AN IMMUNOHISTOCHEMICAL EVALUATION OF THE EFFECT OF SALT (NaCl) ON ADRENAL ADRENOMEDULLIN CONTENT IN DAHL RATS.

by

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DECLARATION

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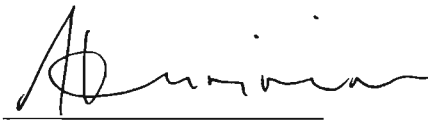
Dear Sir

I, Arvind Hariram, registration number 9602355, hereby declare that the dissertation entitled **AN IMMUNOHISTOCHEMICAL EVALUATION OF THE EFFECT OF SALT (NaCl) LOADING ON ADRENAL ADRENOMEDULLIN CONTENT IN DAHL RATS** is the result of my own investigation and research and that it has not been submitted in part or in full for any other degree or to any other University.



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ABSTRACT

Adrenomedullin (ADM) is a 52 amino acid vasodilator peptide isolated, in 1993, from human pheochromocytoma. It has been demonstrated in the adrenal medulla of several mammalian species, including humans and rats. There have been conflicting results of the tissue distribution in the adrenal cortex. Hypertension is a complex trait with multiple genetic and environmental influences. Furthermore, salt-sensitive hypertension is characterized by a cluster of renal, hormonal, and metabolic derangements that might favour the development of cardiovascular and renal complications. Therefore the objective of this study was to investigate the adrenal distribution of ADM as well as to semi-quantitatively assess the adrenomedullin secretory capacity of the adrenal gland in the rat model of salt sensitive hypertension.

Fourty-four male weanling rats were divided into 4 experimental groups and placed on a dietary regimen for 6 weeks viz. Dahl salt sensitive (DSS) rats on a high sodium diet (8% NaCl), DSS on a normal sodium diet (1% NaCl) matched with normotensive Dahl salt resistant (DSR) rats on the same dietary treatments.

Blood pressure was monitored by tail-cuff readings and by the end of the six weeks, the DSS rats developed hypertension with tachycardia irrespective of the diet they were fed. The normal sodium diet was found to delay the development of hypertension, whilst the high sodium diet exacerbated the development of hypertension. Kidney weights and heart weights were greater in DSS rats than DSR rats probably due to their renal pathology or cardiac hypertrophy.

Adrenomedullin immunopositivity was found predominantly in the adrenal medulla, and to varying degrees in the zona glomerulosa and zona reticularis of the adrenal cortex. The semi-quantitative analysis indicate that there was a 6.3 fold increase in ADM content of DSS rats compared to the DSR rats, where both consumed the 1% NaCl supplemented diet (DSR : 5.98 ± 0.3 vs. DSS : 37.85 ± 0.5, $p < 0.0001$). Similarly, there was a 4.1 fold increase in the ADM content of DSS on 8% NaCl compared to the DSR on 8% NaCl diet (DSR : 18.49 ± 0.5 vs. DSS : 75.13 ± 1.6), which was considered extremely significant.

Dahl salt-sensitive hypertensive rats, on either a normal or high NaCl diet, displayed an increase in adrenomedullin content, which suggests a possible compensatory role of this hormone in experimental hypertension. Therefore, the Dahl rat may be considered an appropriate model for the testing of new therapeutic agents, which may stimulate the secretion of adrenomedullin, in future hypertension research.

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1. INTRODUCTION

Hypertension, which has been plaguing our generations for thousands of years, can be traced back as far as the Nei Ching, which is the oldest of the extant medical writings, where the Yellow Emperor observed, "Hence, if too much salt is in the food, the pulse hardens, tears make their appearance, and the complexion changes" (Rettig *et al.*, 1989). This association of high salt intake and hypertension has been exhaustively documented for decades, but to date this still proves to be a "disease" affecting numerous communities leading to increased mortality. Hypertension, which is also referred to as the "silent killer", as the symptoms of high blood pressure can often go undetected until it is too late, may lead to strokes and heart attacks which are the main causes of death in South Africa (Villiers, 1995).

Human essential hypertension appears to be a salt related disease. Societies consuming low amounts of salt have no hypertension, yet if such people migrate to a society with high salt intakes, ~30% of them show a significant rise in blood pressure. Similarly, certain strains of rats develop a significant rise in blood pressure when placed on a high sodium diet. Thus it is likely that the mechanisms that cause a high NaCl intake to raise blood pressure in animals are the same mechanisms, that cause a high NaCl diet to raise blood pressure in susceptible people.

The unique animal model of experimental hypertension developed by Dahl has provided unequivocal evidence that several environmental stimuli produce hypertension only in the presence of the appropriate genetic trait. The Dahl salt-sensitive (DSS) rat is genetically

predisposed to become hypertensive, whilst the Dahl salt-resistant (DSR) rat is genetically predisposed to resist hypertension when exposed to a high sodium diet.

The human body, however, is essentially a complex of regulatory mechanisms, which is able to assess the physiological status of the body, and make the required adjustments to restore the normal state, once it has been disturbed. Over the last couple of decades, the concept of endocrinology has undergone numerous changes. One important change being the secretion of peptide hormones from organs not actually referred to as "classical" endocrine organs. Some organs now referred to as endocrine organs include the brain, heart, vascular tissue and adipose tissue. With the recent discovery of adrenomedullin (ADM), this "idealistic" concept of endocrinology has been exponentially revolutionized due to its ubiquitous nature.

This novel hypotensive peptide, adrenomedullin, was discovered in human pheochromocytoma by monitoring the elevation of cyclic-AMP by Kitamura *et al.* (1993). Adrenomedullin has a diverse range of biological actions, which include vasodilation, cell growth, regulation of hormone secretion and natriuresis. It has been established as being a ubiquitous regulatory peptide, produced in a great number of cell types and tissues.

ADM originally identified in the adrenal medulla has binding sites in the adrenal gland, however, its role in the adrenal medulla is unclear. Studies conducted on the tissue distribution of adrenomedullin in the adrenal gland have been proven to be rather

contradictory. Furthermore, no preliminary quantification studies have been implemented with the specific purpose of assessing the adrenomedullin secreting capacity of the adrenal glands.

Since the DSS rats has many features in common with human essential hypertension it therefore, provides a unique experimental model with which to unravel mechanisms concerning the role of sodium in human essential hypertension, and in this study, the response of adrenomedullin to increased blood pressure.

Owing to the lack of scientific evidence on the adrenal tissue distribution of adrenomedullin, this study was designed to shed more light on this topic by using immunoreactive methods as well as to semi-quantitatively analyze its ADM content in salt loaded Dahl rats.

2. LITERATURE REVIEW

2.1 HYPERTENSION

2.1.1 Definitions

It has been known for over a century that high arterial pressure lessens life expectancy and for half that time in most patients the raised pressure and its consequences constitute the disease (essential hypertension) (Laragh and Brenner, 1990). A more general operational definition which states that hypertension is that level of blood pressure at which detection and treatment does more good than harm, offers a new challenge to define hypertension as a risk factor rather than a pathological condition (Evans and Rose, 1971). The World Health Organization classified hypertension as systolic pressure of 140 mm Hg or greater and diastolic blood pressure of 90 mm Hg or greater. Table 1 shows the classification system to rank blood pressure values for adults.

Table 1 : Classification system to rank blood pressure values for adults (Tortora and Grabowski, 1996).

	SYSTOLIC	*DIASTOLIC*
OPTIMAL	Less than 120mm Hg	Less than 80 mm Hg
NORMAL	Less than 130 mm Hg	Less than 85 mm Hg
HIGH-NORMAL	130 - 139 mm Hg	85 - 89 mm Hg
HYPERTENSION	140 mm Hg or greater	90 mm Hg or greater
Stage 1 (mild)	140 - 159 mm Hg	90 - 99 mm Hg
Stage 2 (moderate)	160 - 179 mm Hg	100 - 109 mm Hg
Stage 3 (severe)	180 - 209 mm Hg	110 - 119 mm Hg
Stage 4 (very severe)	210 mm Hg or higher	120 mm Hg or higher

2.1.2 Types and causes of Hypertension

Hypertension, also known as "hardening of the pulse" has been suspected as the underlying cause for stroke and myocardial infarction. Humans develop hypertension very spontaneously and approximately 90 - 95% of all cases are primary (essential) hypertension. The remaining 5 - 10% of cases is secondary hypertension. Hypertension is a common abnormality in humans, and can be produced by many diseases as reflected in Table 2.

Table 2 : Principal causes of sustained hypertension in humans (Ganong, 1989).

Principal causes of sustained diastolic hypertension in humans
1. Unknown
2. Adrenocortical diseases
a. Hypersecretion of aldosterone (Conn's syndrome)
b. Hypersecretion of other mineralocorticoids (hypertensive form of congenital virilizing adrenal hyperplasia; 17α - hydroxylase deficiency)
c. Hypersecretion of glucocorticoids (Cushing's syndrome)
3. Catecholamine-secreting tumors of adrenal medullary or paraganglionic origin (pheochromocytoma)
4. Tumors of juxtaglomerular cells
5. Narrowing of one or both renal arteries (renal hypertension)
6. Renal disease
a. Glomerulonephritis
b. Pyelonephritis
c. Polycystic disease
7. Narrowing (coarctation) of the aorta
8. Severe polycythemia
9. Oral contraceptives

Primary (essential) hypertension is of unknown etiology : its diverse hemodynamic and pathophysiologic derangements are unlikely to result from a single cause (*http://www.geocities.com/CollegePark/Den/5701/hypertension.htm*). Several factors combine to predispose a person to hypertension, including diet, lack of exercise, metabolic effects and stress. Heredity is a predisposing factor, but the exact mechanism is unclear. Environmental factors (eg, dietary Na, obesity, stress) seem to act only in genetically susceptible persons.

Early in the course of essential hypertension, the blood pressure elevations are intermittent and there is an exaggerated pressor response to stimuli such as cold and excitement that produce only a moderate blood pressure elevation in normal individuals (Ganong, 1989). This suggests that overactive autonomic reactions are responsible for the arteriolar spasm, and treatment with drugs that block sympathetic outflow markedly slows the progress of the disease. Later the blood pressure elevation becomes sustained. The baroreceptor mechanism is "reset" so that the blood pressure is maintained at the elevated level (Ganong, 1989).

Irvine H. Page, a former director of the Cleveland Clinic Foundation and pioneer in the study of cardiovascular diseases, developed the "mosaic" theory of hypertension. This theory suggested that instead of being traceable to a single cause, high blood pressure resulted from complex interactions of many regulatory systems. Page was also one of the first scientists to recognize that hypertension was a disease, and was treatable.

2.1.3 Salt and hypertension

Excessive dietary salt has long been touted as a primary cause of hypertension. Ancient predecessors made the association of salt in the diet and hedonistic eating patterns with apoplexy and hardening of the pulse.

Although many controversies still exist regarding the role of dietary sodium intake in the pathogenesis of hypertension, it is accepted that there is a strong positive correlation between sodium intake and hypertension, and even modest dietary sodium excess induces hypertension in salt-sensitive individuals who are genetically susceptible (Laragh and Brenner, 1990).

Two international studies (Intersalt and WHO-CARDIAC) were set up to examine the relationship between salt and hypertension. In the Intersalt study fifty-two centers in 32 countries each recruited 200 subjects in whom sodium intake was estimated from 24-hour urinary sodium excretion (INTERSALT Cooperative Research Group, 1988). This study provided both negative and positive data on the salt/blood pressure relationship. It was concluded from the study that "lower average sodium intake might have a favourable influence on blood pressure, on change of blood pressure with age, and hence on cardiovascular mortality."

The other study, WHO-CARDIAC, looked at various other dietary factors in addition to sodium and potassium excretion, was based on about 10,000 people aged 48-56 years in

46 communities around the world. Significantly, BP was positively related to sodium excretion in men and not in the women.

2.1.3.1 Salt Sensitivity

"Salt sensitivity" is defined by the observed changes of arterial pressure as daily salt intake is changed, which has been called the "chronic pressure-natriuresis relationship" (Guyton, 1986). It is a common trait in patients with essential hypertension and seems to have both an inherited and an acquired component (eg. is influenced by aging and renal insufficiency). Oparil *et al.* (1989) categorized patients with essential hypertension as salt sensitive when blood pressure was raised by more than 10% in response to a high salt diet.

The blood pressure response, for a given load of dietary salt, is variable amongst hypertensives and normotensives. Salt-sensitive hypertensives experience a significant rise in blood pressure when switching from a low-salt to a high-salt diet compared to the salt-resistant hypertensives who experience a minimal change in blood pressure.

A genetic sensitivity of blood pressure to alterations in dietary sodium is apparent from studies in animals and humans (Ely, 1997). The development of animal models reinforced the concept of varying degrees of salt sensitivity of blood pressure in different types of hypertension. Dahl *et al.* (1962) developed the Dahl S and R rat strains, which showed marked sensitivity of blood pressure and resistance, respectively, to the ability of high-salt intake to raise blood pressure.

Although salt sensitivity and sodium sensitivity of blood pressure are often thought of interchangeably, considerable evidence suggests that both sodium and chloride must be provided to fully express salt sensitivity (Laragh and Brenner, 1990). Thus, in animal models such as the Dahl-S, DOCA-salt and stroke-prone spontaneously hypertensive rat (SHR-sp), feeding sodium without chloride or chloride without sodium does not raise blood pressure to the extent that NaCl feeding does (Boegehold and Kotchen, 1991).

2.1.3.2 Some mechanisms suggested in the pathogenesis of salt sensitivity

2.1.3.2.1 Volume

Kotchen *et al.* (1989) postulated that the observation that the concomitant administration of both sodium and chloride is required for the development of salt sensitive hypertension may provide a clue to the mechanism of salt sensitivity. Volume mechanisms have been implicated in the pathogenesis of NaCl-sensitive hypertension in both man and experimental models, including the Dahl S rat (Overbeck, 1981; Schackow and Dahl, 1966; Roman and Osborn, 1987).

Increasing evidence suggest that hypertension in the Dahl S rat is related to an impaired natriuretic capacity of the kidney and an expanded extracellular fluid volume or plasma volume on a high NaCl intake (Overbeck, 1981; Roman and Osborn, 1987).

Kotchen *et al.* (1989) concluded that expansion of the extracellular fluid volume by dietary NaCl contributes to the pathogenesis of salt sensitive hypertension; and that

failure of selective sodium loading (without chloride) to increase blood pressure may be related to a redistribution of sodium between the extacellular and intracellular spaces.

2.1.3.2.2 Neural Activity

There is increasing evidence for the participation of the sympathetic nervous system in the pathogenesis of salt sensitive hypertension. One proposal suggests that sodium exerts its hypertensive action by decreasing the affinity of alpha-adrenergic receptors in the medullary centers, resulting in disinhibition of sympathoinhibitory neurons (Gavras, 1986)

2.1.3.2.3 Renal Hemodynamic Effect of Chloride

Chloride itself may act as a direct renal vasoconstrictor. Schnermann *et al.* (1976) concluded that a specific renal hemodynamic effect of chloride, resulting in a decreased filtration fraction and hence a natriuretic "handicap" might contribute to the expansion of extracellular fluid volume and hypertension in NaCl-loaded animals.

2.1.3.3 Physiological Adaptations to varying Salt intakes

The body copes astonishingly well, at least in the short or medium term, with a huge range of sodium intake, from less than 10mmol to 400mmol or more (Laragh and Brenner, 1995).

Known short-term adaptations to a high salt intake include the following: extracellular fluid (ECF) is expanded; cardiac output is higher; plasma epinephrine, norepinephrine,

and dopamine fall; glomerular filtration rate (GFR) increases; plasma renin activity (PRA), angiotensin II, aldosterone fall; plasma ANP rises; and prostacyclin production increases (Laragh and Brenner, 1995).

The purpose of these adjustments is presumably to make sure that sodium output equals intake, to keep body sodium as near as possible to a standard norm, and perhaps to minimize the amount of disturbance in hemodynamics. There seems to be an impression that body sodium is kept virtually constant in spite of large changes in sodium intake. However, the evidence is to the contrary: body sodium is higher on a high sodium intake than on a low one. It is evident that the level of sodium intake affects many physiological systems and the question arises, as to whether the control systems of some people on long term salt intake develop secondary changes which are difficult to reverse (Laragh and Brenner, 1995).

2.1.3.4 Mechanisms of Sodium Homeostasis

Much has been learned about the mechanisms responsible for the relation between salt intake and arterial blood pressure (Cowley, 1997). Many known physiologic mechanisms allow us to respond to increased salt intake. An increase in salt intake results in a reduction in the activity of the renin-angiotensin-aldosterone system (salt-retaining hormones) and an increase in the release of atrial natriuretic peptide (salt-losing hormones).

Each of these systems interacts in turn with other paracrine systems within the kidney, such as the kallikrein-kinin system and prostaglandins, which either enhance or buffer the responses (Laragh and Brenner, 1995). In addition, an increase in salt intake results in a reduction in the sympathetic nerve activity to the kidneys. The net effect of these responses is an increased daily excretion of sodium.

Physical factors such as plasma colloid osmotic pressure (COP) and renal arterial blood pressure have an important influence on sodium excretion. The most important physical factor related to long term achievement of sodium and water balance is the arterial blood pressure in the kidneys. An increase in blood pressure to the kidney results in an increased excretion of sodium and water, known as pressure natriuresis. The kidney's response to changes of arterial pressure defines the salt sensitivity of individuals.

2.1.3.5 The kidneys as a common element in salt sensitive hypertension

Salt-sensitive hypertension is characterized by a cluster of renal, hormonal, and metabolic derangements that might favour the development of cardiovascular and renal complications (Ferri *et al.*, 1998). Although there is strong evidence that the kidney is the final common pathway in the long-term control of pressure, the initial abnormality of the kidney need not be intrinsic to the development of hypertension (Cowley *et al.*, 1995). A variety of factors can lead to a reduction of renal excretory function and result in hypertension. These include circulating hormones such as angiotensin II, aldosterone, atrial natriuretic peptide, renal sympathetic nerve activity which have an important influence on the pressure-natriuresis relation and lead to various forms of hypertension.

In a series of studies carried out in anaesthetized rats it was found that changes of various hormones and the renal sympathetic nerves influence the acute pressure-natriuresis relation and thereby predict salt sensitivity to long-term changes in sodium intake (Cowley, 1997). Factors that resulted in salt-sensitive forms of hypertension are high aldosterone, high adrenergic hormones, high COP, low prostaglandin E2, low kinins, and low atrial natriuretic factor. These are largely sodium- retaining hormones and paracrine systems that influence tubular reabsorption of filtered sodium.

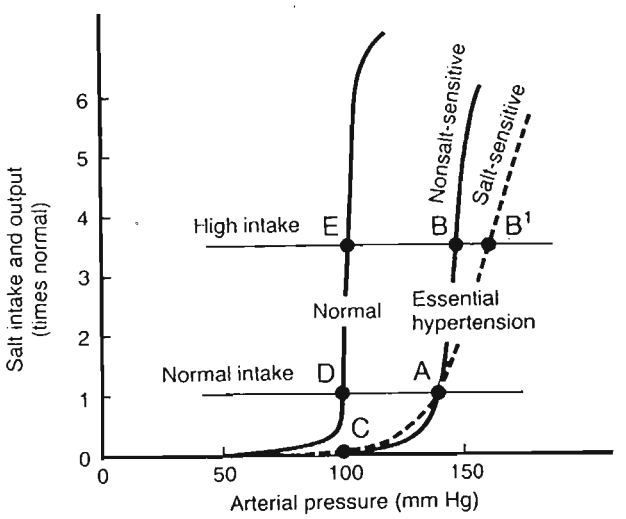


Figure 1 **:** Analysis of arterial pressure regulation in non-salt sensitive essential hypertension and salt-sensitive essential hypertension (Guyton, 1981).

The reasons for the failure of the kidneys of essential hypertensive people to excrete salt and water at normal pressure levels is unknown (Guyton, 1981). Figure 1. illustrates arterial pressure regulation in non-salt sensitive essential hypertension. In both instances the curves are shifted to the right, to a much higher pressure level than for normal people. In the case of the person with non salt-sensitive essential hypertension, the arterial

pressure does not increase significantly when changing from normal salt intake to high salt intake. However, in patients who have salt-sensitive essential hypertension, the high salt intake exacerbates the hypertension. The reason for the difference between nonsalt-sensitive essential hypertension and salt-sensitive hypertension is presumably structural or functional differences in the kidneys of these two types of hypertensive patients (Guyton, 1981).

2.1.4 Animal models in Hypertension

Humans are the only mammals to spontaneously develop hypertension. Unfortunately, there are no animal models that are "naturally" hypertensive because all mammals continually lose fluids due to evaporation and obligatory excretion by the kidney.

Over the past 50 years various animal models of hypertension have been developed.

Characteristics of an ideal animal model

"An ideal animal model should have five characteristics:

i) mimic the human disease,

ii) allow studies in chronic, stable disease,

iii) produce symptoms which are predictable and controllable,

iv) satisfy economical, technical and animal welfare considerations, and

v) allow measurement of relevant cardiac, biochemical and hemodynamic parameters. "(Doggrell and Brown, 1998).

Many species of laboratory animals have been used in hypertension research. Some of the procedures that have been reported to produce sustained hypertension in experimental animals are listed in Table 4. The majority of genetic and induced forms of experimental hypertension have been studied in rats. The major advantage of this species seems to be the availability of strains with different genetic predispositions to the occurrence or induction of experimental hypertension.

2.1.4.1 Rat Models of Hypertension

The use of rat models is rational, due to the costs of maintenance and also due to the fact that a wide range of techniques may be used to explore certain relevant parameters. However, the use of animal models is limited by ethical concerns and opposed opinions within the community on the necessity for the use of animals in research. Some rat models develop terminal illness and death due to certain procedures and this is inevitable if these rats are valid models of human diseases with high morbidity and mortality.

The study of hypertension, or to be more specific, salt-sensitive hypertension has been helped by the introduction of inbred rat models, especially the Dahl/Rapp salt sensitive and salt resistant rats.

2.1.4.2 Dahl Rats

2.1.4.2.1 *History*

In the 1950s, the effects of a high salt diet on the blood pressure were studied (Meneely and Bail, 1958). They remarked that "there was a marked degree of individual variation" in the blood pressure response to salt ingestion.

Rapid evolution of salt sensitivity is strongly supported by the observations of Dahl and Shakow's animal model (Dahl and Shackow, 1964). They showed that artificial selection of rats could, in only three generations, produce one strain with extreme salt sensitivity and another with extreme salt resistance.

Dahl and colleagues noted that an unselected population of Sprague-Dawley rats demonstrated striking variation in the effect of a high salt intake on blood pressure. To test if this was related to genetic factors, his group examined the effect of selective breeding on the blood pressure response to salt.

First the blood pressure response to high dietary salt intake was determined in an unselected group of rats. Matings were produced between a pair with the lowest change in blood pressure with salt loading, the ancestors of the salt-resistant (SR) strain, and another pair with the greatest increase in blood pressure, the ancestors of the salt-sensitive strain.

Table 3 : Procedures that produce hypertension (Ganong, 1989).

Procedures that produce sustained hypertension in experimental animals
1. Interference with renal blood flow (renal hypertension)
a. Constriction of one renal artery ; other kidney removed (one-clip, one-kidney Goldblatt hypertension)
b. Constriction of one renal artery ; other kidney intact (one-clip, two-kidney Goldblatt hypertension)
c. Constriction of aorta or both renal arteries (two-clip, two-kidney Goldblatt hypertension)
d. Compression of kidney by rubber capsules, production of perinephritis, etc
2. Interruptions of afferent input from arterial baroreceptors (neurogenic hypertension)
a. Denervation of carotid sinuses and aortic arch
b. Bilateral lesions of nucleus of tractus solitarius
3. Treatment with corticosteroids
a. Deoxycorticosterone and salt
b. Other mineralocorticoids
4. Partial adrenalectomy (adrenal regeneration hypertension)
5. Genetic
a. Spontaneous hypertension in various strains of rats
b. Salt-induced hypertension in genetically sensitive rats

The first generation of these selective matings demonstrated a significant difference in the blood pressure-raising effects of high salt intake. This process was continued for two more generations, at which time there was no overlap between SS and SR responses to the blood pressure-raising effects of high dietary salt. In this way 2 strains of rats that were either susceptible or resistant to the hypertensive effects of a high salt diet were developed.

These rats were termed the Dahl (SS/Jr) and Dahl (SR/Jr). Harlem Sprague Dawley, Inc has maintained S and R rats with a program of strict inbreeding since receiving these strains from Dr John Rapp in 1986. By 1991, the inbreeding of both these strains for more than 50 generations resulted in a very high level of genetic homogeneity within each strain and, as a result, very reliable physiological responses.

2.1.4.2.2 Characteristics of Dahl rats

The salt sensitive Dahl rats develop severe and fatal hypertension when fed high salt diets, whereas salt resistance rats do not develop such a severe hypertension upon salt loading. Also when fed normal salt diets, the salt sensitive rats become hypertensive, demonstrating that this is a model of genetic hypertension, with the extra feature of salt sensitivity (Rapp, 1982).

Simchon *et al.* (1991) suggested that initiation of hypertension induced by 8% dietary salt for 4 weeks in Dahl S rats was due to an increased cardiac output (CO) accompanied by an expansion of blood volume. Also, they found that progression of hypertension after 8 weeks on this salt diet was associated with an increase in total peripheral resistance (TPR), whereas CO decreased to below normal.

A recent study conducted in this laboratory collaborated with the findings of Simchon *et al.* (1991). When the Dahl S rat is fed a high salt diet (8% NaCl), mean arterial pressure typically increases by 20 mmHg within 24 hours and continues to rise to about 170 mmHg or higher around 2 weeks (Channa, unpublished). The initial rise in arterial

pressure appears to be triggered by sodium retention. The animal gains about 7% in body weight and plasma volume and cardiac output increases significantly. Later, the cardiac output returns towards control values and the hypertension is maintained by increased peripheral resistance.

The age at which a high salt diet is started partly determines the magnitude of the blood pressure response in Dahl S rats. When Dahl salt sensitive rats were placed on a high salt (8% NaCl) diet at weaning (21-23days of age), they rapidly developed hypertension and all died by the 16^{th} week of salt feeding. If high salt feeding was delayed until 3 months of age, the hypertension developed less rapidly and blood pressure went to about 185 mmHg by 16 - 20 weeks (Dahl *et al.*, 1968).

Renal injuries appear in Dahl S rats after 2 to 3 weeks of a high salt diet; the lesions are of a focal nature and comparable to malignant hypertensive renal disease seen in humans (Karlsen *et. al.*, 1997). When Dahl salt sensitive rats are placed on a high salt diet early in life, they typically die after 4 to 8 weeks (Rapp and Dene, 1985). The reason for the rapid development of end-stage renal disease in the Dahl S rat is unknown, however it has been suggested that the glomeruli are exposed to the damaging effect of an elevated pressure caused by a decrease in afferent arteriolar resistance (Azar *et al.*, 1979). There is a widespread misconception that Dahl salt sensitive rats become hypertensive only when placed on a high NaCl diet. In fact, on normal rat chow that contains 1% NaCl rats become markedly hypertensive, but it just takes a longer time (months instead of weeks) (Sustarsie *et al.*, 1981).

