between diet and sex ($F= 3.94$, $P= 0.05$) on birthweight. * indicates a significant difference to females of the same group ($P= 0.0006$), § indicates a significant difference to dietary control females ($P= 0.05$)

Figure 4.1. **Systolic blood pressures (SBP) of 6 week old female offspring from MADX rats fed either a control or low protein diet during pregnancy.**

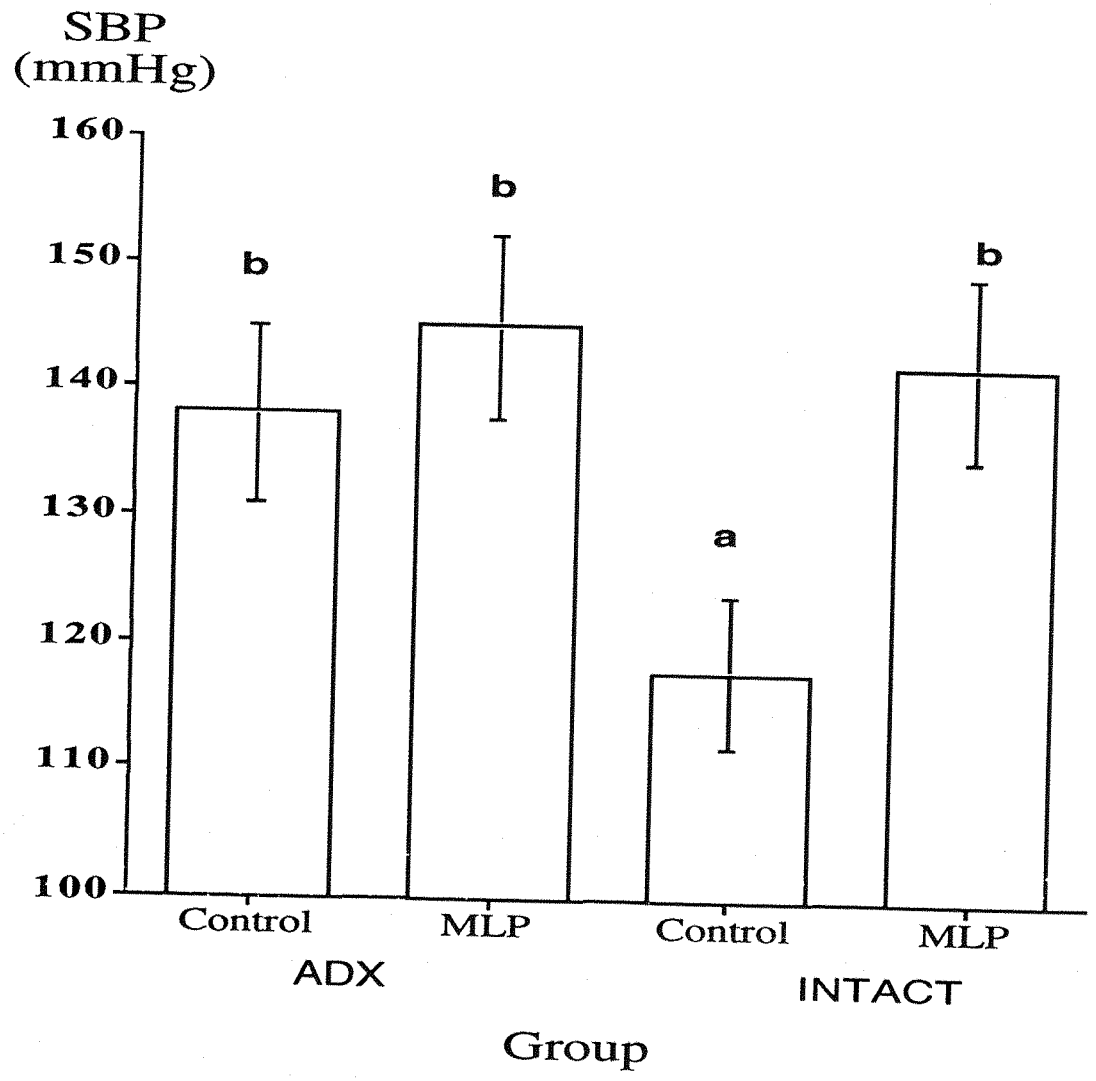


Data are Mean ±SEM for n=10 offspring in each treatment group from n=5 litters per diet group. 12 rats were bilaterally adrenalectomized 2 months prior to mating. Upon conception rat dams received either a control (18% casein n=6) or low protein (9% casein;MLP n=6) diet throughout pregnancy. At birth the diet was substituted for standard chow and litters culled to 8 pups (4 male/4 female). Offspring were weaned onto chow at 4 weeks of age. Systolic blood pressure was determined on offspring at 6 weeks of age using the indirect tail cuff method and compared to age matched offspring from adrenal-intact control dams. Two way analysis of variance (ANOVA) indicated a significant effect of diet ($F= 11.99$, $P= 0.002$) and sex ($F= 16.21$, $P= 0.0001$) upon the blood pressure of the offspring. Bars sharing a similar superscript are not significantly different at $P<0.05$.

4.3.4. The blood pressure of 10 week old female rats after maternal ADX

Systolic blood pressure was assessed at 10 weeks of age as described in *Section 2.6*. The blood pressures of control MADX females were, again, significantly higher than age-matched offspring of adrenal-intact controls (P= 0.005). The difference between MADX MLP females and control MADX females was not apparent at this age. This was due to an increase in the SBP of MADX MLP females and a fall in the SBP of MADX-control females over the four week period (from 6-10 weeks) such that the blood pressure of these animals was not significantly different to age-matched offspring from adrenal-intact MLP dams.

Figure 4.2. **Systolic blood pressures (SBP) of 10 week old female offspring from MADX rats fed either a control or low protein diet during pregnancy.**



Data are Mean ±SEM for n=10 offspring in each treatment group from n=5 litters per diet group. 12 rats were bilaterally adrenalectomized 2 months prior to mating. Upon conception rats received either a control (18% casein n=6) or low protein (9% casein;MLP n=6) diet throughout pregnancy. At birth the diet was substituted for standard chow and litters culled to 8 pups (4 male/4 female). Offspring were weaned onto chow at 4 weeks of age. Systolic blood pressure was determined at 10 weeks of age using the indirect tail cuff method and compared to age matched offspring from adrenal-intact control dams. Two way analysis of variance (ANOVA) indicated a significant effect of sex ($F= 13.69$, $P= 0.001$). Bars sharing a similar superscript are not significantly different at $P<0.05$.

SECTION 2

THE EFFECT OF MATERNAL ADRENALECTOMY AND CORTICOSTERONE OR ALDOSTERONE REPLACEMENT DURING GESTATION, ON LOW PROTEIN-INDUCED HYPERTENSION

4.4. PROTOCOL

43 female rats weighing approximately 234 ±2g were either bilaterally ADX (n=37) or SHAM (n=6) operated as outlined in section 2.4.5 and maintained on 0.9% saline, drinking water and standard chow diet *ad libitum*. After a 1 month recuperation period the surviving rats (n=42) were mated and assigned to control or MLP dietary groups, upon conception, as determined by the presence of a plug on the floor of the cage. ADX rats from each dietary group were assigned to one of 3 groups that received twice daily injections of either corticosterone, aldosterone or vehicle:

1. corticosterone replacement (10mg/kg bodyweight/day *s.c.*) AC: (18% casein controls, 18AC n=6; 9% casein MLP rats, 9AC n=6)
2. aldosterone replacement (100µg/kg bodyweight/d *s.c.*) AA: (18% casein, 18AA n=6; 9% casein, 9AA n=6)
3. vehicle replacement (0.05ml polyethylene glycol *s.c.*) AV: (18% casein, 18AV n=6; 9% casein, 9AV n=6) twice daily throughout pregnancy.

The doses were chosen with the objective of replicating the physiological range of plasma concentration for each hormone based on previous data obtained within this laboratory (Langley 1990) . Two further groups were generated to act as non-adrenalectomized vehicle injected controls; 18% casein sham-operated (18SV n=3) and 9% casein sham-operated (9SV n=3). All steroids were dissolved and injected in vehicle (0.05ml polyethylene glycol). Corticosterone was injected in two differing doses (0.2mg/kg at 9.00am, 0.8mg/kg at 5.00pm) to duplicate the normal diurnal rhythm in corticosterone secretion (Martin *et al* 1978). All

semi-synthetic diets were substituted for standard laboratory chow at d21 (term, 21.9 ±0.7days (mean ±1SD)) of gestation.

At littering steroid replacement was stopped and thereafter rats were not handled nor were offspring weighed until postnatal d3. Litters were therefore not reduced to n=8/litter. This regimen was adopted, successfully, to avoid the high neonatal mortality rate noted in the previous MADX study. Following this protocol a total of 28 rats gave birth from an initial total of 42 that conceived. Of the 14 that failed to give birth 13 reabsorbed their pregnancies, either in early gestation (n=8) or late gestation (n=5). 1 animal died during the experiment. The loss of one third of the pregnancies from this experiment was slightly higher than normal for our colony where approximately 16-25% of pregnancies are unsuccessful. The higher rate of loss reflected the severity of the surgical procedure followed by pregnancy. Therefore it necessarily follows that the *n* of groups in SBP measurements made at 5 weeks of age on the offspring were reduced and thus the data do not carry as much statistical power. Unfortunately the replacement protocol could not be repeated due to time restraints.

Offspring were weaned onto chow at 3-4 weeks of age, whereupon the dam was sacrificed and visually checked for the presence of a regenerated adrenal. Following this examination a further 8 litters were excluded from the analysis after a positive identification of vestigial adrenal tissue. Thus for determination of offspring blood pressures there were a total of: n=2 litters for 18SV, 18AA and 9SV; n=3 litters for 18AV and 9AC; n=4 litters for 9AV and 9AA. Vestigial adrenal tissue was found in all litters from group 18AC which were therefore excluded. SBP of all offspring from each remaining group were determined at 5 weeks of age using the indirect tail-cuff method.

4.5. RESULTS

4.5.1. Maternal weight gain after MADX and hormone replacement

Pre-pregnancy weight gain was slightly greater in SHAM-operated animals than MADX (55 ±6 *vs* 47 ±3g, P= NS), with average weights on day 0 of gestation of 289 ±9g (SHAM n=4) and 281 ±4g (MADX n=16). Food intake was significantly lower in the

MADX animals over the recuperation period (SHAM 18.2 ±1.2g/d, MADX 12.3 ±0.6g/d, P< 0.01) and is sufficient to account for lesser weight gain in MADX animals. Upon conception, rats were divided into groups to receive either control or MLP diets with corticosterone, aldosterone or vehicle. However due to the number of resorptions within dams from each group a meaningful analysis of the effect of each treatment on food intake and weight gain could not be performed and therefore these data are not included.

4.5.2. Reproductive success after MADX with hormone replacement

Litter size was unaffected by maternal dietary manipulation or surgical procedure with large variations within groups (Table 4.3). For the male offspring, 2-way ANOVA indicated a significant effect of diet (F= 20.47, P<0.0001) and steroid treatment (F= 15.26, P<0.0001) on weight at day 3. MLP males were lighter than control males in all groups receiving hormone replacement. For the female offspring, 2-way ANOVA indicated a significant effect of steroid treatment only upon weight at day 3 (F= 9.00, P< 0.0001). Thus, female pups from MADX dams were lighter than female pups from SHAM dams. In the MLP groups, aldosterone or vehicle treatment reduced weight at day 3 of both males and females. This effect was greater in females.

4.5.3. Systolic blood pressures of control or MLP offspring from MADX/SHAM dams

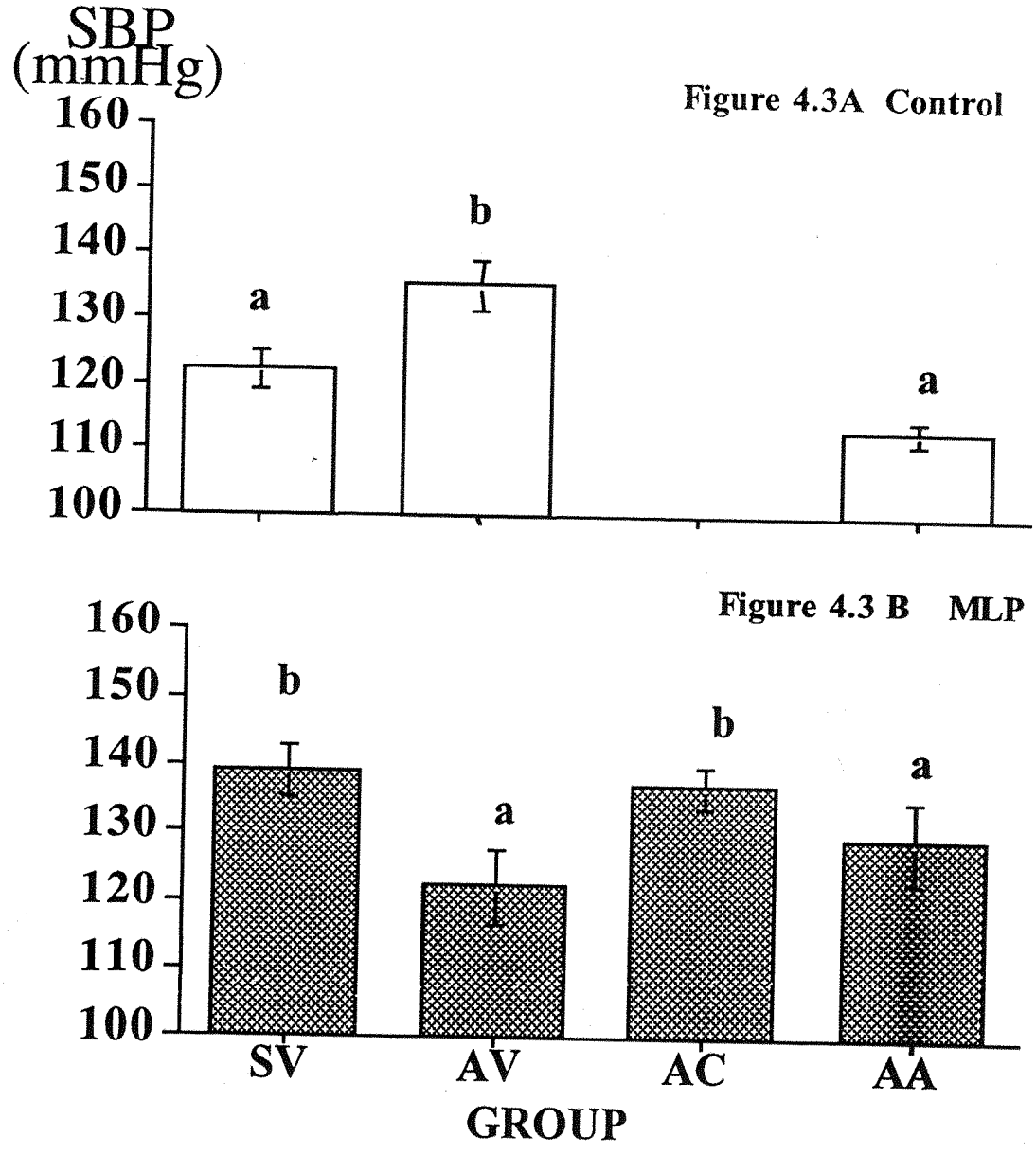
At 5 weeks of age SBP of the MLP offspring from adrenal-intact dams was greater than in the offspring of dietary controls (group 9SV *vs* 18SV) in males (an elevation of 18mmHg, P= 0.002) and females (an elevation of 10mmHg) (Figures 4.3 & 4.4). Maternal adrenalectomy (MADX) with vehicle replacement resulted in an elevation of SBP in both male and female offspring exposed to the protein replete diet throughout pregnancy (18AV *vs* 18SV, P< 0.05). In contrast, MADX reversed the hypertensive effect of maternal protein restriction (9AV *vs* 9SV) in male (P< 0.01) but not female animals.

