STUDIES ON THE SYMPATHETIC NERVOUS SYSTEM IN EXPERIMENTAL RENOVASCULAR HYPERTENSION

A thesis submitted for the degree of
Doctor of Philosophy to the University of Leicester

by

STEPHEN M. WALKER

Department of Medicine,
University of Leicester,
Leicester.

July, 1987.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

To my parents

PREFACE

Experimental renovascular hypertension was first produced by bilateral renal artery constriction in dogs by Goldblatt and co-workers. Two subsequently derived models, one-kidney, one-clip (1K1C) (renal artery constriction with contralateral nephrectomy) and two-kidney, one-clip (2K1C) (renal artery constriction and opposite kidney untouched) hypertension, have been the subject of intensive research ever since. The rat was the animal chosen for the studies described in this thesis, both for convenience and because 1K1C and 2K1C hypertension can be induced in this species.

The present thesis consists of three sections, and section I (General Introduction) is in turn divided into three chapters. Chapter 1 discusses the salient early studies in hypertension, together with the experiments leading to the assignment of both prohypertensive and antihypertensive functions to the kidney. A thesis concerned with renal hypertension would not be complete without some reference to these early studies; with limited resources and little in depth knowledge of the cardiovascular system, remarkable work was performed. Before considering specific questions regarding sympathetic nervous system (SNS) activity in 1K1C and 2K1C hypertension, SNS involvement in normal blood pressure homeostasis, and in clinical and experimental hypertension in more general terms, is considered in Chapter 2. Methods of assessing SNS activity in experimental hypertension are also discussed in this chapter. The final part of section I (Chapter 3) discusses the factors postulated to play a role in the development, maintenance and reversal of both 1K1C and 2K1C hypertension. The degree of blood pressure elevation is similar in the two models, but the mechanisms underlying the hypertension are different and the areas of

difference are stressed. Moreover, none of the factors proposed can completely explain the blood pressure elevation, or blood pressure fall after surgical reversal, in either model. Many questions concerning experimental renovascular hypertension, therefore, remain unanswered. It is for this reason that two series of experiments were designed to study SNS activity in 1K1C and 2K1C hypertension, before and after surgical reversal by unclipping the renal artery. These experiments are reported and discussed in sections II and III.

ACKNOWLEDGEMENTS

First and foremost I would like to express my gratitude to my supervisor, Dr. R.F. Bing for help and guidance throughout this work. I would also like to thank Professor J.D. Swales for giving me the opportunity to work in the Department of Medicine. I am grateful to Mr. J. Strupish for invaluable advice during the development of the HPLC catecholamine assay and to Mr. N. Godfrey for technical assistance. I would like to express my appreciation to Mrs. L.D. Allan for typing this manuscript, to Mr. C.J. Walker for help in the preparation of the figures, to Dr. P. Lloyd for proof-reading and to Miss H.E. Wheeler for assistance with referencing. Thanks are also due to the Department of Medical Illustration, Leicester Royal Infirmary for the preparation of photographs and half-plates.

Finally, I am indebted to the Department of Medicine for financial support.

ABBREVIATIONS

BP, blood pressure

1K1C, one-kidney, one-clip

2K1C, two-kidney, one-clip

NA, noradrenaline

SNS, sympathetic nervous system

HPLC, high performance liquid chromatography

Additional abbreviations are indicated in the text.

Journal title abbreviations in the bibliography are as given in : List of Journals Indexed in Index Medicus (1986)

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SECTION I

GENERAL INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION AND HISTORICAL BACKGROUND

1.1 The Sympathetic Nervous System and Hypertension

The autonomic nervous system (ANS), particularly the sympathetic nervous system (SNS) component, is of great interest in the study of clinical and experimental hypertension because of its involvement in blood pressure (BP) homeostasis. The preservation of adequate perfusion of vital organs under a wide variety of conditions is largely dependent on the autonomic control of the cardiovascular system (De Champlain, 1976 and 1977a). Traditionally the ANS was thought to be characterised by gross, indiscriminating responses, but it has since been demonstrated to be capable of the fine control of vascular and glandular responses (Miller, 1969).