2.1.4.2.3 Suitability of Dahl salt sensitive rats in salt-sensitive hypertension

Understanding salt-sensitive hypertension in humans has been helped by the introduction of the inbred rat models, especially the Dahl/Rapp salt-sensitive and salt-resistant rats. The development of hypertension and heart failure in the Dahl/Rapp salt-sensitive rat can be controlled by titration of the amount of salt in their diet (Dahl and Heine, 1975). Therefore, when one considers a NaCl-related type of hypertension such as essential hypertension in humans or in the Dahl salt sensitive rat, it is usually the combination of a high-NaCl diet plus a kidney with slow Na excretion which brings about a rise in blood pressure.

In Dahl S rats the rate of hypertension development is partly related to the daily salt intake. A 4% NaCl diet brings about 30% rise in blood pressure by the 11^{th} week of feeding while in the same period the 8 % NaCl diet induces a 70% rise in blood pressure (Dahl *et al.*, 1968). This allows for the Dahl S rat to serve as a model of hypertension which can be studied both in its acute and chronic forms.

In conclusion, the Dahl S rats have many features in common with human essential hypertension and provide a unique experimental model with which to unravel mechanisms concerning the role of sodium in human essential hypertension.

2.2 ADRENOMEDULLIN

2.2.1 History

In 1993, a group of scientists in Japan "accidentally" discovered a new hypotensive peptide while screening a panel of peptides extracted from a pheochromocytoma. This new regulatory peptide was discovered by monitoring its stimulating action on platelet cAMP production. Since it was derived from the adrenal medulla, it was termed "adrenomedullin".

Their findings was reported in a seminal paper entitled "Adrenomedullin : a novel hypotensive peptide from human pheochromocytoma" (Kitamura *et al.*, 1993), and since this report, several hundred papers have been published regarding the regulation of its secretion and multiplicity of its actions.

2.2.2 Structural Identification of Adrenomedullin

Human ADM consists of 52 amino acids and has one intermolecular disulphide bond (Kitamura *et al.*, 1993). Figure 2 shows the amino acid sequence of human ADM.

The carboxy-terminal tyrosine residue is amidated, a feature that is often observed in other biologically active peptides. The sequence homology of ADM with human cGRP and amylin is not so high, although they share a 6-residue ring structure formed by an intramolecular disulphide linkage and the C-terminal amide structure (Kitamura *et al.*, 1993). Therefore, adrenomedullin is thought to belong to the cGRP superfamily.

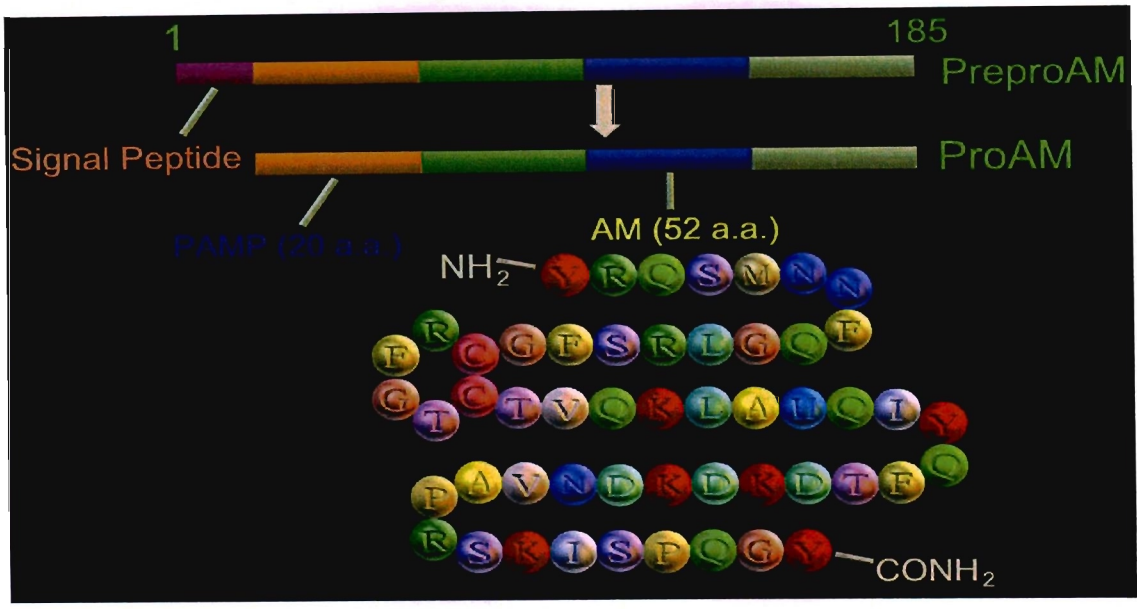


Figure 2 **:** Amino acid sequence of human ADM.

(http://www.fantom3.com/Eric/gallerypages/AMseq.htm)

Porcine adrenomedullin is nearly identical to the human peptide, with only a single substitution (glycine for asparagine) at position 40 (Kitamura *et al.*, 1994). Rat ADM consists of 50 amino acids similar to, but distinct from human ADM. Compared with the human peptide, 2 residues have been deleted and 6 residues substituted in rat adrenomedullin (Figure 3). The ring structure and carboxy-terminal amide structure, both of which are essential for the hypertensive activity of ADM, are consistent between species.

2.2.3 Synthesis of Adrenomedullin

Adrenomedullin is synthesized as part of a larger precursor molecule, termed preproadrenomedullin. As shown in Figure 4, human preproadrenomedullin consists of 185 amino acids, which include the adrenomedullin sequence (Kitamura *et al.*, 1993). The predicted sequence of proadrenomedullin contains a Gly-Lys-Arg segment

Human	YRQSMNNFQGLRSFGCRFGTCTVQKLAHQIYQFTDKDKDNVAPRSKISPQGY-NH_2
Pig	YRQSMNNFQGLRSFGCRFGTCTVQKLAHQIYQFTDKDKDGVAPRSKISPQGY-NH_2
Bovine	YRQSLNNFQGLRSFGCRFGTCTVQKLAHQIYHFTDKDKDGSAPRSKISPQGY-NH_2
Rat	YRQSMN--QGSRSTGCRFGTCTMQKLAHQIYQFTDKDKDGMAPRNKISPQGY-NH_2
Mouse	YRQSMN--QGSRSNGCRFGTCTFQKLAHQIYQLTDKDKDGMAPRNKISPQGY-NH_2
Dog	YRQSMNNFQGPRSFGCRFGTCTVQKLAhqiyqftdkdkdnvaprskispqgy-NH_2

Figure 3 : A comparison of the amino acid sequences of adrenomedullin from different species (Hinson *et al.*, 2000).

immediately adjacent to the carboxy-terminal Tyr residue of adrenomedullin. Gly-X-Y, where X and Y are basic amino acid residues, can serve as a signal for carboxy-terminal amidation (Kitamura *et al.*, 1993).

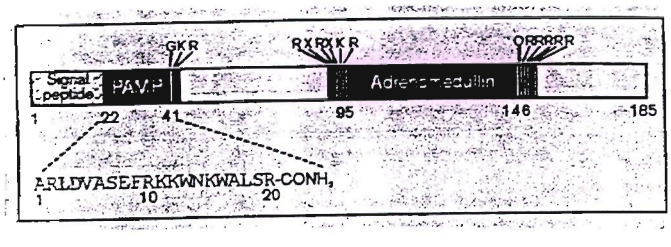


Figure 4 : Schematic structure of human adrenomedullin precursor, and amino acid sequence of human proadrenomedullin amino-terminal 20 peptide (PAMP). (Kangawa *et al.*, 1996).

Preproadrenomedullin contains a 21-amino acid N-terminal signal peptide, designated proadrenomedullin N-terminal 20 peptide or PAMP (Kitamura *et al.*, 1993). It has also been suggested that a further biological active peptide, termed adrenotensin, may be a product of the adrenomedullin gene (Gumusel *et al.*, 1995).

2.2.4 Genomic structure of adrenomedullin

The gene for human adrenomedullin was isolated from a human genomic library and its structure was determined (Ishimitsu *et al.*, 1994). The gene encoding preproadrenomedullin is termed the adrenomedullin gene and has been mapped and localized to a single locus of chromosome 11. The genomic DNA of human adrenomedullin consists of 4 exons and 3 introns, and the 5'-flanking region contains TATA, CAAT and GC boxes (Ishimitsu *et al.*, 1994). There are several binding sites for activator protein-2 (AP-2) and a cAMP-regulated enhancer element.

Studies have shown that the adrenomedullin gene is expressed in a wide range of tissues. The highest levels of adrenomedullin mRNA expression were seen in the adrenal medulla, ventricle, kidney, and lung but recent studies show that it is highly expressed in endothelial cells than even in the adrenal medulla (Sugo *et al.*, 1994). Although adrenomedullin is widely regarded as being a product of vascular endothelial cells, it appears that not all endothelial cells synthesize the peptide. This is noticeable in the rat adrenal gland, where adrenomedullin immunostaining is absent in the vascular endothelial cells and smooth muscle cells (Satoh *et al.*, 1996). It is still questionable as to whether some cell types store adrenomedullin. However, evidence shows that the secretory granules in the pancreas store adrenomedullin (Martinez *et al.*, 1996), but this has not been established in other endocrine tissues.

2.2.5 Circulating adrenomedullin

After numerous studies to measure plasma adrenomedullin levels, it was concluded with some certainty that the normal plasma concentration of adrenomedullin is in the range of 1 - 10 pM, with most values between 2 and 3.5 pM (Kohno *et al.*, 1996; Lewis *et al.*, 1998).

Evidence suggests that the major circulating form of adrenomedullin is a carboxy-terminal glycine-extended peptide, which is converted to mature adrenomedullin by enzymatic amidation (Kitamura *et al.*, 1998). The plasma concentration of glycine-extended adrenomedullin (iAM) is 2.7 ± 0.18 pM, and mature adrenomedullin (mAM) is 0.48 ± 0.05 pM (Kitamura *et al.*, 1998).

Adrenomedullin has also been measured in rat plasma, using an antiserum that recognizes both rat and human forms of adrenomedullin (Sakata *et al.*, 1994). Adrenomedullin levels measured in rat plasma were comparable to that measured in man, at 3.6 ± 0.34 pM.

Numerous studies have been conducted to measure circulating adrenomedullin in disease. In many cardiovascular disorders, plasma adrenomedullin is reported to be elevated, suggesting that increased adrenomedullin is part of the homeostasis of blood pressure, released to compensate for elevated blood pressure (Hinson *et al.*, 2000).

2.2.6 Receptors and Signal Transduction

Adrenomedullin binding has been demonstrated in most cell types and tissues of the body (Hinson *et al.*, 2000). Adrenomedullin receptors have always been closely associated with receptors for the related peptide, CGRP. Although CGRP receptors mediate some of the effects of adrenomedullin, recent studies show the presence of receptors with a higher affinity for adrenomedullin than CGRP (Ishizaka *et al.*, 1994; Eguchi *et al.*, 1994). Examination of specific ^{125}I-adrenomedullin binding sites showed high levels of abundant and specific binding for ADM in rat heart, lung, spleen, liver, skeletal muscle and spinal cord (Owji *et al.*, 1995).

Adrenomedullin seems to have little affinity for receptors for the other two members of the peptide family, calcitonin (Disa *et al.*, 1997) and amylin (Vine *et al.*, 1996).

The effects of adrenomedullin may be mediated by both specific adrenomedullin binding sites and by CGRP binding sites. The actions of adrenomedullin have been inhibited using $CGRP_{8-37}$ and $adrenomedullin_{22-52}$, a $CGRP_1$ receptor antagonist and an adrenomedullin receptor antagonist, respectively. However, neither of these peptides is especially potent or specific.

Two receptor clones have been proposed to have specific adrenomedullin binding properties, L1 (Hanze *et al.*, 1997) and the CRLR-RAMP2 combination (Buhlmann *et al.*, 1999). However, there are experiments in which neither of these candidates appears to account for specific adrenomedullin binding.

Evidence reflect that the major effect on adrenomedullin-stimulated cells is an elevation of cAMP (Takahashi *et al.*, 1997; Coppock *et al.*, 1999), which is characteristic of the calcitonin family of peptides. Therefore, the initial mechanism of action of adrenomedullin (and CGRP) is in most cases via G-protein linked receptor activation of Gs, adenylyl cyclase, and protein kinase A (PKA). However, data suggest alternative signal transduction pathways for ADM may exist.

Adrenomedullin has also been shown to activate other signal transduction mechanisms including K^+- ATP channels (Sakai *et al.*, 1998) and c-fos expression (Moody *et al.*, 1997). However, a lot more needs to be learnt about adrenomedullin signalling before its mechanisms of actions can be conclusively determined.

2.2.7 Tissue distribution of Adrenomedullin

Radioimmunassay of plasma and tissue extracts, immunohistochemical studies and detection of tissue ADM mRNA has made it possible to investigate the potential sites of synthesis and distribution of this ubiquitous peptide.

The adrenal medulla was found to display a high concentration of immunoreactive adrenomedullin. High concentrations of immunoreactive adrenomedullin was also found in the atrium, lung, pancreas and small intestine whilst the brain cortex and heart ventricle displayed smaller amounts. Table 4 summarizes the distribution of immunoreactive ADM in human tissues.

Table 4 **:** Regional distribution and plasma concentration of immunoreactive ADM in humans. Results are expressed as fmol/mg wet tissue. Plasma concentration of ADM is expressed as pg/ml. (Kangawa *et al.*, 1994).

REGION	*IMMUNOREACTIVE ADM*
Adrenal medulla	47.7 ± 26.1
Pheochromocytoma	92.1 ± 101.6
Heart atrium	1.68 ± 1.58
Heart ventricle	0.15 ± 0.02
Aorta	0.42 ± 0.09
Lung	0.80 ± 0.37
Kidney	0.35 ± 0.12
Pancreas	1.04 ± 0.35
Small intestine	0.97 ± 0.45
Liver	0.20 ± 0.06
Spleen	0.53 ± 0.10
Brain cortex	0.31 ± 0.15
Thyroid gland	0.28 ± 0.12
Plasma	17.2 ± 6.4

Jougasaki *et al.* (1995) demonstrated the immunohistochemical localization of ADM in glomeruli, cortical distal tubules, and medullary collecting ducts but not in proximal tubules of canine kidney.

The concentration of immunoreactive adrenomedullin in aorta, ventricle, kidney and lung was found to be less than 3% of that in adrenal medulla, yet high levels of adrenomedullin messenger RNA were found in the former tissues (Kitamura *et al.*, 1994;

Sakata *et al.*, 1993). This discrepancy may be explained by the possibility that adrenomedullin biosynthesized in these tissues may be rapidly and constitutively secreted into the blood, or may function as an autocrine or paracrine regulator (Bean *et al.*, 1994).

In contrast, adrenomedullin synthesized in the adrenal medulla is thought to be stored in the granules and secreted in the regulatory pathway. Therefore, the biosynthetic and excretory pathways of adrenomedullin may be different in each tissue.

2.2.8 Brief overview of the biological actions of Adrenomedullin

Initially, adrenomedullin was thought to be exclusively a vasodilator, however recent studies suggest that its more than simply a vasodepressor. This hormone has been shown to have a wide range of actions, from regulating cellular growth, actions on the cardiovascular system, central nervous system, and endocrine system.

2.2.8.1 Hemodynamic actions

Ishiyama *et al.* (1993) studied the hemodynamic effects of human adrenomedullin, using anesthetized rats. They found that administration of adrenomedullin resulted in a significant decrease in total peripheral resistance accompanied by a fall in blood pressure. Their findings indicated that human adrenomedullin is a potent vasodilator and may have some role in the regulation of blood pressure. The hypotensive activity of adrenomedullin is comparable to that of CGRP, which has been established as one of the potent vasorelaxants, indicating that adrenomedullin may serve as a hypotensive peptide that controls circulation (Kitamura *et al.*, 1995).

These effects were also noticeable in a study using hypertensive rats (Khan *et al.*, 1997). Furthermore, the hypotensive effect of adrenomedullin on mean arterial pressure in the anesthetized rat is not inhibited by $CGRP_{8-37}$, indicative that this effect is not mediated via CGRP receptors (Nandha *et al.*, 1996).

Adrenomedullin was found to lower vascular resistance in rat lung, heart, kidney and adrenal gland (He *et al.*, 1995). Ebara *et al.* (1994) demonstrated that adrenomedullin caused an increase in renal blood flow (RBF), suggesting it to be a potent vasodilatory peptide with a diuretic effect. They concluded that adrenomedullin may play an important role in the regulation of renal function.

A study using the isolated perfused rat lung preparation, showed that human adrenomedullin caused a decrease in preconstricted vascular tone with no effect on resting tone (Heaton *et al.*, 1995).

Adrenomedullin appears to have direct effects on the heart and coronary circulation and was found to exhibit inotropic effects by increasing heart contractility via a specific adrenomedullin, Ca2+ -dependent mechanism in the rat heart (Szokodi *et al.*, 1998).

2.2.8.2 Growth

Numerous studies suggest the possibility of adrenomedullin being an autocrine/paracrine growth factor in tumors and normal cells. One study found that Swiss 3T3 cells produced correctly processed adrenomedullin, which is regulated by cytokines and growth factors (Isumi *et al.*, 1998).

Also, adrenomedullin and CGRP stimulated DNA synthesis and cell proliferation in rat vascular smooth muscle cells (Iwasaki *et al.*, 1998). These results provide strong evidence for a growth-promoting effect of adrenomedullin, possibly mediated via cAMP.

Adrenomedullin has also been identified as an important factor in embryogenesis and differentiation (Montuenga *et al.*, 1997; Yotsumoto *et al.*, 1998). However, the exact effects of adrenomedullin on growth and proliferation are debatable, and further research is needed to clarify this phenomenon.

2.2.8.3 Endocrine Effects

2.2.8.3.1 The pituitary

In studies conducted, adrenomedullin was found to inhibit ACTH from rat anterior pituitary cells in a dose-dependent manner and also attenuated CRH-stimulated ACTH production (Samson et *al.*, 1995). Samson *et al.* (1995) also demonstrated the ability of angiotensin II to antagonize the actions of adrenomedullin. Another study found that intravenous infusion of adrenomedullin into conscious sheep, caused significant reduction in plasma ACTH which continued to fall after cessation of the infusion (Parkes and May, 1995), suggesting that adrenomedullin plays a role in inhibiting ACTH release.

2.2.8.3.2 The adrenal gland

Adrenomedullin was found to affect the secretory activity of the adrenal cortex in both rat and human (Nussdorfer, 1996). Furthermore, it was demonstrated that adrenomedullin significantly inhibited aldosterone production in rats (Yamaguchi *et al.*, 1996).

Also, several different adrenal tissue preparations were used to investigate the effects of adrenomedullin on steroid secretion, which have produced conflicting results. Whilst some studies report the inhibitory effect of adrenomedullin on angiotensin II-stimulated aldosterone secretion in man (Andreis *et al.*, 1997), others reported that adrenomedullin stimulates aldosterone secretion (Hinson *et al.*, 1998). In intact rats adrenomedullin also causes an increase in adrenal blood flow (He *et al.*, 1995). This observation that adrenomedullin also acts as a vasodilator in the adrenal vascular bed suggests that adrenomedullin can stimulate corticosterone secretion by the rat adrenal. Studies conducted on intact perfused rat adrenal preparations suggest that this is the case (Kapas and Hinson, 1998 ; Andreis *et al.*, 1997).

A study conducted of adrenomedullin on adrenal catecholamine release in dogs (Masada *et al.*, 1999), revealed that adrenomedullin did not affect catecholamine levels, however further studies need to be conducted in this field.

2.2.8.3.3 The pancreas

The effects of adrenomedullin on the pancreas appear to be contradictory. An initial study reported the stimulatory effects of adrenomedullin on insulin secretion from isolated rat islets (Mulder *et al.*, 1996). A later study revealed the inhibitory role of adrenomedullin on insulin secretion (Martinez *et al.*, 1996).

2.2.8.4 Renal Effects

Studies have reported the role for locally produced adrenomedullin in tubular function. Intrarenal adrenomedullin administration in anesthetized rats increased renal blood flow, urine output, and urinary Na^+ excretion in a dose-dependent manner, which is indicative of direct preglomerular and postglomerular arteriolar effects (Ebara *et al.*, 1994). In the anesthetized rat, intrarenal adrenomedullin infusion lead to increases in renal blood flow, glomerular filtration rate (GFR), Na^+ excretion, and urine flow (Hirata *et al.*, 1995; Haynes *et al.*, 1995). These effects were not inhibited by CGRP8-37. Mean arterial blood pressure was lowered and RBF, GFR, and urine flow were raised by bolus administration of adrenomedullin (Vari *et al.*, 1996)

Studies have been performed in the rat model of heart failure. It was found that intravenous infusion of a low dose of adrenomedullin to normal rats or those with heart failure led to significantly increased urine volume and Na^+ excretion without changing GFR, RBF or any other hemodynamic parameter (Nagaya *et al.*, 1999). High dose adrenomedullin infusion decreased mean arterial pressure and increased cardiac output in both rat groups. They also showed that adrenomedullin reduced right ventricular systolic pressure in heart failure rats with pulmonary hypertension.

Hinson *et al.* (2000) suggested that adrenomedullin could also play a regulatory role in the endocrine function of the kidney. A study revealed that adrenomedullin elevated plasma renin levels in rats, a response thought to be secondary to the hypotensive action of adrenomedullin (Jensen *et al.*, 1997). Furthermore adrenomedullin also stimulates intrarenal renin release where renin release was shown to be from juxtaglomerular cells (Jensen *et al.*, 1997).

2.2.8.5 Respiratory effects

Adrenomedullin was found to cause pulmonary vasodilation as well as inhibiting bronchoconstriction in rats (Yang *et al.*, 1996). Also, adrenomedullin may have an anti-inflammatory role in the lung (Zhang and Phan, 1996).

2.2.8.6 CNS effects

The exact sites of adrenomedullin action in the CNS still remain unclear. However, the paraventricular nuclei and supraoptic nuclei are the most likely sites of action, since previous studies have shown adrenomedullin immunoreactivity and binding sites exist here (Satoh *et al.*, 1995).

Adrenomedullin is present in the central nervous system and may have some role in the modulation of salt appetite, thirst, and sympathetic activity. Samson and Murphy (1997) found that intracerebroventricular administration of ADM inhibits salt appetite in the rat possibly through a central action that mirrors the natriuretic effects of the peptide in the

kidney and the adrenal effects to inhibit aldosterone secretion. Earlier, Murphy and Samson (1997) reported that adrenomedullin inhibits water drinking in the rat.

The central hypertensive actions of adrenomedullin in conscious rats are dose-dependent and not antagonized by CGRP8-37 (Saita *et al.*, 1998). Direct effects of adrenomedullin on sympathetic nerve activity in conscious rats showed that adrenomedullin induced an increase in preganglionic sympathetic discharge (Saita *et al.*, 1998). To further understand the CNS effects of adrenomedullin, further attention needs to be addressed in this area.

2.2.9 Adrenomedullin and Hypertension

Due to the high specificity and sensitivity of the radioimmunoassay for adrenomedullin, a study was conducted to determine plasma levels of immunoreactive adrenomedullin in healthy volunteers and in patients with hypertension (Kitamura *et al.*, 1994). The patients were grouped according to the WHO classification of stages of hypertension, on the basis of their clinical characteristics. The plasma concentrations of immunoreactive adrenomedullin in patients with severe (WHO stage III) hypertension was significantly ($p < 0.05$) higher than that in normotensive volunteers and in patients with mild (WHO stage I) hypertension. In addition, the mean plasma level of immunoreactive adrenomedullin in patients with mild and moderate hypertension showed a nonsignificant tendency to increase in comparison with that in normotensive individuals. Thus, the augmentation of plasma adrenomedullin in patients with hypertension seems to be progressive and proportional to the severity of the disease.

Ishimitsu *et al.* (1994) studied plasma levels of adrenomedullin in patients with essential hypertension or chronic renal failure. Compared with healthy individuals, plasma adrenomedullin was increased by 26% ($p < 0.05$) in hypertensive patients without organ damage and by 45% ($p < 0.005$) in those with organ damage. The increase in plasma adrenomedullin levels was more prominent in patients with renal failure than in patients with hypertension. Adrenomedullin levels were also found to show intimate correlation with noradrenaline (norepinephrine), atrial natriuretic peptide and cAMP levels in plasma (Sugo *et al.*, 1994). Thus, it may be postulated that plasma adrenomedullin levels increase in association with changes in sympathetic nervous activity and body fluid volume in hypertension and renal failure (Kitamura *et al.*, 1995).

The plasma level of adrenomedullin in patients with congestive heart failure was 5.4 ± 0.3 pmol/L, which was significantly higher than that of healthy control individuals ($p < 0.05$) (Tanaka *et al.*, 1994).

To date, plasma adrenomedullin levels have been found to be elevated in patients with hypertension, renal failure and congestive heart failure. Considering adrenomedullin's potent hypotensive and vasodilatory effect, adrenomedullin could be expected to counteract the arteriolar vasoconstriction found in these disorders and may therefore be of benefit.

In conclusion, it is possible that adrenomedullin plays a role in the pathophysiology of hypertension (Chao *et al.*, 1997). Plasma levels of AM are found to be elevated in

In conclusion, it is possible that adrenomedullin plays a role in the pathophysiology of hypertension (Chao *et al.*, 1997). Plasma levels of AM are found to be elevated in hypertension in proportion to the severity of blood pressure elevation and to the degree of renal impairment (Ishimitsu *et al.*, 1994; Kohno *et al.*, 1996). Studies in animal models of hypertension have demonstrated that short and long term adrenomedullin infusion lowers arterial blood pressure (Khan *et al.*, 1997) as does gene delivery of human AM gene in spontaneously hypertensive rats (Chao *et al.*, 1997).

2.3 STRUCTURAL ORGANIZATION OF THE ADRENAL GLAND

2.3.1 Gross Anatomy

The paired adrenal glands occupy the superior borders of the kidneys. They are composed of two parts viz. an outer cortex and an inner medulla - which function as two separate glands. The adrenal cortex is responsible for secreting the steroid hormones, which participate in the regulation of mineral balance, energy balance, and reproductive function. The adrenal medulla secretes the hormone epinephrine and norepinephrine, which complement the sympathetic nervous system in the "fight or flight" reaction.

2.3.2 General Histology

2.3.2.1 Adrenal cortex

The cortex consists of three concentric zones:

- zona glomerulosa - thin, outermost zone
- zona fasiculata - thick, middle zone

- zona reticularis - thin, inner zone

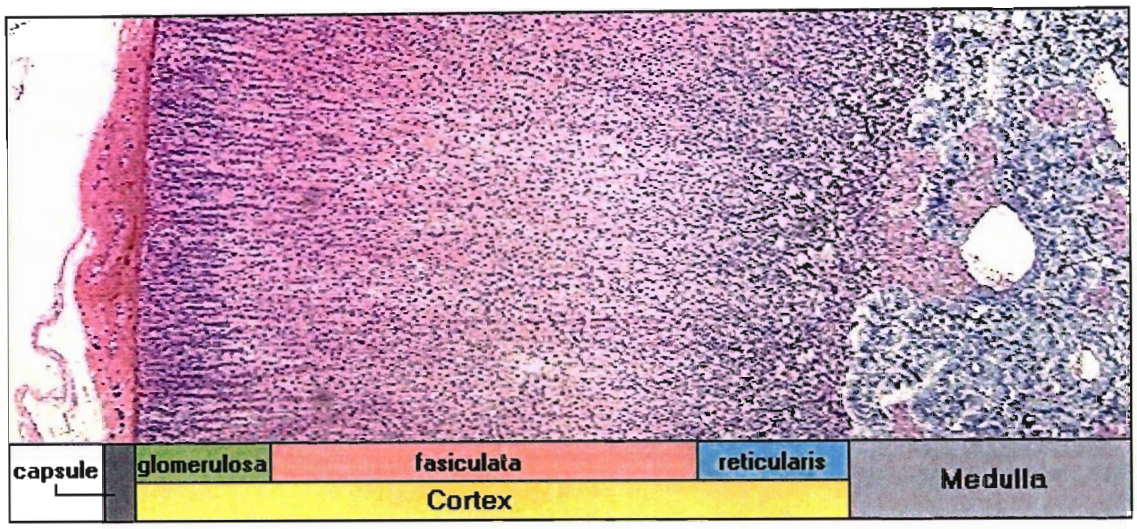


Figure 5 : Rabbit adrenal gland (H&E stain)

(http://arbl.cvmbs.colostate.edu/hbooks/pathphys/endocrine/adrenal/histo_overview)

Figure 5 shows an example of the three cortical zones and medulla in a section of a rabbit adrenal gland. The cells in the adrenal cortex consist of three concentric zones. The outermost zone is named the zona glomerulosa. The cells present in this zone appear columnar in shape and are arranged in irregular cords.