Table 4.3. **Reproductive success of MADX/SHAM control or MLP rats following hormone replacement**

	Neonatal weights at day 3							
	Sham Vehicle		ADX Vehicle		ADX CORT		ADX ALDO	
	Male	Female	Male	Female	Male	Female	Male	Female
Control	8.9 ±0.1	8.5 ±0.3	8.5 ±0.1*	7.7 ±0.2	-	-	7.8 ±0.3	7.8 ±0.4
MLP	8.7 ±0.3§	8.7 ±0.8§	7.1 ±0.4	7.6 ±0.4*	7.1 ±0.1	6.8 ±0.1	6.9 ±0.2	6.6 ±0.1
Diet..	Control	MLP	Control	MLP	Control	MLP	Control	MLP
Litter size	13,7	10,9	11 ±6	6 ±3	-	11 ±5	11 ±4	7 ±4

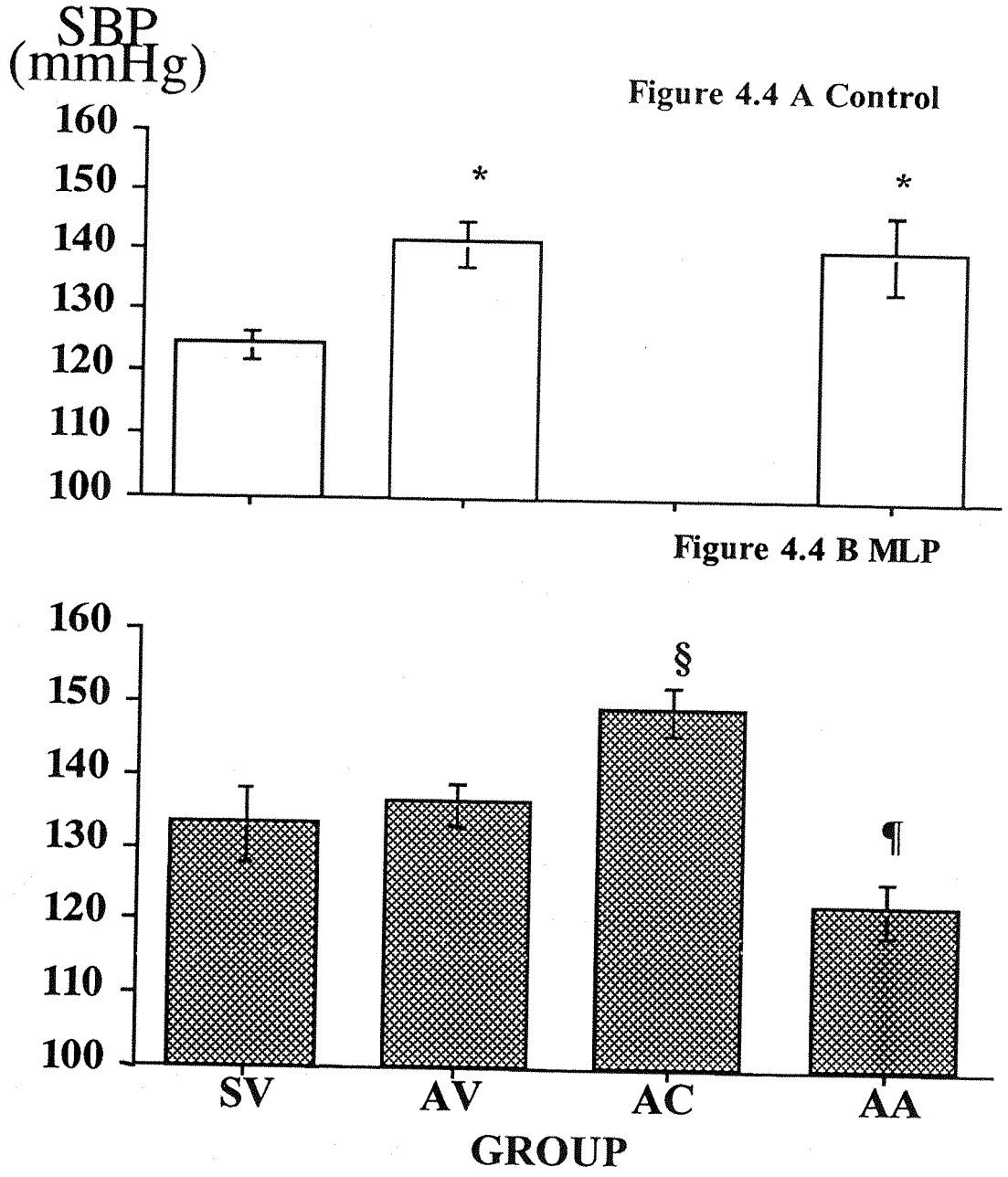
All data are mean ±SEM for between 2-4 litters, and 7-30 offspring (neonatal d3 weights). Rats were bilaterally adrenalectomized (ADX) or sham operated 1 month prior to mating. Upon conception rats were allocated to receive either 18% casein (control) diet or 9% casein (low protein) diet and either twice daily injections *s.c.* of corticosterone (CORT) (10mg/kg/d in two doses of 0.2mg/kg in the morning or 0.8mg/kg, evening), aldosterone (ALDO) (100µg/kg/d) or vehicle (0.05ml polyethylene glycol). All steroids were dissolved in vehicle. At d21 (term= 21.9 ±0.7d) gestation semi-synthetic diet was replaced with laboratory chow diet. Neonates were weighed on day 3 to minimise disturbance of the dam in the immediate postpartum period. For males 2-way ANOVA indicated a significant effect of diet ($F= 20.47$, $P<0.0001$) and steroid treatment ($F= 15.26$, $P<0.0001$). For females 2-way ANOVA indicated a significant effect of steroid treatment ($F= 9.00$, $P< 0.0001$) and suggested a trend for an interaction between diet*steroid treatment ($P= 0.06$). * indicates a significant difference to animals within the same group ($P<0.05$), § indicates a significant difference to all MADX groups ($P<0.05$).

Figure 4.3. **The systolic blood pressure of male offspring from control (A) and low protein (B) exposed MADX/SHAM dams following hormone replacement during pregnancy**



All data are mean ±SEM for n= 5-10 offspring/group. Refer to Table 4.3 for details of methodology. Systolic blood pressures (SBP) were determined in the male offspring at 36 ±1.6 days using the tail-cuff method. Two way analysis of variance (ANOVA) indicated a significant effect of steroid treatment (F= 3.89, P= 0.02) and suggested an interaction between diet*steroid treatment (F= 8.68, P= 0.0005). Groups: SV, sham-operated vehicle injected; AV, MADX vehicle injected; AC, MADX corticosterone injected; AA, MADX aldosterone injected.*a* is significantly different to *b* (P<0.05).

Figure 4.4. **The systolic blood pressure of female offspring from control (A) and low protein (B) exposed MADX/SHAM dams following hormone replacement during pregnancy**



All data are mean ±SEM for n= 4-10 offspring/group. For details of groups and methodology refer to Figure 4.3. Two way analysis of variance (ANOVA) indicated a significant interaction between diet*steroid treatment only (F= 5.66, P= 0.006). *indicates a significant difference to 18SV (P<0.05), § indicates an effect of corticosterone replacement (P= 0.01), ¶ indicates a significant effect of aldosterone replacement (P<0.05).

Corticosterone replacement to MADX-MLP rats (9AC) significantly elevated SBP of the female offspring relative to SHAM-operated MLP rats (Figure 4.4), and effectively negated the hypotensive effect of MADX in male animals (Figure 4.3). In the offspring of MADX-control diet fed dams different responses to prenatal aldosterone were observed. In males (18AA) SBP was similar to pressures observed in group 18SV (Figure 4.3 A) whilst in females, higher pressures, as in group 18AV, were observed (Figure 4.4 A). In MADX-MLP rats, aldosterone replacement (9AA), partially restored the higher SBP of male but not female offspring (Figures 4.3 B and 4.4 B). However caution must be exercised when attributing a role of maternal corticosterone in the programming of elevated SBP in MLP since no comparison can be made to corticosterone replaced control rats (Group 18AC).

4.6. DISCUSSION

Development from conception to maturity is characterised as a 'vulnerable period' in the life of the organism (Dobbing 1981). Cells are more vulnerable during periods of relatively rapid growth i.e. mitotic activity as opposed to increases in cell size (Winick & Noble 1966) and exposure to a 'programming' stimulus during this time may have irreversible consequences (Lucas 1991). The ability of steroid hormones to irreversibly programme homeostatic mechanisms has been well characterised (Arai & Gorski 1968; Besa & Pascual-Leone 1984; Harris & Levine 1962; Sloop *et al* 1983). For example, postnatal injection of androgen into female rats at 5 days of age results in a permanent masculinisation of hypothalamic mechanisms regulating ovulation (Arai & Gorski 1968) and an acyclical hormone release pattern that resembles the male pattern rather than female (Harris & Levine 1962). Edwards *et al* have proposed that exposure to an increased level of bioactive maternal glucocorticoids programmes hypertension in adult life (Edwards *et al* 1993) and suggest that maternal glucocorticoid (GC) exposure underpins the fetal origins of adult hypertension hypothesis (Barker 1995).

As previously indicated (Chapter 3), maternal protein restriction elevates the SBP of the adult offspring and similarly lowers the activity of placental 11-HSD2 in late gestation. It is inferred, albeit indirectly, that fetuses exposed to maternal protein restriction may be

exposed to excess maternal bioactive GC during gestation. The MLP diet programmes increased activity of enzymes that are GC-inducible in the resultant offspring (Chapter 3), and the hypertensive state of the offspring maybe dependent upon the presence of maternal GC (Langley-Evans 1997a) since metyrapone injections to MLP rat dams for the first two weeks of gestation abolishes the elevated SBP of MLP relative to controls. Carbenoxolone injections to rat dams receiving a protein replete diet reduces the birthweight and elevates SBP of the resultant offspring (Langley-Evans 1997b). Thus, carbenoxolone largely reproduces the effects of the MLP diet in terms of birthweight and SBP. Indeed carbenoxolone and dexamethasone treatment may influence nutrient transfer across the placenta. Furthermore similarities exist between the nutritional model of Langley & Jackson (1994) and the hormonal model of Edwards *et al* (1993) in terms of a reduction in birthweight (in the order of 20% in both models when a large sample size is included in the analysis) and the degree of insulin resistance (Pickard *et al* 1996). The degree of hypertension in MLP is greater than that exhibited by rats exposed to prenatal dexamethasone (Benediktsson *et al* 1993) indicating that other factors may contribute to the generation of hypertension in MLP, such as an effect on the kidney and RAS of the MLP diet (Welham *et al* 1996, Sherman *et al* 1996). The influence of the maternal adrenal upon MLP-induced hypertension was therefore assessed in the studies described in this chapter.

Maternal adrenalectomy could only be confirmed as being successful retrospectively, either through analysis of maternal plasma corticosterone concentrations (MADX without replacement study[4]), which were non-detectable in all MADX animals following weaning of the rat dams under the conditions of the assay, or visual examination for adrenal regeneration (MADX with replacement study). Corticosterone was not measured in MADX-with-replacement study and thus it is not known whether the regenerated adrenal tissue noted was actually functional.

The weight gain of MADX rats that did not receive hormone replacement throughout pregnancy was reduced compared to intact controls. This may reflect the influence of

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[4] Interpretation of this study is made difficult and the strength of it weakened by the lack of sham-operated animals and intact male animals with which to compare. This provides a basis for further study in this area to explore the interactions of MADX and hormone replacement upon the SBP of Control and MLP animals

glucocorticoids on food intake. ADX of rats decreases food intake, which low-dose corticosterone replacement is able to restore (Dallman *et al* 1993). The onset of feeding behavior, elicited by sympathetic (noradrenaline) activity in the brain, is dependent upon the presence of circulating corticosterone (Liebowitz *et al* 1984) reflecting the permissive role of GC upon homeostatic functions. MADX, eliminating all circulating corticosterone in early gestation promotes reduction in food intake and hence weight gain.

The perinatal mortality of MADX offspring was higher than in adrenal-intact controls. The MLP diet further compounded this effect as neonatal mortalities were greater in MADX-MLP than MADX-controls, thus demonstrating the nutritional compromise of MLP during gestation. Most of the mortalitys in litters that received only vehicle replacement can be attributed to the MADX dams eating the pups. This may be a consequence of the MLP diet. When laboratory chow containing 18.9% protein was substituted for MLP diet at day 21 gestation and litters were not handled or normalised to 8 pups at delivery in the present study birthweights were reduced yet neonatal mortality was totally absent. Experiments by Langley & Jackson (1994) in which litters were neither handled nor normalised at birth suggest this procedure has no effect upon the SBP of the offspring. Thus limited availability of protein immediately prior to parturition may influence the dams behaviour towards the newborn pups. In dams receiving hormone replacement the mortalities can be entirely attributed to fetal resorptions. The potential bias that the variation in litter size may introduce to the study is acknowledged but ultimately could not be controlled for.

Pharmacological ADX (PHADX) using metyrapone, which inhibits 11-hydroxylase on both sides of the placenta (Baram & Schultz 1990), prevents MLP-induced hypertension in male and female offspring. Metyrapone, however, may increase maternal SBP and therefore potentially confound the results. SBP was reduced in MLP male offspring by MADX and re-established following corticosterone replacement. Replacement with aldosterone did not increase SBP in male and female offspring from MADX-MLP dams. However, given that aldosterone replacement to MADX-control male rats resulted in a reduction in SBP, a possible masking of the hypertensive effect of MADX-MLP in males may have been produced with aldosterone replacement and cannot be ruled out.

Surprisingly and unexpectedly both male and female offspring of MADX-control dams had elevated SBP from 5 weeks of age which rather confuses interpretation of the data. It would be expected that should MADX be inherently hypertensive as it is in controls then the SBP of MADX-MLP would be higher. In similarity to the effect of MADX in control animals PHADX (during the first two weeks of pregnancy only) lowers SBP of MLP, but also raises SBP of male and female offspring from control dams (Langley-Evans 1997a; Langley-Evans *et al* 1996e). It is therefore indicated that a lack of maternal GC during early to mid-pregnancy may elevate the SBP of control rats suggesting that some tonic exposure to maternal glucocorticoid in early gestation may be necessary to regulate processes governing the cardiovascular 'setting' of blood pressure.

Alternatively given that maternal corticosterone concentrations following MADX appear normal in late gestation and correlate with the number of fetuses carried *in utero* (Chatelain *et al* 1980; Dupouy *et al* 1975), then following maturation of fetal adrenal function, over-exposure to corticosterone of fetal origin in the third week of gestation may also programme later hypertension in MADX and PHADX-control rats. If rats are maintained on an adequate (18%) protein diet during early gestation followed by a relative *overexposure* to maternal glucocorticoid in late gestation, either through maternal protein restriction (Langley-Evans 1997a), which reduces placental 11-HSD2 (*section 3.3.1.5.*) or exogenous administration (Levitt *et al* 1996) then the resultant adult offspring develop hypertension. Similarly following a period of suppression by PHADX the fetal adrenal may oversecrete corticosterone which may programme elevated SBP during late gestaton in control rats. It has been demonstrated in sheep that glucocorticoids may influence fetal cardiovascular control through a permissive effect on the action of angiotensin II (Tangalakis *et al* 1992). The attenuated effect of MADX/PHADX on SBP in MLP may be explained by an effect of MLP upon fetal adrenal maturation and the ability of the fetal adrenal to compensate for maternal lack.

The SBP of MADX-MLP male rats was significantly lower than the SBP of MADX-control males. Thus, in the face of an absence of glucocorticoid in early gestation the 'normal setting' of cardiovascular control has been maintained in males. The mechanism by which MLP diet may 'protect' against the hypertensive effect of low corticosterone is not known,

but an effect of MLP upon fetal corticosteroid production may be inferred. Exposure to both excess and too little maternal glucocorticoid therefore appears able to programme hypertension. It was unfortunate that all of group 18AC dams had regenerated adrenals and therefore had to be excluded from the study, since a comparison between 18AC and 18SV would have allowed investigation of this possibility.

The hypertensive state of female offspring of MLP dams is abolished by PHADX (Langley-Evans 1997a). Corticosterone replacement to MLP dams following either MADX or PHADX (Langley-Evans 1997a) results in a significant elevation of SBP. However, hypertension in the female offspring of MADX-MLP was not prevented by MADX. The data therefore suggest that development of fetal adrenal function may differ between MLP males (whose SBP was lowered by MADX) and females (whose SBP was not convincingly lowered by MADX). The mechanism for this effect is unclear. Sex-specific differences have been noted in the placental transfer and metabolism of corticosterone (Montano *et al* 1993). Perhaps it is an effect of MLP to unmask the sex-specific response to MADX.

Alterations in the ontogeny of GC-receptors in response to MADX may differ in males and females. Certainly, the activity of central GPDH correlates with SBP in females but not males, suggesting differences in the central regulation of GC-dependent functions (Langley-Evans *et al* 1996f). Central differences in GC-activity have been proposed to explain the sexually dimorphic response of male or female rat fetuses to fetal alcohol exposure (FAE) following MADX (Redei *et al* 1993). As a tentative hypothesis, female adrenal glands may be prematurely activated relative to male adrenal glands following MADX. Early activation of adrenocortical function may influence hypothalamic cardiovascular control areas towards elevated SBP in adult life. Since PHADX prevents hypertension in female rats (Langley-Evans 1997a) and PHADX abolishes all corticosterone synthesis in maternal and fetal adrenal glands suggests that such a phenomenon may occur. Alternatively the differences in the SBP response of rats to PHADX and MADX may relate to the fact that PHADX may increase the SBP of rat dams and thus influence placental blood flow and maternal metabolism.

CHAPTER 5

MAINTENANCE OF AN ELEVATED SYSTOLIC BLOOD PRESSURE IN LOW PROTEIN EXPOSED ANIMALS DURING ADULT LIFE

5.1. INTRODUCTION

Hypertension of fetal origin is the adult manifestation of a dynamic interaction between nutritional factors (Campbell *et al* 1996; Godfrey *et al* 1996) and hormones (Benediktsson *et al* 1993; Lindsay *et al* 1996) acting during pregnancy. The rat model used in the present studies illustrates this, in that a maternal low protein diet induces disproportionate fetal growth patterns leading to lower birthweight and programmes functions of the offspring glucocorticoid axis *(Chapter 3)*. Furthermore, the nutritionally-induced hypertensive state *(section 3.3.3.1.)* may be dependent upon the presence of maternal glucocorticoids *(Chapter 4)* and Langley-Evans (1997a, 1997b) However, the nature of the association between fetal glucocorticoid exposure and the programming of adult blood pressure remains uncertain.

Glucocorticoid excess, whether experienced in adult life as a consequence of Cushings syndrome (Ross & Linch 1982) or infused *in utero* (Hill *et al* 1988) raises blood pressure. Glucocorticoids have many 'hypertensinogenic' actions within the vasculature (Whitworth *et al* 1995) mediated either directly through GR (Komel 1993; Pirpiris *et al* 1992) or indirectly through permissive effects on pressor agents (Grünfeld & Eloy 1987). Maternal glucocorticoids influence fetal cardiovascular control by raising tissue sensitivity to endogenous angiotensin II and thus increasing peripheral resistance (Tangalakis *et al* 1992). Increased intrauterine glucocorticoid exposure, as a consequence of maternal protein restriction, may therefore subtly alter the development of the fetal cardiovascular system, rendering the individual more responsive to pressor actions in adult life, and chronically raising systolic blood pressure. The mechanism for this steroid-dependant effect may be receptor based (Bian *et al* 1992; Langley-Evans *et al* 1996c; Meaney *et al* 1993).