The influence of the SNS in hypertension is suggested by the fact that some of the most potent antihypertensive drugs have their action by interfering with sympathetic function (Abrahams, 1969; Kuchel, 1977 and 1983; Davis, 1977). Many drugs that were first thought to act on the peripheral SNS may produce their effects mainly by acting on the vasomotor centres of the brain stem (Laverty, 1973), raising the possibility of the involvement of a central component in the development of hypertension. It seems likely that the ANS is in some way involved in hypertension, if not directly at least by a failure to adjust appropriately cardiovascular functions to oppose an overt hypertensive stimulus (Kuchel, 1983).

1.1.1 Early Studies Investigating the SNS Influence on the Cardiovascular System

The tone of blood vessels has been known to be influenced by the SNS since 1851 when Claude Bernard demonstrated that section of the

cervical sympathetic chain caused vasodilation of the blood vessels of the face. Subsequently, stimulation of the cervical sympathetic chain was shown to cause vasoconstriction of the same vessels (Brown-Séquard, 1854). In 1866 Cyon & Ludwig noted that vasomotor reflexes were mediated via the aortic depressor nerve, but it was almost 60 years until it was demonstrated that denervation of the carotid sinus region caused an abrupt rise in arterial pressure (Hering, 1924). Later Koch & Mies (1929) demonstrated persistent hypertension following sectioning of the aortic depressor and carotid sinus nerves.

The first hormone identified was adrenaline (N-methyl-3,4-dihydroxyphenylethanolamine), the pressor secretion of the adrenal glands (Abel & Crawford, 1897). The similarities in the actions of adrenaline and sympathetic nerve stimulation observed by Langley (1901), led to the hypothesis that an adrenaline-like substance was released from sympathetic nerve endings (Elliot, 1905). Experimental evidence in favour of this followed later (Loewi, 1921; Cannon & Uridil, 1921), but the nature of the SNS chemical neurotransmitter substance remained unclear until discovered to be noradrenaline (NA) by von Euler (1948). NA is the major neurotransmitter of peripheral post-ganglionic sympathetic fibres (Axelrod & Weinshilboum, 1972). The heart and blood vessels contain specific receptors for catecholamines (Ahlquist, 1948) and can therefore be influenced by NA released from sympathetic nerve endings, or by circulating catecholamines released from the adrenal medulla (De Champlain, 1976).

The only form of clinical hypertension directly attributable to catecholamines is the elevated arterial pressure associated with a catecholamine producing tumour, phaeochromocytoma. Tumours of the

adrenal medulla were first recognised by Frankel (1886), and a phaeochromocytoma is characterised by hypertension which is variable in onset and paroxysmal. Hypertensive attacks are due to the release of pressor catecholamines by the tumour (Engelman, 1977), which can arise from the chromaffin tissue of the adrenal medulla or at extra-adrenal sites of sympathetic neural tissue. The incidence of phaeochromocytoma is low, between 0.1-1.0% of the hypertensive population of which the lower value probably represents the most accurate estimate (Engelman, 1977). Surgical removal of the tumour usually cures this form of secondary hypertension (Kuchel, 1977).

SNS involvement in other forms of clinical hypertension is less clear (Kuchel, 1977). Recently a comprehensive review of 78 studies investigating plasma catecholamines in essential hypertension (Goldstein, 1983a) revealed that the majority of studies demonstrated increased plasma noradrenaline (NA) concentrations, indicative of increased SNS activity in at least some patients with essential hypertension. Studies involving relatively young patients invariably showed increased plasma NA levels compared to normotensive controls, and these patients could represent a sub-group in the hypertensive population in which there is increased sympathetic neural activity. The possible role of neurogenic mechanisms in clinical renal hypertension has also been investigated. Oral administration of clonidine, a predominantly centrally acting sympatholytic agent, lowered BP in patients with hypertension secondary to either unilateral renal parenchymatous disease or unilateral renal artery stenosis. It has been suggested therefore that clinical renal hypertension may be maintained by a central pressor mechanism mediated by increased SNS activity (Mathias, Wilkinson, Stone & Peart, 1985).

There is, however, no evidence that clinical renal hypertension is associated with elevated plasma (Christensen & Christensen, 1972; Kuchel, 1977; Skrabal, Auböck, Hörtnagl & Brücke, 1981) or urinary (Kuchel, 1977) catecholamine levels.

In the late 1940s extensive excision of the paravertebral sympathetic ganglia was used as a treatment of hypertension (Pickering, 1968), based on the assumption that an overactive SNS was responsible for the raised BP. Smithwick (1948) advocated the success of surgical sympathectomy, although it appears to have in general limited effectiveness in reducing arterial pressure and is much inferior to drug treatment. This procedure can have a spectacular effect in a few patients but the difficulty is to identify these beforehand (Pickering, 1968). Sympatholytic drugs were used with similar effects although side effects were a major limitation. However, adrenoreceptor blocking drugs are still major therapeutic agents in the management of hypertension.