Figure 6 is a picture showing the zona glomerulosa from the adrenal of a cat and a rabbit.

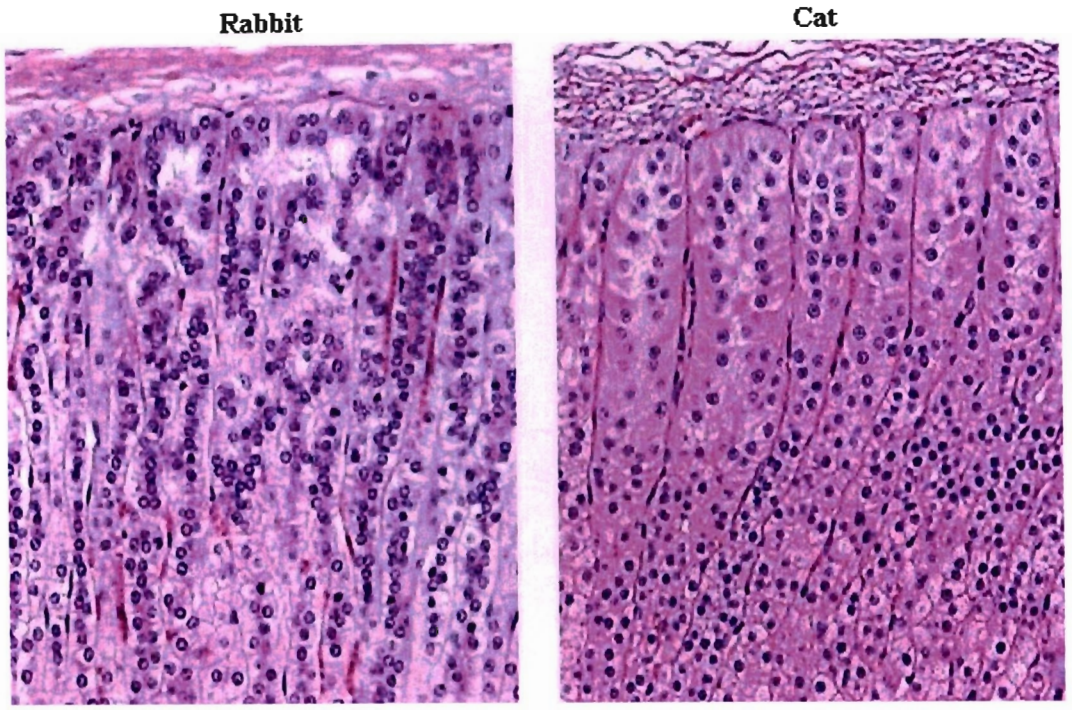


Figure 6 **:** The zona glomerulosa region of the adrenal cortex of the rabbit (left) and the cat (right) ((H&E stain)

(http://arbl.cvmbs.colostate.edu/hbooks/pathphys/endocrine/adrenal/histo_cortex)

The middle zone, which is termed the zona fasciculata, is the largest of the three zones. The cells present in this zone are polyhedral in shape, are abundant with lipid droplets, and are arranged in straight cords that radiate toward the medulla. Cortical capillaries are usually prominent within the fasiculata.

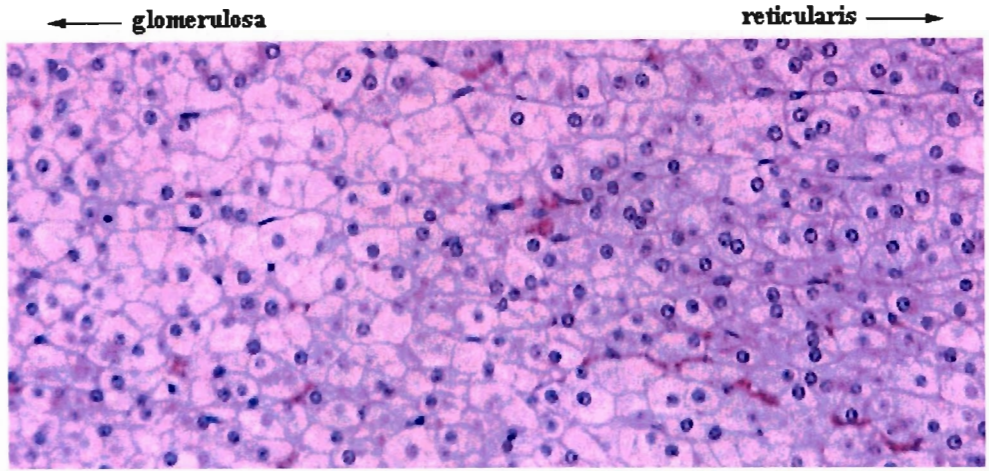


Figure 7 **:** The zona fasiculata region of the rabbit adrenal gland (H&E stain)

(http://arbl.cvmbs.colostate.edu/hbooks/pathphys/endocrine/adrenal/histo_cortex)

Figure 7 above shows the zona fasiculata in a rabbit adrenal. The innermost zone of the cortex is the zona reticularis. These cells are arranged in cords that project in different directions and anastomose with one another. Figure 8 below shows the reticularis centrally, with part of fasiculata on the left and the medulla on the right.

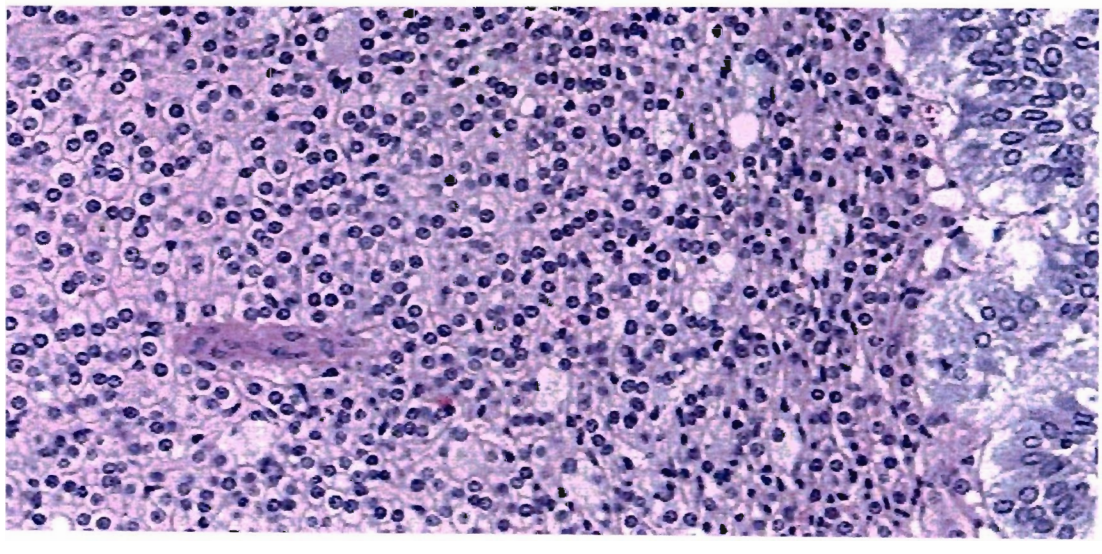


Figure 8 **:** The zona reticularis of the rabbit adrenal gland (H&E stain)

(http://arbl.cvmbs.colostate.edu/hbooks/pathphys/endocrine/adrenal/histo_cortex)

2.3.2.2 Adrenal Medulla

The most abundant cell in the adrenal medulla is the chromaffin cell. These cells are columnar in shape and rather basophilic. At high magnification, they are seen to have a granular cytoplasm due to hormone-containing granules. They are arranged in clusters, usually around medullary veins, as seen below in a picture of rabbit adrenal (Figure 9).

The adrenal medulla is richly innervated by preganglionic sympathetic fibers and small numbers of sympathetic ganglion cells can be observed in the medulla. Ganglion cells are round or polygonal with prominent nuclei.

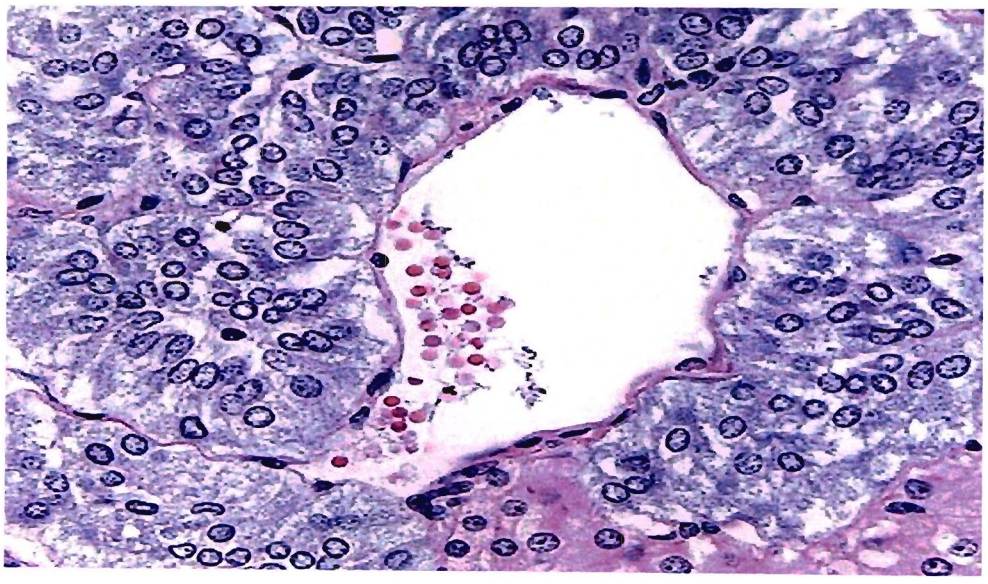


Figure 9 : The medullary region of the rabbit adrenal gland (H&E stain)

(http://arbl.cvmbs.colostate.edu/hbooks/pathphys/endocrine/adrenal/histo_medulla)

2.3.3 Vascularization and Innervation

A connective tissue capsule encases the adrenal gland and it is this capsule that extends septae into the substance of the gland. This organ is richly vascularized and capsular blood vessels, nerves and lymphatics penetrate along with the connective tissue septae.

Histological examination of the adrenal gland reveals a rich vasculature.

Ramifying the surface of the gland are numerous small arteries from several sources, which penetrate the gland in two ways:

- Cortical arteries and arterioles branch into capillary beds within the cortex to supply that area, then coalesce into veins at the corticomedullary junction.
- Medullary arteries and arterioles penetrate the cortex without branching, then form capillary beds in the medulla.

Blood from both cortical and medullary veins empties through a single large central vein, which leaves the adrenal and anastomoses with either the vena cava or renal vein.

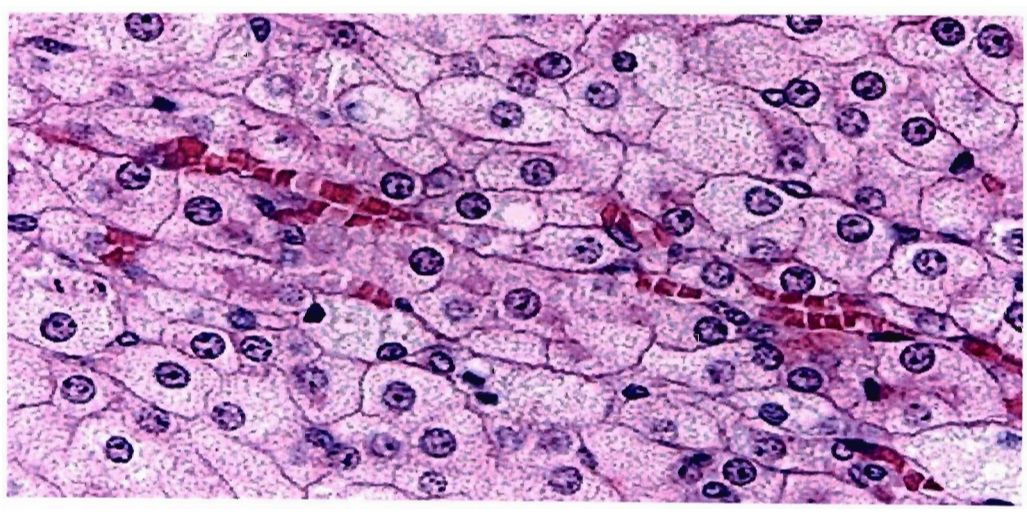


Figure 10 **:** Cortical capillaries in the zona fasiculata of rabbit adrenal gland (H&E stain)

(http://arbl.cvmbs.colostate.edu/hbooks/pathphys/endocrine/adrenal/histo_overview)

2.4 PHYSIOLOGICAL TECHNIQUES

During the course of this study, several specialized physiological techniques were employed. This subchapter aims to discuss very briefly the advantages, disadvantages and more importantly the choice of the method used. Techniques discussed include immunohistochemistry, non-invasive blood pressure recordings as well as the use of the AIN-76 nutrtional diet.

2.4.1 Priciples of Immunohistochemistry

2.4.1.1 Intoduction

Immunohistochemistrty, is a technique that employs the use of antibodies to locate antigens in tissues. Because molecules are restricted in distribution to specific cell types, the ability to identify molecules through antigenic sequences has become a powerful technique in diagnostic surgical pathology (True, 1990). The goal of immunohistochemistry is a more precise characterization of cells, infectious agents, and macromolecules that nonimmunologically based histochemical stains can usually provide. Bancroft and Stevens (1990) defined immunohistochemistry as a technique which identifies cellular or tissue constituents (antigens) by means of antigen-antibody interaction, with the site of antibody binding being identified either by direct labeling of the antibody, or by use of a secondary labeling method.

2.4.1.2 Theoretical Aspects

2.4.1.2.1 *Antigens*

Any molecule that has generated an antibody response is called an antigen. The part of the antigen that reacts with a given antibody is the specific antigenic determinant or epitope. A molecule possesses several different potential epitopes, although an antibody will bind with only one specific epitope. Although most antigens are proteins, any type of chemical can be antigenic.

Antigenicity

Antigenicity refers to the presence of antibody binding activity. Because antigenicity is dependent on the physicochemical nature of the three-dimensional structure of the antigen, it is influenced by the chemical and physical forces of tissue processing (True, 1990). "Optimal" antigenic preservation refers to retention of antigenicity that is identifiable with available antibodies. The various procedures of tissue handling which may alter antigenicity are (True, 1990):

1. Auotlysis

Ischemia and cell death, which occur after tissue is devitalized, initiate the release of lysosomal proteolytic enzymes. By metabolizing cell components, these enzymes, irreversibly alter their molecular structure and alter their antigenicity. To minimize autolysis of devitalized tissue, the endogenous proteolytic enzymes must be rapidly inactivated, either by fixation or by maintaining the tissue at a sufficiently low temperature.

2. Fixation

The purpose of fixation is threefold : inactivation of autolytic mechanisms, with retention of tissue architecture; immobilization of molecules to prevent their artifactual relocalization to other cell compartments or to prevent diffusion from the tissue; and increase in tissue rigidity for possible sectioning without embedding (True, 1990).

The degree of loss of antigens from tissue varies with the fixative. Protein losses of up to 6%, 8%, and 10% have been reported with 10% formalin, Carnoy's solution, and ethanol, respectively (Mays, 1984). Conditions that accelerate fixation - heat, agitation, and the more rapid delivery of fixative to tissues, such as by perfusion - optimize localization of antigens. The degree to which antigenicity is altered depends on both fixative and antigen. However, neither formaldehyde nor ethanol changes the antigenicity of peptide hormones to readily available antibodies (True, 1990). Furthermore, the length of fixation correlates with the degree of alteration of antigenicity (Battifora and Kopinski, 1986). The conditions of fixation also affect antigenicity, therefore fixation in an isotonic solution at neutral pH optimizes retention of native antigenicity (True, 1990).

The most common protocols and agents for fixation are:

Freezing - Freezing optimizes retention of native antigenicity and, when done rapidly, precisely localizes antigen.

Alcohols - Alcohols and acetone fix antigens and enzymes by denaturation. Antigenicity is altered minimally.

Aldehydes - Aldehydes fix by primarliy covalently cross-linking proteins between α and ε-amino groups.

Osmium - Osmium tetroxide, which is a tetrapolar compound, cross-links lipids and proteins through chelation of multiple sites (Bullock, 1984).

3. Embedding

Both the conditions of embedding and the type of embedding medium potentially alter antigenicity. Heat which is necessary for infiltration of tissues by paraffin can potentially affect antigenicity. The type of embedding material may also have a differential affect on retention of molecules. Common embedding methods are :

Freezing - Freezing provides a sufficiently rigid structure for sectioning; its advantage over other media is that it alters antigenicity the least.

Paraffins - The paraffins are complex hydrocarbons that are selected for their melting point, hardness, and ease of removal. The great advantage of paraffin is that it enables cutting of many sections with efficiency.

Plastics - Plastics possess greater hardness, and therefore facilitate thinner sectioning. Most of the plastics, as embedding media, can be removed after sectioning to regain antigenicity. (True, 1990)

2.4.1.2.2 Antibodies

Antibodies belong to the class of serum proteins known as immunoglobulins. They are glycoproteins that bind with high affinity and specificity to antigens. Antibodies are formed in the humoral immune system by plasma cells, the end cell of B lymphocyte

transformation after recognition of a foreign antigen. There are five types of antibody found in the blood of higher vertebrates, IgA, IgD, IgE, IgG and IgM. IgG is the commonest and the most frequently used antibody for immunohistochemistry. The IgG molecule is composed of two pairs of light and heavy polypeptide chains linked by disulphide bonds to form a Y shaped structure. The terminal regions of each arm vary in amino-acid sequence and are known as 'variable domains'. This variability in amino acids provides specificity for a particular epitope and enables the antibody to bind specifically to the antigen against which it was raised.

Polyclonal Antibodies

These are produced by immunizing a host animal with a purified specific molecule (immunogen) bearing the antigen of interest. The animal will mount a humoral response to the immunogen and the antibodies so produced can be harvested by bleeding the animal to obtain immunoglobulin-rich serum.

It is likely that the animal will produce numerous clones of plasma cells (polyclonal), with each clone producing an antibody with a slightly different specificty to the variety of epitopes present in immunogen. Some of these antibodies may cross-react with other molecules and will need to be removed by absorption with the appropriate antigen.

Polyclonal antiserum maximises the number of antibodies bound to a molecule that has little repetitiveness of epitopes (True, 1990). Polyclonal antisera are quicker and more readily available than monoclonal antibodies.

Monoclonal Antibodies

Monoclonal antibodies are produced by clones of plasma cells. Antibodies from a given clone are immunohistochemically identical and react with a specific epitope on the antigen against which they are raised.

The development of the hybridoma technique, by Kohler and Milstein (1975), to produce monoclonal antibodies has revolutionised immunohistochemistry by increasing enormously the range, quality and quantity of specific antisera. This approach to antibody production has dramatically increased the number of antisera available for immunohistoochemistry and has allowed for further evolution with the ability to identify more antigens in paraffin sections (Bancroft and Stevens, 1990). However, on comparison with polyclonal antibodies, they have a lower sensitivity.

2.4.1.3 Affinity

Affinity is the binding strength of an antibody to a specified antigen and is characterised as an affinity constant. The derivation of the affinity constant is as follows :

$$K \text{ (affinity constant)} = \frac{[\text{AbAg complex}]}{[\text{Ab}] \times [\text{Ag}]}$$

$$\text{Ab} + \text{Ag} \qquad \text{AbAg complex}$$

For immunohistochemistry, the higher the affinity constant, the better the antibody, because the antibody is more likely to remain bound to the antigen sought during the procedure (True, 1990).

2.4.1.4 Antibody-antigen binding

The amino acid side chains of the variable domain of an antibody form a cavity which is geometrically and chemically complementary to a single type of antigen epitope. The analogy of a lock (antibody) and key (antigen) has been used, and the precise fit required explains the high degree of antibody-antigen specificty seen. The associated antibody and antigen are held together by a combination of hydrogen bonds, electrostatic forces and van der Waal's forces.

2.4.1.5 Detection Systems

Direct fluorescent antibody methods

Indirect fluorescence techniques

Enzyme-labelled antibody methods

Unlabelled antibody-enzyme method

2.4.1.5.1 Direct Fluorescent Antibody Methods

Principle

In this method a known antigen in a section or smear of tissue is localized by virtue of its combination with fluorescently labelled molecules of its antibody. However, this method

is not capable to localize antigens at low concentrations in a tissue and due to this lack of sensitivity, it is not often used.

2.4.1.5.2 Indirect Fluorescence Techniques

Principle

In indirect methods it is possible to identify either antigens or antibodies in a tissue. The reagent common to the procedures of this type is a fluorescently labelled antiserum to immunoglobulin: an anti-antibody. This technique is superior to the direct method however some antigens occur in tissues in quantities too small to be detected by the indirect fluorescent antibody method. Greater sensitivity is achieved when the label is a histochemically demonstrable enzyme rather than a fluorochrome.

2.4.1.5.3 Enzyme-labelled Antibody Methods

Principle

In these techniques antibodies are labelled by conjugation with enzymes. Numerous methods employing enzymes as tracers for immunohistochemistry have been developed over the past two decades.

These techniques are based on the principle that enzymes may be attached to antibodies either directly or indirectly and that sites of antibody attachment to tissue sections can be demonstrated by the addition of an appropriate enzyme substrate, cofactors that are necessary for enzymatic activity, and a chromogen or capture reagent that is deposited at sites of antibody attachment (Nakane, 1975).

Horseradish peroxidase (HRP) is the most widely used enzyme and in combination with the most favoured chromogen, i.e. 3,3'-diaminobenzidine tetrahydochloride (DAB), it yields a crisp, insoluble, stable, dark brown reaction end product which is capable of enhancement (Graham and Karnovsky, 1966).

2.4.1.5.3.1 Direct Method

This is the simplest and shortest method of antigen detection (Bancroft and Stevens, 1990). The primary antiserum is conjugated directly with a tracer molecule such as the enzyme horseradish peroxidase. It is the least sensitive method.

2.4.1.5.3.2 Indirect Method

In this technique, the primary unconjugated antibody is allowed to bind to the antigen in the tissue section. A second tracer-conjugated antibody, raised in another animal host and specific for the animal and immunoglobulin class of the primary antibody, is applied to the section and allowed to bind to the primary antibody. This method is more sensitive than the direct technique and is still rapid and inexpensive.

The advantage of these techniques over the indirect fluorescent antibody method lies in its higher sensitivity. However, the next method described is much more sensitive than the indirect enzyme-labelled antibody procedure. Its sensitivity is considerably greater than that of any other immunohistochemical technique and this was the method employed during our study.

2.4.1.5.4 Unlabelled Antibody-Enzyme Method

Principle

This method localizes the primary antibody with a secondary antibody, termed the "bridging" antibody. When incubated with the section in excess, this secondary antibody "bridges" the primary to a tertiary complex, which contains the label. The tertiary peroxidase-antiperoxidase (PAP) complex is developed in the same species of animal as is the primary antibody for successful " bridging". A schematic representation of this immunostaining method is illlustrated in Figure 11.

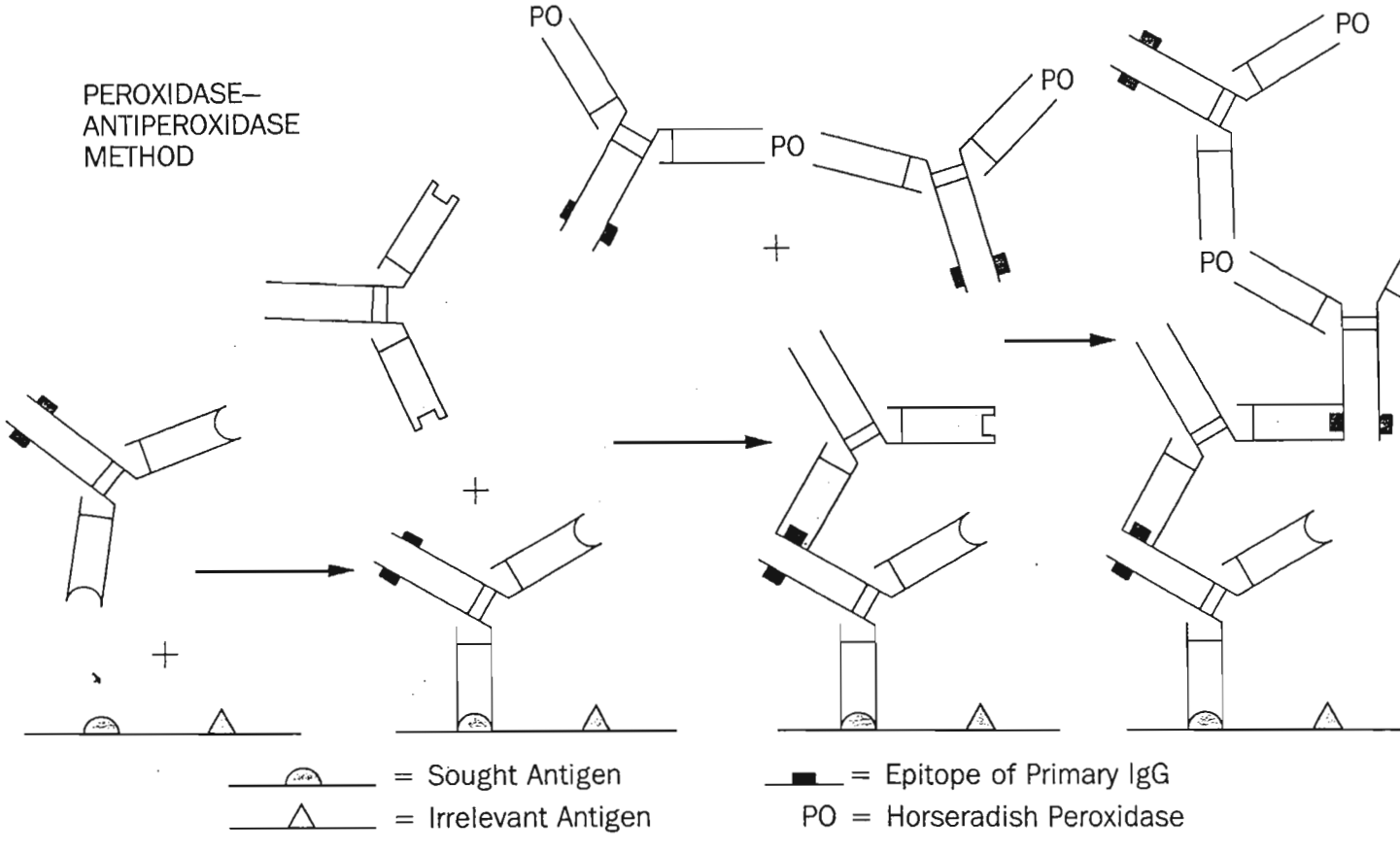


Figure 11 : Immunostaining method - Peroxidase-antiperoxidase (True, 1990).