The following chapter determines whether nutritionally-induced hypertension is dependent upon glucocorticoid action in adult life, as in the spontaneously hypertensive rat (Ruch *et al* 1984; Suzuki *et al* 1996). Furthermore, the peripheral action of angiotensin II is explored to determine whether glucocorticoid exposure *in utero* may increase peripheral vascular reactivity to a given pressor agent. A number of other factors including nor-adrenaline, arginine vasopressin, NO may mediate blood pressure increases in MLP rats. The renin-angiotensin-system (RAS) was selected for study on the basis of an observed role for angiotensin-converting enzyme (ACE) in the maintenance of raised SBP (Langley-Evans & Jackson 1995, Sherman & Langley-Evans 1998). Moreover elements of the RAS (angiotensinogen and ACE) are subject to regulation by glucocorticoids (Mendelsohn *et al* 1982, Campbell & Habener 1986).

5.2. PROTOCOL

Animals were generated as described in section 2.2.1 from a breeding population of n=6 dams/dietary group. At approximately 47 ±1 days of age, the male offspring from each dietary group (18% casein, control n=24; 9% casein, MLP n=20) were randomly chosen for either bilaterally adrenalectomy (A) or sham operation (S) under sodium pentobarbitone anaesthesia as described in section 2.4.5. Post-operatively adrenalectomized animals had free access to 0.9% NaCl solution to maintain physiological electrolyte concentrations. The four groups generated (18% casein-A, 18% casein-S, 9% casein-A and 9% casein-S) were then subdivided into two further groups that received either corticosterone (C) replacement (20mg/kg/d in 0.1ml arachis oil) or vehicle (V) (0.1ml arachis oil) s.c.twice daily (9.00am and 4.00pm) for 14 days post surgery. A twice daily replacement protocol was adopted to mimic the daily rhythm in corticosterone secretion that follows the light/dark schedule. A total of 8 groups were thus studied 18AC, 18AV, 18SC, 18SV, 9AC, 9AV, 9SC and 9SV (controls n=6/group, MLP n=5/group). The body weights of all animals were determined daily throughout the treatment period. The systolic blood pressures of all animals were determined prior to surgery (d 0), and then 7 and 14 days after replacement began. SBP was measured following replacement injections in the morning and prior to the afternoon injections. On Day

15 animals were injected in the morning and 1hr later were placed under non-recoverable anaesthesia and blood and organs collected.

SECTION 1

THE EFFECT OF POSTNATAL ADRENALECTOMY ON OFFSPRING EXPOSED TO EITHER A MATERNAL CONTROL OR MLP DIET

5.3. RESULTS

5.3.1. Effect on body weight.

Prior to surgery there was no difference in body weight between the two maternal dietary groups (control; 190 ±7.8g n=24 *vs* MLP; 180 ±2.8g n=20) (Table 5.1). Throughout the experimental period vehicle injected ADX animals maintained a steady weight gain similar to the weight gain in vehicle injected sham-operated controls. Corticosterone replacement significantly reduced the weight gain of all corticosterone treated animals and by day 14, treated animals were lighter (75 ±7.5g (control), 65 ±4.8g (MLP)) than vehicle treated control animals. Body weight gain was not influenced by maternal diet, except for 9AC, which gained significantly more weight than 18AC (Table 5.1).

5.3.2. Corticosterone measurements.

Corticosterone concentrations in sham-operated rats were similar to concentrations in non-operated intact rats (refer to Figure 3.7). Twice daily corticosterone injections elevated plasma corticosterone concentrations 2-3-fold above concentrations in vehicle treated controls. Adrenalectomy reduced the corticosterone concentration to non-detectable levels when measured at day 14. Maternal diet had no effect on the plasma corticosterone concentration (Table 5.2).

Table 5.1. **The effect of postnatal adrenalectomy and corticosterone replacement on body weight gain of 6 week old rats exposed to either a maternal control or low protein diet**

	Body Weight (g)				
	Initial	**7 days**	**14 days**	**Gain (g)**	**n**
Control					
18SV	210±18	255±17	302±14	91.8±5.7 *	6
18AV	197±26	232±26	279±28	82.2±8.3 *	6
18SC	177±5	191±5	195±5	17.3±5.2	6
18AC	180±8	184±8	192±6	12.0 ±3.4	6
MLP					
9SV	181±7	223±7	272±7	91.0±2.2 *	5
9AV	186±4	217±6	268±9	82.0±5.4 *	5
9SC	173±4		190±6	17.4±3.2	5
9AC	183±6	191±6	207±9	24.8 ±9.0	5

Values are means ±SEM for offspring derived from a total of 6 litters/dietary group. Analysis of variance (ANOVA) showed a significant effect of corticosterone replacement (F= 74.2, P= 0.0001) relative to vehicle injected controls. Groups; 18- exposed to 18% casein control maternal diet, 9- 9% casein MLP diet, A- bilaterally adrenalectomized, S- sham operated, C- received corticosterone replacement (10mg/kg in 0.1ml arachis oil) twice daily for 14 d, V- vehicle injected (0.1ml arachis oil) twice daily for 14 d. * indicates a significant difference to vehicle-injected rats (P <0.05). n, animals in each group.

Table 5.2. **The effect of postnatal adrenalectomy and corticosterone replacement on plasma corticosterone levels of 8 week old rats exposed to either a maternal control or low protein diet**

Group	CORT (ng/ml)	n
18SV	22.0 ±2.8	6
	nd	6
18SC	57.5 ±6.0*	6
18AC	65.3 ±4.5*	6
9SV	22.7 ±3.9	5
9AV	nd	5
9SC	50.3 ±7.7*	5
9AC	59.6 ±2.4*	5

Values are means ±SEM for offspring derived from a total of 6 litters/dietay group. Analysis of variance (ANOVA) showed significant differences for corticosterone replacement ($F=185.5$, $P<0.0001$), and an interaction between ADX and corticosterone replacement ($F=11.6$, $P<0.01$). For details of groups refer to Table 5.1. CORT-corticosterone; nd-not detectable (detection limit = 30pg/100µl plasma). * indicates a significant difference to group SV in each dietary group ($P<0.05$). n, animals in each treatment group.

5.3.3. Blood pressure measurements.

Prior to the experimental procedure, the MLP rats had significantly higher blood pressure than the control animals (MLP;165 ±3.8 mmHg n=20, controls; 142 ±3.3 mmHg n=24 P <0.0001). Table 5.3 shows the blood pressure response of each dietary group to the experimental protocol. The higher blood pressure exhibited by the MLP animals at day 7 was reduced by ADX to yeild pressures similar to those of 18% casein exposed controls (group 9AV). No significant effect of ADX on blood pressure was observed in the parallel 18AV group. Corticosterone replacement to ADX animals increased blood pressure, in both dietary groups (18AC and 9AC) at day 7, although group 9AC exhibited a tendency towards an increased sensitivity (mean blood pressure increase after 7 days corticosterone injections; group 18AC = 31 ±8mmHG, group 9AC = 37 ±6mmHg; P= >0.05). Corticosterone replacement raised blood pressure in group 18SC, but had no effect on the blood pressure of group 9SC. The increase in blood pressure observed at day 7, following corticosterone replacement, was transient in all groups and was absent at day 14 in all but group 18AC (Table 5.3).

5.3.4. Glycerol-3 phosphate dehydrogenase (GPDH)

GPDH activity in the rat brain has been shown to be a reliable index of GC stimulation at the cellular level (Kitraki *et al* 1995), and consequently was assayed as a marker of GC status in the animals. In the hippocampus of MLP exposed rats the activity of GPDH was significantly reduced (37%) by adrenalectomy. Hippocampal GPDH activity decreased by 24% in group 18AV relative to the sham controls (18SV) (Figure 5.1).

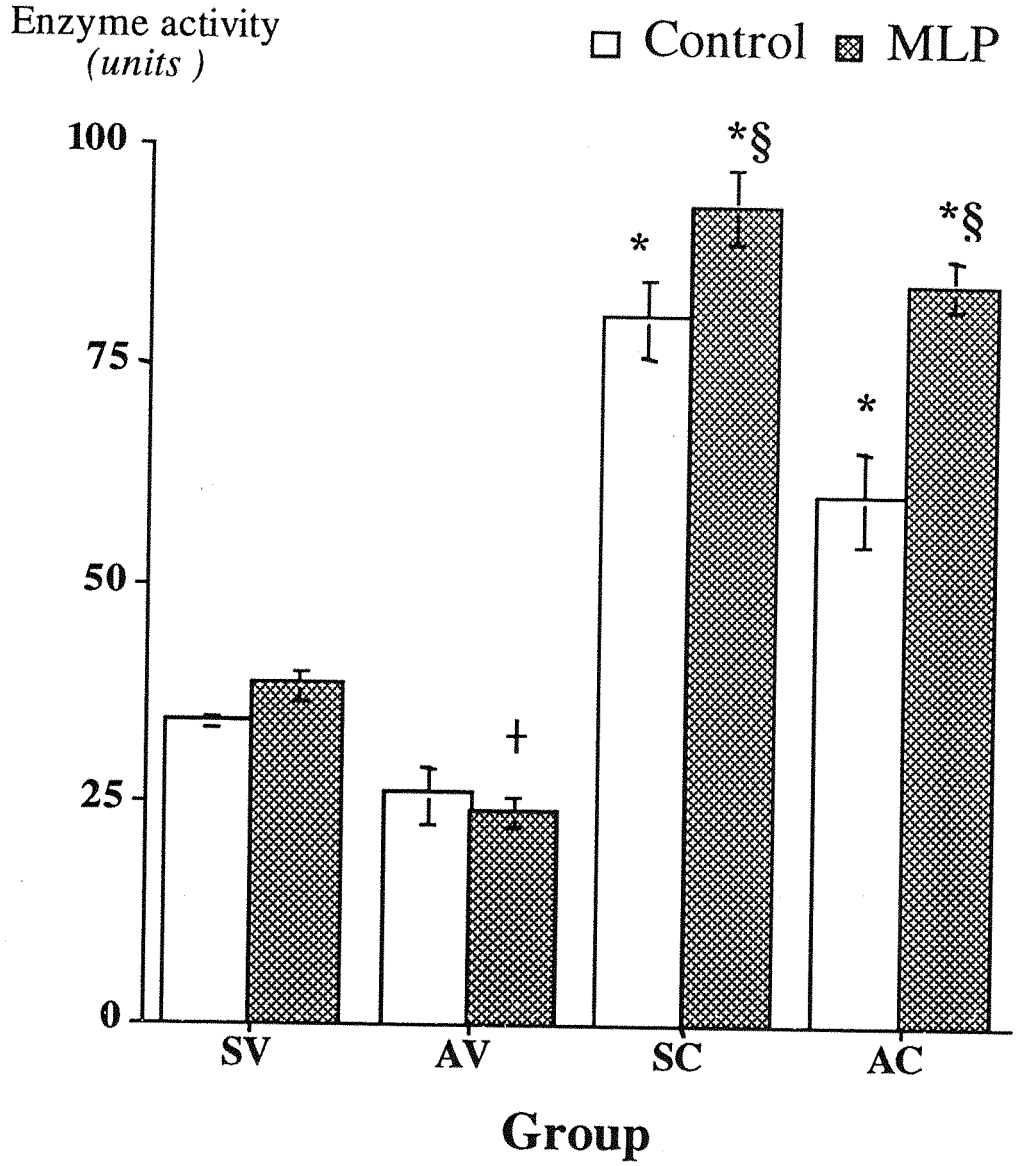
Corticosterone replacement to both dietary groups and in both SHAM/ADX groups significantly elevated hippocampal GPDH activity (132% and 135% for 18AC & 9AC respectively, P< 0.0001; and 251% and 143% for 18SC & 9SC respectively, P< 0.0001), illustrating the sensitivity of this enzyme to corticosterone. However, the low protein exposed groups appeared more sensitive to the corticosterone replacement protocol, in terms of hippocampal GPDH activity, since the activities measured following corticosterone injections were significantly higher in groups 9SC and 9AC than in group 18SC and 18AC (P< 0.001).

Table 5.3. **The effect of postnatal adrenalectomy on the blood pressure of animals exposed to either a maternal control or low protein diet**

	Systolic blood pressure (mmHg)					
	Control			MLP		
Group	Initial	7d	14d	Initial	7d	14d
SV	147 ±4	149 ±5	137 ±7	161 ±7*	168 ±10*	153 ±11
AV	142 ±5	149 ±2	137 ±10	162 ±12*	143 ±9	131 ±6
SC	146 ±8	166 ±6¶	146 ±7	175 ±9*	165 ±4§	171 ±5*
AC	143 ±7	174 ±3¶	168 ±4¶	165 ±6*	202 ±8*¶	185 ±9*¶

Refer to Table 5.1 for details of groups. The SBP of rats was determined by the indirect tail cuff method prior to surgery (initial) and subsequently at day 7 and 14 following either ADX or SHAM (day 0). Values represent mean ±SEM for n=5-6 observations (control), n=4-5 (MLP) rats in each treatment group. The results were analysed by four way analysis of variance (ANOVA) followed by a suitable post hoc test (Bonferroni test) where differences were indicated. Main effects were: diet $F=29.85$, $P<0.0001$; corticosterone replacement $F=50.67$, $P<0.0001$; an interaction between diet & corticosterone replacement $F= 3.47$, $P= 0.06$; and an interaction between diet & ADX & corticosterone replacement $F= 2.89$, $P= 0.09$. *indicates a significant difference to respective values in control rats ($P<0.05$), ¶ indicates a significant effect of corticosterone replacement ($P<0.05$), § indicates a significant difference to initial value ($P<0.05$).

Figure 5.1. **The effect of adrenalectomy and corticosterone replacement in animals exposed to either a maternal control or low protein diet on hippocampal glycerol-3 phosphate dehydrogenase (GPDH) activity**



For details refer to Table 5.1. GPDH activity was determined on brain homogenates as described in section 2.6.2. Values represent mean ±SEM for n=5-6 offspring (Control), n=4-5 offspring (MLP) rats in each treatment group. Analysis of variance (ANOVA) indicated a significant effect of adrenalectomy (F= 27.67, P= 0.0001), corticosterone replacement (F= 406.2, P= 0.0001) and an interaction between diet and corticosterone replacement (F=12.78, P= 0.001). Where significant differences were indicated a suitable post hoc test (Bonferroni test) was conducted. * indicates a significant difference to groups SV and AV ($P < 0.001$), § indicates a significant difference to control rats ($P<0.001$). + indicates a significant difference to 9SV ($P<0.05$).*units*, of enzyme activity are nmoles/min/mg protein.

In both dietary groups ADX significantly reduced GPDH activity in the cerebellum ($P<0.0001$) whilst corticosterone replacement to both SHAM and ADX animals increased activity ($P< 0.0001$). No effect of maternal diet was noted following the replacement protocol (Figure 5.2). Significant correlations between GPDH activity in both the cerebellum ($r = 0.56$, $P= 0.0002$) and hippocampus ($r = 0.57$, $P= 0.0003$) with blood pressure were noted.

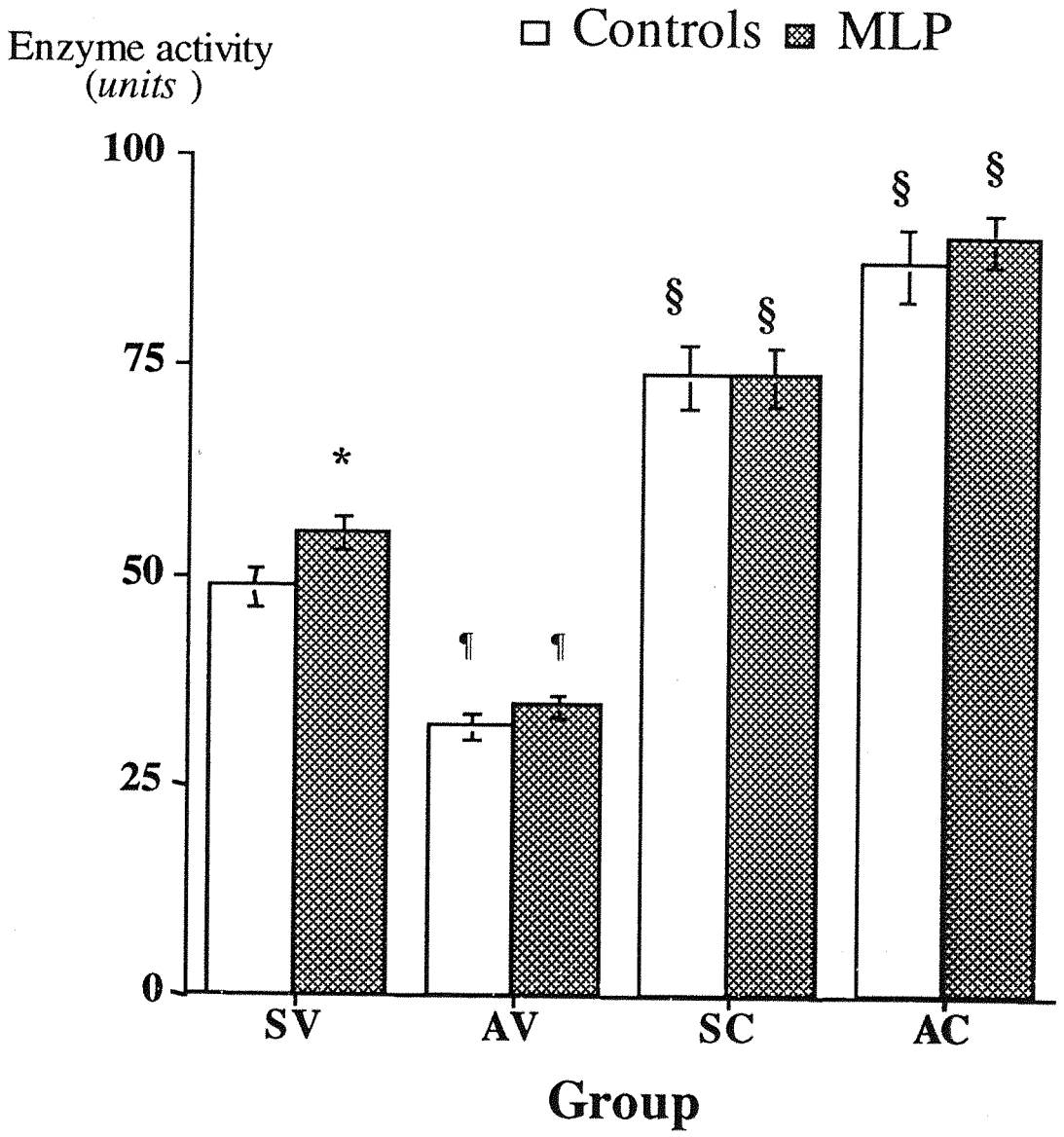
5.3.5. Tyrosine aminotransferase (TAT)

Hepatic TAT represents a peripheral GC-inducible enzyme (Shargill *et al* 1983). In MLP, sham operated controls (group 9SV), the activity of hepatic TAT (Figure 5.3) was significantly higher (a 55% increase) than in the sham operated dietary controls (group 18SV) ($P= 0.01$). The activity of TAT increased ($P< 0.0001$) in response to corticosterone replacement in all groups measured (18AC, 18SC, 9AC and 9SC). Adrenalectomy did not exert any significant influence upon the activity of hepatic TAT in either dietary group (Figure 5.3).