1.2 Historical Background to the Study of Hypertension

Stephen Hales (1733) proved experimentally, in the horse, that flowing blood exerted a pressure on the walls of vessels. Further advances in BP measurement were restricted in man until a satisfactory method for indirect measurement was developed (Riva-Rocci, 1896; von Recklinghausen, 1901; 1930; Korotkoff, 1905).

In the period before satisfactory techniques were available for the measurement of BP, the relationship between renal disease and cardiac hypertrophy was first observed by Bright (1836). Post-mortem studies

on patients who had died from diseases characterised by dropsy and albumin in the urine, revealed shrunken diseased kidneys invariably associated with cardiac enlargement; this became known as Bright's disease. Traube (1856) postulated that in Bright's disease the general shrinkage of the kidneys reduced renal blood flow and BP increased to restore adequate renal perfusion, causing the left ventricle to first dilate and then hypertrophy. It is now known that the part of the hypothesis concerning how the diseased kidneys cause BP elevation is incorrect, but Traube was the first to note that Bright's disease was associated with raised arterial pressure.

Tigerstedt and Bergman (1898) made a major breakthrough in the study of the relationship between the kidney and BP when they discovered and characterised renin, the first hypertensive chemical extract, obtained from homogenised rabbit kidneys. Renin is formed in the renal cortex and released into the blood stream via the renal venous effluent. In an attempt to link this discovery with Bright's disease, they postulated that in certain circumstances there may be an over-production of pressor substance in the kidney and increased release into the circulation. The resistance in the blood vessels would thus be constantly raised above normal, leading eventually to hypertrophy of the heart (Tigerstedt & Bergman, 1898). Further studies on renal involvement in hypertension were limited by the lack of an experimental model.

However, Mahomed (1874) and Allbutt (1896) described cases of elevated BP without renal involvement. Enlargement of the heart in these cases was proposed to be due to increased arteriolar resistance, not damaged capillaries of diseased kidneys as proposed by Traube (1856).
George Johnson (1868) had described thickening of minute and larger

arteries in Bright's disease, and Leyden (1881) proposed that narrowing of the lumen of the arterial branches in the renovascular bed could cause arterial hypertension in the absence of disease of the renal parenchyma.

It has since become apparent that the concept of primary or essential hypertension is the norm rather than the exception. This has a different aetiology to hypertension secondary to renal disease (Julius & Hansson, 1983). Almost 95% of patients suffering from an elevated arterial pressure have no obvious underlying cause for this (Berglund, Andersson & Wilhelmsen, 1976). The contribution of the kidney to hypertension in the absence of apparent renal disease is controversial; even when renal vascular changes are present there remains debate as to whether these changes pre-date hypertension, or are merely a consequence of the elevated BP.

1.2.1 A Model of Experimental Renal Hypertension

One of the most significant advances in the study of renal hypertension was the production of a reproducible model of renovascular hypertension, without concomitant renal failure, in dogs by reducing the blood flow to the kidneys (Goldblatt, Lynch, Hanzal & Summerville, 1933; 1934). Earlier attempts to produce a model of renal hypertension were always associated with various degrees of renal failure (see Goldblatt, 1937).

The application of constricting clamps to both renal arteries of a dog produced persistent hypertension; whilst partial occlusion of one of the renal arteries led to only a transient elevation of arterial pressure, which returned to normal within a few days. Removal of the

constricting clamps from the two main renal arteries was followed by a prompt normalisation of BP (Goldblatt, 1937). This form of hypertension was proposed to be produced by the release of a renal pressor substance from the ischaemic kidneys into the circulation via the renal veins; neurogenic mechanisms were not thought to be involved (Goldblatt, 1937).