The PAP complex is thus a valuable and versatile immunohistochemical reagent for the detection of the sites of binding of anti-antibodies. Several studies have demonstrated that the PAP technique has a higher sensitivity than the direct and indirect conjugate methods or the unlabelled antibody bridge technique (Azar, 1988)

2.4.1.6 Quantitative Immunohistochemistry

Quantitative immunohistochemistry is the determination of the concentration of an antigen at the light-microscopic level within tissues or cells or at the electron-microscopic level within cell compartments, in either relative or absolute units (True, 1990).

The concentration of an antigen is most easily accessible by estimating relative visual intensity of a chromogenic label. Successful quantitative immunohistochemistry should satisfy the following conditions (True, 1988) :

- All antibody-antigen and enzyme-substrate reactions must be conducted under conditions where binding is saturated.
- The distribution of reagents must be homogenous.
- The matrix (tissue or embedding medium) and the antigen density should have a predictable effect on immunostaining intensity.
- Quantification should be done objectively.

2.4.1.7 Variables and Controls in Immunohistochemical procedures

The interpretation of any immunohistochemical staining procedure is dependent on a number of important variables, which include the specificity of the immunological

reagents, the sensitivity of the detection system, the adequacy of control studies, and the experience of the observer (Azar, 1988)

2.4.1.7.1 Choice of Technique

The methodology and choice of technique in immunohistochemistry is most frequently governed by the nature of the antigen to be detected. The benefits of using the indirect technique are the short time required to complete the staining and the low cost of the antisera.

2.4.1.7.2 Antiserum Specificity

The specificity of the antiserum is of paramount importance in the interpretation of an immunohistochemical reaction. For optimal staining to occur it is necessary to use the primary specific antiserum at the correct dilution since incorrect dilutions can give rise to false negative results.

2.4.1.7.3 Blocking Endogenous Enzymes

If enzymes similar to those used as the tracer are present in the tissue they may react with the substrate used to localise the tracer and give rise to problems in interpretations (Bancroft and Stevens, 1990). False positive reactions produced in this way can be eliminated by inhibiting the endogenous enzyme activity prior to staining.

Incubation in absolute methanol containing 0.3% hydrogen peroxide for 10-30 minutes at room temperature has been reported to produce an almost complete abolition of endogenous peroxidase activity, without affecting the immunoreactivity of antigens.

2.4.1.7.4 Controls

It is essential that any method using immunohistochemical principles includes controls to test for the specificity of the antibodies involved. Polyclonal antibodies usually contain antibodies specific for several antigenic determinants on the antigen and, as many related molecules have components in common, false positive results can be obtained.

For immunohistochemical staining to be claimed as specific it must be shown firstly, that no staining occurs in the absence of the primary antiserum and secondly, that staining is inhibited by adsorption of the primary antiserum with the relevant antigen prior to its use, but not by adsorption with other related or unrelated antigens.

In practice, to evaluate the results of immunohistochemical staining, the following immunological and non-immunological specificity controls are usually undertaken.

Negative control: This involves either the omission of the specific primary antiserum from the staining schedule or the replacement of the specific primary antiserum by an immunoglobulin that is directed against an unrelated antigen. The immunoglobulin must be of the same class, source and species.

Positive control: As the absence of staining in a test section does not necessarily imply that the antigen is not present, the use of a section of known positivity is advisable.

Absorption control: The ideal negative control is to demonstrate that immunoreactivity is abolished by pre-absorption of the specific primary antiserum with purified antigen, but not by preabsorption with similar molecules. This type of control is necessary in the characterisation and evaluation of new antibodies. It is rarely used in diagnostic work, where well characterised antisera are available, because it is wasteful of reagents and expensive.

2.4.2 NON-INVASIVE BLOOD PRESSURE MEASUREMENTS

In 1733, Stephen Hales became the first person to measure arterial pressure directly. He did this when he sacrificed his horse in his back yard and measured the height of a column of blood extending from the carotid artery into a glass tube from the time of çannulation until the horses' death. This was the first step in the development of direct and indirect means with which to measure arterial pressure. Table 5 represents the various landmarks which led up to our current means of noninvasive blood pressure measurement.

With the proper tools anyone can record mean arterial pressure reliably in anaesthetized rats, but when blood pressure is to be monitored repeatedly in awake rats over several weeks or months, then the technical difficulties become almost insurmountable (Bunag, 1984).

Three methods are commonly used to measure blood pressure in the rat and mouse: tail cuff, telemetry and indwelling catheter. Table 6 briefly compares the advantages and disadvantages of these three techniques.

Table 5 : First blood pressure measurements.

(http://www.mcphu.edu/continuing/cme/medicine/med_study.htm)

First BP Measurements
• Late 1700s ,Stephen Hales. First direct arterial pressure measurement. • Early 1800s Poiseuille introduced mercury hydrodynometer. Smaller height of column needed. • 1846 Carl Ludwig. Added a float to the mercury manometer with a connecting arm which inscribed arterial pulse wave on a moving smoked drum, gave permanent record. • 1880s. First noninvasive measurements. VonBasch recorded systolic BP needed to obliterate the arterial pulse. • 1889 vonHemholtz improved upon vonBasch instrument. • 1896 Riva-Rocci. Wrap around inflatable rubber cuff. • 1900s vonRecklinghausen increased width of the cuff from 5 to 13 cm • 1905 Nikolai Sergeyevich Korotkoff described the sounds heard with a stethoscope placed over the brachial artery below the Riva-Rocci-vonRecklinghausen cuff during slow deflation.

2.4.2.1 Indirect Measurement in Awake Rats

Direct measurements are undeniably more accurate, yet most chronic studies still rely almost exclusively on indirect measurements (Bunag, 1984). Over the past three decades, the tail-cuff method has been the method of choice for indirect measurement. Of these two methods direct recording is often continuous and more accurate, but also technically

more demanding. By contrast, indirect tail-cuff measurements, though less accurate, do not require surgery and can be repeated almost indefinitely.

Table 6 : Comparison of the tail-cuff, telemetry and indwelling catheter measurement techniques. (from Deng, 1998)

METHODS	*ADVANTAGES*	*DISADVANTAGES*
TAIL CUFF	Relatively inexpensive Noninvasive Fast in obtaining systolic pressure	Less accurate Immobilization stress imposed by physical restraint
INDWELLING CATHETER	Accurate and direct in measuring SBP, DBP, and MAP Long term and continuous More economical than telemetry	Invasive
TELEMETRY	Accurate for SBP, DBP, MAP Direct measurement Allows chronic, long term and continuous monitoring	Very expensive in costs of purchasing running and maintaining the instrument Invasive

2.4.2.1.1 The Tail-Cuff Method

Bunag (1984) aptly stated in a review that "a rat's tail is a slender appendage of which the weight of so much research in hypertension hangs". Accuracy of measurement with any indirect method is greatly influenced by the length of the arterial segment that is compressed, which in turn depends on a balance between width of the occluding cuff and

diameter of the extremity on which the cuff is applied (Bunag, 1984). Most methods employ preheating to dilate the tail vessels, but the amount of preheating required varies from one rat to another and is therefore difficult to standardize.

Two reasons argue against the use of a tail cuff as a blood pressure measuring method (Deng, 1998). (1) When conducting measurements, the tail cuff must be immobilized and the animal heated. Thus, the blood pressure can be stress induced. If two groups of rats being compared respond differently to this stress, the blood pressure differences between them will have an added element of environmental artefact. (2) The tail cuff measures systolic pressure, but does not reliably give diastolic and mean arterial pressure measurements.

2.4.2.1.2 Validation of tail-cuff measurements

In studying a large number of rats simultaneously, the tail-cuff method can be used as a screening tool initially, but a direct measurement should be helpful afterwards. Validation is usually done by comparing indirect measurements with those recorded directly from an arterial catheter (Bunag, 1984).

2.4.3 NUTRITIONAL DIETS

In 1973, the Ad Hoc Committee was formed by the Council of the American Institute of Nutrition (AIN) to develop recommendations on nutritional methodology which would serve as guidelines for scientists who have limited experience in experimental nutrition techniques (American Institute of Nutrition, 1977).

2.4.3.1 Recommended Diets for Rats

2.4.3.1.1 Cereal Based Diets

The National Institute of Health developed an open formula cereal-based diet in 1972. It has been found to be satisfactory for reproduction, lactation, and maintenance of both rats and mice.

2.4.3.1.2 Purified Diet

In developing a formula for a purified diet, the committee decided that the diet should not contain quantities of vitamins and minerals highly in excess of the requirements for rats and mice as set forth by the committee on Animal Nutrition National Research Council.

The mineral mixture was designed to contain all trace elements known to be required by rats and mice when maintained under conventional conditions, and when used with a conventional protein source. In the proposed vitamin mix, the amounts provided are considered to be several times the requirements, and several vitamins are higher to allow for possible losses during mixing and storing.

The formula for the diet is shown in Table 4. The diet contains 50% sucrose - this primary carbohydrate was selected in view of the fact that no one-carbohydrate source could be considered ideal for all studies. However, high sucrose diets are cariogenic, and after 6 months, teeth of all rats can be expected to be carious, although this does not seem to otherwise affect their general health and performance.

The Committee believed that the AIN-76 Purified Diet meets the nutritional requirements of rats and mice and supports, growth , reproduction and lactation comparable to those from cereal based diets.

3. MATERIALS AND METHODS

3.1 EXPERIMENTAL ANIMALS, HOUSING AND TREATMENTS

A total of 44 male weanling rats of Dahl salt resistant (DSR) and Dahl salt sensitive (DSS) strains, weighing about 35-40g each, were bred in the Biomedical Resource Centre (BRC) at the University of Durban-Westville, South Africa. Thereafter they were divided into 4 categories and fed special diets as follows:

	1% NaCl	*8% NaCl*
DSR	Group 1 (n=10)	Group 2 (n=10)
DSS	Group 3 (n=12)	Group 4 (n=12)

The animals were individualized by ear notching. Rats were given deionised water *ad libitum* and during the course of the baseline week, which was a week prior to commencement of the experimental procedures, these weanlings were maintained on ordinary rat chow containing 0.4% NaCl.

They were housed in individual metabolic cages (Nalgene) in a temperature (22 °C), humidity (55%) and day/night controlled environment in the BRC. All experiments were conducted in accordance with the guidelines for the care and use of animals approved by the Ethics Committee of the University of Durban-Westville.

3.2 DIETARY TREATMENT- AIN 76 diet

The animals were maintained on the AIN 76 diet which is recommended for optimal growth since it contains all essential nutrients (in correct proportions) to ensure normal physiological functioning of the rat (American Institute of Nutrition recommended Diet, 1977). However, a 1% NaCl and 8% NaCl load was included into the diet, and these two loads of dietary NaCl could be considered as either "normal" or "high", respectively.

3.2.1 Diet Composition

Specifications of the American Institute for Nutrition were strictly adhered to. Table 7 shows the ingredients and their respective concentrations in the basal diet.

Table 7 : American Institute of Nutrition recommended diet (1977)

INGREDIENTS	*PERCENTAGE*	*G/kg mixture*
Choline Bitartrate	0.2	4g
DL - Methionine	0.3	6g
AIN 76 Vitamin Mix	1.0	20g
AIN 76 Mineral Mix	3.5	70g
Alphacel	5.0	100g
Corn Oil	5.0	100g
Corn Starch	15.0	300g
Casein	20.0	400g
Sucrose	50.0	1000g

The two diets were similar in all respects except for the NaCl content. For the preparation of the 1% NaCl diet, 10g of NaCl per kg of diet was included into the mixture. Similarly, for the 8% NaCl diet 80g of NaCl per kg of diet was included in the mixture.

Table 8 and Table 9, shows the vitamin and mineral mixtures of the diet.

Table 8 : AIN 76 Vitamin Mixture

VITAMIN	*Per kg of mixture*
Thiamine Hydrochloride	600 mg
Riboflavin	600 mg
Pyridoxine Hydrochloride	700 mg
Nicotinic acid	3 g
D- Calcium Pantotenate	1.6 g
Folic acid	200 mg
D - Biotin	20 mg
Cyanocobalamin (Vitamin B12)	1 mg
Retinyl palmitate (Vitamin A)	400 000 IU
α - tocopheryl acetate (Vitamin E)	5 000 IU
Cholecalciferol (Vitamin D3)	2.5 mg
Menaquinone (Vitamin K)	5.0 mg
Sucrose, finely powdered	To make up 1000 g

Table 9 : AIN 76 Mineral Mix

MINERAL	*G/kg*	*For 3 kilograms*
Calcium Phosphate Dibasic	500.0	1500 g
Potassium Citrate Monohydrate	220.2	660 g
Potassium Sulphate	52.0	156 g
Magnesium oxide	24.0	72 g
Magnesium Carbonate (43-48%Mn)	3.5	10.5 g
Ferric citrate (16-17% Fe)	6.0	18 g
Zinc carbonate (70% ZnO)	1.6	4.8 g
Cupric carbonate (53-55% Cu)	0.3	0.9 g
Potassium iodate	0.01	0.03 g
Chromium potassium sulphate	192.0	1.65 g
Sucrose, finely powdered	To make 1000g	576 g

3.2.2 Diet Preparation

All the diets were prepared in the Department of Physiology and Physiological Chemistry. Three kilograms of mineral mixture was calculated to be sufficient to last the entire duration of the project, and this was prepared initially.

The diets were made in 2x2kg batches on a weekly basis i.e. 4 kilograms of high sodium diet as well as a normal sodium diet were prepared weekly.

Mixing of the diet was performed manually, with dietary components being added in increasing quantities so as to ensure the homogeneity and consistency of the diet. A similar procedure was followed during the preparation of the mineral mix. Alphacel, which is a very amorphous substance, was first stabilized to corn oil before addition to the diet. After manual mixing, each 2 kg batch was transferred to a mechanical food mixer (Gyphron) for 30 minutes to ensure equal distribution of all the individual components in the mixture. The final diets were stored and refrigerated at 8°C.

3.3 FOOD CONSUMPTION AND BODY WEIGHTS

Feeding of the animals was performed at 9:00am from Monday to Friday. The prepared diets were dispensed in 25g quantities into the food trays. At the end of five days, a load of 75g was dispensed to accommodate for the weekend. Daily food consumption was calculated as the difference between the quantity remaining in the food trays from the initial quantity (25g). As the rats grew, the quantity of the diet was increased to 30g, but the same principle for monitoring food consumption was used.

Body weights were monitored twice a week with the use of an electronic balance (Mettler). Weight measurements and daily food consumption was monitored in the BRC.

3.4 BLOOD PRESSURE MONITORING

3.4.1 Blood Pressure Equipment

The IITC Model 31 NIBP software was used to capture noninvasive blood pressure test recordings. The system comprises of an automatic scanner and pump, a tail cuff with a

photoelectric sensor and amplifier to measure and count the pulse rate in the animal's tail. The principle of operation is related to the Riva-Rocci method used in humans.

Small, medium and large sizes of restraining devices were used. This was to compensate for the increase in mass and size during the 6-week duration of the experiment. These restrainers are constructed of sturdy transparent, 1/4" wall acrylic, which fit the rats snugly so as to minimize movement, which could have affected the blood pressure values.

Restrainers allowed for the ventilation of the head at one end and protrusion of the tail through the tail cuff at the other end. Two tail cuff sizes, 10mm and 15mm, were used during the duration of the experiment.

3.4.2 Training Protocol

Training sessions were carried out for 5 consecutive days i.e. sessions of unrecorded measurements using the tail-cuff method. During this period, rats were acclimatized at 28°C in restrainers for blood pressure measurements. Handling of rats, confinement in restrainers, and repeated inflation and deflation of the tail cuff was also incorporated into the training session. The aim of the sessions was to habituate the rats to blood pressure monitoring conditions so that when actual recordings were taken, stress reactions would be minimized ensuring that reliable values could be obtained.

3.4.3 Experimental measurements of Blood Pressures

Blood pressure was measured weekly between 10h00 and 13h00. Measurement of blood pressures at the same time on the day of the recording was performed to minimize the variables related to circadian rhythms. Heaters were switched on 30 minutes prior to blood pressure measurements to ensure that a room temperature of 28°C-29°C was maintained. Due to its sensitivity, the II TC apparatus allows direct measurement of systolic and mean arterial pressure without external preheating as long as the room temperature is maintained at 28°c or above. This is 1°C above the normal 26°C-27°C ambient temperature for rats. Therefore rats were warmed for 10 to 15 minutes at 28°C, to make pulsations of the tail artery detectable, prior to measurements for blood pressure.

Conscious, unanaesthetised rats were allowed to enter the restrainer by manipulating the head gate and the boot straps of the end plate. Animals were then preheated at ± 28°C for 5 minutes. Thereafter the appropriate tail-cuff was applied at the base of the tail. The pump automatically inflated the tail cuff, which resulted in arterial blood supply to the tail to be occluded. The cuff was then deflated and the reappearance of pulsations, as detected by the photoelectric sensor, was taken as the systolic blood pressure. As the pressure continued to fall, the computer stored the value of each high pulse point, which was accepted as a mean pressure if there was no higher pulse pressure within the next two seconds. The end point selection is automatic for systolic, heart rate while the diastolic values are automatically computed using the equation: Diastolic = (3 mean - systolic)/2. The computer is programmed to commence the test when maximum cuff pressure is achieved and to end the test at a designated termination point. Also, this model has an

artifact filter to reduce the problem of artifacts due to movement. The results were displayed as data plots and summary data of systolic, diastolic and mean blood pressure and heart rate on the computer screen. The test information was in 2 forms - plots of analog waveforms and digital values (Figure 12).

Blood pressure was calculated from the average of three readings obtained while each animal was resting and motionless but alert. Blood pressures were determined weekly, with a final measurement taken at 6 weeks of age. Validation of the method was previously carried out in this laboratory (Somova *et al.*, 1998).

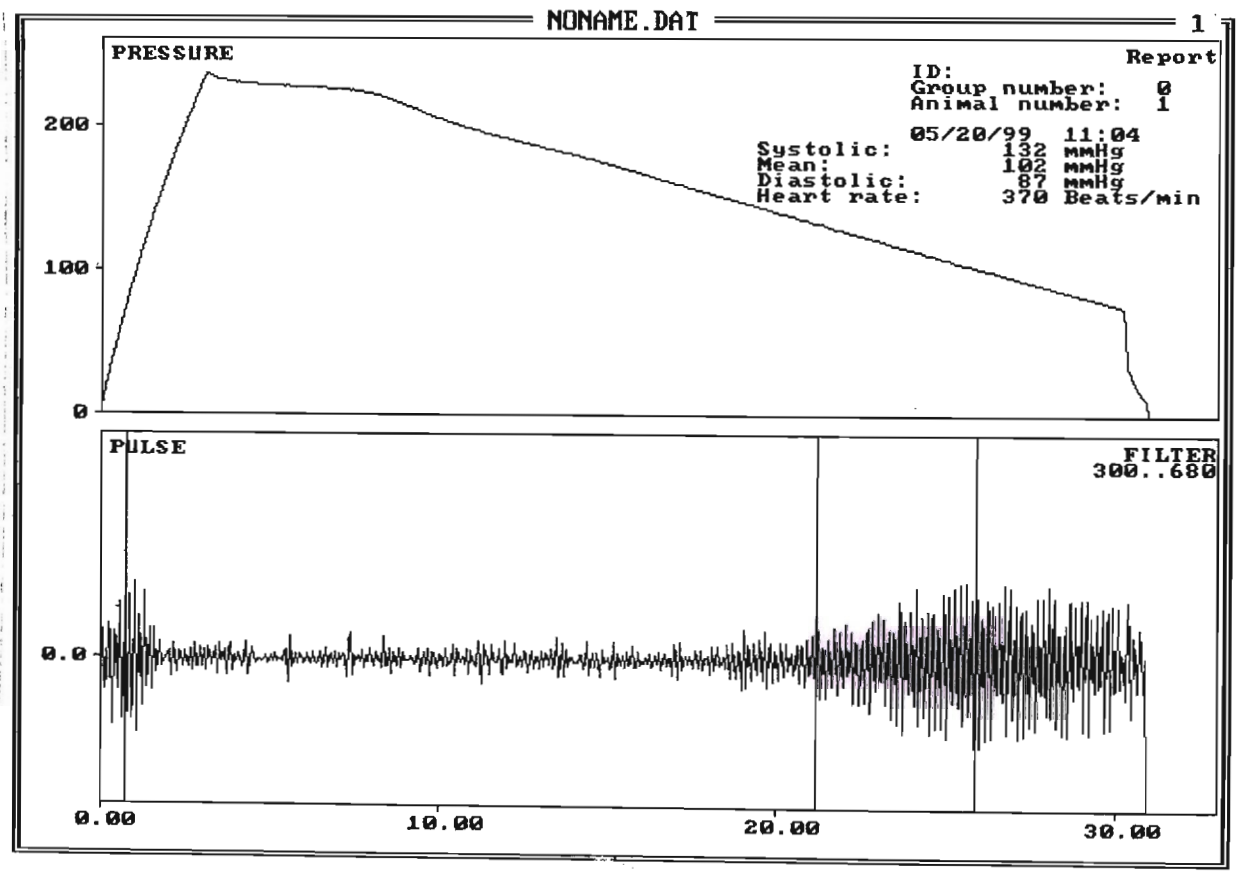


Figure 12 : Typical tail pulse recording using the II TC BP monitor

3.5 TISSUE SAMPLE COLLECTION

After the desired experimental duration (6 weeks) had expired, the rats were fasted overnight, and sacrificed the following morning. Animals were anaesthetised with sodium thiopentone (30 mg/kg) and the adrenal glands from all animals were excised and immediately placed in 10% buffered neutral formalin.

3.6 MICROTECHNIQUE

3.6.1 Tissue Processing

Processing comprises of many steps viz. fixation, dehydration, clearing and infiltration. The different chemical solutions, alcohols, clearing reagents and supporting mediums used together with the stipulated duration are tabulated below.

Processing Steps	*Chemicals/Reagents*	*Time Duration*
1. FIXATION	10 % Buffered neutral formalin	24 hours
2. DEHYDRATION	70 % alcohol	1 hour
	80 % alcohol	1 hour
	95 % alcohol	1 hour
	95 % alcohol	1 hour
	100 % alcohol	1 hour
	100 % alcohol	1 hour
	100 % alcohol	1 hour
3. CLEARING	Xylene	45 minutes
	Xylene	45 minutes
4. INFILTRATION	Melted Paraffin	1.5 hours
	Melted Paraffin	1.5 hours

3.6.2 Embedding

After infiltration, the tissues were embedded by use of an embedding machine (Shandon Histocentre 2).

3.6.2.1 Embedding Equipment and procedures

The equipment comprises of a paraffin tank, hot plate, cold plate, base moulds and a warming tank.

Processed tissue specimens in cassettes were placed initially in a stainless steel holding container in molten paraffin. Cassettes were thereafter removed and placed on the warming tank. An appropriate base mould was selected from the prewarmer area housing an assortment of base mould sizes. The mould was placed on the hot plate and paraffin wax from the paraffin tank was dispensed to about a quarter of the level of the mould.
The specimen from the warming tank was orientated in the base mould, and holding the tissue in the desired position with a pair of warmed forceps was transferred to the cold plate section, to anchor the tissue firmly into place. Thereafter the cassette was replaced on top of the base mould, transferred to the hot plate and filled completely with paraffin wax. The mould was transferred back onto the cold plate for rapid chilling. After chilling the case specimen was removed from the mould.

3.6.3 Sectioning

Paraffin blocks were cut using a rotary microtome, at a thickness of 5μm The paraffin-embedded serial sections obtained were immediately floated out on a constant temperature water bath.

3.6.4 Mounting

The ribbon of sections floating on water was split into individual or groups of sections. Sections were picked from the water bath with slides coated with poly-*L*-lysine. Poly-*L*-lysine is a general-purpose section adhesive without any apparent production of background staining.

The slides were thereafter placed on a warming table (Ransom Warming plate) and then transferred to the oven for complete drying. Slides were left overnight in an oven at 37°C so as to allow the sections to adhere firmly to the slides.

3.6.5 Selection

Six slide specimens from each experimental group were randomly selected for immunohistochemical staining.

3.7 Immunohistochemical Staining

A detailed description of the preparation of buffers, reagents and solutions is given in Appendix A-1.

3.7.1 Staining Procedure

After drying overnight at 37°C the slides were incubated for 5 minutes at 70°C. Slides were thereafter deparaffinized with graded concentrations of xylene and ethanol.

The following concentrations of xylene to ethanol were prepared -
(30:70) ; (50:50) ; (70:30) ; (90:10). (100% xylene). The tissue sections were ringed with a DAKO pen (Dako Corporation, USA) which created an invisible well for the activity of the reagents used in subsequent steps.

Endogenous peroxidase activity was blocked by incubation with 0.6 % H_2O_2 in methanol for 30 minutes at room temperature. Slides were washed with 2 % BSA in TBS, 3 changes of 2 minutes each.

After washing, they were incubated with 40 µl 5% goat serum (Dako Corporation, USA) for 10 minutes at room temperature. This was to reduce nonspecific background staining.

Sections were washed with 2% BSA in TBS (2 changes of 2 minutes), slides were incubated with 40 µl rabbit polyclonal anti ADM antiserum (Penninsula Laboratories, USA) at a dilution of 1: 500 in a humidified chamber for 48 hours. The humidified chamber was used to prevent evaporation of the antisera.

The control slides were treated with 40 µl 5% non-immune rabbit serum (Dako Corporation, USA) for 45 minutes at room temperature. All the slides, including the

control slides, were rinsed with 2% BSA in TBS (3 changes of 2 minutes) and thereafter incubated with 40 µl second antibody - goat-anti-rabbit HRP (Dako Corporation, USA) at a dilution of 1: 100 for 30 minutes at room temperature.

After washing with 2% BSA in TBS (3 changes of 2 minutes), the final reaction was achieved by incubating with 40 µl fresh DAB solution for 10 minutes at room temperature. The DAB-hydrogen peroxidase reaction is used for demonstration of peroxidase activity. The reaction was stopped by rinsing with distilled water (3 changes of 2 minutes.)

The sections were counterstained with Mayer's haemotoxylin for 5 minutes at room temperature and washed with running tap water (2 changes of 3 minutes).

Dehydration : the slides were hydrated and cleared through the following solutions.

Alcohols/Xylene	*Changes*	*Time Duration*
70% EtOH	1 time	2 minutes
80% EtOH	1 time	2 minutes
95% EtOH	1 time	2 minutes
100% EtOH	2 times	2 minutes
Xylene	2 times	2 minutes

The sections were immediately mounted using DPX and viewed using an Olympus microscope. Stained sections were photographed under the microscope at different magnifications, using a digital camera (Zeiss Auxio photomicroscope).