5.3.6. Glutamine synthetase (GS).

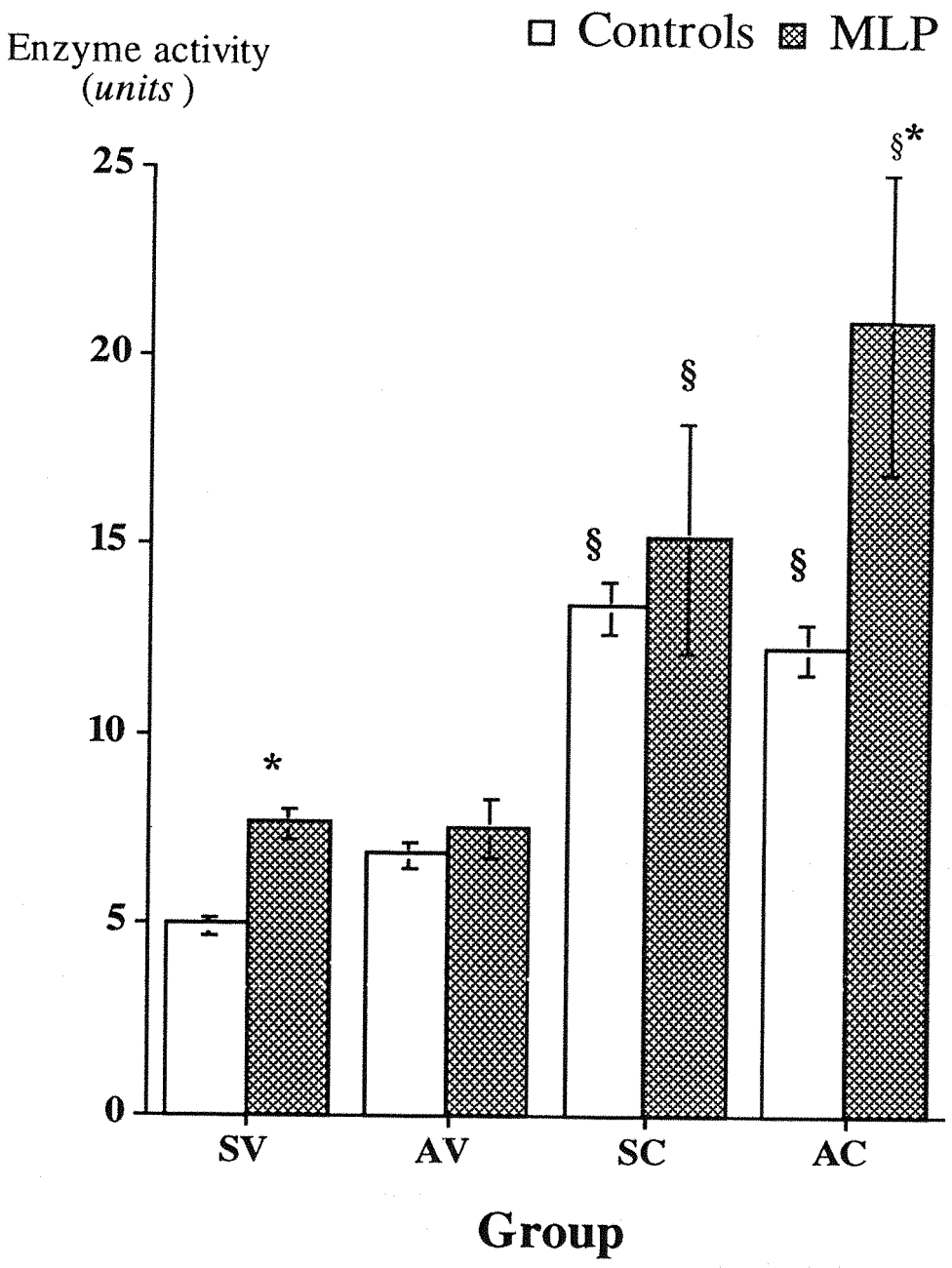
GS is a known GC target gene (Kumar *et al* 1986; Patel *et al* 1983) and in the liver, is believed to be specifically localised in perivenous cells (Desai & Hales 1997). In agreement with the GC-sensitivity of hepatic GS, activity increased (23%) after corticosterone replacement ($P= 0.01$), but only in group 18SC (Figure 5.4). No effect of diet on GS activity was observed in any other group.

Figure 5.2. **The effect of adrenalectomy and corticosterone replacement in animals exposed to either a maternal control or low protein diet on glycerol-3 phosphate dehydrogenase (GPDH) activity in the cerebellum**



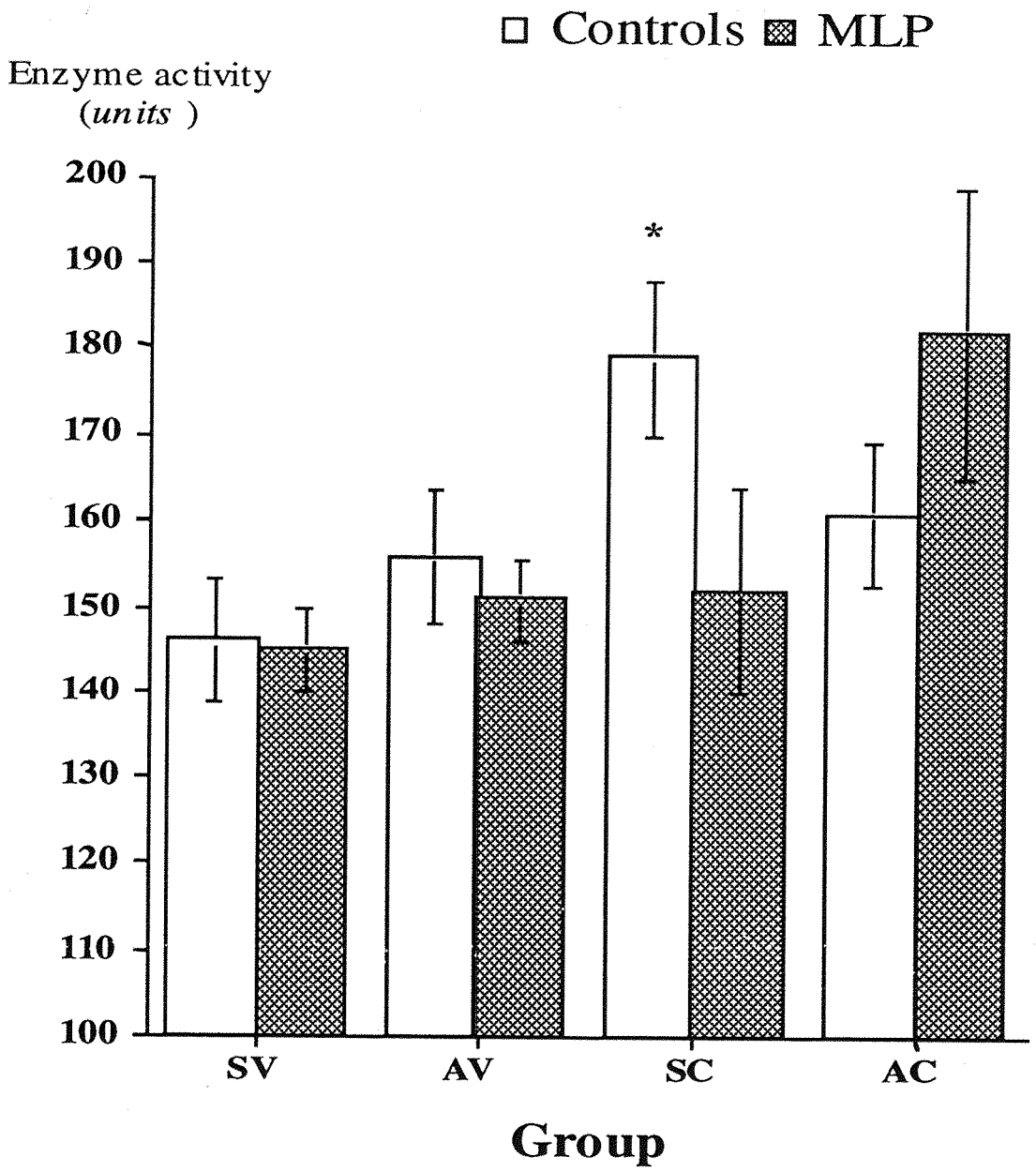
For details refer to Table 5.1. GPDH activity was determined on brain homogenates as described in section 2.6.2. Values represent mean ±SEM for n=5-6 offspring (control), n=4-5 offspring (MLP) rats in each treatment group. Analysis of variance (ANOVA) indicated a significant effect of corticosterone replacement (F= 340.2, P= 0.0001) and an interaction between ADX and corticosterone replacement (F=62.0, P= 0.0001). Where significant differences were indicated a suitable post hoc test (Bonferroni test) was conducted. * indicates a significant effect of diet (P<0.05), § indicates a significant effect of corticosterone replacement (P<0.05), ¶ indicates a significant effect of ADX (P<0.05). *units*, of enzyme activity are nmoles/min/mg protein.

Figure 5.3. **The effect of adrenalectomy and corticosterone replacement in animals exposed to either a maternal control or low protein diet on hepatic tyrosine aminotransferase (TAT) activity**



For details refer to Table 5.1. TAT activity was determined on liver homogenates as described in section 2.6.4. Values represent mean ±SEM for n=5-6 offspring (control), n=4-5 offspring (MLP) rats in each treatment group. Analysis of variance (ANOVA) indicated a significant effect of corticosterone replacement (F= 48.65, P= 0.0001) and diet (F=7.57, P= 0.01). Where significant differences were indicated a suitable post hoc test (Bonferroni test) was conducted. * indicates a significant effect of diet ($P<0.05$), § indicates a significant effect of corticosterone replacement (P<0.001). *units*, of enzyme activity are nmoles/min/mg protein.

Figure 5.4. **The effect of adrenalectomy and corticosterone replacement in animals exposed to either a maternal control or low protein diet on hepatic glutamine synthetase (GS) activity**



For details refer to Table 5.1. GS activity was determined on liver homogenates as described in section 2.6.3. Values represent mean ±SEM for n=5-6 offspring (control), n=4-5 offspring (MLP) rats in each treatment group. Analysis of variance (ANOVA) indicated a significant effect of corticosterone replacement (F= 7.91, P= 0.01). Where significant differences were indicated a suitable post hoc test (Bonferroni test) was conducted. * indicates a significant effect of corticosterone replacement ($P < 0.05$). *units*, of enzyme activity are nmoles/min/mg protein.

5.3.7. Malate dehydrogenase (MD)

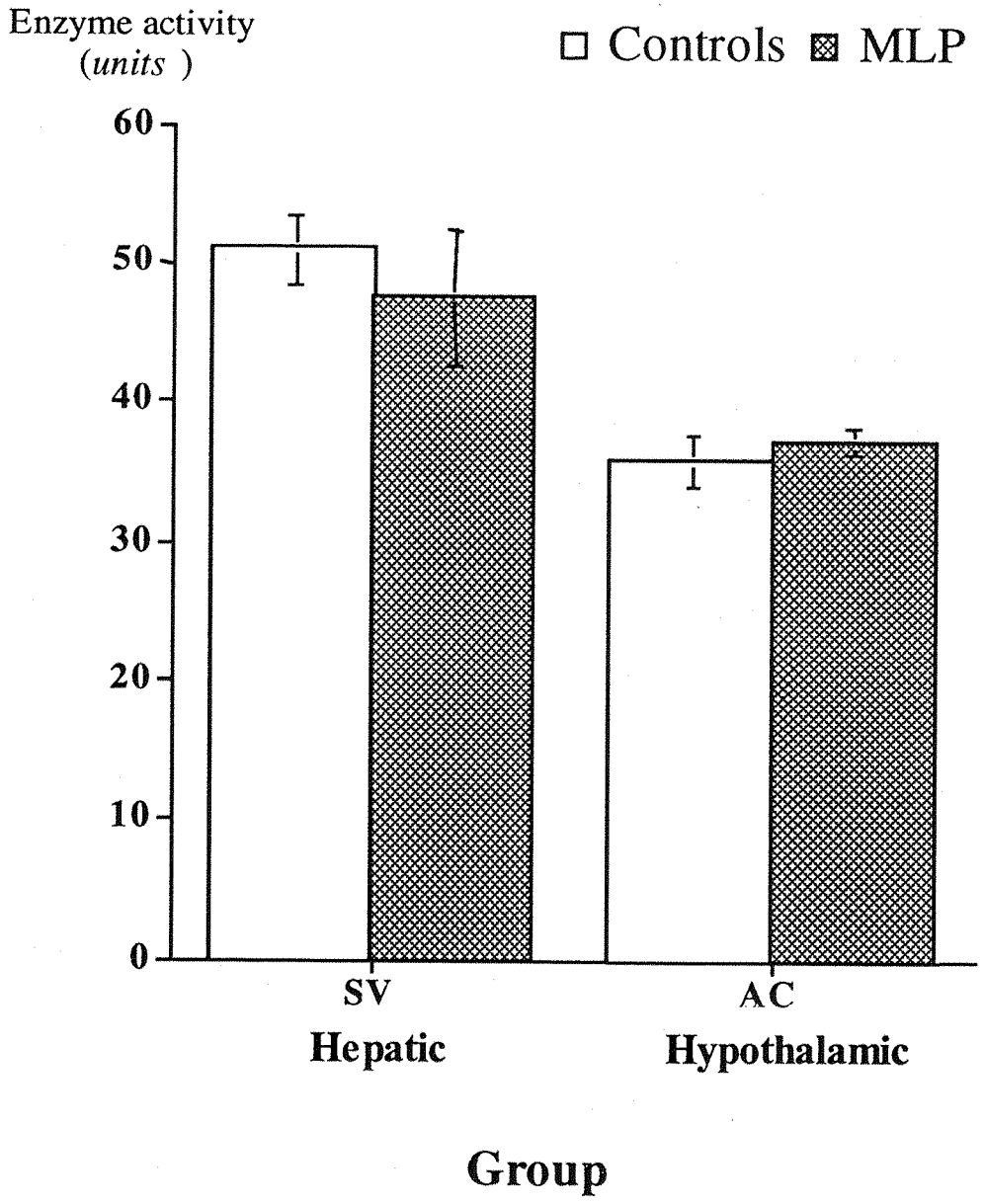
MD is a steroid-insensitive enzyme (Langley & York 1990) and consequently was assayed to determine whether the effects on GC-inducible enzyme activities represented a steroid-specific effect or alternatively a general up-regulation of all enzyme systems in the brain or liver. Previous data has demonstrated no effect of prenatal diet upon the activity of MD in hypothalamic tissue from adrenal-intact animals (*Table 3.*14). In both central (hypothalamic) and peripheral (hepatic) tissue the activity of MD was similar between dietary groups following corticosterone replacement (Figure 5.5).

5.4. DISCUSSION

Whilst adrenalectomy (ADX) itself had little effect on the weight gain of rats following surgery, injections of corticosterone to either ADX or sham-operated rats significantly reduced weight gain. The activity of central glycerol-3-phosphate dehydrogenase (GPDH) mirrored glucocorticoid (GC) status, whilst the activities of hepatic tyrosine aminotransferase (TAT) and glutamine synthetase (GS) were sensitive to increased corticosterone action but not ADX. The SBP of MLP rats was lowered by ADX whereas the lower SBP of control rats was unaltered. Corticosterone replacement to ADX-MLP rats negated the SBP lowering effect of ADX and significantly increased the SBP of control rats. The results suggest that maintenance of hypertension in MLP is dependent upon an intact adrenal gland and, in particular, corticosterone activity.

Successful ADX was confirmed by analysis of plasma corticosterone concentrations, which were non-detectable in all ADX rats at 14 d post surgery. In sham-vehicle injected rats corticosterone concentrations were within the normal range for the rat in our laboratory (Langley 1990) and others (Martin *et al* 1978). The dose of corticosterone replacement chosen was based on previous experience where 10mg/kg/day was ineffective in inducing increased activity of glucocorticoid-inducible enzymes (Langley 1990). However, it is apparent from the observed reduction in body weight, following corticosterone treatment, that the replacement dose used in the present study can be considered pharmacological rather than physiological and thus may obscure a

Figure 5.5. **The effect of adrenalectomy and corticosterone replacement in animals exposed to either a maternal control or low protein diet on hepatic and hypothalamic malate dehydrogenase (MD) activity**



For details refer to Table 5.1. MD activity was determined on liver and brain homogenates as described in section 2.6.1. Values represent mean ±SEM for n=5-6 offspring (control), n=4-5 offspring (MLP) rats in each treatment group. No differences in either hepatic or hypothalamic MD activity were observed between the dietary groups. *units*, of enzyme activity are nmoles/min/mg protein.

specific GR (glucocorticoid receptor) mediated effect. The response seen is consistent with the catabolic nature of high dose glucocorticoids (Devenport *et al* 1989) and some caution should be used in attributing responses to classical glucocorticoid action. For example, the corticosterone replaced animals may have been rendered insulin resistant which could have influenced their blood pressure. Given that the plasma corticosterone concentrations were similar between groups 18AC and 18SC suggests that the HPA axis was effectively 'switched off' by the corticosterone replacement protocol.