Similar models of experimental renovascular hypertension were later produced in the rat. Unlike the situation in the dog, partial occulsion of only one renal artery leads to persistent hypertension (Byrom & Wilson, 1938). A second model was produced in the rat when the application of a constricting clip to one renal artery was combined with removal of the opposite kidney (Byrom & Dodson, 1949). Clinical hypertension secondary to unilateral renal disease in the absence of reduced renal function, was reported by Butler (1937). Moreover, removal of the diseased kidney was found to reverse the elevated arterial pressure in some of these patients. Hypertension secondary to unilateral renal artery stenosis is the clinical equivalent to hypertension produced in a rat by partially occluding the renal artery of one kidney, leaving the contralateral kidney untouched (Pickering, 1968; Swales, 1979a). Removal of the ischaemic kidney or surgical reconstruction of the stenosed renal artery have been found to be effective in reducing BP in this form of clinical hypertension (Mackay, Brown, Lever & Robertson, 1980).

1.3 The Kidney and Hypertension

The kidney has been demonstrated to exert both prohypertensive and antihypertensive effects and renal involvement in hypertension could

therefore involve a pressor mechanism, a failure to exert an antihypertensive function, or a combination of both these actions. The experimental evidence leading to these suggestions is considered in this section. Discussion of the evidence for the assignment of a pressor role to the kidney demands a consideration of the experiments leading to the discovery and characterisation of the renin-angiotensin system, the potent pressor mechanism originating from the kidney.

1.3.1 Pressor Function of the Kidney

a) Release of a Pressor Substance

Saline extracts from the ischaemic kidneys of 2-kidney, 1-clamp (2K1C) hypertension in dogs caused a greater pressor response than extracts from contralateral untouched kidneys, when introduced into the circulation of unanaesthetised recipient dogs (Harrison, Blalock & Mason, 1936; Prinzmetal & Friedman, 1936). Goldblatt (1937) demonstrated the necessity of an intact ischaemic kidney for the development of hypertension. Furthermore, hypertension did not develop in a dog if there was complete occlusion of both main renal veins (Goldblatt, 1937). These observations suggested that ischaemic kidneys released a pressor substance into the circulation via the renal veins, which was capable of producing a marked and persistent elevation of arterial pressure. The pressor substance was proposed to be renin (Prinzmetal & Friedman, 1936). Pickering, Prinzmetal & Kelsall (1939) demonstrated that in rabbits with 1K1C hypertension of less than 8 days duration, the renal renin content was abnormally high. In hypertension of greater duration (both 1K1C and 2K2C hypertension of 2-17 months duration) however, renal renin content was normal. Furthermore,

excision of the ischaemic kidney within 8 days of onset abolished 1K1C hypertension within a few hours, but was ineffective at lowering BP after 7 weeks of sustained hypertension (Pickering, 1945). These observations are consistent with the hypothesis that the early stages of renal hypertension are due to the release of renin from the ischaemic kidney, but a non-renal mechanism is chiefly responsible for the maintenance of prolonged hypertension. However, it is necessary to determine the substance which is released into the circulation rather than what is stored in the renal parenchyma (Fasciolo, Houssay & Taquini, 1938a).

Early studies failed to demonstrate a greater pressor effect of extracts of plasma from renal hypertensive compared to normal dogs (Page, 1937; Prinzmetal, Friedman & Oppenheimer, 1938). Pickering et al (1939) failed to recover any renin-like activity from the blood of rabbits with established renal hypertension. Conversely, when large quanitites of blood were transfused between renal hypertensive and bilaterally nephrectomised dogs, the BP of the latter increased (Solandt, Nassim & Cowan, 1940). Transfusions between normal and nephrectomised dogs caused no change in the BP.

Ischaemic kidneys from renal hypertensive dogs when grafted into the neck of bilaterally nephrectomised dogs, caused the BP of the recipient animals to rise within a few minutes; whereas normal kidneys produced no change in BP (Houssay & Fasciolo, 1937). Furthermore, renal venous blood from an ischaemic kidney produced an immediate and intense vasoconstrictor response in the isolated vascular bed of the hind limb of the toad, Bufo parenarum. Venous blood from a normal kidney, and from other organs of dogs with 2K1C hypertension, was only slightly vasoactive in this preparation (Houssay & Taquini, 1938).