3.8 Quantification of adrenomedullin granules

For the quantitative analysis, three different areas in the medulla were randomly selected. Therefore for each animal, the number of immunopositive granules within the medulla was obtained by direct counting of granules in 18 fields using a 40X magnification. A previous investigation conducted in this laboratory has found that the use of a computerised programme viz. Kontron Systems 300, was not extremely selective in its quantitative analysis, therefore a manual counting method was employed (Hariram, unpublished).

3.9 Statistical Analysis

Values are expressed as mean ± standard deviation. For statistical analysis, the GraphPad Instat V3.00 program was used, including 1-way ANOVA and Turkey-Kramer multiple comparison test. Comparison between groups was performed using Student's unpaired t test and Kolmogorov and Smirnov assumption test.

4. RESULTS

4.1 PHYSICAL AND BEHAVIOURAL OBSERVATIONS

Four animals did not survive the 6 week experimental period and have not been included in the data analysis. The number of rats surviving at each time interval is given in Table 10. One of these subjects was from the Dahl salt resistant lines and the remainder from the Dahl sensitive strain, although all animals were maintained on the high (8% NaCl) diet. It is evident that the high (8% NaCl) diet was more severe to these rats.

Some of the experimental rats on the 8% NaCl diet displayed facial abrasions and experienced bleeding from their noses. Their movements within the metabolic cages were rigid and lethargic on comparison with the slightly friskier rats maintained on the 1% NaCl diet.

Table 10 : Number of Dahl salt resistant (DSR) or Dahl salt sensitive (DSS) rats surviving on Normal (1% NaCl) or High (8% NaCl) Salt Diets.

Weeks on Diet	DSR on 1% NaCl	DSS on 1% NaCl	DSR on 8% NaCl	DSS on 8% NaCl
0	10	12	10	12
2	10	12	10	12
4	10	12	10	12
6	10	12	9	9

4.2 FOOD CONSUMPTION, BODY AND ORGAN WEIGHTS

The results for the average weekly food consumption are tabulated in Table 11 and graphically represented in Figure 13. In the DSS lines, the first 4 weeks showed a gradual increase in food consumption. Weeks 1-2 displayed a low dietary intake and this was attributed to a period of adjustment of the rats to their new environment (metabolic cages) as well as the diets (salt diets). A steady increase in food consumption is noticeable during weeks 2 to 4, however while this trend continued in Dahl sensitive rats on 1% NaCl, the DSS on 8% NaCl showed a steady decline in the remaining two weeks. This was probably a result of the degree of severity pertaining to the hypertensive state becoming severe and these animals became weak, stopped growing and lost weight. Therefore, at the culmination of the experiment, the final mean body weights of the DSS rats on a high salt diet (241 ± 4.2g) was found to be lower than the DSS on a normal salt diet (299 ± 3.7g).

Table 11 : Average Weekly Food Consumption (grams)

Weeks on Diet	DSR on 1% NaCl	DSS on 1% NaCl	DSR on 8% NaCl	DSS on 8% NaCl
1	97.00	106.58	89.70	100.58
2	103.90	112.92	86.30	103.00
3	102.10	129.92	99.20	118.75
4	102.30	135.50	103.50	130.42
5	98.30	140.75	98.33	135.00
6	99.60	136.83	104.44	100.11

In the Dahl resistant lines, the low dietary intake in the first 2 weeks could also be indicative of the impact of the new environment and salt diets. However, whilst a gradual increase in food consumption was noticeable during weeks 2-4 of the DSR rats on 8% NaCl diet, this was absent in the DSR on 1% NaCl diet. Food consumption in Dahl resistant rats on 1% NaCl diet remained relatively constant throughout the remainder of the experiment at ± 100g/week. However, during weeks 2-4 of the DSR on 8% NaCl diet, there was a steady increase in food consumption to a maximum of ± 104g/week. The remaining 2 weeks displayed fluctuating results, with week 5 showing a decrease in food consumption to a low of ± 98g/week, whilst week 6 showing a gradual increase to ± 104g/week. The final mean body weights of the DSR rats on 8% NaCl diet (192 ± 4.1g) was found to be lower than the DSR on 1% NaCl (236 ± 6.0g).

The results for the average weekly mass gain are tabulated in Table 12 and illustrated in Figure 14. The heaviest group over the 6 week experimental period was the DSS on 1% NaCl diet, followed closely by DSS on 8% NaCl diet, thereafter DSR (1% NaCl) and DSR (8% NaCl).

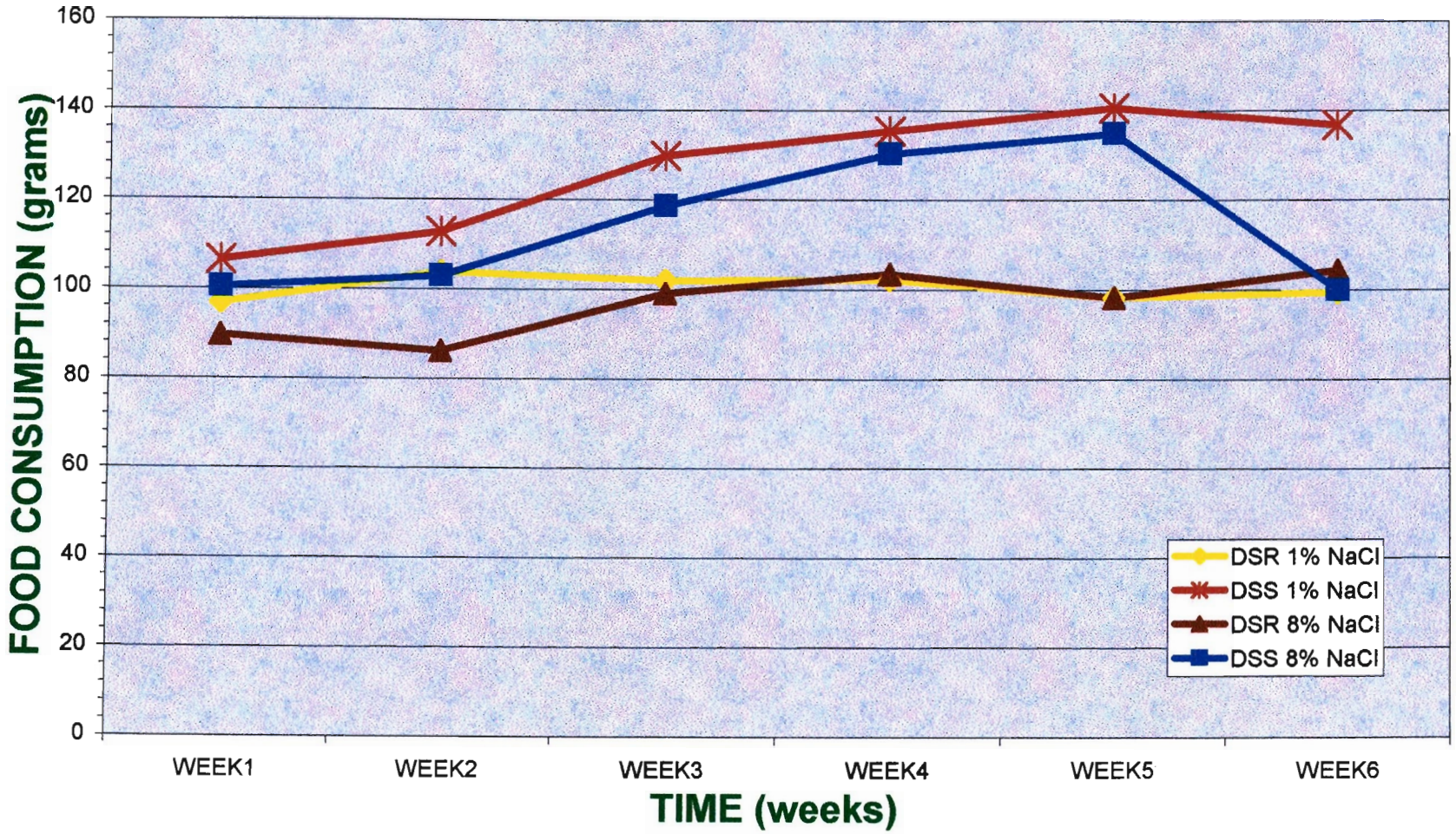


Figure 13 **: Cumulative Food Consumption vs. Time**

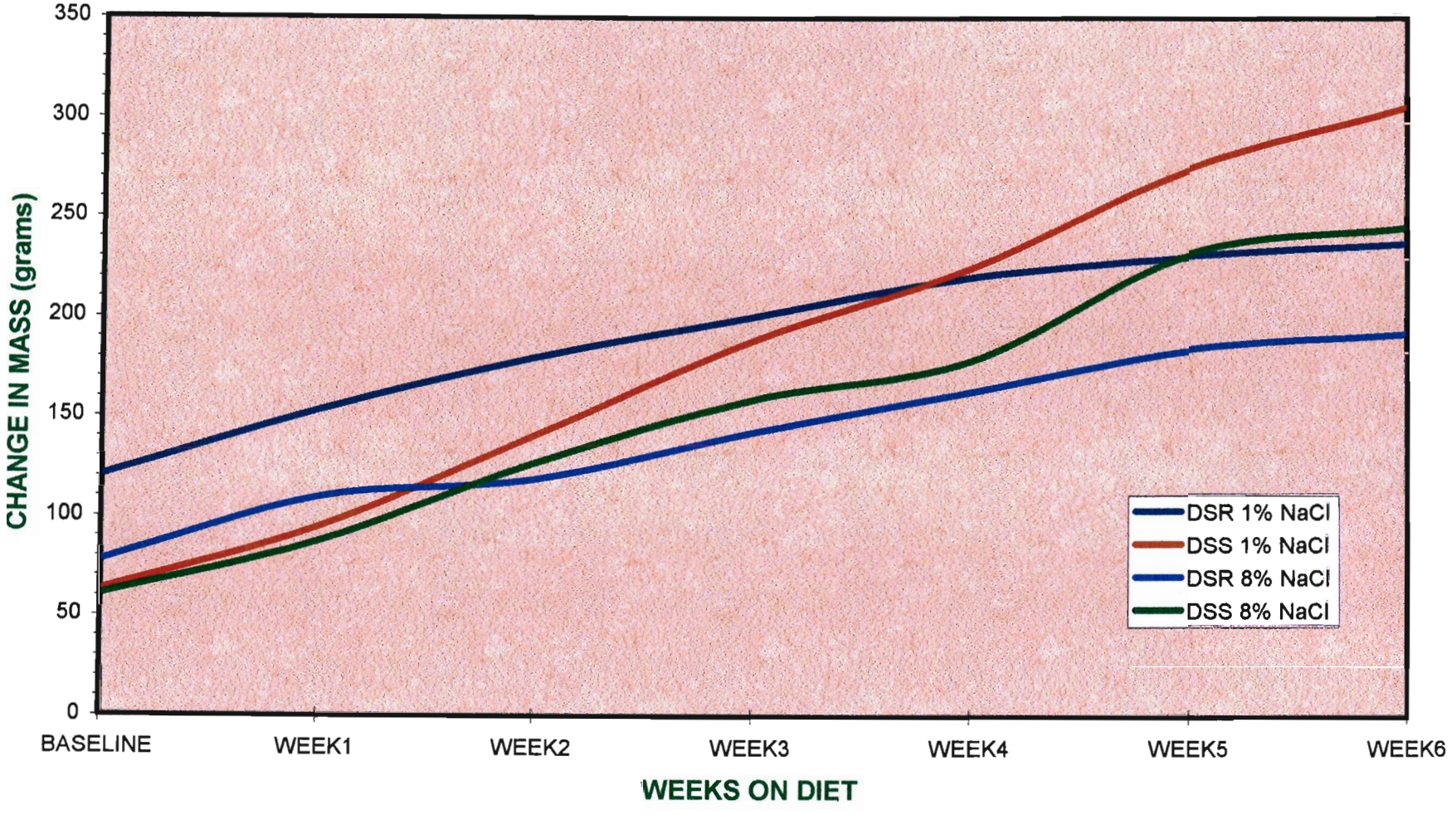


Figure 14 **: Growth curves of Dahl salt resistant (DSR) and Dahl salt sensitive (DSS) rats on normal (1% NaCl) or high (8% NaCl) diets.**

Table 12 : Average weekly mass gain (in grams)

Weeks on Diet	*DSR on 1% NaCl*	*DSS on 1% NaCl*	*DSR on 8% NaCl*	*DSS on 8% NaCl*
1	153.50	95.00	110.20	87.83
2	179.80	140.67	118.80	127.00
3	200.50	188.58	142.70	159.00
4	220.50	225.08	163.22	178.50
5	230.50	274.30	184.00	231.67
6	236.30	304.42	191.00	244.27

Table 13 contains results pertaining to body and organ weights, which include changes in heart weight/body weight, left ventricle weight/body weight and paired kidney weight/body weight ratios of Dahl rats on normal and high salt diets. At weaning (1 month old), both salt resistant (DSR) and salt sensitive (DSS) rats had similar body weights (35-40g), but at the end of the experimental period (3 months), the DSS line showed a significant reduction in body weight gain.

Kidney weights were higher in Dahl sensitive rats than Dahl resistant rats either on the 8% NaCl diet or 1% NaCl diet (DSS = 2.679 ± 0.09, DSS = 2.446 ± 0.05 vs DSR = 1.733 ± 0.04, DSR = 1.678 ± 0.07). This increase in weights is probably due to their renal pathology, as McCormick et al. (1989) reported that in contrast to compensatory renal growth, sodium-induced renal growth seems to be due mainly to renal hyperplasia in DSS rats.

The observation that heart weights were greater in Dahl sensitive rats than Dahl resistant rats either on high salt (8% NaCl) diet or normal salt (1% NaCl) diet, is a result of cardiac hypertrophy occurring concomitant with the rise in blood pressure (DSS = 1.254 ± 0.03, DSS = 1.156 ± 0.03 vs. DSR = 0.692 ± 0.01, DSR = 0.827 ± 0.04).

4.3 CARDIOVASCULAR PARAMETERS

The effects of a low and high sodium diets on systolic pressure, diastolic pressure and heart rate were analyzed. All blood pressure results are tabulated in Table 14. The effects of an 8% NaCl and 1% NaCl supplemented diet on systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rates (HR) are shown in Figures 15,16 and 17 respectively.

The Dahl resistant rats remained normotensive on both the 1% NaCl and 8% NaCl diet for the 6 weeks with only a moderate increase in blood pressure corresponding to their age.

At 2 weeks, DSS rats on normal-salt (1% NaCl) had systolic blood pressures which were significantly increased compared to control values of the same group (120 $\pm$ 2.1mm Hg vs 116 $\pm$ 1.9mm Hg, $P < 0.05$). The DSS rats treated with a high salt (8% NaCl) diet developed Stage 1 hypertension (systolic BP 156 $\pm$ 3.4mm Hg and diastolic BP 93 $\pm$ 3.3mm Hg).

The DSS rats treated with normal NaCl (1%), at the age of 4 weeks had above normal blood pressures (systolic BP 162 $\pm$ 7.8mm Hg and diastolic BP 106 $\pm$ 7.1mm Hg) and the DSS on a high NaCl (8%) diet also had high blood pressures (systolic BP 162 $\pm$ 7.6mm Hg and diastolic BP 109 $\pm$ 8.0mm Hg). These values fall under Stage 2 classification of hypertension and were significant compared to control values of their respective groups ($p < 0.05$).

At 6 weeks, the Dahl sensitive rats on 1% NaCl had decreased blood pressures on comparison with the 4 week interval (SBP 150 ± 7.3mm Hg and DBP 106 ± 7.4mm Hg). The DSS rats on the 8% diet became severely hypertensive developing Stage 3 hypertension (systolic BP 174 ± 3.3mm Hg and diastolic BP 124 ± 7.1mm Hg). Both the results were significant compared to control values of their respective group further emphasizing sensitivity to dietary salt intake ($p < 0.05$).

***Table 14*:** Changes in blood pressure(mm Hg) and heart rate(beats/min) of Dahl Salt Resistant(DSR) and Dahl Salt Sensitive(DSS) rats treated with normal(1% Na) and high(8% Na) diet.

Group/ Parameter	*CONTROL*			*2 WEEKS DIET*			*4 WEEKS DIET*			*6 WEEKS DIET*		
	SBP	**DBP**	**HR**	**SBP**	**DBP**	**HR**	**SBP**	**DBP**	**HR**	**SBP**	**DBP**	**HR**
DSR 1% Na diet	128 ± 2.7	80 ± 1.7	459 ± 9.2	138 ± 4.1	88 ± 3.7	462 ± 9.0	128 ± 1.5	84 ± 3.5	474 ± 12.7	134* ± 3.5	78 ± 3.9	440 ± 10.2
DSR 8% Na diet	115 ± 2.0	70 ± 3.9	477 ± 8.8	127 ± 4.3	74 ± 4.5	486 ± 11.5	133* ± 5.0	92* ± 7.1	423 ± 12.3	132*± 5.9	82 ± 4.1	435 ± 7.3
DSS 1% Na diet	116 ± 1.9	77 ± 1.3	445 ± 18.6	120* ± 2.1	81 ± 2.0	436 ± 14.4	162*+ ± 7.8	106*+ ± 7.1	496* ± 12.1	150*+ ± 7.3	106*+ ± 7.4	481*+ ± 14.0
DSS 8% Na diet	116 ± 2.5	78 ± 1.2	478 ± 19.2	156*+ ± 3.4	93*+ ± 3.3	461 ± 19.8	162*+ ± 7.6	109* ± 8.0	531*+ ± 14.6	174*+ ± 3.3	124*+ ± 7.1	487 ± 18.9

Mean ± SEM

* Significant compared to control value of the same group

\+ Significant compared to the respective DR group

SBP Systolic Blood Pressure

DBP Diastolic Blood Pressure

HR Heart Rate

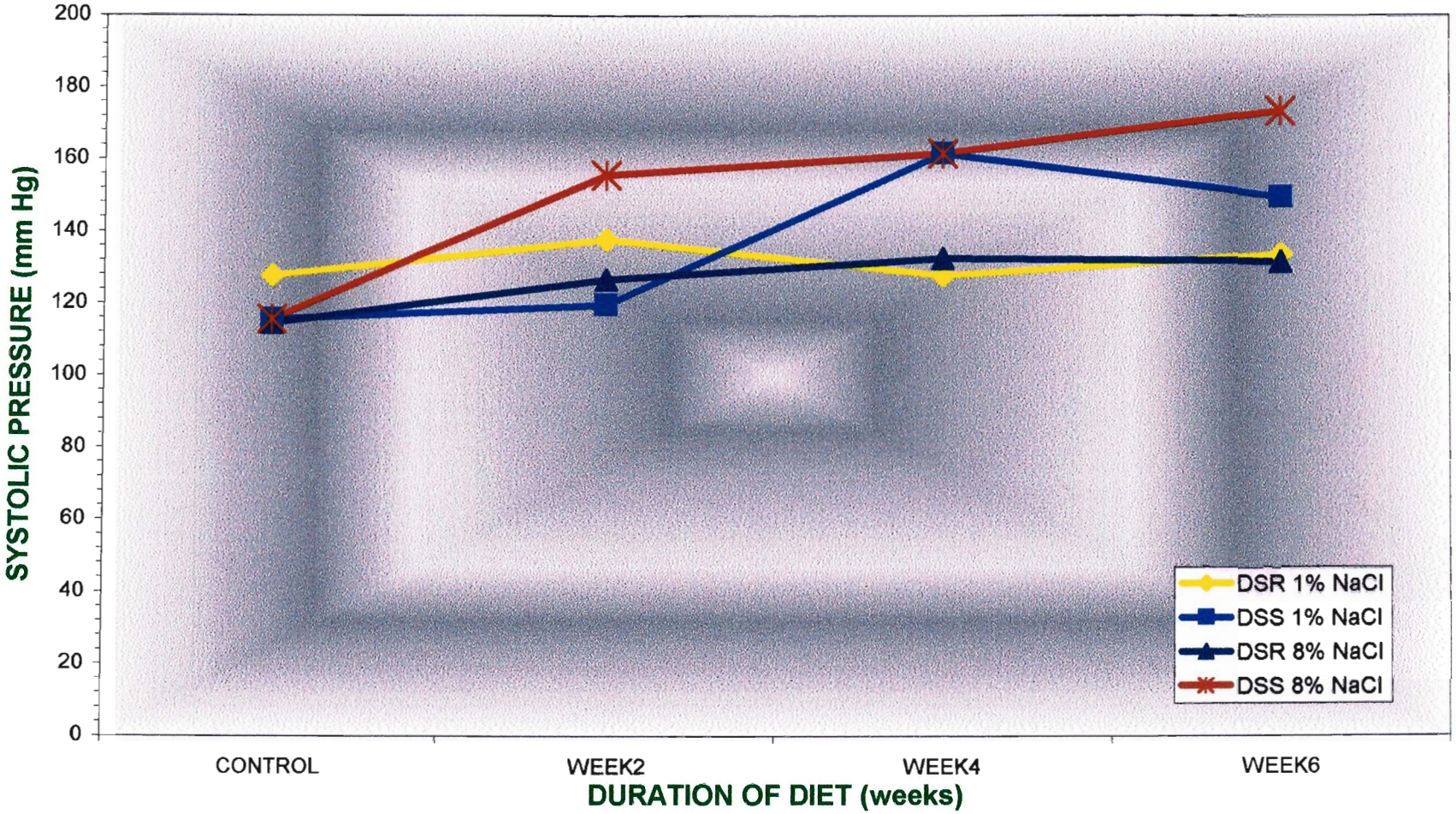


Figure 15 **: Systolic blood pressure responses of Dahl salt-resistant (DSR) and Dahl salt-sensitive (DSS) on normal salt (1% NaCl) or high salt (8% NaCl) diets from weaning**

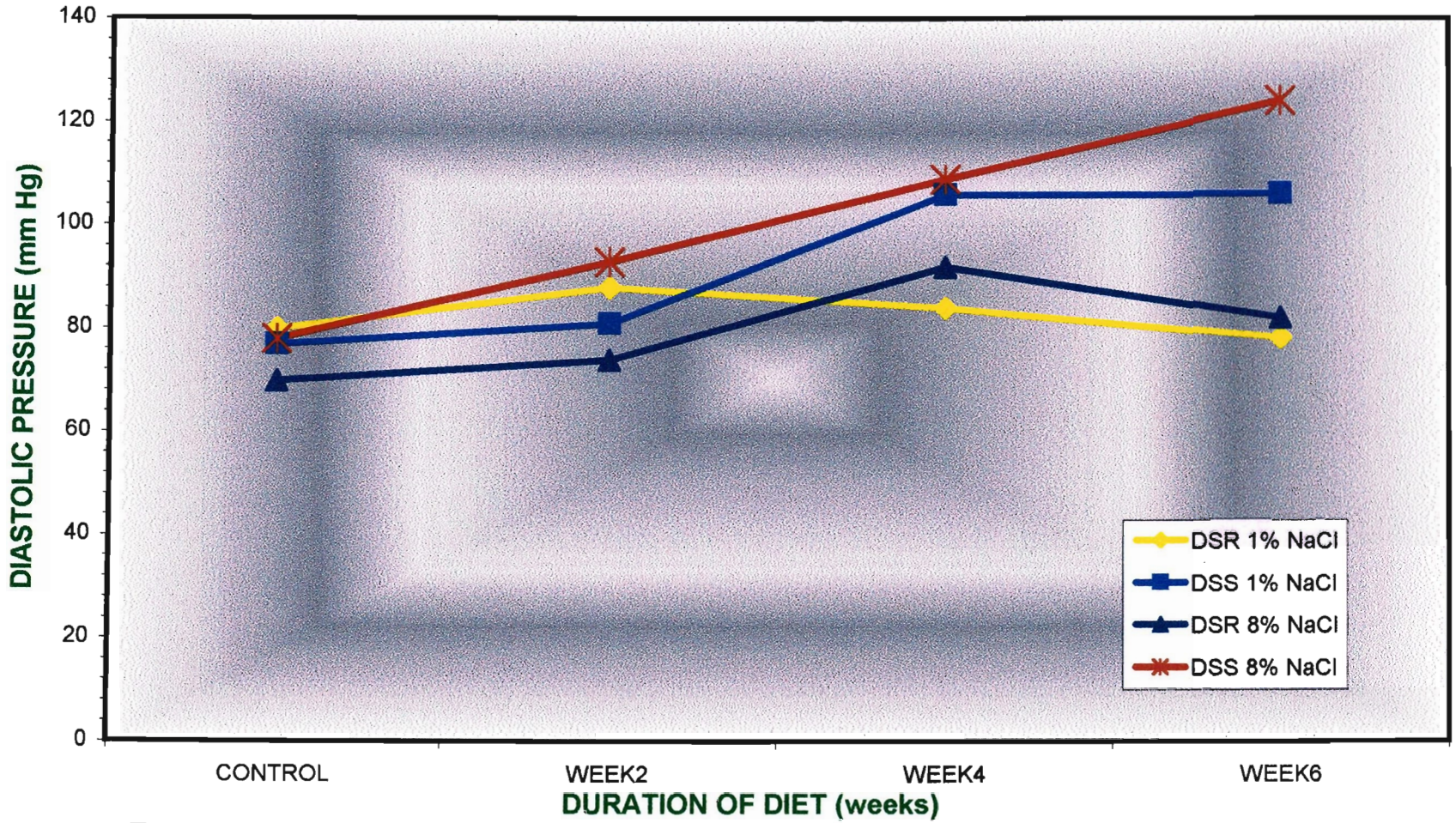


Figure 16 **: Diastolic blood pressure responses of Dahl salt-resistant (DSR) and Dahl salt-sensitive (DSS) rats on normal salt (1% NaCl) or high salt (8% NaCl) diets from weaning.**

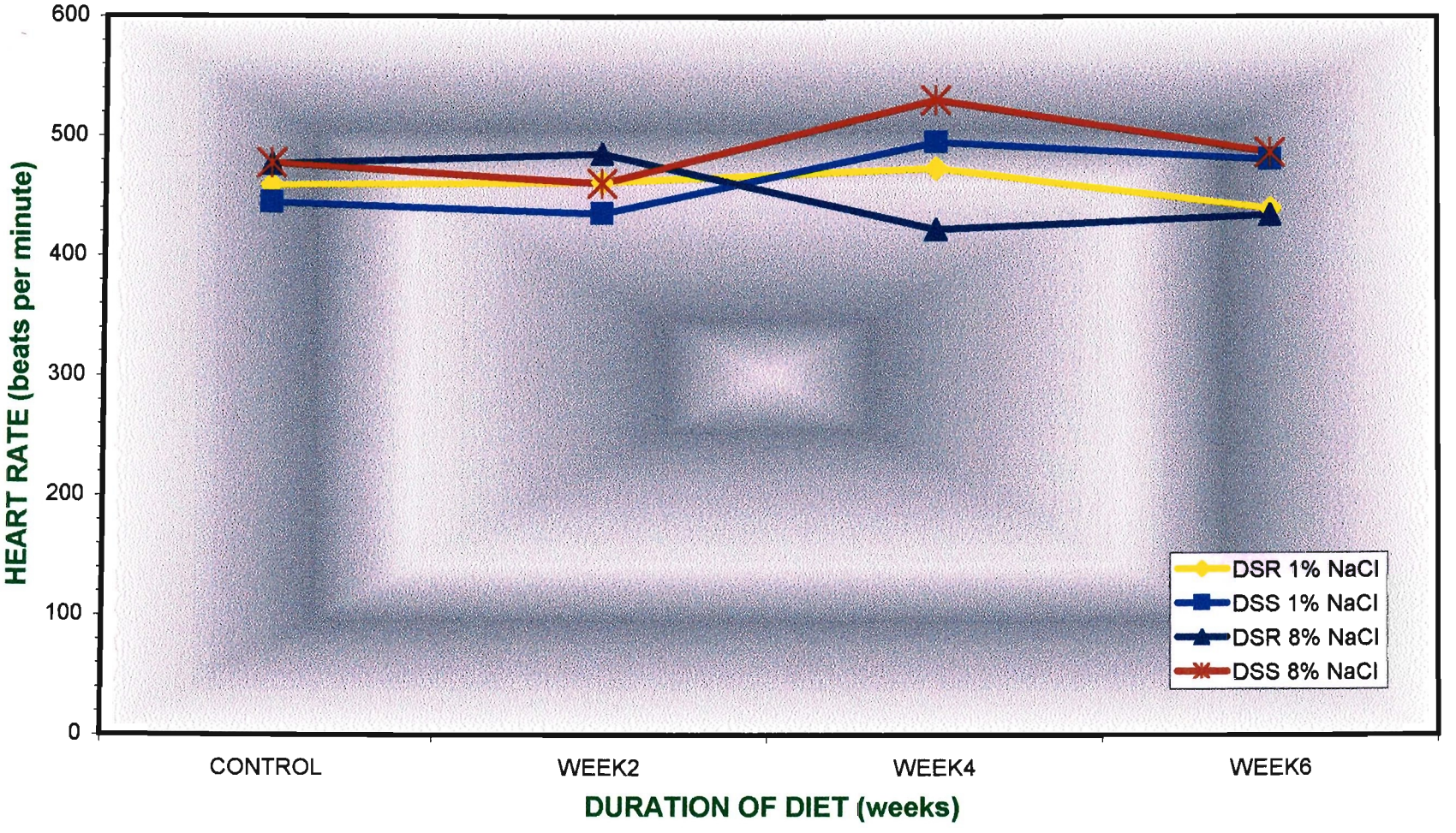


Figure 17 **: Heart rates of Dahl salt-resistant (DSR) and Dahl salt-sensitive (DSS) rats on normal salt (1% NaCl) and high salt (8% NaCl) diets from weaning**

4.4 IMMUNOHISTOCHEMISTRY

The reaction of horseradish peroxidase with 3,3 diaminobenzidine (DAB) substrate, resulting in a dark stain, at sites of adrenomedullin antibody binding was used to detect and visualize the zonal distribution of adrenomedullin on paraffin sections of the rat adrenal. Staining for the hormone was observed sporadically in the zona glomerulosa of the adrenal cortex (Figure 19). In addition, positive immunostaining seemed to be also present in the zona fasiculata and zona reticularis (Figure 20 and Figure 21), although differentially distributed. However, intensity of the staining was greatly increased in the adrenal medulla (Figures 22-27). The control adrenal specimens treated with non-immune rabbit serum showed no ADM immunoreactivity (Fig 18).