Nevertheless, maintenance of ADX animals on 0.9% saline had no significant effect on blood pressure, group 18AV (Imms & Neame 1974). Therefore the response in group 9AV (significantly decreased SBP over 14 days) can be attributed to the absence of GC stimulation. Any possible effect ascribed to a lack of circulating adrenaline in group 9AV cannot be evaluated but did not appear to influence SBP in group 18AV. In the absence of a feedback mechanism in the hypothalamus the concentrations of adrenocorticotrophic hormone (ACTH) and corticotrophin releasing hormone (CRH) will dramatically rise following ADX (Widmaier 1992). ACTH and CRH have cardiovascular actions but these are subservient to the vasoconstrictor function of corticosterone (Whitworth *et al* 1995), emphasised by the observation that corticosterone replacement restores the SBP of MLP rats to the level observed prior to surgery. This clearly implicates a functional role for the adrenal gland in maintaining the hypertensive state induced by the MLP diet.

Corticosterone replacement elevated SBP in all rats except group 9SC. The increase in SBP was most pronounced in group 9AC perhaps reflecting altered regulation of GR in these animals (Langley-Evans *et al* 1996c). A slight decrease in SBP, observed at post-operative day 14 relative to day 7, occurred in all corticosterone treated animals (except group 9SC) indicating an apparent vascular accommodation to the high GC replacement dose. GC regulate their own receptor population (Svec 1985) and the observed lowering of SBP following 7 days corticosterone injections may indicate a down-regulation of vascular GR. Corticosterone replacement to adrenal-intact MLP (group 9SC) failed to elevate SBP of this group in the first 7 days. Similarly, the feeding of a high salt diet to the offspring of protein replete controls significantly elevates SBP whilst having no effect on the SBP of MLP (Langley-Evans & Jackson 1996). Whether the apparent lack of effect of known hypertensive agents upon the

SBP of animals with established high blood pressure reflects maintainence of SBP at an upper level whereby the MLP rats are insensitive to further hypertensive agents is not known.

As with the present model, the integrity of the adrenal gland is essential for the full expression of the hypertensive state in spontaneously hypertensive rats (SHR). ADX and subsequent maintenance on 0.9% saline, reduces the SBP of SHR to pressures observed in Wistar Kyoto (WKY) controls, (Suzuki *et al* 1996). Replacement of GC (dexamethasone) to these animals restores pressures back to previously observed values and the SHR exhibit a degree of hypersensitivity to GC replacement (Suzuki *et al* 1996). Moreover, early ADX of SHR prevents the development of hypertension (Ruch *et al* 1984). The full expression of the hypertensive phenotype of both SHR and MLP is therefore dependent upon the continued presence of glucocorticoid hormone.

GPDH activity in the brain provides a marker of GC action (Kitraki *et al* 1995). The cerebellum, hippocampus and hypothalamus are GC target regions as reflected by their high densities of Type II (GR) receptors (McEwen *et al* 1986). The higher activity of GPDH found in group 9SV relative to 18SV is consistent with our previous reports (Langley-Evans *et al* 1996c). Increased basal activity of central GPDH in the absence of raised circulating corticosterone may reflect a tonic over-stimulation of GR mediated through increased GR densities (Langley-Evans *et al* 1996c). Alternatively pre-receptor mechanisms (11-HSD) may have a role in mediating the increased GPDH activity. The limited evidence to date, however, suggests that central 11-HSD1 and 11-HSD2 activities are unaffected by prenatal dietary experience (Langley-Evans *et al* 1996c). However given that the 11-HSD assay conducted within this lab is fundamentally flawed then the similarity of central 11-HSD1 and 11-HSD2 activities between dietary groups should be treated with caution.

The activity of hepatic tyrosine aminotransferase (TAT) was higher in group 9SV than in 18SV, suggesting increased hepatic GC action in MLP. Increased TAT activity combined with reduced hepatic GR numbers (Langley-Evans *et al* 1996c) may suggest an increased affinity and sensitivity of hepatic GR for GC. However, receptor affinities are similar in both dietary groups (Langley-Evans *et al* 1996c) suggesting increased post GC-receptor sensitivity i.e. increased sensitivity of GC-induced gene expression. The hepatic expression of phosphoenolpyruvate carboxykinase (PEPCK) is indeed permanently increased

by a maternal low protein diet (Desai *et al* 1997). Given that the activity of TAT was not decreased by ADX in both dietary groups suggests that hepatic TAT may be constitutively regulated by factors other than GC. Shargill *et al* demonstrated the sensitivity of TAT to changes in food intake and tryptophan (Shargill *et al* 1983).

The activity of hepatic glutamine synthetase (GS), whilst being responsive to high dose corticosterone replacement in groups 9AC and 18SC was not sensitive to variations in GC status in any other group. The apparent dichotomy between central (*sections 3.3.3.5. & 3.3.3.6.*) and peripheral GS activity is readily explained by functional differences in GS within different tissues. In hepatic tissue GS is more sensitive to growth hormone than GC reflecting its function in providing amide nitrogen for anabolic processes (Wong *et al* 1980). In the brain GS is predominately involved in amino acid neurotransmitter metabolism and detoxification of ammonia (Patel *et al* 1983).

Clearly the activity of GPDH in particular, and to a limited extent, the activity of hepatic TAT parallel the changes in GC status in the present study, emphasising the utility of these enzymes as markers of GC action. However, the changes in GC status were extreme and thus a further analysis of the degree of suppression of ACTH would have provided an additional index of GC action. As the activity of central MD is not altered by the manipulations in GC status suggests that the changes in GPDH are specifically GC-directed and not the result of a general up-regulation of all enzyme systems within the brain. Specific increases in the basal activities of central and peripheral GC-inducible enzymes in the face of low-normal circulating glucocorticoid concentrations, concomitant with increased central and peripheral GR (Langley-Evans *et al* 1996c), suggests nutritionally-induced programming of GR populations.

Permanent alterations to central and peripheral GR densities (Langley-Evans *et al* 1996c) and expression of GC-regulated gene products (Desai *et al* 1997) are manifestations of a poor nutritional environment encountered *in utero*. Relatively mild neonatal stress elicited by daily handling permanently alters the subsequent expression and responsiveness of central GR populations (Meaney *et al* 1992). The nutritional regimen used in this study indicates a comparable physiological stress given that the normal doubling of body weight observed in control animals over the last 2 days gestation is severely curtailed in the MLP group (*Figure*

3.3). Alterations to GR populations programmed by the prenatal diet may influence the adaptive range of GR in the immediate postnatal environment. This may affect both the long term regulation of the HPA axis in MLP, as has been demonstrated (Chapter 3), and, perhaps, influence the generation of hypertension in MLP, since MLP rats demonstrate greater vascular GR binding (Langley-Evans *et al* 1996c). Certainly altered GR densities in either central or peripheral regions may predispose the individual to high SBP. At the central level, GC decrease and mineralocorticoids increase blood pressure via their respective receptors (van den Berg *et al* 1990). The changes in GC-inducible enzymes in the brain and secretion of adrenocorticotropic hormone suggest a central programming phenomenon. Increased binding to GR within vascular tissue (Langley-Evans *et al* 1996c) which may influence peripheral vascular sensitivity to GC through a potentiation of other pressor agents such as angiotensin II and noradrenaline (Grünfeld & Eloy 1987; Whitworth *et al* 1995).

In summary, corticosterone replacement was sufficiently high to be considered pharmacological, based on the attenuated weight gain. Following ADX the high SBP exhibited by MLP animals was reduced to levels observed in control rats. The SBP lowering effect of ADX in MLP was reversed by corticosterone replacement. The activity of GPDH mirrored the changes in GC status and correlated with SBP. This indicates the role of GC in blood pressure regulation. An intact adrenal gland is essential for the full expression of the hypertensive state in MLP. An increased peripheral sensitivity to GC action may mediate the observed responses.

SECTION 2

THE ROLE OF VASCULAR SENSITIVITY TO ANGIOTENSIN II IN THE MAINTENANCE OF HYPERTENSION IN RATS EXPOSED TO A MATERNAL LOW PROTEIN DIET

5.5. PROTOCOL

Animals were generated as outlined in *Methods*. All female offspring aged 10 weeks (n=5 offspring from a total of n=5 litters/dietary group) were anaesthetised (72mg/kg bodyweight sodium pentobarbitol) and the right carotid artery and right femoral vein were cannulated (*Methods* 2.5.) for blood pressure recordings and angiotensin II (AII) injections respectively. A period of 30mins following vascular cannulation was allowed before a direct estimate of systolic blood pressure was determined and the AII administration protocol began. The effects of randomised, bolus *i.v.* injections of 1, 5, 10, 20 and 40ng AII, delivered intravenously in 0.1ml 0.9% NaCl maintained at 37°C, on blood pressure were determined in all animals. Injections were carried out in duplicate in random order and for each concentration of AII. A period of at least 5min was allowed between successive AII injections. The dose of AII at which the systolic pressure response was maximal was determined by injections of between 20-60ng AII. The plasma half-life of AII was not established, however preliminary studies indicated (Appendix 4) that, following intravenous AII injection, baseline values of SBP were reestablished within 2-3mins indicating a half-life of 1-2 mins.

5.6. RESULTS

5.6.1. The vascular response of control or MLP exposed female rats to intravenous angiotensin II (AII)

Prior to the first dose of intravenous AII, basal SBP, was significantly (P= 0.04) higher in the low protein exposed group (143 ±4 mmHg, n=3 *vs* 130 ±3 mmHg, n=5). There was no difference in heart rate between the two groups (controls; 374 ±21 b/min, MLP; 390

±14 b/min). As Figure 5.6 illustrates, the dose response curve of pressor responsiveness to AII injection was shifted to the left in the low protein exposed animals. The maximal response to AII was greater in the MLP rats than in controls at all doses of AII. Two way analysis of variance indicated a significant effect of diet (F= 5.54, P= 0.02) and AII dose (F= 195.4, P <0.0001). Statistical significance for the maximal pressor response to AII in low protein exposed rats was achieved at the lower (1 and 5ng) doses of AII. The dose which gave the maximal pressor response to AII was significantly lower in MLP than controls (MLP, 37 ±1ng AII n=5; Controls, 44 ±2ng AII n=5, P= 0.01).

Two way analysis of variance indicated a significant effect of diet on systolic blood pressure at 1ng (F= 4.40, P= 0.04) and 5ng (F= 12.62, P= 0.0008) AII only. Thus it is suggested that doses of AII within the physiological range for rats (Langley-Evans & Sherman - unpublished observations) the response to AII in MLP rats was both initially greater and more prolonged than in control animals. Figures 5.7A & B illustrate the pressor response to AII at 1ng (A) and 5ng (B) respectively.

5.7. DISCUSSION

Systolic blood pressure (SBP) when measured directly was higher in MLP females than controls. The higher SBP exhibited by MLP rats is programmed by prenatal undernutrition and, in adult life, appears to be dependent upon an intact and functional adrenal gland. The mechanism through which corticosterone acts to raise SBP in MLP appears to involve the potentiation of the action of other vasomodulatory compounds, such as AII. Female offspring of MLP dams exhibited a significantly greater pressor response to AII injection at physiological levels compared with control rats. The dose of AII which gave the maximal pressor effect was lower in female MLP rats than controls.

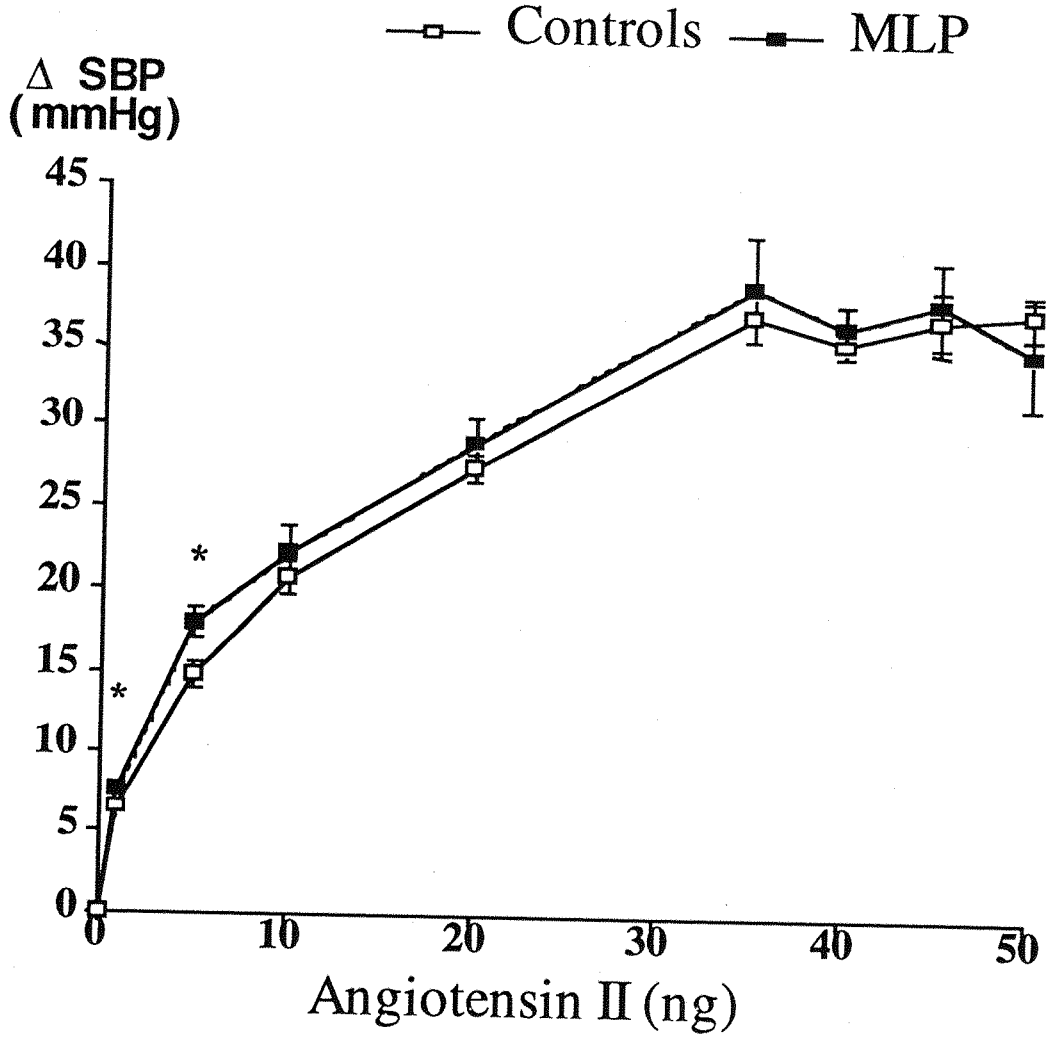
Under conditions of stress SBP rises, thus facilitating the fight or flight response. One criticism of the MLP-induced model of hypertension has been that blood pressure was measured by an indirect method. It has been suggested that the higher SBP of MLP rats represents an altered cardiovascular response to the tail-cuff procedure (van den Berg *et al* 1994). When measured directly under anaesthesia, the SBP of MLP-female were significantly

higher than in control rats, which shows clearly that MLP-induced hypertension is not simply a product of the tail-cuff procedure. Physiological measurements under anaesthesia may not necessarily reflect the normal *in vivo* state since pentobarbitone is a cardiovascular depressor and further studies measuring SBP in free living, chronically catheterised rats would be desirable.

The female offspring of MLP dams exhibit an increased peripheral reactivity to the action of AII, at all the studied doses of AII that were studied. Thus, the dose response curve for AII was shifted to the left in MLP rats relative to control rats, implying an increased physiological sensitivity to the pressor effect of AII. This, together with the data reporting increased densities of GR in vascular tissue of MLP rats (Langley-Evans *et al* 1996c) and the observation that an intact adrenal is essential for the full expression of the hypertensive state of MLP (*section 4.5.3.*), is suggestive of an interaction between glucocorticoids and AII in the maintenance of MLP-induced hypertension. The importance of AII in the development of the hypertensive state is emphasised by the finding that blockade of AII synthesis in early postnatal life (2-4 weeks) prevents the elevation of SBP in MLP rats some 8 weeks after cessation of treatment (Sherman & Langley-Evans 1998) . However, a role for corticosterone in mediating the vascular reactivity to pressor agents other than AII e.g. catecholamines, arginine vasopressin, adrenomedullin, NO cannot be excluded by the present data and should be the subject of future studies.