Attempts to extract the pressor substance from renal blood led to the isolation of 'hypertensin', a substance pharmacologically and chemically distinct from renin (Braun-Menéndez, Fasciolo, Leloir & Munoz, 1939). Renin was proposed to be a proteolytic enzyme secreted by the kidney, which acts on a pseudoglobulin in the plasma to produce the pressor peptide 'hypertensin' (Fasciolo, 1977). Working independently, Page's laboratory simultaneously discovered that renin did not act directly on blood vessels, and postulated that it required activation by a kinase present in plasma or whole blood (Kohlstaedt, Helmer & Page, 1938; Page, 1939). The reaction between renin and 'renin activator' was supposed to produce a highly active pressor substance - 'angiotonin' - which acted directly on blood vessels (Page & Helmer, 1940). Subsequently, hypertensin and angiotonin were realised to be synonymous and the two groups agreed to amalgamate the terms; the functional pressor peptide being renamed angiotensin and the plasma substrate angiotensinogen (Braun-Menéndez & Page, 1958). The proteolytic action of renin, as proposed by Braun Menéndez et al (1939), was confirmed when the incubation of angiotensinogen with pepsin was demonstrated to produce a polypeptide with very similar properties to angiotensin (Croxatto & Croxatto, 1942).

Angiotensin when isolated and purified was found to be a decapeptide consisting of 9 different amino acids (Skeggs, Marsh, Kahn & Shumway, 1954a). During attempts to obtain pure angiotensin, two separate pressor peptides were differentiated by countercurrent distribution (Skeggs et al, 1954b); the complete pattern of events thus began to emerge. The action of renin on its substrate in the plasma produces angiotensin I, which is rapidly converted to angiotensin II through the

action of a converting enzyme (Skeggs, Lentz, Kahn, Shumway & Woods, 1956). A pressor decapeptide from ox serum was sequenced (Elliot & Peart, 1956) and subsequently found to possess 1.5-3.0 times the pressor activity of NA in an anaesthetised rat (Peart, 1956). Determination of the amino acid sequence of the octapeptide angiotensin II soon followed (Skeggs et al, 1956), as did the elucidation of the structure of the part of the substrate molecule from which angiotensin I is generated (Skeggs, Kahn, Lentz & Shumway, 1957). Bumpus, Schwarz & Page (1957) synthetised the functional pressor octapeptide angiotensin II, but it was 20 years before pure renin was isolated from hog kidney (Inagami & Murakami, 1977). Recently models have been constructed to represent the 3-dimensional structure of human renin, based on the known structure of aspartyl proteases (Akahane et al, 1985; Carlson, Karplus & Haber, 1985). The biochemical pathway illustrating the formation and metabolism of angiotensin II is shown in Figure 1.1.

Renin was found to be present in the renal cortex rather than the renal medulla (Tigerstedt & Bergman, 1898; Prinzmetal & Friedman, 1936). Acidophil and basophil granules within specialised smooth muscle cells of the afferent arteriole in close proximity to the glomerulus, the juxtaglomerular (JG) cells, were proposed as the site of renin localisation (Goormaghtigh, 1939). The JG cells are in close proximity to a specialised segment of the distal tubule, the macula densa, which consists of long narrow epithelial cells and is contiguous with both the afferent and efferent arterioles. This complex of the JG cells and the macula densa, together with the interstitial or lacis cells is known as the juxtaglomerular apparatus (JGA) (Heptinstall, 1974; Oparil & Haber, 1974). Renin was found to be mainly located in the outer cortex, an area containing most of the glomeruli, whilst none was found in the