Photographs of the adrenal medulla show cords of cells and interspersed capillaries. In the adrenal glands of Dahl rats, the medullary parenchymal cells have ADM cells which seem to have a regular appearance with a slightly dense core bounded by a membrane. In addition to the appearance of ADM immunoreactive granules in the medullary region, it was also observed that there was a tendency to accumulate around the capillaries (Figure 23).

According to the semiquantitative evaluation, the number of adrenomedullin cells seemed greater in adrenals of rats treated with 8% NaCl than those treated with 1% NaCl. Quantitative data obtained to support this observation is represented in Table 15 and graphically in Figure 28 and 29. Compared with the rats treated with 1% NaCl diet, there was an increase in the number of adrenomedullin granules in the animals treated with an

8% NaCl diet, irrespective whether it pertained to the Dahl salt sensitive or Dahl salt resistant strains.

Table 15 **:** The number of adrenomedullin cells per $0.16mm^2$ # in rat adrenal gland tissue after different salt treatments.

Experimental Group	*Number of adrenomedullin cells/0.16 mm^2*
DSR on 1% NaCl	5.98 ± 0.3
DSS on 1% NaCl	37.85 ± 0.5†
DSR on 8% NaCl	18.49 ± 0.5
DSS on 8% NaCl	75.13 ± 1.6 ‡

Results are expressed as mean ± SEM
† $P < 0.0001$, DSR 1% NaCl vs. DSS 1% NaCl
‡ $P < 0.0001$, DSR 8% NaCl vs. DSS 8% NaCl
# Refer to Appendix F-2

There was a 6.3 fold increase in the proportion of ADM content in the DSS rats treated with a 1% NaCl diet when compared with DSR treated with 1% NaCl diet which is considered extremely significant (DSR : 5.98 ± 0.3 vs. DSS : 37.85 ± 0.5). Also, compared with the DSR rats consuming 8% NaCl diets, there was a 4.1 fold increase in ADM content of DSS on 8% NaCl diets which is considered extremely significant (DSR : 18.49 ± 0.5 vs. DSS : 75.13 ± 1.6). The data also revealed that adrenomedullin content was considerably greater after the exposure to the high sodium chloride diet which is evident graphically in Figure 30 by the skewing of adrenomedullin content in rats fed with 8% NaCl diet. Arrow heads in Figures 19-27 indicate ADM immunopositive cells

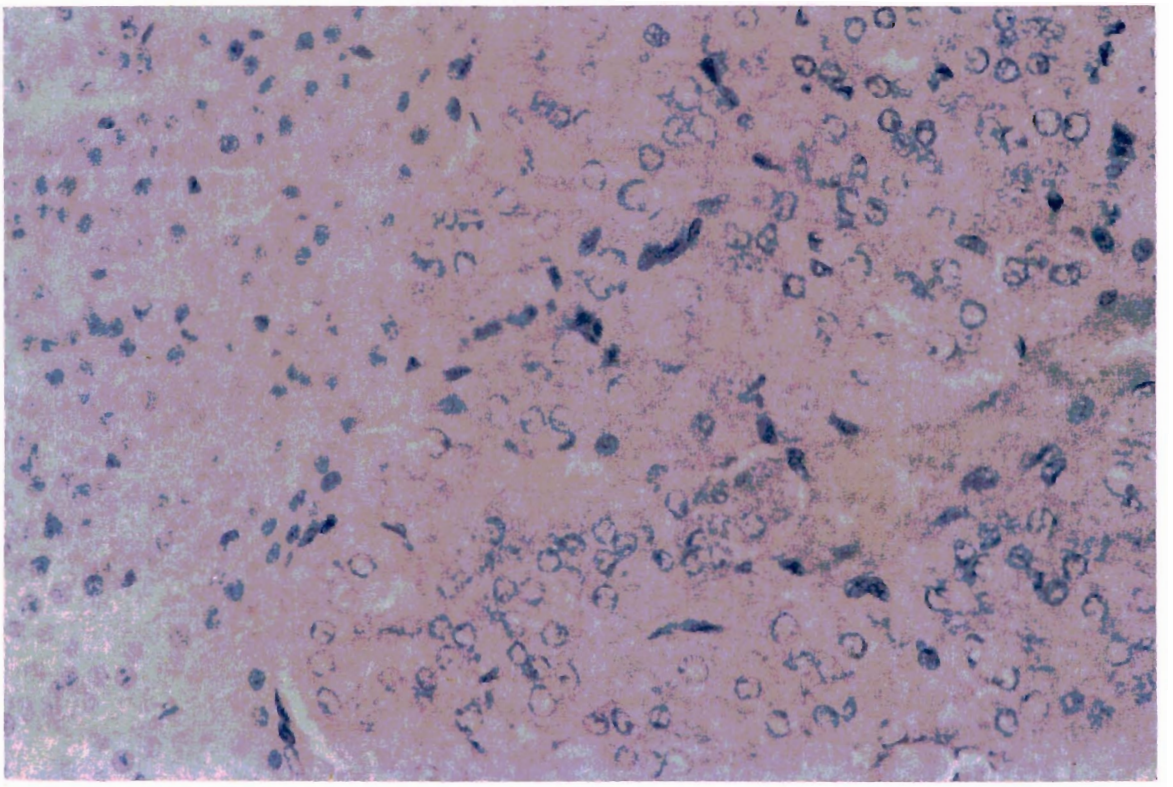


Figure 18 : Immunohistochemical staining of control adrenal gland tissue - NON IMMUNE RABBIT SERUM. (magnification, X 40)

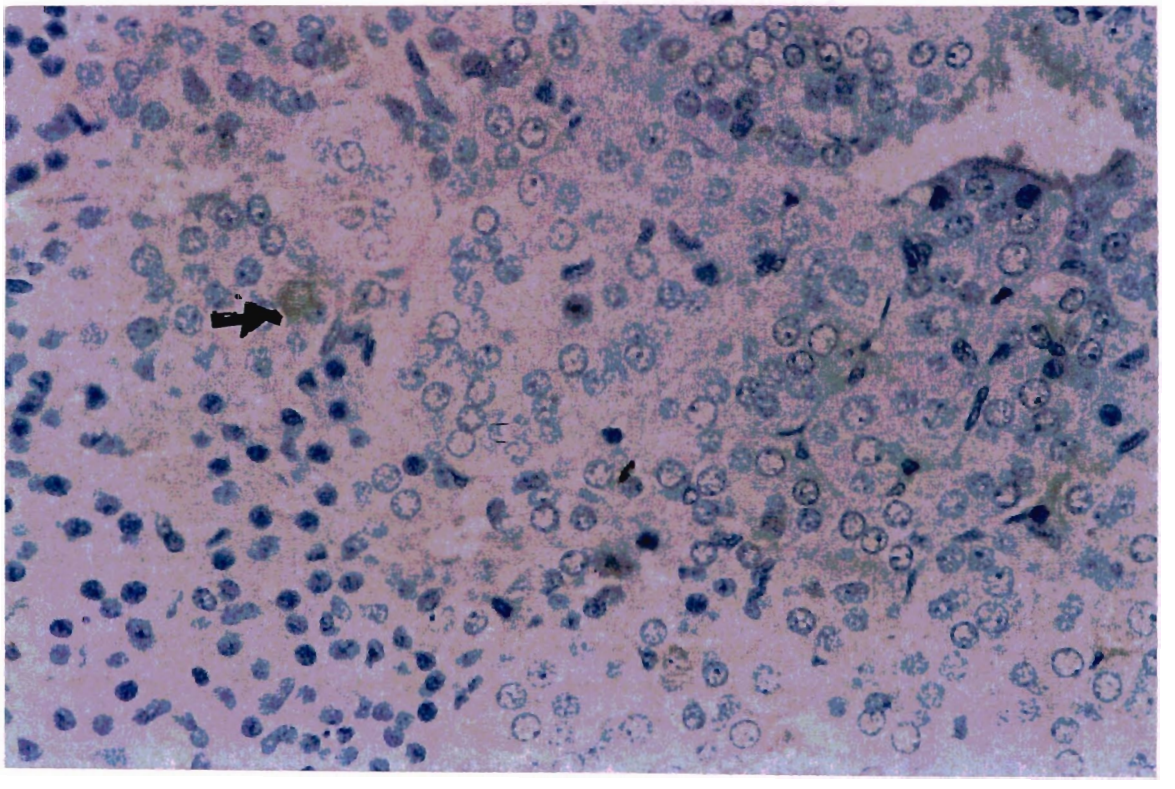


Figure 19 : Immunohistochemical localization of ADM in zona glomerulosa cells of rat adrenal gland tissue. (magnification, X 40)

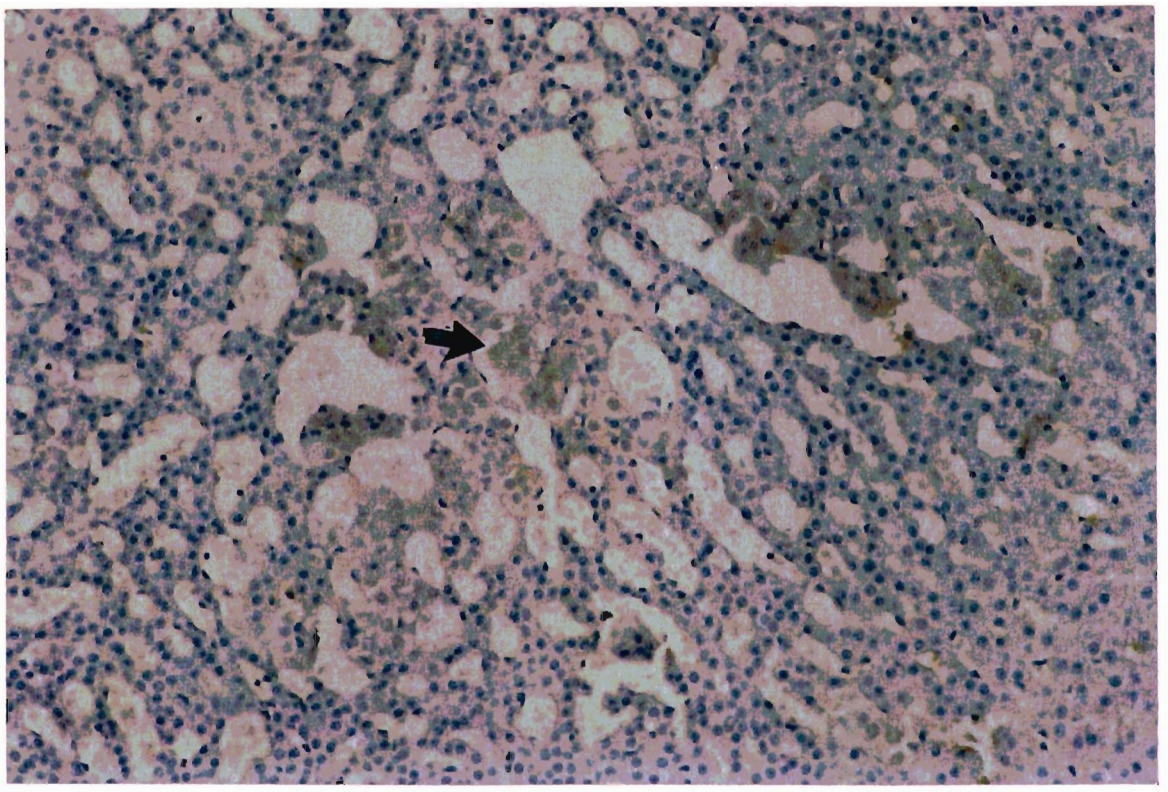
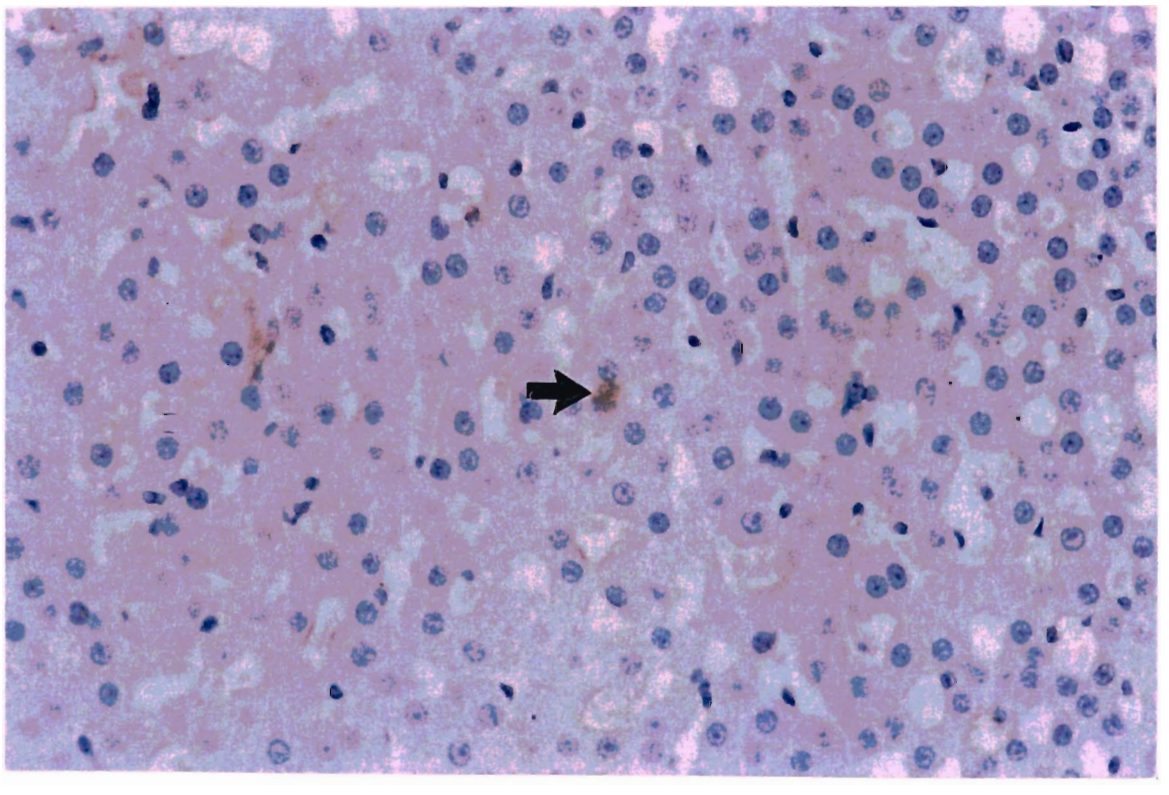


Figure 20 : Immunohistochemical localization of ADM in the zona reticularis region of the rat adrenal gland tissue. (magnification, X 20)



Figures 21 **:** Immunohistochemical localization of ADM in the zona reticularis region of the rat adrenal gland tissue. (magnification, X 40)

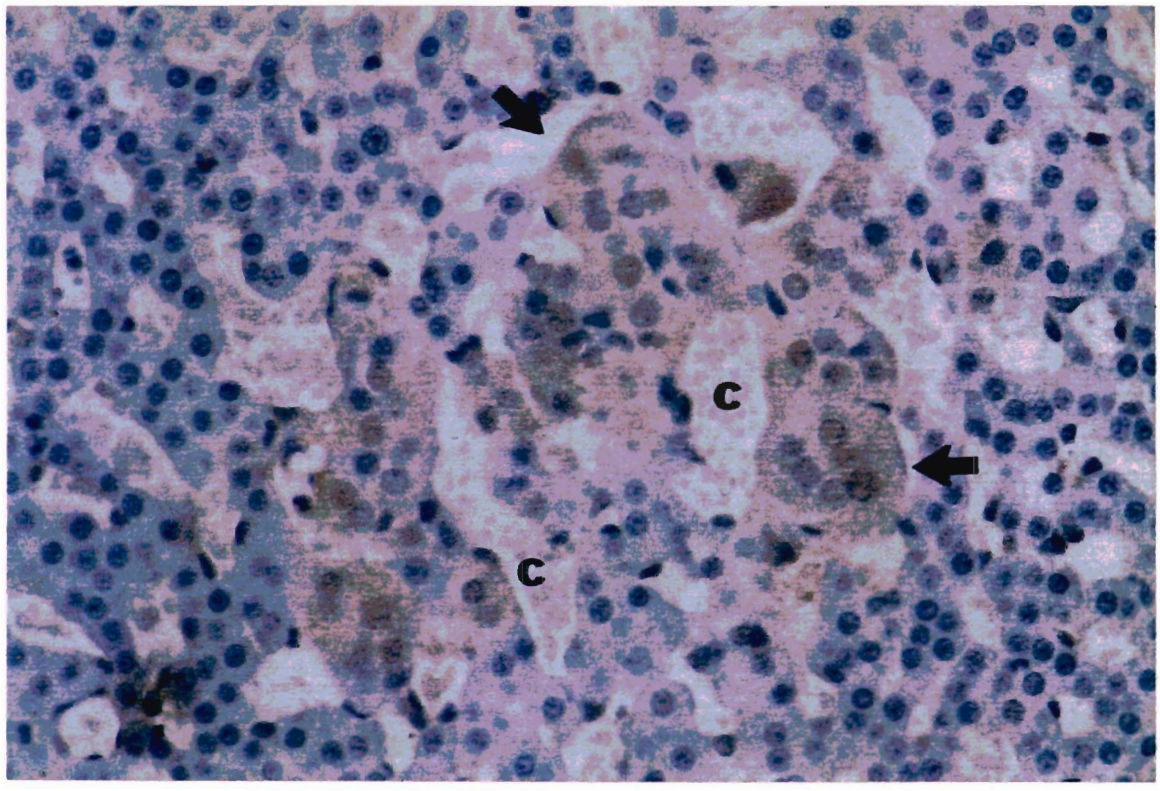


Figure 22 : Immunohistochemical localization of ADM around capillaries in the medullary region of adrenal gland tissue. (magnification, X 40)

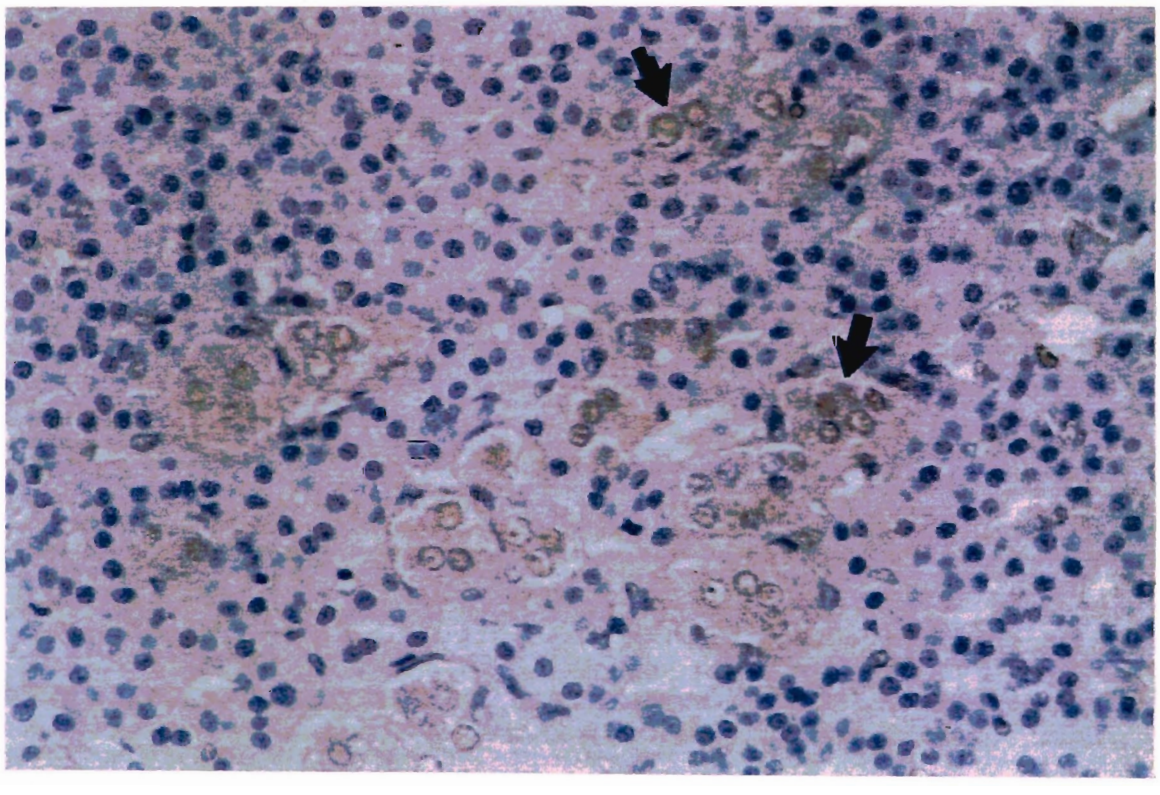


Figure 23 : Immunohistochemical localization of ADM in the medullary region of the rat adrenal gland tissue. (magnification, X 40)

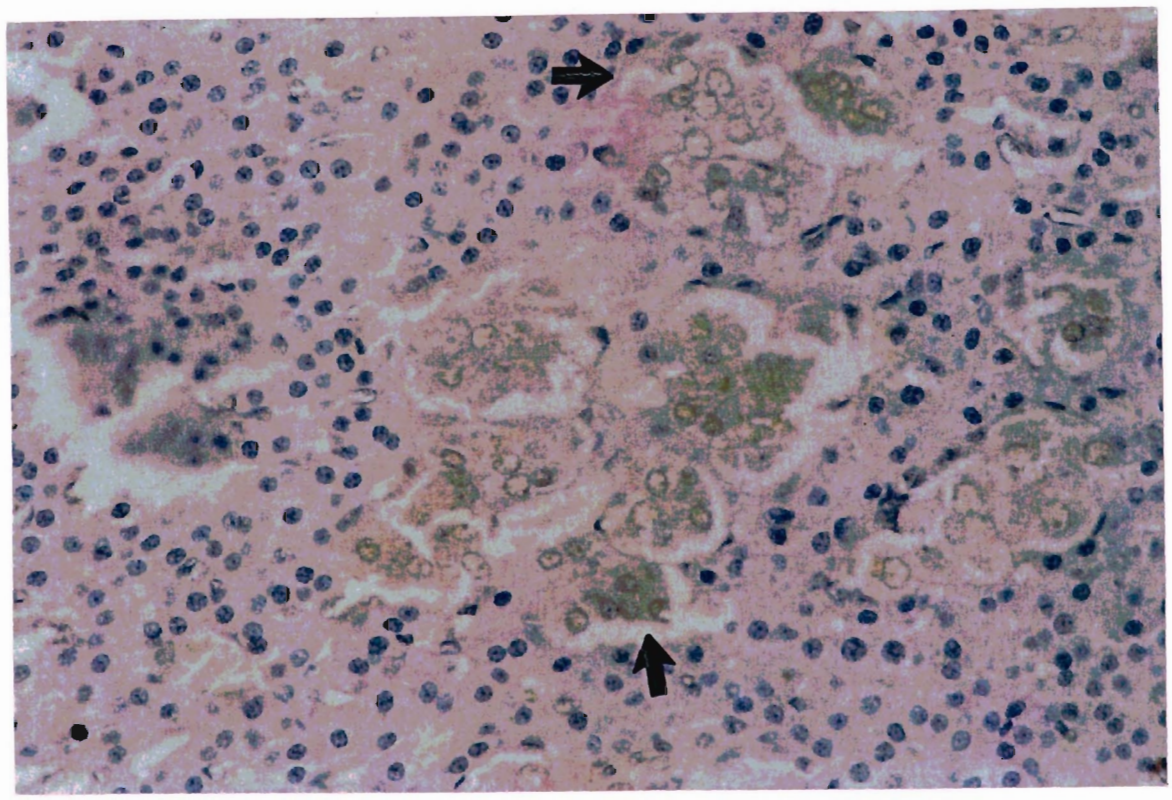


Figure 24 : Immunohistochemical localization of ADM in the medullary region of the rat adrenal gland tissue. (magnification, X 40)