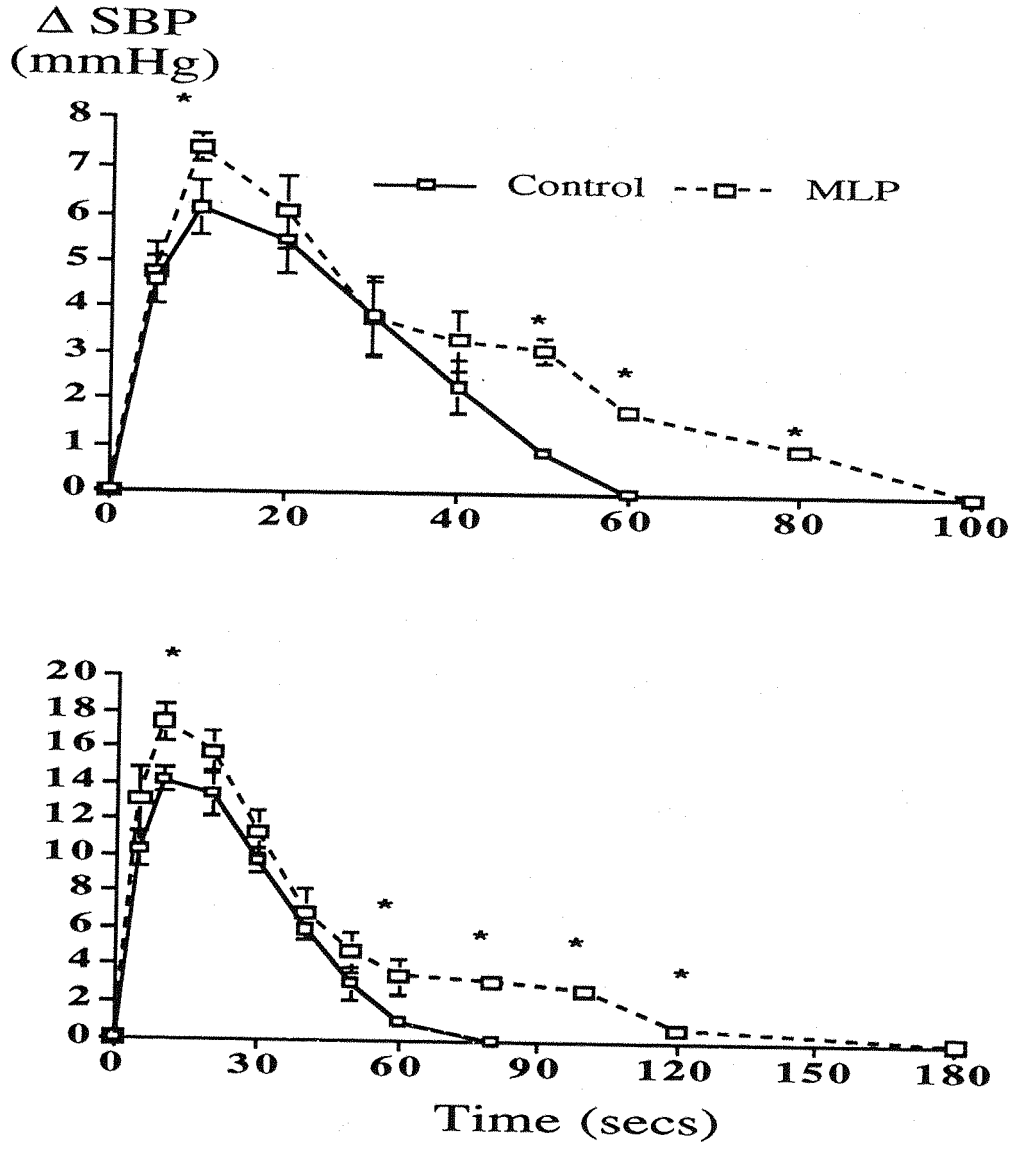
Administration of the angiotensin-converting-enzyme (ACE) inhibitor, captopril, results in a significant reduction of SBP in MLP, to levels similar to controls (Langley-Evans & Jackson 1995). Glucocorticoids influence the renin-angiotensin system at many levels. Thus the expression of angiotensinogen, the precursor of angiotensin I (Campbell & Habener 1986), and the synthesis of ACE (Mendelsohn *et al* 1982), are stimulated by glucocorticoids. In the SHR, binding of AII to its receptor is increased by glucocorticoids (Provencher *et al* 1995). The thoracic aorta of MLP rats exhibit significantly higher binding of tritiated corticosterone to GR (Langley-Evans *et al* 1996c). Possible mechanisms which underlie these observations are suggested by studies on vascular smooth muscle. Glucocorticoids operating

Figure 5.6. **A curve illustrating the maximal pressor response to angiotensin II (AII) of female rats exposed to either a maternal control or low protein diet**



The figure demonstrates the maximal change in SBP to varying bolus doses of *i.v.* injection of AII. Data presented are mean ±SEM for n=5 offspring per dietary group from a total of n=5 litters per dietary group and at each dose of AII. Animals were generated as outlined in *Methods*. At 10 weeks of age rats were administered sodium pentobarbitone anaesthetic (72mg/kg bodyweight) *i.p.* and the carotid artery and femoral vein cannulated as described in *Methods* 2.5. After at least 30mins a direct estimate of systolic blood pressure was obtained on a calibrated chart recorder, attached to a pressure transducer and amplifier, and the infusion protocol started. Briefly, AII was delivered intravenously dissolved in a total volume of 0.1ml, 0.9% NaCl that was maintained at 37°C. Randomised doses (0, 1, 5, 10, 20, 40ng) of AII were administered allowing a period of least 5mins between each injection. Data were analysed by two way analysis of variance (ANOVA) which indicated a significant effect of diet ($F= 5.54$, $P= 0.02$) and AII dose ($F= 195.4$, $P <0.0001$). * indicates a significant difference to control rats ($P< 0.05$).

Figure 5.7. **A curve illustrating the pressor response over time to 1ng (A) and 5ng (B) angiotensin II (AII) infusion of female rats exposed to either a maternal control or low protein diet**



All data presented are mean ±SEM for n=5 offspring per dietary group from a total of 5 litters per dietary group and at each dose of AII. Animals were generated as outlined in *Methods* 2.2.1. For details refer to Figure 5.6. Doses (0, 1, and 5ng) of AII were administered allowing a period of least 5mins between each injection. Data were analysed by two way analysis of variance (ANOVA) for diet and time. The initial vascular response to AII injection in the MLP group was significantly greater at both levels of AII and, at both doses, the recovery to baseline values appeared delayed. Thus, at both 1ng and 5ng AII injection there was a significant effect of diet (1ng; $F= 4.40$, $P= 0.04$: 5ng; $F= 12.62$, $P= 0.0008$) and AII time (1ng; $F= 11.95$, $P< 0.0001$: 5ng; $F= 39.15$, $P< 0.0001$). *indicates a significant difference to control rats ($P< 0.05$).

through the GR (Kornel *et al* 1993a), increase transluminal Na^+ and Ca^{2+} transport in cultured vascular smooth muscle (VSM) (Kornel *et al* 1993b) and *in vivo* (Kornel *et al* 1995). Thus both the contractility and reactivity of VSM prior to stimulation by vasoconstrictors is increased by glucocorticoids. Increased VSM responsiveness to AII, mediated by glucocorticoids, may be the crucial process in the development of hypertension in MLP. AII, operating through its receptors (AT-1&2), mediates contraction and proliferation of VSM and release of catecholamines and prostacyclin (Griendling & Alexander 1994). Increased AII action *in utero* potentiated by the glucocorticoid environment may initiate second messenger processes leading to smooth muscle hypertrophy, perhaps due to alterations to intracellular electrolytes (Berk *et al* 1989), and/or hyperplasia (Daemen *et al* 1991). Such processes may then provide a positive feedback mechanism causing further increases in blood pressure in later life (Folkow 1978).

Determining the primary cause or secondary responses to hypertension is extremely difficult. There is, however, sufficient evidence to implicate a role for AII in the earliest stages of development of hypertension in MLP. In the SHR captopril is more effective at reducing VSM hypertrophy than other treatments despite equivalent reductions in SBP (Owens 1987). AII may therefore mediate the hypertrophic/hyperplastic growth processes which have been precipitated by raised SBP. The observation that blockade of ACE early in life (Sherman & Langley-Evans 1998), rather than later (Langley-Evans & Jackson 1995), permanently reduces the SBP of MLP, indicates a role for AII in the positive feedback mechanism leading to secondary hypertension. Similarly, GC may be involved in the secondary processes associated with hypertension in SHR since early ADX of SHR prevents the development of the hypertensive state (Ruch *et al* 1984). Whether early (e.g. 2 weeks old) ADX, as with AII blockade, prevents the development of hypertension in MLP animals is not known. Interestingly early treatment with a Ca^{2+} antagonist has no effect on MLP-induced hypertension suggesting specificity of the effect to AII (Sherman & Langley-Evans - unpublished observations).

Whilst altered reactivity of systemic blood vessels to AII may initiate structural changes (Folkow 1978), increased reactivity to AII within vascular beds such as the fetal kidney, following glucocorticoid exposure, may influence renal development and predispose

MLP rats to progressive increases in blood pressure. Late gestation cortisol infusion in fetal sheep elicits increases in renal blood flow and glomerular filtration rate (Hill *et al* 1988). Decreased renal blood flow relative to controls has also been noted in adult MLP rats (Welham *et al* 1996) perhaps reflective of earlier intrauterine events. Alterations to the renal vascular system and renin-angiotensin system dynamics *in utero* may therefore potentially influence kidney development given that AII, via the AT1 receptor, regulates nephrogenesis and the nephrovascular network (Tufro-McReddie *et al* 1995). Prenatally programmed deficits of renal growth, as a secondary consequence of a poor maternal diet may thus influence the functional capacity of the kidney leading to metabolic conflicts in later life predisposing to elevated SBP (Mackenzie & Brenner 1995). Unilateral nephrectomy of MLP and longitudinal measurements of SBP may test this hypothesis experimentally .

CHAPTER SIX

6.0. GENERAL DISCUSSION

A progressive rise in blood pressure to a point where the elevated blood pressure increases the risk of pathophysiology is termed hypertension. Hypertension is a multi-factorial disease. In only a small percentage (<5%) of the population can the hypertensive state be attributable to a single cause such as a gene defect (AME) (Stewart *et al* 1988), or secondary to pathophysiology such as Cushing's syndrome (Ross & Linch 1982). For the majority (>90%) the underlying aetiology of hypertension cannot be readily explained and is termed 'essential hypertension'. Hypertension is a major cardiovascular risk factor for stroke or coronary heart disease (Thom *et al* 1992). 50 years of research has established many factors contributing towards elevated blood pressure such as; low physical activity, obesity, or the intake of saturated fat, salt and alcohol (Ward 1990). Together these factors still only explain a proportion of cardiovascular risk.

Systolic blood pressure (SBP) tracks from early life to adulthood i.e. a rank order for SBP is maintained from infancy to adulthood after allowing for an effect of increased body size (Law *et al* 1993) and is related to the degree of increase of SBP in childhood (Lever & Harrap 1992). Factors that may influence SBP in early life may, therefore, determine future cardiovascular risk. Barker and colleagues have identified a negative relationship between birthweight and SBP in childhood (Law *et al* 1991). The relationship remains into adult life and, furthermore, is amplified with ageing (Barker *et al* 1989a). Thus determinants of intrauterine growth leading to an asymmetric or a proportionately small baby at term, also influence future cardiovascular risk (Barker 1994).

Whilst genetic factors account for a small proportion of the variation in birthweight, non-genetic factors such as the maternal environment have an overriding importance (Brooks *et al* 1995; Milner & Gluckman 1996; Walton & Hammond 1938). Intrauterine growth is an anabolic process, requiring nutrients, which the fetus obtains from the mother via the placenta. Maternal nutrition can therefore influence the intrauterine growth process. Indeed birthweight is related to the intake of specific nutrients within the maternal diet such as

protein and carbohydrate (Godfrey *et al* 1996) and indices of maternal nutritional state such as skinfold thickness (Godfrey *et al* 1994) and maternal iron stores (Godfrey *et al* 1991). A relatively low intake of protein and reduced skinfold thickness and iron stores all predict lower weight at birth.

SBP in childhood and adult life are related to the same indices of maternal nutrition. Maternal anaemia (Godfrey *et al* 1991) and reduced maternal skinfold thickness (Godfrey *et al* 1994) are associated with a higher SBP. A low intake of protein with an associated high intake of carbohydrate during gestation leads to an elevated SBP in the offspring when measured 45 years later (Campbell *et al* 1996). The propensity towards hypertension and coronary heart disease, is therefore, partially determined *in utero* by nutritional factors. The underlying physiological processes relating maternal nutrition, intrauterine growth patterns and adult hypertension are as yet unknown.

The growth and development of an organism is characterised by an increase in size and an increase in complexity (Milner & Gluckman 1996). An increase in size is accomplished by a net deposition of material and thus an increase in both the number and size of cells. An increase in complexity involves cellular differentiation from a population of uniform, undifferentiated totipotent cells to form populations of diverse cells specialised for distinct functions. Nutrients are the building blocks of growth and nutrient restriction may disturb the growth process.

The disturbance of the growth process in the short term, in response to nutrient restriction, involves adaptations in the fetal environment which serve to maintain functions necessary for continued development. Blood flow and consequently, the nutrients within the blood, are diverted preferentially towards the brain and heart which may compromise optimal growth of peripheral organs such as spleen, kidney, liver, and muscle (Rudolph 1984). If the nutrient restriction is particularly severe then the fetal compromise will be evident as intrauterine growth retardation. If the nutrient restriction is mild, then a developmental compromise may not be evident from organ masses alone, but the decrease in cellular differentiation and/or cell-cell interactions may reduce the functional capacity of cells, tissues, organs and the organism as a whole. Decreased functional integrity of organs may, in the face

of a challenging environment, manifest in later life as disease. The challenging environment may merely represent the metabolic handling of excess nutrients in adult life given the 'thrifty' metabolism adopted, and thereafter programmed, in response to mild nutrient restriction. This is the framework of the thrifty phenotype hypothesis (Hales & Barker 1992).

Data from animal models lend support to the hypothesis. A maternal low protein diet programs aspects of hepatic biochemistry (Desai & Hales 1997) and pancreatic function (Dahri *et al* 1993) that may influence the propensity towards non-insulin-dependent-diabetes mellitus. Similarly, prenatal protein restriction alters aspects of adult kidney and renin-angiotensin function (Langley-Evans & Jackson 1995; Welham *et al* 1996; Zeman 1968) that may contribute towards elevated SBP. The present work supports a model of hypertension programmed by maternal nutrition and suggest an influence of maternal glucocorticoids in the programming of the hypertensive state. Assessment of the endogenous flux of corticosterone into the fetal compartment in MLP during gestation should help determine a clear role for maternal glucocorticoid in programing elevated SBP. It has been demonstrated that *de novo* synthesis of a product of the adrenal gland, likely glucocorticoid in origin, in postnatal life is necessary for the maintenance of the hypertensive state.

Maternal protein restriction induced early hypertrophy of the placenta and together with increased total placental 11-HSD2 activity at day 14 gestation may have facilitated, in part, an accelerated early intrauterine growth rate in MLP rats relative to controls. The influence of maternal hormones and growth factors in mediating the growth response of MLP in early gestation needs to be established unequivocally. Continuation of the low protein diet into late gestation, when fetal demands for nutrients are increased, results in disproportionate patterns of growth that are interpreted as an adaptive response to a reduced substrate supply. For example the growth curve of the fetal brain over late gestation was maintained in proportion to body weight, whereas the growth of the liver and lungs was retarded. However interpretation of the 11-HSD2 data presented and that of Seckl *et al* (1997b) must be treated with some caution due to methodological issues.

The mechanism of fetal adaptation to late gestational undernutrition may underpin later susceptibility towards hypertension. The fetal HPA axis assumes autonomy at

approximately day 16 in the rat (Chatelain *et al* 1980). The MLP diet induced a reduction in the activity of total placental 11-HSD2 at day 20. It is inferred that this allows an increased flux of maternal steroid across the placenta during a critical period of fetal HPA development. Whilst requiring confirmation by detailed measurements of steroid flux across the placenta, this inference is supported by a recent observation that brain GPDH is significantly elevated in fetuses exposed to MLP from d15 gestation onwards (Langley-Evans & Nuwagwu 1998). Altered diurnal profiles of adrenocorticotrophic hormone (ACTH) in plasma and increased activities of glycerol-3-phosphate dehydrogenase (GPDH) and glutamine synthetase (GS) in postnatal life of MLP indicate long term programming effects of glucocorticoid exposure on HPA axis function. Interestingly, early menarche is associated with reduced birthweight (Cooper *et al* 1996) and growth hormone secretion in adulthood is related to growth in infancy (Fall *et al* 1997). Both observations are suggestive of programming of hormonal axes within the brain in early life.

Reduced secretion of ACTH and increased activity of glucocorticoid-inducible enzyme activities are not the causal factor in the programming of hypertension in MLP but rather reflect a generalised greater sensitivity to glucocorticoid action. The finding that the blood pressure of MLP in adult life is lower following adrenalectomy suggest a role for adrenal products in maintaining the raised SBP of MLP. Corticosterone is the likely mediator since corticosterone replacement blocked the reduction in SBP following ADX. Furthermore, vascular tissue of MLP-female rats appears to be more responsive to the pressor action of angiotensin II (AII). The development of hypertension in MLP is dependent upon adequate circulating AII in early life since blockade of AII production by captopril prevents development of hypertension in MLP (Sherman & Langley-Evans 1998). A glucocorticoid role in modulating the pressor responsiveness of vascular tissue to AII has been noted in humans (Whitworth *et al* 1995) and in the present results and thus the development of hypertension in MLP may involve a synergistic interaction between glucocorticoid and the RAS.

The basis for increased central and peripheral sensitivity to glucocorticoids (as reflected in the enzyme studies) does not appear to be due to an increased concentration of steroid, since corticosterone concentrations were similar between offspring of the two dietary

groups. However differences in the metabolism of corticosterone between dietary groups has not been addressed by the present studies. Babies who were relatively thin at birth, indicative of late gestational growth retardation, exhibit increased excretion of cortisol metabolites at 9 years of age (Clark *et al* 1995). This may either suggest increased circulating plasma glucocorticoid concentrations or altered metabolic handling and breakdown of cortisol.