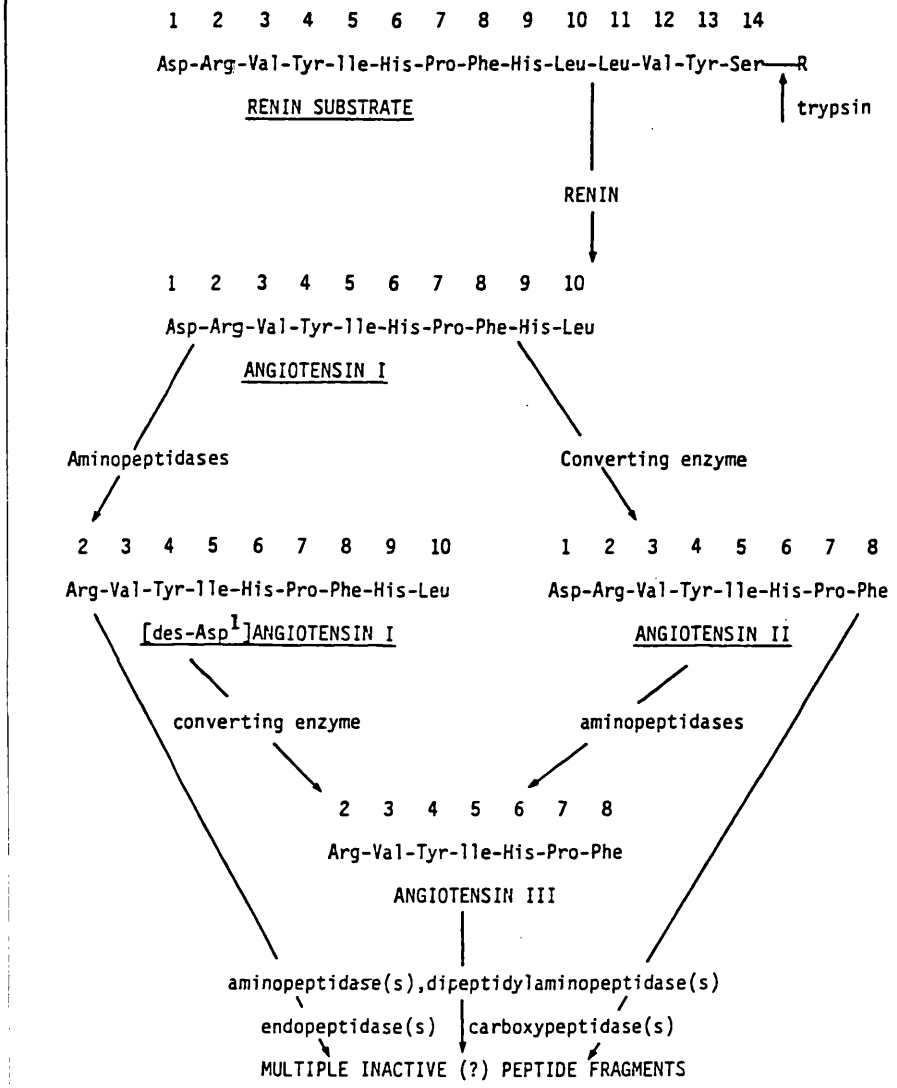


FIGURE 1.1

Biochemical pathway for the formation and metabolism of angiotensin II (Peach, 1977).

sub-capsular region where there are no glomeruli (Cook, Gordon & Peart, 1957). Moreover, a striking correlation was obtained between the amount of JG granulation (JG index) and the amount of extractable renin in the kidney (Tobian, Janecek & Tomboulian, 1959). The localisation of renin to the granules within the JG cells was finally confirmed by Edelman & Hartroft (1961) using specific fluorescence labelled antibodies.

Renal secretion of renin is increased by factors which decrease extracellular fluid volume and BP or which increase sympathetic nervous system (SNS) activity, and is controlled by the following mechanisms (Davis & Freeman, 1976; Peach, 1977; Swales, 1979a):

1) The juxtaglomerular (JG) baroreceptor responds to changes in stretch of the afferent arteriolar wall, such that a decrease in intravascular pressure in the afferent arteriole stimulates the release of renin. Conversely an increase in pressure within the afferent arteriole inhibits renin secretion.

2) The macula densa chemoreceptor detects changes in the tubular fluid composition in the distal tubule. When glomerular filtration rate (GFR) is reduced the filtered load of sodium and chloride arriving at the macula densa is decreased and the neighbouring JG cells are stimulated to secrete renin. The mechanism of this response is not clear, but either an alteration in the rate of delivery of sodium and chloride to the distal tubule, or the rate of transport of one or both of these ions across the cells of the macula densa may be important in determining renin release.

3) The JGA is richly innervated by efferent adrenergic nerve fibres (Nilsson, 1965). Catecholamines, either circulating in the blood or released locally from sympathetic nerve endings, stimulate the release of renin by a direct action on specific adrenergic receptors on the surface of the JG cells. Intrarenal beta-adrenoceptors cause renin release, whilst alpha-adrenoceptors are probably inhibitory (Vandongen & Peart, 1974; Pettinger, Keeton, Campbell & Harper, 1976). Sympathetic efferent renal nerve stimulation can also increase renin secretion indirectly by causing vasoconstriction of the afferent arteriole. This will stimulate the JG baroreceptor by decreasing the stretch within the baroreceptor wall, and the macula densa chemoreceptor by reducing GFR; both of which will increase renin secretion.

In addition to these stimuli circulating angiotensin II inhibits renin release by a negative feedback mechanism. Potassium and antidiuretic hormone (ADH) reduce renin secretion (Bunag, Page & McCubbin, 1967; Davis & Freeman, 1976; Swales, 1979a), and prostaglandins synthesised in renal cortical structures may also be involved in the regulation of renin secretion, renal blood flow and GFR (Weber & Siess, 1980).