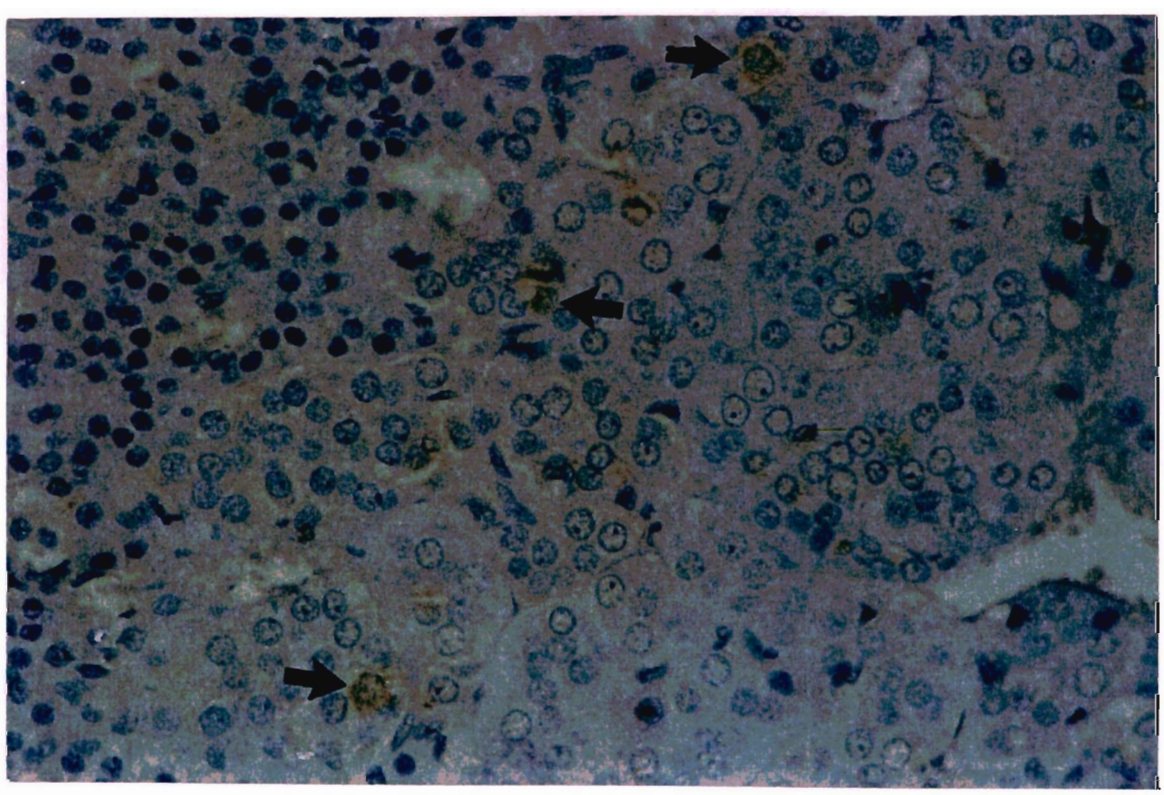


Figure 25 : Immunohistochemical localization of ADM in the medullary region of the rat adrenal gland tissue. (magnification, X 40)

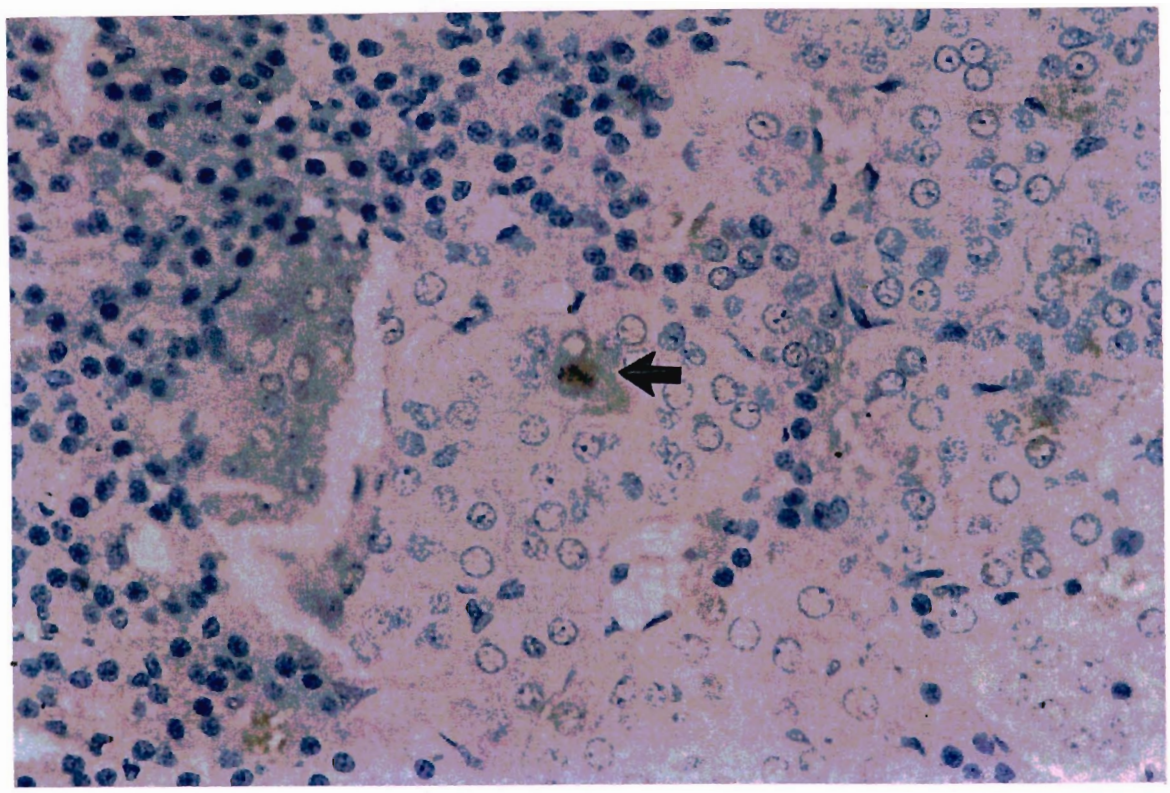


Figure 26 : Immunohistochemical localization of ADM in the medullary region of the rat adrenal gland tissue. (magnification, X 40)

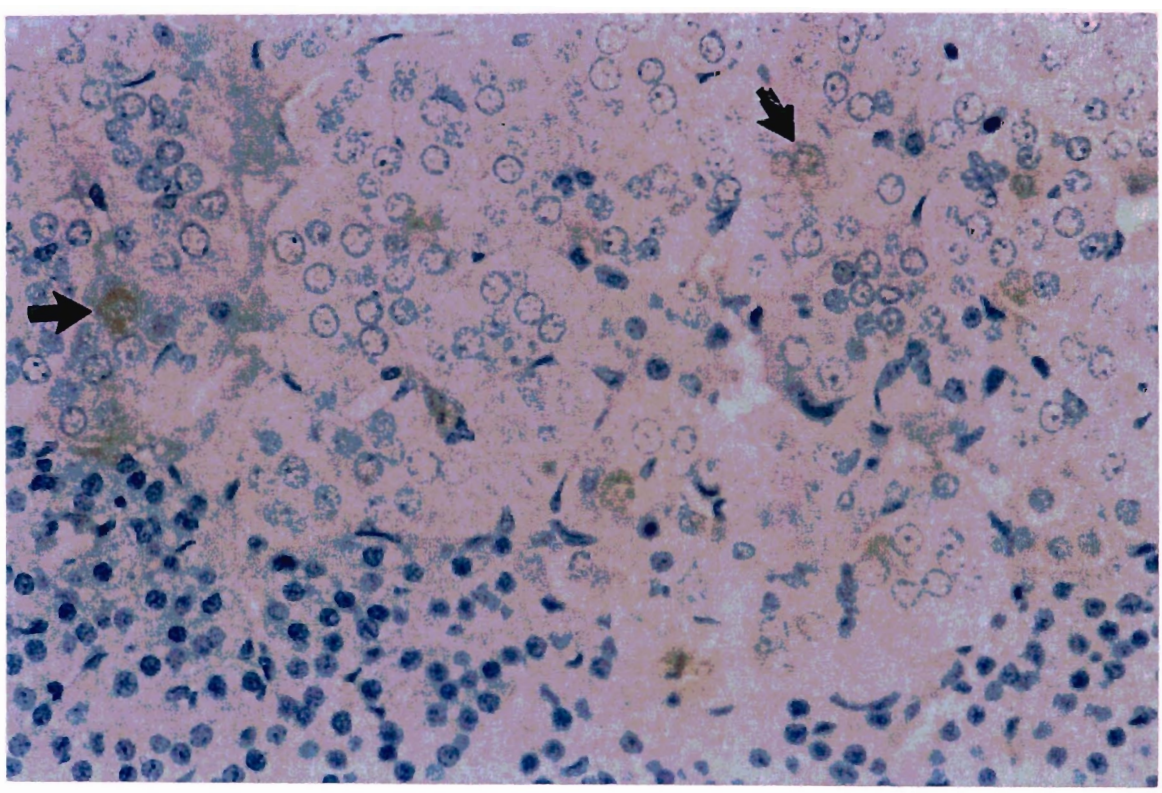


Figure 27 : Immunohistochemical localization of ADM in the medullary region of the rat adrenal gland tissue. (magnification, X 40)

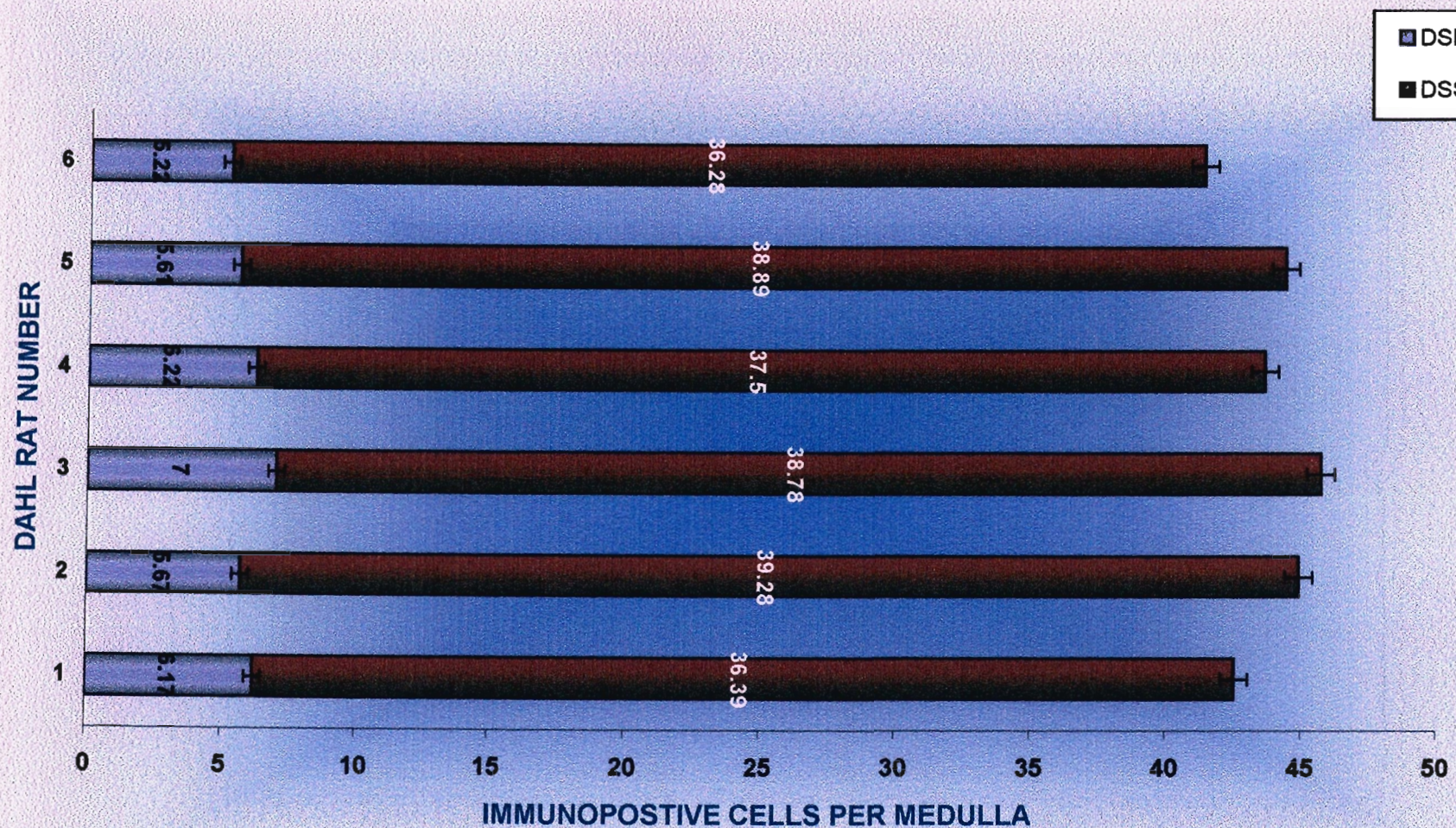


Figure 28 **: Immunopositive cells per medulla of Dahl salt sensitive (DSS) or Dahl salt resistant (DSR) rats consuming a 1% NaCl diet**

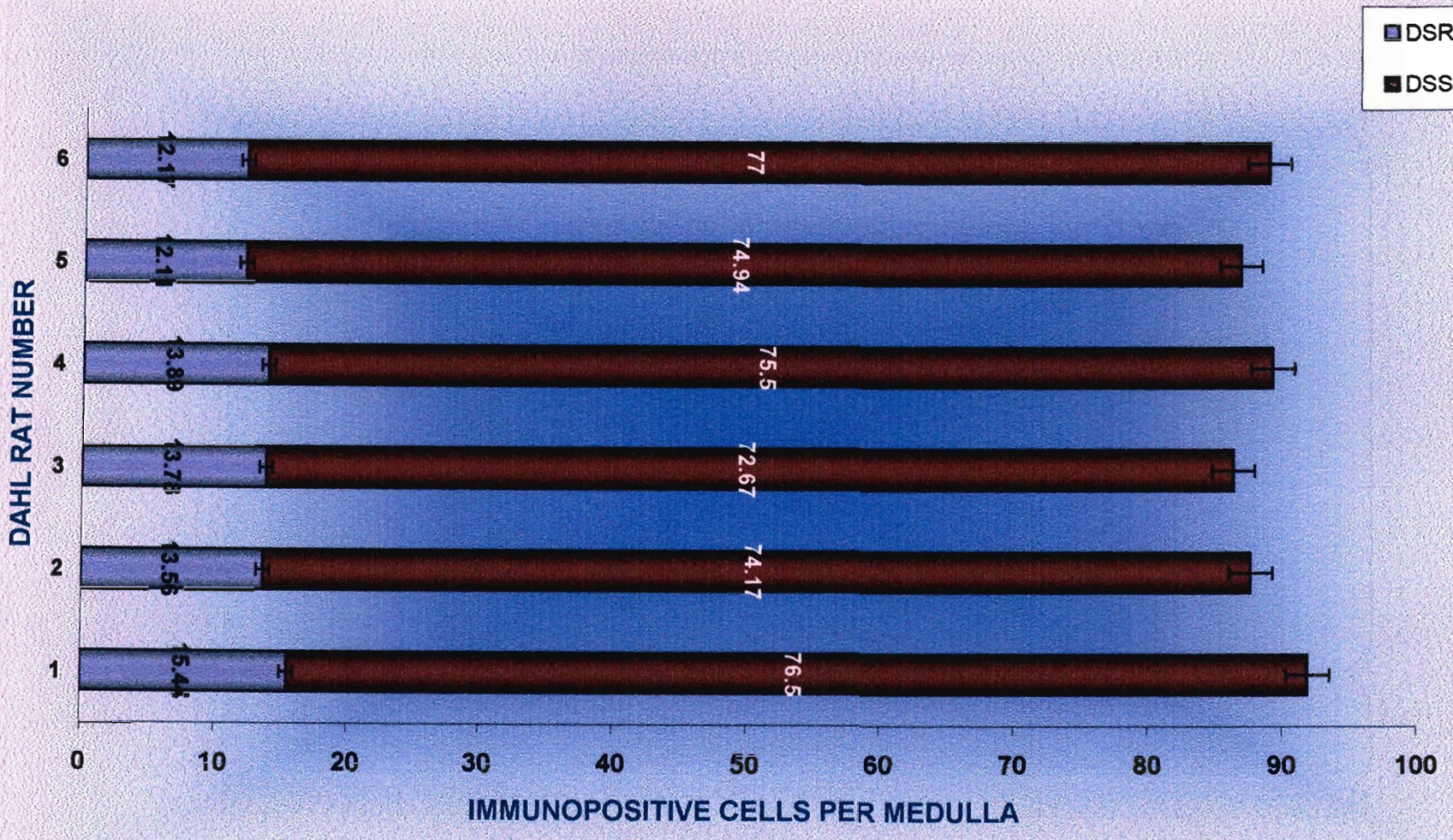


Figure 29 **: Immunopositive cells per medulla of Dahl salt sensitive (DSS) or Dahl salt resistant (DSR) rats consuming a 8% NaCl diet.**

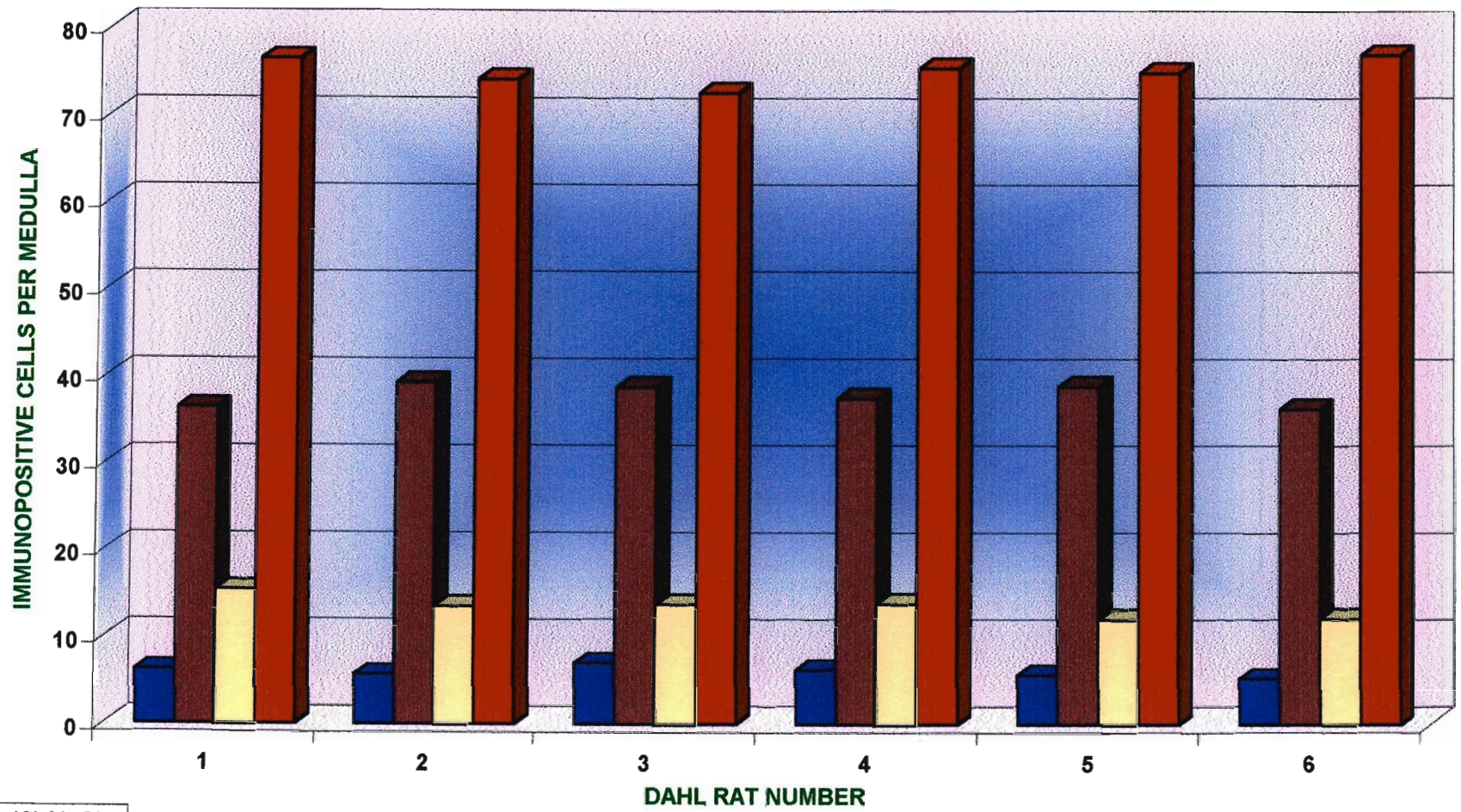


Figure 30 **: The number of adrenomedullin cells immunostained in the adrenal medulla of Dahl salt-resistant (DSR) and Dahl salt-sensitive (DSS) rats on normal salt (1% NaCl) and high salt (8% NaCl) diets**

5. DISCUSSION

Hypertension is a complex trait with multiple genetic and environmental influences (Kurtz and Spence, 1993). Hormones and their actions are important in the maintenance of blood pressure and considerable evidence suggest that adrenomedullin, a recently discovered hypotensive hormone, contributes to the control of fluid and electrolyte homeostasis (Samson, 1999). Adrenomedullin, which is a 52-amino-acid vasodilator peptide, was isolated in 1993 from human pheochromocytoma (Kitamura *et al.*, 1993). It is a known multifunctional hormone, which affects cellular growth, the cardiovascular system, the central nervous system, and the endocrine system. In the central nervous system, ADM may have a role in the modulation of salt appetite, thirst, and sympathetic activity (Samson, 1999 ; Saita *et al.*, 1998). Adrenomedullin has been measured in a wide range of disease states and in many cardiovascular disorders, it has been reported to be elevated, possibly suggesting that increased adrenomedullin participates in the regulation of blood pressure, released to compensate for elevated blood pressure (Hinson *et al.*, 2000). Although the exact mechanisms by which adrenomedullin produces vasodilation are not clearly defined, evidence suggests that it acts indirectly through NO and through cAMP-mediated mechanisms and via the inhibition of angiotensin II and endothelin production (Troughton *et al.*, 2000). Adrenomedullin also acts in the kidney in animals, relaxing both efferent and afferent glomerular arterioles, increasing glomerular filtration, and inducing natriuresis (Ebara *et al.*, 1994).

Currently, it is believed that the adrenal medulla is one of the major production sites of adrenomedullin. The adrenal medulla also produces several known regulating factors of

aldosterone secretion such as epinephrine, norepinephrine, vasopressin and dopamine. Whereas previous studies have reported immunoreactivity in the rat adrenal gland (Satoh *et al.*, 1996), little is known about the medullary secretory capacity of adrenomedullin. The results obtained from the present study demonstrate to some extent the precise sites of secretion of this hormone as well as the increased rate of secretion of adrenomedullin to compensate for the elevated blood pressure.

In order to produce exaggerated and more severe hypertension in which to evaluate the secretion of adrenomedullin, half of the rats were maintained on a high salt (8% NaCl) diet. The Dahl rats were chosen as a model of salt-sensitive hypertension, because the salt-sensitive rats (DSS) are selectively bred to develop hypertension after a high salt diet while the salt-resistant strain (DSR) remain normotensive. Our results reveal that a high salt (8% NaCl) diet is detrimental to the health of these animals, which is evident in the fact that four animals did not survive the duration of the project. This has been known to occur since similar results were observed in a previous study conducted by Rapp and Dene (1985). They found that when DSS rats are placed on a high salt diet early in life, death ensued after 4 to 8 weeks. This suggests that the time and duration of the high salt diet determine the survival of DSS rats.

Food consumption in the DSS strain on 8% NaCl diet decreased as the severity of the hypertensive state increased, and these animals became weak, stopped growing and lost weight. This observation further emphasizes the detrimental effects to a high salt diet, which conforms to other studies which found that when DSS rats were put on a high salt

diet early in life, they were found to become severely ill by the 4th week of food consumption (Rapp and Dene, 1985).

The changes in the kidney and heart weights are consistent with a previous study which reported that increasing salt intake resulted in marked hypertension with left ventricular and renal enlargements in salt sensitive rats (McCormick *et al.*, 1989). In this study, high salt consumption also resulted in retardation in body weight gain in the DSS rats, which are in keeping with the results of previous investigators (Somova *et al.*, 1999; Laakso *et al.*, 1998) but contrasted with others who found a greater growth rate on a high sodium diet (Channa M.L, unpublished).

Due to the fact that the DSS rats on 8% NaCl developed hypertension as early as the 2nd week as reflected in the systolic and diastolic blood pressures suggests that DSS rats are susceptible to the development of hypertension even at a young age which is in agreement to the findings put forth by Dahl *et al.* (1968). By weeks 3-4 the DSS rats on 1% NaCl also became hypertensive further emphasizing the high susceptibility of this strain of rat to the development of hypertension.

The reason for the decrease in blood pressures during weeks 4-6 in DSS rats on 1% NaCl diet, could be indicative of the maximising of the pressure natriuretic effect which has been reported to peak at 160mm Hg (Tobian, 1995). Channa (unpublished) reported that the initial rise in blood pressure in DSS rats appears to be triggered by sodium retention which accompanies an increased plasma volume and cardiac output. Thereafter the

cardiac output returns towards normal and hypertension is maintained by an increased peripheral resistance (Wilson *et al.*, 1997). At the culmination of this study, no significant differences in heart rates between both groups of Dahl salt-sensitive rats were observed indicating that increased peripheral resistance is responsible for maintaining the elevated blood pressure. Thus it can be concluded that even a low NaCl diet is sufficient to induce hypertension in DSS rats as reflected by their high systolic pressures but the hypertensive status of DSS rats on a high NaCl diet, was further exacerbated as depicted by their high systolic and dystolic blood pressures.

Whether the rats consumed a normal or a high salt diet, they gradually developed hypertension (BP above 150/95 according to WHO criteria) with tachycardia at the age of 2 months. The period for the development of hypertension was reduced due to the salt loading, with the DSS on 8% NaCl rapidly developing severe hypertension at the end of 6 weeks. Therefore this study also confirmed the genetic predisposition for the development of hypertension and the well known positive correlation between high blood pressure and the sodium chloride intake in the Dahl salt sensitive model.

To date, no studies have examined quantitatively, the adrenomedullin secretory capacity of the adrenal gland in either animal or human models of hypertension. It is known that cells of the adrenal medulla accumulate and store their hormones (epinephrine and norepinephrine) in granules and the data obtained from this study suggest that adrenomedullin is also stored in secretory granules in the adrenal medulla.

In the present study, adrenomedullin immunopositivity was found predominantly in the adrenal medulla, and to varying levels in the adrenal cortex or more precisely, the zona glomerulosa and zona reticularis. In agreement with our results, Kapas *et al.* (1998) and Mulder *et al.* (1996), found immunoreactive adrenomedullin present in the rat adrenal medulla. However, previous studies have produced conflicting results in localizing the presence of adrenomedullin in adrenal cortex. We observed minimal staining for adrenomedullin in the zona glomerulosa which are in keeping with the results of previous investigations (Kapas *et al.*, 1998). However, our study also found groups of two to three cells displaying minimal-to-moderate staining for adrenomedullin in the zona reticularis, which has not been documented previously to show immunoreactivity.