A number of observations suggest increased glucocorticoid sensitivity in MLP: Firstly, the activities of GC-sensitive marker enzymes are elevated relative to control animals which is a specific GC effect since the activities of non GC-sensitive enzymes (MD and PK) are unaltered by prenatal diet. Secondly, the circulating plasma corticosterone concentration is low-normal in MLP. Finally, the pattern of enzyme induction and receptor alterations in MLP closely mirrored that observed in obese Zucker rats which represents a clearly defined GC-dependent paradigm (Langley & York 1990). Furthermore the increased GC-sensitivity of Zucker rats is receptor based (Langley & York 1990). Thus the suggestion is that the increased GC sensitivity in MLP is also receptor based, an assertion supported by the fact that MLP exhibit increased binding to GR in central regions and vascular tissue (Langley-Evans *et al* 1996c).

Whilst increased vascular GR may mediate increases in SBP increased central GR populations may underlie the blunted secretion profile of ACTH in MLP rats. Thus the secretion of ACTH, whilst predominantly under the influence of hypothalamic peptides, is also responsive to neural projections from the hippocampus which form a negative feedback circuit (Seckl & Olsson 1995). Enhanced sensitivity to corticosterone feedback mediated by increased GR in the hippocampus, as has been noted in MLP (Langley-Evans *et al* 1996c), results in a greater inhibitory signal to the hypothalamus resulting in reduced output of ACTH.

Whilst glucocorticoids are involved in the programming of hypertension in MLP they do not solely account for the increase in SBP noted, reflecting the situation in the human population where hypertension can only be described as being multifactorial. Inhibition of angiotensin converting enzyme, in similarity to adrenalectomy of adult MLP, normalises SBP in MLP (Langley-Evans & Jackson 1995). Hypertension in MLP, therefore, is the consequence of a graded rise in SBP over and above that in control rats due to many

components operating in concert, but involving centrally programmed factors and peripheral HPA, renin-angiotensin and programmed alterations to kidney structure and function. The MLP diet specifically and consistently impairs the growth of the kidney as a whole (Langley-Evans *et al* 1996f) and more specifically the formation of nephrons (S.J.M. Welham - *unpublished observations*). A reduced nephron complement at birth is associated with an increased susceptibility to hypertension (Mackenzie & Brenner 1995). The influence of other factors that are known to associate with essential hypertension in humans (Reaven & Hoffman 1987) and rats (Langley *et al* 1994a, Pickard *et al* 1996), such as insulin resistance, have not been addressed by the present studies but may have an, as yet undefined, role.

During mid-late gestation the impact of maternal protein restriction becomes apparent as reduced maternal weight gain relative to controls. Interestingly MLP-fetuses enter the third week of gestation on an accelerated early growth curve which may render them more vulnerable to limited nutrient availability (Harding *et al* 1992). In order to minimise the impact of protein restriction MLP fetuses appear to respond in late gestation by reducing growth of the trunk. At term, control pups, either exceed or match the birthweight of MLP pups. A supply of nutrients that does not match the fetal demand results in a shift in metabolism favouring a continued supply to organs essential for continued survival which may be mediated by endocrine changes.

Fetal adaption to undernutrition involves alterations to circulatory dynamics (Rudolph 1984). Changes in the vascular system are mediated by changes in the effectors that control the fetal cardiovascular system such as glucocorticoids and angiotensin II (Tangalakis *et al* 1992). Glucocorticoids, whilst enhancing the pressor action of AII, also serve to mobilise fuel and maintain glucose supply to the brain. Glucocorticoid receptors mediate the cellular action of glucocorticoids (McEwen *et al* 1986). GR expression and function are demonstrable in the developing fetal rat brain from day 15 to term (Kitraki *et al* 1996). An altered fetal metabolic environment produced through undernutrition may promote inappropriate expression of GC-inducible genes that, in the short term, facilitates the fetal response to intrauterine stress. Stress encountered at an early age permanently programmes GR populations (Meaney *et al* 1988). Inappropriate glucocorticoid action during sensitive periods of brain growth influences neuronal growth, maturation and density (McEwen *et al*

1986) and may alter the function of the existing neural cell membranes (membrane potential, excitability, receptor density) in regions controlling blood pressure within the hypothalamus. Thus the 'set point' for blood pressure equilibration may be permanently altered in MLP. For example, increased hypothalamic GS activity may indicate a general disturbance of hypothalamic glutamate metabolism. Glutamate is excitatory in the nucleus tractus solitarus, the cardiovascular control centre in the medulla (Talman *et al* 1984). Similar effects of glucocorticoids upon the metabolism and membrane responsiveness to centrally acting monoamines, serotonin and noradrenaline (McEwen *et al* 1986) may further render cardiovascular control regions within the brain hyperreactive to afferent inputs. The present study, however, has not explored any possible role for the sympathetic nervous system or altered central monoamine metabolism in mediating the elevation of SBP in MLP. Interestingly, transplantation of hypothalamic tissue from day 16 SHR embryos to normotensive adult WKY rats significantly elevates the SBP of the WKY rats (Eilam *et al* 1991). Hypothalamic factors therefore, underlie, in part, hypertension in SHR and may contribute to the rise in SBP of MLP.

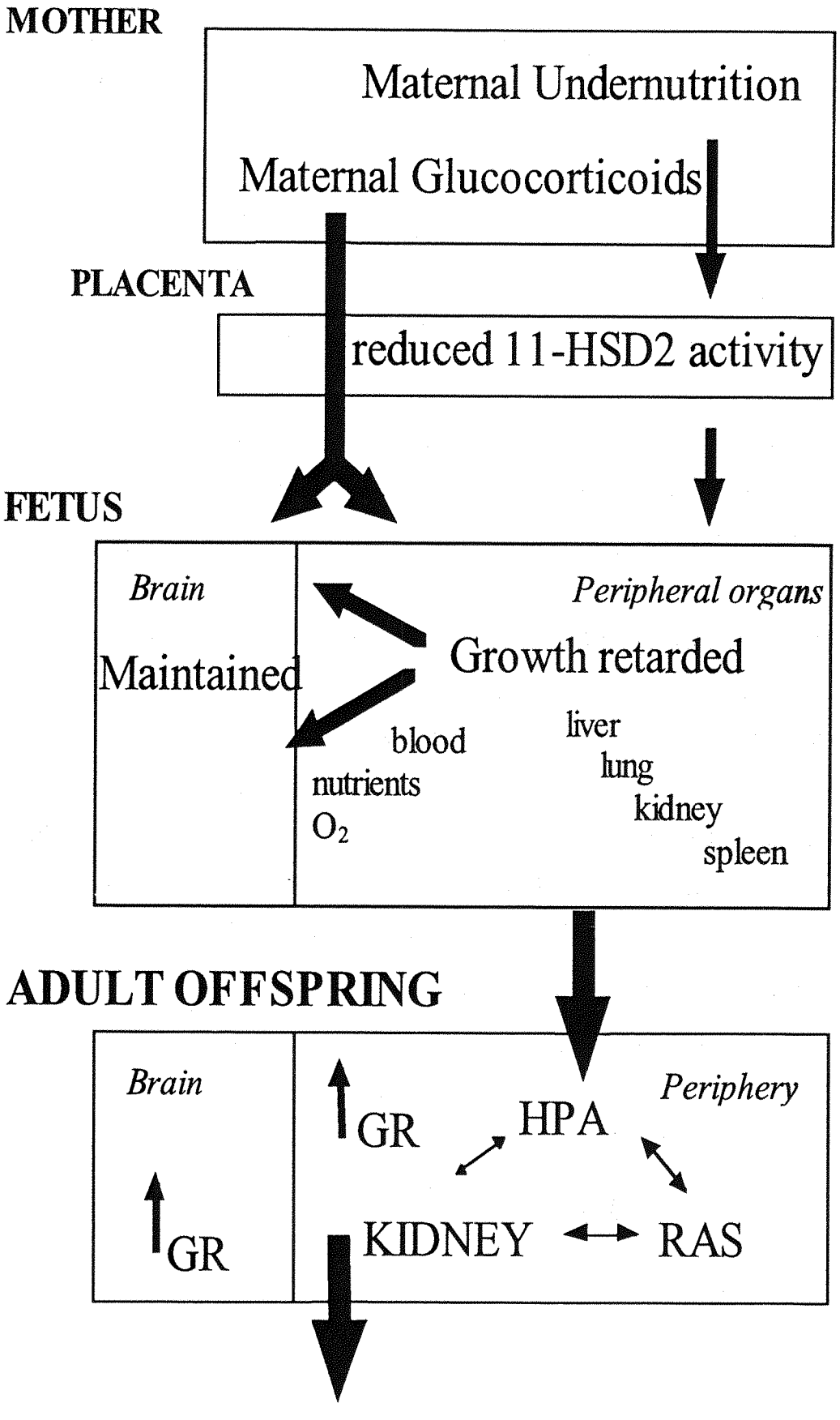
The primary stimulus promoting elevated SBP in later life may be comprised of a combination of factors. These include redistributed intrauterine blood flow profiles in response to the metabolic challenge that maternal protein restriction represents and programmed alterations to receptors in the vasculature that control the ability of blood vessels to redistribute flow. GC may enhance the action of pressor agents such as AII and nor-adrenaline upon the VSM. In postnatal life an exaggerated response of VSM to pressor activity persists perhaps facilitating structural cardiovascular adaptation which may predispose to higher blood pressure and secondary hypertension. Structural adaptation within the arterial, cardiac and renal baroreceptors in response to the chronically elevated SBP may reset the 'barostat' function of baroreceptors at a higher level, further contributing to the hypertensive state (Sleight *et al* 1975).

The present thesis has started to explore the mechanisms which underlie the development of high SBP in MLP offspring but leaves a number of questions unresolved. These results, with others, help create a working hypothesis that may explain the intrauterine programming of adult hypertension (Figure 6.1). The feeding of a low protein diet appears to

have a down-regulating effect upon placental 11-HSD2 activity in the later stages of gestation. It is suggested that this results in an increased flux of glucocorticoid from mother to fetus. The collective findings of experiments involving maternal ADX, pharmacological ADX (Langley 1997a), carbenoxolone inhibition of 11-HSD (Lindsay *et al* 1996, Langley-Evans 1997b) and dexamethasone treatment (Benediktsson *et al* 1993) suggest that an increased flux of glucocorticoid would promote increased SBP in the offspring and that the hypertensive effect of MLP is dependent upon intact maternal adrenal function. The immature fetal hypothalamus may be a primary target for the programming effects of glucocorticoids resulting in long term adaptations and alterations to HPA axis function. Effects of nutrient restriction, that may or may not be glucocorticoid mediated, upon development of peripheral organs may have additional effects upon late cardiovascular function that is independent of any centrally mediated mechanism (Langley-Evans *et al* 1996b). Postnatal interaction of central and peripheral effects result in a greater rate of increase in SBP during early life leading towards hypertension in later life. If the mechanisms such as, angiotensin II, facilitating secondary structural adaptation in postnatal life are blocked then hypertension does not develop (Sherman & Langley-Evans 1998). Furthermore the early rise in SBP is exacerbated by conditions of nutritional excess. The effects of early exposure to a low protein diet upon SBP followed by exposure to a cafeteria-diet in later life are cumulative (Petry *et al* 1997).

The present data support the fetal origins of adult disease hypothesis proposed by Barker (Barker & Osmond 1986). The implications of the hypothesis for human health are considerable. As yet there is not sufficient evidence to make any recommendations about the nutrition of pregnant women nor suggest any prenatal nutritional interventions, yet these remain the ultimate goal of the body of research designed to elucidate the mechanisms underlying the hypothesis. Of particular concern is the increasing tide of westernisation in developing countries and the concommitant increase in the intake of energy dense foodstuffs. Overnutrition in persons and populations adapted to chronic levels of poor nutrition represents a time bomb in terms of the future incidence of degenerative diseases such as diabetes and coronary heart disease.

Figure 6.1. Glucocorticoid programming of Adult Disease



6.1. FUTURE DIRECTIONS

There are a number of important directions indicated by the present work. In the first instance a measure of the placental flux of corticosterone from maternal to fetal compartments during mid-late gestation should be determined. This is central to the mechanism proposed for MLP-induced hypertension. In association with this, a measurement of intrauterine glucocorticoid action at the level of gene expression, such as glycerol 3-phosphate dehydrogenase/tyrosine hydroxylase mRNA in fetal brain, is desirable to confirm an increased GC signal in the fetal compartment. Determining the expression and activity of tyrosine hydroxylase in fetal and adult tissues would serve a dual function. Firstly, it would determine whether the enzymic programming by glucocorticoids is glial cell specific, since tyrosine hydroxylase is primarily a neuronal marker. Secondly, tyrosine hydroxylase is a rate limiting step in the biosynthesis of catecholamines and therefore would provide a useful marker for catecholamine metabolism in central regions. Furthermore, determination of the hypothalamic/medulla content of catecholamines, serotonin, angiotensin II and perhaps other important neurotransmitters in the control of blood pressure may provide an indication of the excitability of the hypothalamic cardiovascular control regions. As yet, no measure has been made of corticotrophin-releasing hormone secretion which may be an important contributor to the blunted ACTH secretion profile.

Postnatally, as yet, we have only partially characterised one aspect of the control of blood pressure. With an increase in SBP and no change in heart rate we have assumed that total peripheral resistance is increased. However this is an assumption and should be tested experimentally through measurements of heart rate and blood flow to determine peripheral resistance. A measure of cardiac output would help determine whether there is a role for increased cardiac output in maintaining increased SBP.

The framework around which the hypothesis is based assumes some degree of structural cardiovascular adaptation leading to secondary hypertension. Early interventions utilising either surgical or pharmacological techniques should attempt to prevent the development of the hypertensive state. Blockade of specific adrenergic receptors or early ADX may achieve such a function. Similarly other, albeit downstream, mechanisms involved in the regulation of SBP could also be characterised. Preganglionic and postganglionic

sympathetic blockade utilising relevant pharmacological agents for α and β receptors and a measure of noradrenalin turnover rate may indicate the contribution, if any, of a neurogenic component. In addition it is important to establish a measure of vascular distensibility/contractility/reactivity utilising *in vitro* organ bath techniques to determine the degree to which each vasoactive compound may influence the vasculature of MLP. Components of the VSM that may constitute the material of vascular hypertrophy and/or hyperplasia could also be quantified through specific staining techniques. VSM contents of elastin, actin filaments, the collagens and glycosaminoglycans all contribute to the development of contractile smooth muscle.

A pilot study of the effect of 6 d twice daily treatment with the glucocorticoid antagonist RU486 (at a dose previously established to be an effective anti-glucocorticoid; 30mg/kg/day in 0.05ml polyethylene glycol) or vehicle (0.05ml polyethylene glycol) upon the pressor response to an intravenous bolus injection of AII was carried out in male rats (n=3/dietary group) towards the end of the PhD. Male animals were exclusively chosen both to avoid potentially confounding interactions of RU486 with sex steroids in females (however this may only be of importance during pregnancy) and due to the limitation of female animals generated. Whilst RU486 treatment appeared to raise blood pressure in control animals by approximately 14mmHg (P=NS) when assessed indirectly and by approximately 13mmHg (P=NS) using direct methods, no such effect was observed in MLP rats when measured directly or indirectly. However, GR-mediated responses cannot be concluded to be unimportant in the present study given the low number of animals involved. Where this occurs throughout the thesis (predominantly within the MADX study) data could perhaps have been pooled to increase the strength of analysis. This is the major weakness of these MADX studies and in the future greater numbers will be used to generate more offspring. Given that MLP-females exhibit an exaggerated response to intravenous AII whereas males do not then a potential sex-specific response pattern to an AII bolus may operate in MLP. The basis for this and for the other sexually dimorphic responses noted in MLP, especially following MADX, may warrant further investigation. Further work in this area may proceed by examining similar responses following gonadectomy/hypophysectomy to determine a distinct sex-specific effect of prenatal undernutrition.

APPENDICES

APPENDIX 1

COMPOSITION OF SYNTHETIC DIETS.

	Composition (% by wt)	
Maternal Diet...	**18% Protein (Control)**	**9% Protein (MLP)**
[1]Casein	18	9
[1]Snowflake (cornstarch)	42.5	48.5
[1]Solkafloc (cellulose)	5	5
[2]Sucrose	21.3	24.3
[3]Choline	0.2	0.2
[1]Mineral Mix (AIN-76)	2	2
[1]Vitamin Mix (AIN-76)	0.5	0.5
[3]DL-Methionine	0.5	0.5
[4]Corn Oil	10	10
Gross Energy (MJ/kg)	20.2	19.9

Diet was prepared in the departments own diet kitchen and ingredients were bound together through the addition of water. Diet, as balls (60-100g dry weight), was dried for 24hr at 60°C and stored at -20°C for upto, but no longer than, one month. Gross energy was determined by ballistic bomb calorimetry. Ingredients for semi-synthetic diets were purchased from [1]Special Diet Services, UK; [2]Tate & Lyle, UK; [3]Sigma, UK; [4]Mazola, UK.

APPENDIX 2.

COMPOSITION OF CRMX LABORATORY CHOW DIET.

Nutrient	Composition (%)
Protein	18.8
Total starch	44.8
Total fibre	12.3
Sucrose	4.7
Choline	0.95
Major minerals	3.63
Major vitamins	2.84
Corn Oil	
Trace elements	8.6

Diet was provided to the animals as extruded pellets.

APPENDIX 3

ASSAY REAGENTS

11β-hydroxysteroid dehydrogenase (11HSD) buffer:

118mM NaCl

3.8mM KCl

1.19mM KH_2PO_4

2.54mM $CaCl_2$

1.19mM $MgSO_4$

25mM $NaHCO_3$

The pH was adjusted by bubbling through CO_2.

ACTH radioimmunoassay elution solvents:

Buffer A

1% Triflouroacetic acid (HPLC grade) in distilled water

Buffer B

60% Acetonitrile (HPLC grade) in 1% Buffer A

B.C.A. solution:

50ml Bicinchoninic acid (BCA) solution

1ml 4% copper sulphate

Bis-benzamide solution:

Stock solution of bis-benzamide made upto 0.2mg/ml with analar water

Dilute 100 fold prior to use in assay

CORTICOSTERONE REAGENTS:

Corticosterone Standard (A)

Make a 2mg/ml stock solution of corticosterone in ethanol. This is then diluted 1:100 twice in ethanol. Further dilute it 1:100 in Diluent. This is then diluted with diluent to achieve the following concentrations (ng/ml): 0, 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0.