It has been proposed that renin may be stored in an inactive form and released by an activation process (Boyd, 1972). Inactive forms of renin have been demonstrated in the plasma of several species, which can be activated <u>in vitro</u> by acidification to pH 3.3, trypsin or pepsin digestion, cooling to -5°C, or by kallikrein (Swales, 1979a). Activation of renin from hog and rabbit kidney <u>in vitro</u> is accompanied by a reduction in molecular weight, suggesting it may involve removal of a renin-inhibitor carrier protein or degradation of a higher

molecular weight inactive protein (Peach, 1977). The term 'prorenin' has been suggested for inactive forms of renin to indicate that they may be precursors of renin (Sealey & Laragh, 1975). The physiological importance of prorenin, however, remains to be assessed and it is not known whether activation of renin occurs in vivo. In the absence of evidence of activation in vivo inactive renin cannot be definitely identified as a renin precursor, and it is not known whether inactive forms of renin have any involvement in normal BP control or hypertension (Peach, 1977; Swales, 1979a).

b) Control of Sodium and Water Balance

The involvement of the kidney in the development and maintenance of hypertension has hitherto been regarded to be due to the release of a renal pressor substance. However, the renal involvement in certain forms of hypertension could equally be to retain, or failure to excrete, sodium and water. Braun-Menéndez (1951) found that an increase in extracellular fluid volume followed bilateral nephrectomy in rats, and a close correlation existed between the accumulation of fluid in the extracellular spaces and hypertension. Rats with hypertension secondary to unilateral perinephritis also had an increased blood volume and extracellular fluid volume (Braun-Menéndez, 1951). Dogs made hypertensive by sodium loading and partial nephrectomy had an initial transient increase in cardiac output, followed by a more sustained increase in total peripheral resistance (Coleman & Guyton, 1969).

The best example of clinical hypertension produced by sodium and water retention is the elevated BP found in anephric patients and many patients with terminal renal disease (Vertes, Cangiano, Berman & Gould, 1969; Wilkinson, Scott, Uldall, Kerr & Swinney, 1970; Swales, 1981a).

Under these circumstances the level of BP correlates with expansion of the extracellular fluid volume, and can be controlled by removal of sodium and water with dialysis treatment in most cases (Merril, Giordano & Heetderks, 1961; Swales, 1981a). The pathogenesis of hypertension associated with primary aldosteronism (Conn's syndrome) is also dependent on sodium and fluid retention. Conn's syndrome results from a benign tumour of the zona glomerulosa of the adrenal cortex, which produces excessive quantities of the sodium-retaining hormone aldosterone (see p.17) (Pickering, 1968; Keele & Neil, 1971; Moore, 1976; Ferriss, Brown, Fraser, Lever & Robertson, 1983). Exposure to excess mineralocorticoid activity in patients with primary aldosteronism induces renal sodium retention in exchange for potassium and hydrogen ions. This leads to expansion of extracellular fluid and plasma volume and hence a rise in BP, hypernatraemia and hypokalaemia. After several weeks of hypertension, however, part of the sodium retained initially is excreted and extracellular fluid and plasma volumes decline towards normal, whilst BP remains elevated or rises even further at this stage (Wenting, Veld, Verhoeven, Derkx & Schalekamp, 1977). This is proposed to result from a pressure natriuresis and diuresis, eventually producing a new equilibrium characterised by a reduced exchangeable sodium and plasma volume; although exchangeable sodium remains increased in these hypertensive patients compared to controls (Wenting et al, 1977). Surgical removal of the tumour normally cures the hypertension (Keele & Neil, 1971; Moore, 1976; Wenting et al, 1977; Ferriss et al, 1983).