In the medulla, the pattern of staining is reminiscent of the staining of chromaffin cells possibly suggesting that adrenomedullin is possibly synthesised by either adrenaline-secreting or nor-adrenaline secreting cells or both. However, this study did not attempt to establish which chromaffin subtype synthesised adrenomedullin, but a previous study (Mulder *et al.*, 1996) has reported that the adrenaline-secreting cells may be a more significant source of adrenomedullin than nor-adrenaline secreting cells, but to what extent is unknown. In that study, which sought to determine which subtype of chromaffin cell synthesised adrenomedullin, it was reported that the peptide was found in both phenylethanolamine *N*-methyltransferase (PNMT)-positive and -negative cells. PNMT is the enzyme that converts noradrenaline to adrenaline and is not expressed in nor-adrenaline secreting cells. Therefore, in the study conducted by Mulder *et al.* (1996), staining for adrenomedullin was less intense in the PNMT-negative cells suggesting that

adrenaline-secreting cells may be a more significant source of adrenomedullin than noradrenaline-secreting cells.

The present study demonstrated that on comparison with both the strains (DSR vs. DSS) treated with 8% NaCl diet, there was an increase in the number of adrenomedullin granules in the medullary region of the adrenal glands of DSS rats. Our current findings show that there was a 6.3 fold increase in ADM granules of adrenal glands of DSS rats compared to DSR rats, ingesting a 1% NaCl supplemented diet. Also, there was a 4.1 fold increase in adrenomedullin granules of adrenal glands of DSS rats than DSR rats, consuming an 8% NaCl supplemented diet. Importantly, the number of positive cells and the intensity of staining also increased significantly in animals treated with 8% NaCl in comparison to those treated with a 1% NaCl diet. This shows that the changes in ADM content were not only strain specific but dependant on the sodium chloride intake also. It shows both a genetic and environmental (salt) component in the ADM content increase.

The possible physiological significance underlying the aforementioned changes in the hypertensive state are both complex and varied. Like other regulatory peptides present in the adrenal gland, adrenomedullin affects the secretory activity of the adrenal cortex in both the rat and human (Hinson *et al.*, 2000). Therefore, it is possible that the high secretion and production of adrenomedullin by secretory cells of the adrenal medulla, may modulate the physiologic function of the adrenal cortex possibly by a paracrine mechanism. This assumption may seem plausible since ADM and PAMP are present in the blood, but their concentrations, under both normal and pathological conditions, are in

the pmolar range, thereby ruling out the possibility that they act on zona glomerulosa as circulating hormones (Richards *et al.*, 1996).

Troughton *et al.* (2000) found that plasma renin activity (PRA) rose during high dose-ADM and was accompanied by a small rise in Ang II, whereas aldosterone levels tended to fall consistent with the findings of Yamaguchi *et al.* (1996). In the adrenal glomerulosa cells, cyclic AMP is known to stimulate aldosterone secretion, however Yamaguchi *et al.* (1995) documented from their study that human adrenomedullin did not affect basal aldosterone secretion and inhibited angiotensin II- and potassium-stimulated aldosterone secretion of rat zona glomerulosa cells, probably by impairing Ca^{2+} influx. However, Andreis *et al.* (1997) suggested that not ADM, but proadrenomedullin N-terminal 20 peptide (PAMP) to be considered a physiologic inhibitor of mineralocorticoid secretion, which may play an important role in the regulation of fluid and electrolyte homeostasis.

Previous researchers have demonstrated interactions between ADM and other hormones (Samson *et al.*, 1995; Mulder *et al.*, 1996). Aldosterone which is synthesised and released by the zona glomerulosa cells of the adrenal cortex, is an important factor for the regulation of blood pressure and secretion is subject to regulation by several known vasoactive peptides, including angiotensin II and atrial natriuretic peptide.

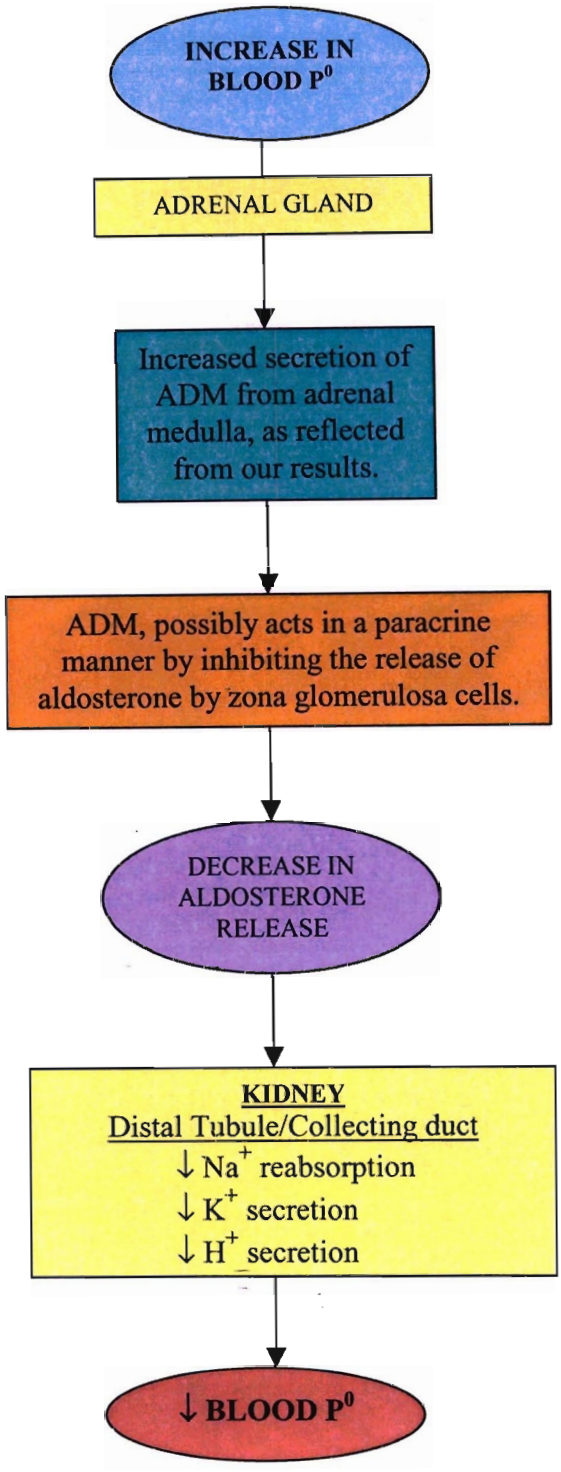


Figure 31 : Schematic representation of the postulated hypotensive actions of adrenomedullin after secretion from the adrenal medulla during hypertension

Aldosterone, the principle mineralocorticoid, causes sodium retention and potassium excretion by the distal nephron. It is known to act within the kidney to stimulate Na^+ absorption and K^+ secretion by the principal cells of the distal tubule and collecting duct. It achieves these effects by entering the cells, binding to a receptor and inducing the synthesis of a number of proteins. These, in turn are involved in increasing the number of Na^+ and K^+ channels in the luminal membrane and in increasing the activity of Na^+/K^+ ATPase in the basolateral membrane. Therefore, sodium retention by the mechanism described above, together with excretory ability, results in increased blood volume and hence blood pressure.

The release of adrenomedullin secreted in large amounts in the adrenal medulla is thought to inhibit the secretion of aldosterone from the zona glomerulosa. The large amounts of ADM, if it does act as a local hormone, seems to inhibit secretion of aldosterone from the zona glomerulosa, ensuring that sodium reabsorption is at a minimum, possibly reducing blood volume as well as blood pressure. Figure 31 illustrates the possible hypotensive effects of ADM after secretion from the adrenal medulla.

To compensate for the increase in blood pressure due to the salt diets, there was a co-ordinated increase in medullary adrenomedullin. The data suggests that the adrenal gland exhibits a compensatory response in rats with developing or full blown hypertension manifested as a co-ordinated localized increase in adrenomedullin.

However, it is still unclear whether systemic increases in adrenomedullin reflect an overflow from local sites of production and action, or whether in certain conditions increased plasma adrenomedullin has a hormonal function causing a general decrease in vascular resistance and a fall in blood pressure (Hinson *et al.*, 2000). Although there is still some debate on the underlying mechanism of action of adrenomedullin, this study provides evidence for a possible compensatory role of adrenomedullin in experimental hypertension.

6. CONCLUSION

The key issues that were addressed in this study, are briefly outlined as follows :

- The Dahl Rat provided a good model for the development of hypertension, as this study also confirmed the genetic predisposition for the development of hypertension and the positive correlation between high blood pressure and sodium chloride in Dahl salt sensitive rats. Our findings indicate that whether the rats consumed a normal or a high salt diet, they gradually developed hypertension with tachycardia after 4 weeks irrespective of the diets.

- Salt loading was found to increase the secretion of ADM from the adrenal medulla in DSS rats compared to DSR rats. Interestingly, DSR on a high sodium chloride diet also had increased ADM levels compared to DSR on a low salt diet. ADM levels therefore seemed to increase in a strain specific manner (ie. DSS vs. DSR) after salt loading (high salt vs. low salt).

Based on the above findings, it was therefore postulated that hypertension induced by salt loading may lead to increased secretion of ADM by the adrenal medulla of Dahl rats which may possibly initiate a compensatory response by acting in a paracrine manner, by inhibiting aldosterone secretion. It is possible therefore that not only is there a genetic component to the alteration of ADM levels but also a salt dependant component.

Furthermore, the Dahl hypertensive rat provides a good model for the evaluation of adrenomedullin in hypertensive states, and may therefore prove an appropriate model for testing of drugs which may influence ADM secretion during hypertension.

- This research was also a pioneering study in the field of immunohistochemistry in this laboratory, and serves as a basis for establishing this method as a routine histological technique. Immunohistochemical methods are known to be reemployed in many fields of biological research and are regularly used in diagnostic pathology. Therefore, this technique will prove a vital "tool" for future research, which may require diagnostic immunopathological methods.

Recommendations for future work are briefly given below :

Some interesting questions on interpreting the immunohistochemical data can be raised regarding the rate of secretion of adrenomedullin following hypertension. It is possible that a more accurate assessment could have been achieved if rate of secretion was monitored on a weekly basis rather than an overall assessment at the completion of the 6 week period. Therefore, if sacrificing of some animals on a weekly basis occurred, with evaluation of ADM secretion being determined directly afterwards, a more concise account of its secretion occurring concurrently with developing hypertension could have been established.

This study has established with some clarity that increased blood pressure does result in a co-ordinated increase in adrenomedullin, which is a known vasodilator. However its sites

of action once released was not confirmed but it has been speculated to act on the glomerulosa cells by inhibiting aldosterone secretion possibly by a paracrine mechanism. Furthermore, no literature documented any occurrence of adrenomedullin in the zona reticularis and therefore significance of the presence of adrenomedullin in the adrenal cortex of the rat adrenal should be further explored to fully understand the role it plays in not only hypertension, but also other diseased states if any.

In a post-genomic era, a vast array of state-of-the-art technologies are now available for the unravelling of complex biological phenomena such as blood pressure regulation. In the light of some of our findings, it is proposed that :

- Transgenic technology may be employed to address the question of intra-adrenal vs. systemic levels of adrenomedullin (by confering the production in a tissue specific manner).

- Protein-protein interactions be evaluated using coimmunoprecipitation with the relevant antibodies, in order to shed more light on the exact role of ADM in sodium reabsorption and transport in the kidney.

- ADM knock-out (KO) mice be employed as negative controls, to provide direct data on the physiological distribution and function.

The information can then be used to provide new targets for therapeutic intervention.

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APPENDICES

APPENDIX A-1

BUFFERS, REAGENTS AND SOLUTIONS

1. BUFFERS

1.1 Tris Buffer (TBS) (0.05M, pH 7.6)

Dissolve 6.1g Tris (hydroxymethyl amino methane) in 50ml distilled water. Add 37ml of 1N hydrochloric acid and make up to 1 liter with distilled water. Store in refrigerator at 6°C.

1.2 Phosphate Buffered Saline (PBS) (0.02M, pH 7.0)

Dissolve 1.92g dibasic sodium phosphate anhydrous (Na_2HPO_4), 0.92g monobasic sodium phosphate monohydrate ($NaH_2PO_4.H_2O$) and 5.90g Sodium chloride (NaCl) in 1 liter of distilled water. Store in refrigerator at 6°C.

2. REAGENTS AND SOLUTIONS

2.1 Hydrogen peroxide (H_2O_2) (0.6%)

Mix 0.1 ml 3 % H_2O_2 and 5 ml absolute methanol.

2.2 Goat Serum (5%)

Add 20 ml TBS to 1 ml Goat serum + 20 ml TBS. Aliquot solution in 1ml eppendorf tubes, and store in refrigerator at 6°C.

2.3 Non-immune rabbit serum (5%)

Dilute 1ml Non-immune rabbit serum with 20 ml TBS. Aliquot solution in 1ml eppendorf tubes, and store in refrigerator at 6°C.

2.4 Rabbit polyclonal anti-adrenomedullin (1:500)

Dilute 50 µl Rabbit polyclonal anti-ADM with 50 µl distilled water, and thereafter add to 25ml TBS. Aliquot solution in 1ml eppendorf tubes, and store in refrigerator at 6°C

2.5 Goat anti-rabbit horseradish peroxidase (1:100)

Dilute 0.2µl goat anti-rabbit HRP in 20ml TBS. Aliquot solution in 1ml eppendorf tubes, and store in refrigerator at 6°C.

2.6 DAB solution

Dissolve 1 DAB tablet in 10 ml TBS. Aliquot solution in 1ml eppendorf tubes, and store in refrigerator at 6°C, and if it becomes discoloured, discard the solution.

2.7 Hydrogen peroxide (H_2O_2) 3%

Add 0.1 ml H_2O_2 + distilled water.

2.8 Working DAB solution

Add 7.5 µl 3 % H2O2 to 1 ml DAB solution. The workimg DAB solution must be prepared freshly.

2.9 Mayer's Hematoxylin

Dissolve 50g ammonium or potassium alum in water. Add and dissolve 1g of hemotoxylin crystals in this solution. To this mixture add 0.2g sodium iodate, 1g citric acid, and 50g chloral hydrate. Shake well until all the components are in complete solution producing a reddish-violet colour. Solution should be changed weekly, to ensure consistency of staining results.

APPENDIX B

APPENDIX B-1

WEEKLY FOOD CONSUMPTION (in grams)

GROUP	RAT NO	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6
Dahl S (1% NaCl)	1	116	123	135	128	125	128
	2	122	109	147	143	142	141
	3	117	108	115	136	118	140
	4	101	113	131	100	149	147
	5	109	115	130	141	146	152
	6	100	105	126	139	138	134
	7	106	121	133	142	146	132
	8	105	109	125	137	139	134
	9	108	118	132	142	151	124
	10	101	106	130	140	151	141
	11	88	110	126	138	146	141
	12	106	118	129	140	138	128
Dahl S (8% NaCl)	1	79	115	118	127	---	---
	2	100	106	125	139	133	131
	3	102	103	122	130	129	51
	4	96	99	112	135	137	135
	5	108	108	123	134	134	---
	6	96	113	123	130	139	137
	7	95	86	104	120	150	129
	8	108	107	123	132	135	36
	9	103	95	119	131	102	---
	10	111	106	118	130	145	125
	11	105	98	120	128	145	53
	12	104	100	118	129	136	104

APPENDIX B-2

WEEKLY FOOD CONSUMPTION (in grams)

GROUP	RAT NO	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6
Dahl R (1% NaCl)	1	84	86	87	78	83	79
	2	122	120	111	119	109	110
	3	78	96	90	94	89	87
	4	106	115	108	107	106	116
	5	125	114	120	116	113	112
	6	89	112	106	104	104	108
	7	96	98	100	104	96	100
	8	111	112	117	109	104	106
	9	69	91	80	94	84	86
	10	90	95	102	98	95	92
Dahl R (8% NaCl)	1	100	92	104	113	94	103
	2	79	83	108	109	104	105
	3	86	89	105	95	83	86
	4	105	75	94	99	105	103
	5	105	76	105	113	109	117
	6	65	83	94	95	98	114
	7	103	90	96	106	---	---
	8	99	97	93	112	98	104
	9	70	89	90	99	97	107
	10	85	89	103	94	97	101

APPENDIX C

APPENDIX C-1

WEEKLY MASSES (in grams)

	RAT NO	DAY 0	BASE LINE	W1	W2	W3	W4	W5	W6
Dahl S (1% NaCl)	1	56	81	109	161	205	238	271	301
	2	61	67	107	157	201	240	295	321
	3	56	79	103	146	193	224	266	287
	4	58	59	79	126	181	214	250	288
	5	43	57	101	150	196	230	287	319
	6	40	53	87	129	171	216	268	295
	7	43	69	105	150	208	245	298	330
	8	39	64	97	138	183	223	271	300
	9	60	77	111	157	206	236	280	305
	10	40	51	80	124	171	219	276	315
	11	36	46	73	114	164	190	260	297
	12	38	57	88	136	184	226	270	295
Dahl S (8% NaCl)	1	41	64	92	109	152	177	245	---
	2	48	64	98	140	174	192	252	268
	3	31	58	91	131	161	177	232	210
	4	38	48	72	118	145	181	225	258
	5	36	65	84	127	160	176	243	232
	6	47	70	104	142	175	201	252	275
	7	38	57	83	118	142	160	204	225
	8	42	60	88	130	169	181	246	246
	9	34	60	83	120	144	168	192	237
	10	44	69	98	140	174	188	245	259
	11	38	57	78	123	156	179	228	242
	12	47	59	83	126	156	162	216	235

APPENDIX C-2

WEELY MASSES (in grams)

	RAT NO	DAY 0	BASE LINE	W1	W2	W3	W4	W5	W6
Dahl R (1% NaCl)	1	102	131	154	170	179	189	199	203
	2	108	144	178	218	239	264	270	277
	3	121	144	166	190	209	224	234	242
	4	101	146	182	210	229	245	255	260
	5	120	156	200	226	254	278	282	290
	6	55	83	123	153	184	214	230	237
	7	57	86	125	151	179	206	220	225
	8	107	130	170	202	223	243	254	265
	9	65	87	106	121	130	144	147	149
	10	76	100	131	157	179	198	211	215
Dahl R (8% NaCl)	1	70	99	127	134	151	171	196	204
	2	76	105	141	138	164	170	173	176
	3	50	81	115	112	138	153	170	198
	4	62	95	126	120	148	174	176	180
	5	79	93	126	137	164	181	204	204
	6	30	43	69	88	116	141	166	172
	7	48	74	104	128	143	---	---	---
	8	41	56	95	113	135	164	194	202
	9	40	63	93	103	125	152	184	187
	10	43	73	106	115	143	163	193	196

APPENDIX D

DATA FOR ADRENOMEDULLIN COUNTS

APPENDIX D-1

SLIDE/RAT #		1			2			3			4			5			6		
DSR 1% NaCl	1	9	8	7	8	8	7	4	7	5	6	5	8	4	3	3	6	5	8
	2	5	9	6	7	5	4	3	6	7	7	4	6	5	4	4	9	5	6
	3	7	9	6	8	5	6	8	9	9	6	8	5	7	5	5	9	6	8
	4	8	6	5	6	5	6	6	8	5	6	6	8	6	5	7	7	6	6
	5	7	8	5	6	4	4	4	5	5	7	6	5	4	7	6	8	4	6
	6	7	5	4	5	4	5	6	5	5	6	8	6	4	5	4	4	7	4

APPENDIX D-2

SLIDE/RAT #		1			2			3			4			5			6		
DSR 8% NaCl	1	17	19	16	18	15	17	14	12	17	14	15	17	14	14	15	14	15	15
	2	12	10	9	12	14	15	11	13	15	16	18	14	17	13	15	13	12	15
	3	14	11	12	15	12	10	13	17	15	12	15	14	17	15	15	14	13	14
	4	16	14	13	14	11	17	17	14	12	13	15	12	15	15	12	13	13	14
	5	14	11	12	12	11	11	12	11	12	10	11	11	13	12	12	14	15	14
	6	11	9	13	17	14	11	15	11	11	10	12	11	10	14	15	12	12	11

APPENDIX D-3

SLIDE/RAT #		1			2			3			4			5			6		
DSS 1% NaCl	1	44	37	39	38	35	42	33	35	34	35	37	35	35	35	35	37	37	33
	2	35	40	46	39	44	41	33	35	38	40	48	37	38	33	41	36	41	42
	3	38	35	40	35	42	41	33	37	34	39	41	43	41	38	37	36	43	45
	4	33	37	34	38	41	39	40	33	38	41	38	35	38	36	41	40	37	36
	5	41	38	39	35	33	37	41	38	39	44	39	41	38	40	41	37	38	41
	6	35	38	33	38	40	37	36	36	35	38	35	40	32	35	38	33	35	39

APPENDIX D-4

SLIDE/RAT #		1			2			3			4			5			6		
DSS 8% NaCl	1	78	72	84	93	78	85	87	81	74	72	74	77	70	69	74	68	68	73
	2	83	74	77	72	77	69	78	76	77	78	70	69	70	74	73	70	72	70
	3	69	75	79	81	78	76	68	72	75	73	72	65	70	73	69	73	70	70
	4	71	78	75	84	78	81	78	80	81	69	70	75	73	76	81	69	69	71
	5	81	76	75	85	83	79	73	78	79	69	70	74	75	73	70	69	70	70
	6	73	77	75	81	80	77	79	76	76	79	76	78	79	80	82	70	73	75

APPENDIX E

STATISTICAL TESTS

APPENDIX E-1

ADM COUNT : DR 1%NaC vs. DS 1% NaCl

Unpaired t test

Do the means of DR 1% NaCl and DS 1% NaCl differ significantly?

P value

The two-tailed P value is < 0.0001, considered extremely significant.

t = 53.480 with 10 degrees of freedom.

95% confidence interval

Mean difference = 31.872 (Mean of DS 1% NaCl minus mean of DR 1% NaCl)

The 95% confidence interval of the difference: 30.544 to 33.199

Assumption test: Are the standard deviations equal?

The t test assumes that the columns come from populations with equal SDs.

The following calculations test that assumption.

F = 4.479

The P value is 0.0627.

This test suggests that the difference between the two SDs is not quite significant.

Assumption test: Are the data sampled from Gaussian distributions?

The t test assumes that the data are sampled from populations that follow Gaussian distributions. This assumption is tested using the method Kolmogorov and Smirnov:

Group	KS	P Value	Passed normality test?
DR 1% NaCl	0.1914	>0.10	Yes
DS 1% NaCl	0.2587	>0.10	Yes

Summary of Data

Parameter:	DR 1% NaCl	DS 1% NaCl
Mean:	5.982	37.853
# of points:	6	6
Std deviation:	0.6236	1.320
Std error:	0.2546	0.5388
Minimum:	5.220	36.280
Maximum:	7.000	39.280
Median:	5.920	38.140
Lower 95% CI:	5.327	36.468
Upper 95% CI:	6.636	39.239

APPENDIX E-2

ADM COUNT : DR 8% NaCl vs. DS 8% NaCl

Unpaired t test

Do the means of DR 8% NaCl and DS 8% NaCl differ significantly?

P value

The two-tailed P value is < 0.0001, considered extremely significant.

t = 75.083 with 10 degrees of freedom.

95% confidence interval

Mean difference = 61.638 (Mean of DS 8% NaCl minus mean of DR 8% NaCl)

The 95% confidence interval of the difference: 59.809 to 63.467

Assumption test: Are the standard deviations equal?

The t test assumes that the columns come from populations with equal SDs.

The following calculations test that assumption.

F = 1.626

The P value is 0.3033.

This test suggests that the difference between the two SDs is not significant.

Assumption test: Are the data sampled from Gaussian distributions?

The t test assumes that the data are sampled from populations that follow Gaussian distributions. This assumption is tested using the method Kolmogorov and Smirnov:

Group	KS	P Value	Passed normality test?
DR 8% NaCl	0.2074	>0.10	Yes
DS 8% NaCl	0.1400	>0.10	Yes

Summary of Data

Parameter:	DR 8% NaCl	DS 8% NaCl
Mean:	13.492	75.130
# of points:	6	6
Std deviation:	1.241	1.582
Std error:	0.5066	0.6460
Minimum:	12.110	72.670
Maximum:	15.440	77.000
Median:	13.670	75.220
Lower 95% CI:	12.189	73.469
Upper 95% CI:	14.794	76.791

APPENDIX E-3

ADM COUNT: Comparison between all groups

One-way Analysis of Variance (ANOVA)

The P value is < 0.0001, considered extremely significant. Variation among column means is significantly greater than expected by chance.

Tukey-Kramer Multiple Comparisons Test

If the value of q is greater than 3.958 then the P value is less than 0.05.

Comparison	Mean Difference	q	P value
DR 1% NaCl vs DS 1% NaCl	-31.872 62.835	***	P<0.001
DR 1% NaCl vs DR 8% NaCl	-7.510 14.806	***	P<0.001
DR 1% NaCl vs DS 8% NaCl	-69.148 136.33	***	P<0.001
DS 1% NaCl vs DR 8% NaCl	24.362 48.029	***	P<0.001
DS 1% NaCl vs DS 8% NaCl	-37.277 73.491	***	P<0.001
DR 8% NaCl vs DS 8% NaCl	-61.638 121.52	***	P<0.001

Difference	Mean Difference	95% Confidence Interval From	To
DR 1% NaCl - DS 1% NaCl	-31.872	-33.879	-29.864
DR 1% NaCl - DR 8% NaCl	-7.510	-9.518	-5.502
DR 1% NaCl - DS 8% NaCl	-69.148	-71.156	-67.141
DS 1% NaCl - DR 8% NaCl	24.362	22.354	26.369
DS 1% NaCl - DS 8% NaCl	-37.277	-39.284	-35.269
DR 8% NaCl - DS 8% NaCl	-61.638	-63.646	-59.631

Assumption test: Are the standard deviations of the groups equal?

ANOVA assumes that the data are sampled from populations with identical SDs. This assumption is tested using the method of Bartlett.

Bartlett statistic (corrected) = 3.584

The P value is 0.3101.

Bartlett's test suggests that the differences among the SDs is not significant.

Assumption test: Are the data sampled from Gaussian distributions?

ANOVA assumes that the data are sampled from populations that follow Gaussian distributions. This assumption is tested using the method Kolmogorov and Smirnov:

Group	KS	P Value	Passed normality test?
DR 1% NaCl	0.1914	>0.10	Yes
DS 1% NaCl	0.2587	>0.10	Yes
DR 8% NaCl	0.2074	>0.10	Yes
DS 8% NaCl	0.1400	>0.10	Yes

Intermediate calculations. ANOVA table

Source of variation	Degrees of freedom	Sum of squares	Mean square
Treatments (between columns)	3	17454	5818.0
Residuals (within columns)	20	30.873	1.544
Total	23	17485	

F = 3769.0 =(MStreatment/MSresidual)

Summary of Data

Group	Number of Points	Standard Mean	Standard Error of Deviation	Mean	Median
DR 1% NaCl	6	5.982	0.6236	0.2546	5.920
DS 1% NaCl	6	37.853	1.320	0.5388	38.140
DR 8% NaCl	6	13.492	1.241	0.5066	13.670
DS 8% NaCl	6	75.130	1.582	0.6460	75.220

			95% Confidence Interval	
Group	Minimum	Maximum	From	To
DR 1% NaCl	5.220	7.000	5.327	6.636
DS 1% NaCl	36.280	39.280	36.468	39.239
DR 8% NaCl	12.110	15.440	12.189	14.794
DS 8% NaCl	72.670	77.000	73.469	76.791

APPENDIX F

APPENDIX F-1

1. Area under microscope (X 40) = πr^2

$= 22/7 \text{ X } (0.45*/2)^2$

$= 22/7 \text{ X } (0.225)^2$

$= 0.16 \text{ mm}^2$

* Refer to Optical Data Form.