Diluent

0.05M Tris-HCl pH 8.0

0.1M NaCl

0.1% NaN_3

0.1% BSA

Dextran Coated Charcoal Suspension (C)

1% Activated Charcoal Powder

0.1% Dextran T70

Dissolve dextran in diluent, add charcoal and stir at 0°C for 1hr and during use.

Radioactive Tracer (D)

^{3}H-corticosterone-minimum 80Ci/mmole prepared in diluent to yeild 150pg/ml.

Take 5µl of present stock, add 495µl diluent and vortex. Of this take 50µl and add 4950µl diluent. Take 2ml and add 14.46ml diluent.

DNA assay buffer (pH 7.4)

50mM Na_2HPO_4

50mM NaH_2PO_4 - Titrate to pH 7

Add;

2M NaCl - Readjust pH to 7.4

Folin Ciocalteau working reagent:

48ml 2% Na_2CO_3 in 0.1M NaOH

0.5ml 0.5% copper sulphate

0.5ml 1% sodium potassium tartrate

1ml 10% sodium deoxycholate

Glucose assay buffer:

0.1M sodium phosphate pH 7.0

Glucose assay reagent:

0.8mg/100mls horse-radish peroxidase

5mg/100mls glucose oxidase

0.1g/100mls ABTS

made up in glucose buffer

Glutamine Synthetase (GS) homogenisation buffer:

0.25 M sucrose

1mM EDTA

Glutamine Synthetase reaction mixture:

80mM Tris-HCL pH 8

20mM $MgCl_2$

20mM β-mercaptoethanol

60mM glutamate (K salt)

40mM hydroxyalanine-HCL

10mM phosphoenol pyruvate

0.5u/ml pyruvate kinase

Glutamine Synthetase colour reagent:

0.37M ferric chloride

0.67M HCL

0.2M Tricloroacetic acid

Glycerol phosphate dehydrogenase homogenisation buffer:

10mM sodium phosphate pH 7.15

1mM EDTA (disodium salt)

100µg/ml BSA

Glycerol phosphate dehydrogenase reaction mixture:

10mM sodium phosphate pH 7.15

1mM EDTA (disodium salt)

100µg/ml BSA

167µM NADH

Malate dehydrogenase homogenisation buffer:

20mM potassium phosphate pH 7.5

0.5mM EDTA

50% glycerol (v/v)

Malate dehydrogenase reaction mixture:

50mM AMP pH 9.5

100µM NAD

5µg/ml glutamate-oxaloacetate transaminase

10mM malate

10mM glutamate (Na Salt)

Pyruvate kinase homogenisation buffer

0.25M sucrose

17mM MOPS pH 7.1

50mM NaF

5mM dithiothreitol

2.5mM EDTA

Pyruvate kinase reaction buffer

270mM Tris-HCl pH 7.1

20mM $MgSO_4$

66mM KCL

2.5mM ADP

0.16mM NADH

5.5 u/ml lactate dehydrogenase

Tyrosine Aminotransferase homogenisation buffer:

125mM KPO_4 pH7.6

1mM EDTA

1mM Dithiothreitol

Tyrosine Aminotransferase reagent:

1.6ml 7.57mM tyrosine

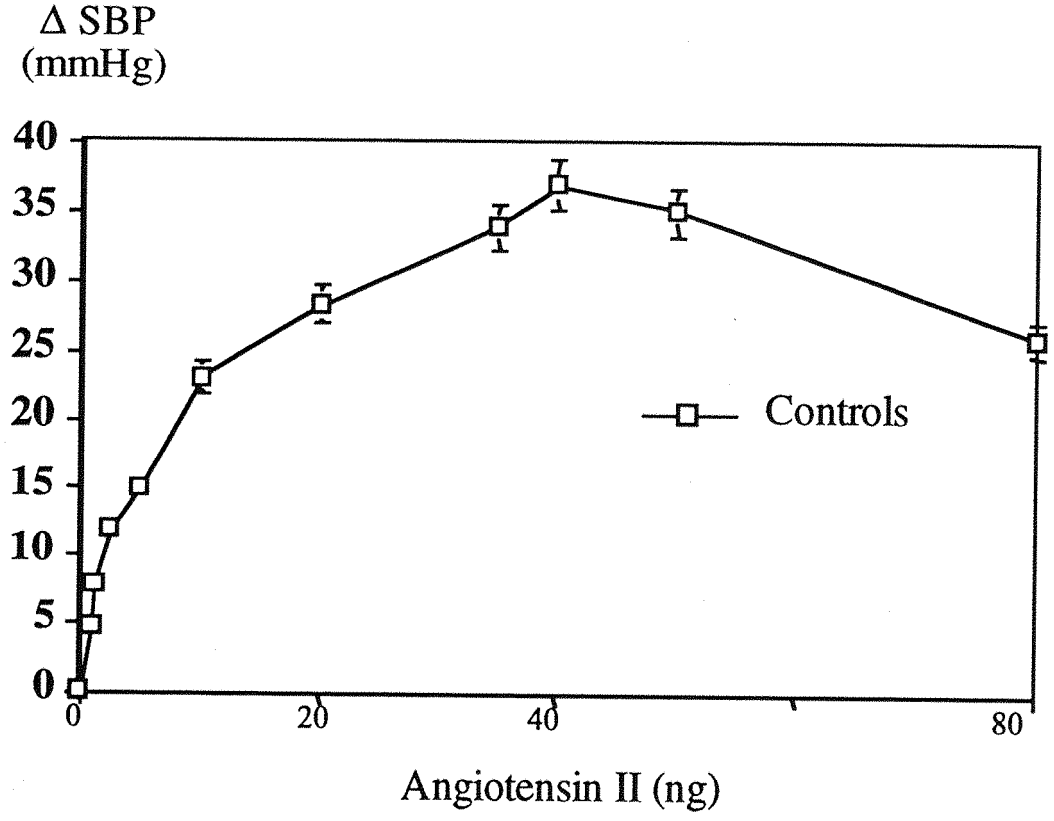
200µl α-ketoglutarate

40µl 5mM pyridoxal 5-phosphate

APPENDIX 4

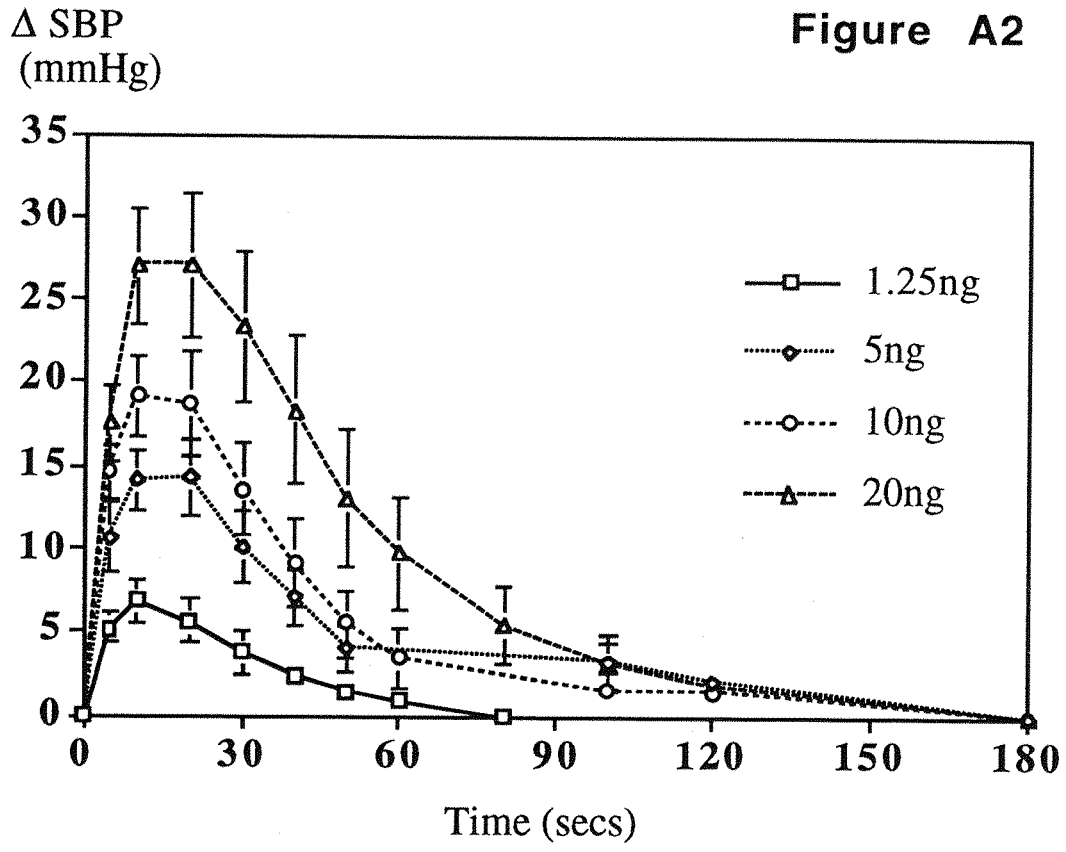
VALIDATION OF ANGIOTENSIN II DOSE

Figure A1. **The dose response curve of chow-fed animals to varying bolus injections of Angiotensin II**



The graph represents data (mean ±SEM) for n=7 animals at each dose of angiotensin II. Angiotensin II (AII) was injected directly into the femoral vein as described in *Methods* 2.5. at doses of between 1.25-80 ng AII. At each dose the animal was given 2-3 injections and the average maximal response taken. A period of at least 5 mins was allowed between doses after which SBP had returned to baseline values. The exact halflife of AII in plasma was not determined in the present study. However, AII was quickly metabolised in the plasma to such an extent that following 5 mins post-injection at the highest doses of AII, SBP of rats had returned to baseline values. The curve indicates that at doses above 40ng AII the mechanisms facilitating increased blood pressure in response to AII were saturated.

Figure A2. **The vascular response over time of chow-fed animals to varying bolus injections of Angiotensin II**

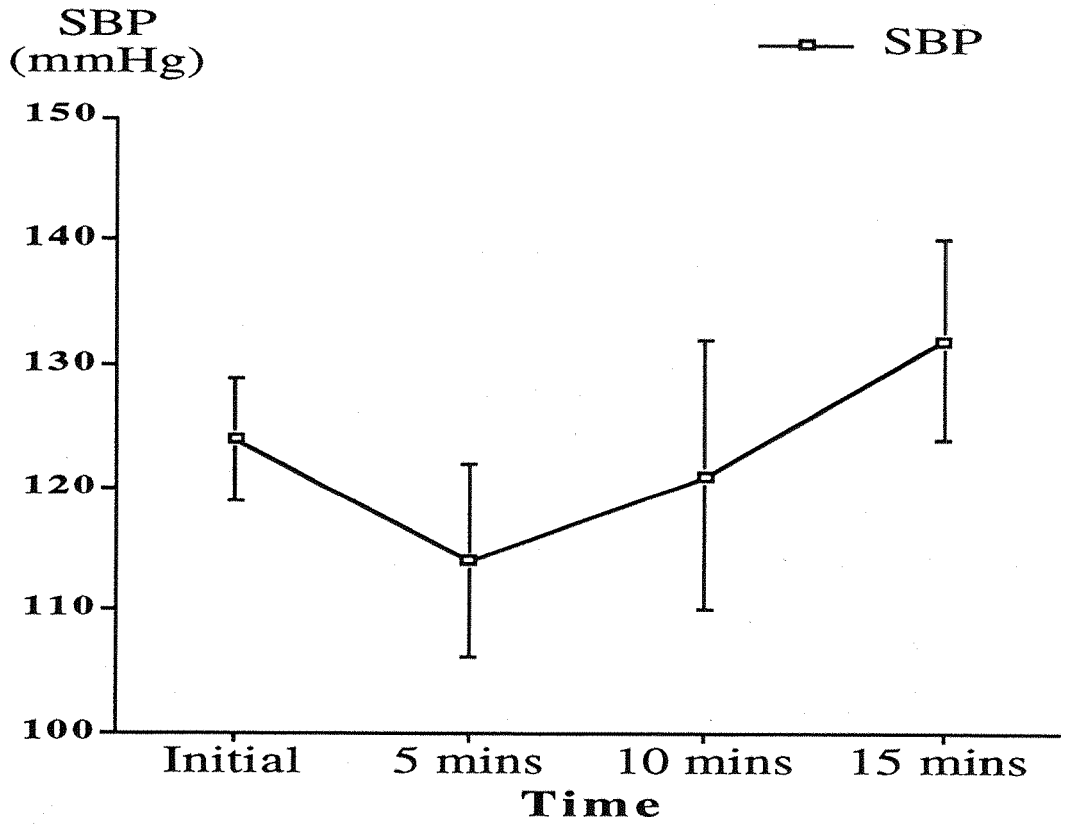


The graph represents data (mean ±SEM) for n=7 animals at each dose of angiotensin II. Angiotensin II (AII) was injected directly into the femoral vein as described in *Methods* 2.5. at doses of between 1.25-20 ng AII. The graph indicates that after approximately 3mins the elevated systolic blood pressure in response to AII infusion had returned to initial baseline values.

APPENDIX 5

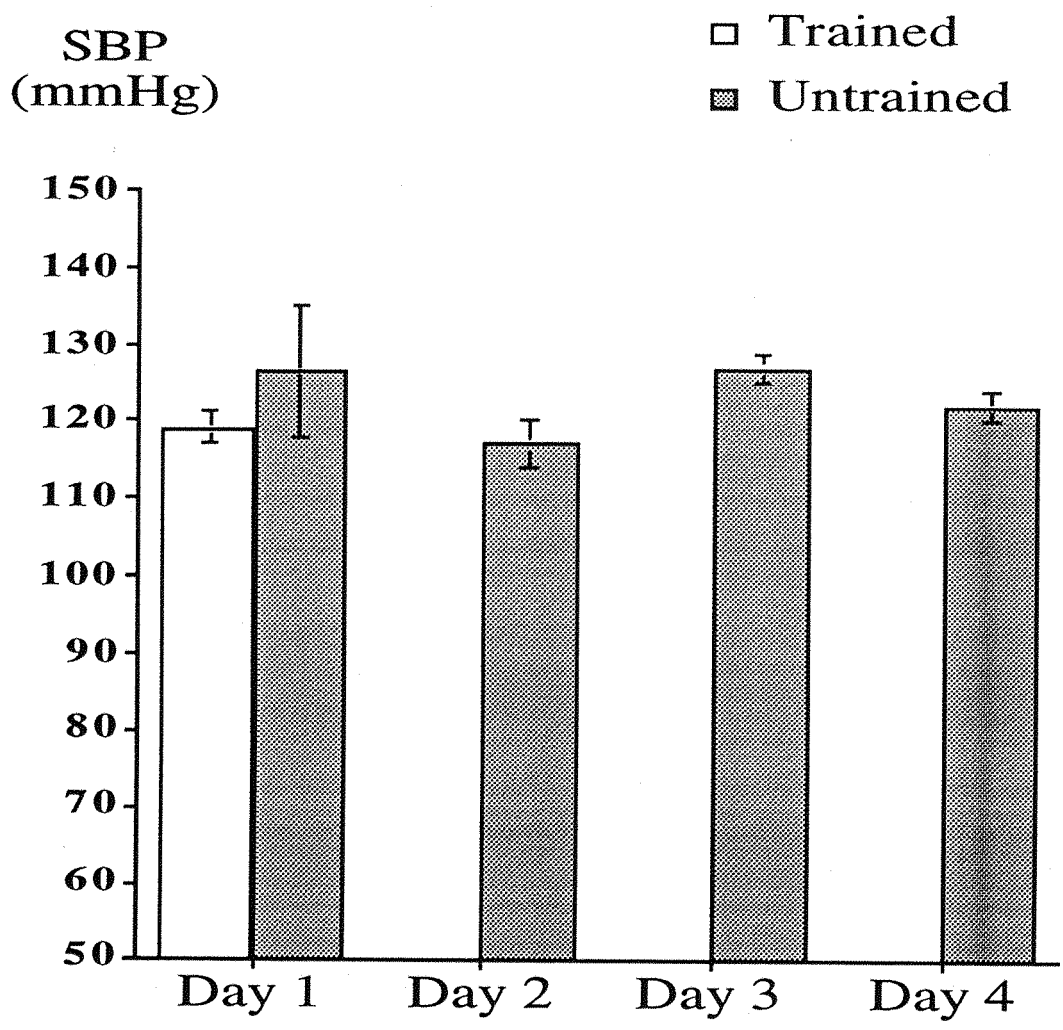
VALIDATION OF BLOOD PRESSURE MEASUREMENTS

Figure A3. **Effect of time spent in the restraint tube**



Data represent the mean ±SEM for n=6 rats. Rats were placed into a restraint tube and the blood pressure was recorded immediately and 5, 10 and 15 minutes later. Blood pressure fell over the initial 3-5mins in the restraint tube. Pressure rose again after 5-7 minutes in the restraint tube. The optimum time therefore for measurement of systolic pressure was established to be approximately 5mins following entry to restraint tube.

Figure A4. **Effect of training**



Rats were either trained to the blood pressure apparatus for 3 days prior to measurement or not introduced to the apparatus until the day of measurement. During training the rats (n=6/group) were twice placed in the perspex restraint tube for approximately 5mins each day. Figure A4 illustrates the effect of training on the initial blood pressure of the animals. The initial systolic blood pressure of untrained animals was not significantly different to that of trained animals (121 ±2mmHg *vs* 128mmHg respectively, P= NS) and further measurements taken at 1, 2, and 3 days later indicated that the blood pressure of previously untrained animals did not differ significantly from their initial value (paired t-test, P= NS).

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