Sodium retention has been proposed to lead to an increased peripheral resistance, and hence an increase in arterial pressure, by the theory of 'autoregulation' (Guyton, 1977). According to this hypothesis the primary abnormality is decreased renal excretion of sodium and water for any given level of systemic arterial pressure compared to normal;

i.e. there is a resetting of the renal pressure-natriuresis curve (Tobian et al, 1978). The resulting sodium and water retention leads to an increase in extracellular fluid and plasma volume, causing an increase in central venous pressure and increased venous return to the heart. Cardiac output consequently increases resulting in an 'over-perfusion' of the peripheral vascular beds. Over a period of days or weeks an autoregulatory arteriolar vasoconstriction restores tissue perfusion to normal, at the expense of elevated peripheral resistance and increased arterial pressure. The raised peripheral resistance returns cardiac output to normal and becomes mainly responsible for the maintenance of established hypertension (Guyton, 1977). Essentially the same series of events have been postulated by Borst & Borst-de-Geus (1963), and Ledingham & Cohen (1963). The 'autoregulation' hypothesis is controversial, being disputed by many workers, and this is discussed in more detail later (Chapter 3). Sodium retention may, however, have some role in certain forms of hypertension, particularly following extirpation of both kidneys.

An important factor in the control of body sodium and water balance is the mineralocorticoid hormone aldosterone. This is released from the glomerulosa cells of the adrenal cortex and stimulates the kidney to retain sodium and water (Pickering, 1968). The release of a mineralocorticoid from the adrenal glands causing sodium retention was first demonstrated in dogs and monkeys by Simpson, Tait & Bush (1952). Increased renin-angiotensin system activity stimulates the release of aldosterone from the adrenal cortex (Laragh, Angers, Kelly & Lieberman, 1960; Ganong & Mulrow, 1961), representing an additional mechanism by which this system could be involved in BP control and hypertension. Angiotensin II appears to stimulate the biosynthesis and

secretion of aldosterone, rather than causing the release of pre-formed aldosterone stores (Boyd & Peart, 1971). Angiotensin II also exerts a direct stimulatory effect on renal tubular sodium reabsorption (Johnson & Malvin, 1977). The renin-angiotensin system is therefore a major factor influencing the bodies response to changes in sodium balance (Peach, 1977).

1.3.2 Antihypertensive Function of the Kidney

The kidney has been proposed to have an antihypertensive function in addition to a pressor role. This is thought to be achieved by the renal control of sodium and water balance, and a specialised endocrine function. As discussed earlier (section 1.3.1), reduced sodium and water excretion would lead to a circulating volume expansion and could give rise to hypertension (Borst & Borst-de-Geus, 1963; Ledingham & Cohen, 1963; Guyton, 1977). However, evidence has also accumulated in favour of an endocrine antihypertensive role for the kidney.

It has been reported that hypertension fails to develop in renoprival human patients if salt and water are controlled by dialysis treatment (Merril, Giordano & Heetderks, 1961). However, this is at variance to the events in a dog, where renoprival hypertension has been observed despite the control of sodium and water balance (Grollman, Muirhead & Vanatta, 1949). Clamping one renal artery of a dog causes only a transient increase in BP, whereas subsequent removal of the untouched kidney leads to a sharp re-elevation of BP which is maintained (Goldblatt et al, 1934; Goldblatt, 1937). Following transplantation of an ischaemic kidney from a renal hypertensive to a normal dog, a greater elevation in BP was obtained if the recipient animal was first bilaterally nephrectomised (Fasciolo, 1938b). Furthermore, the

transfusion of blood between a renal hypertensive and a normal dog caused the BP of the latter to rise only if both kidneys of this animal had been removed (Solandt, Nassim & Cowan, 1940). Therefore, whilst an ischaemic kidney secretes a vasoconstrictor substance into the circulation capable of producing permanent hypertension, the presence of a normal kidney is capable of reducing the action of this pressor substance (Fasciolo, Houssay & Taquini, 1938a).

Several experiments have demonstrated that the kidney exerts an antihypertensive function which is independent of its excretory role. The elevated arterial pressure of a bilaterally nephrectomised, over-hydrated dog was lowered when its blood was perfused through a pair of normal kidneys from a donor animal, even if the urinary fluid and salt loss was replaced by intravenous saline infusion (Kloff & Page, 1954). Renoprival hypertension did not develop in dogs (Grollman, Muirhead & Vanatta, 1949) or rats (Floyer, 1955) following removal of one kidney and implantation of the remaining ureter into the small intestine or anterior vena cava, despite the fact that renal excretion was completely abolished in these animals. The kidney was thus proposed to maintain normal BP by inhibiting an extra-renal pressor mechanism, and that this was independent of its excretory function (Floyer, 1955). Furthermore, if the remaining ureter of uninephrectomised rats was ligatured, rather than anastomosed into the small intestine or vena cava, hypertension did develop (Floyer, 1955). This suggests an antihypertensive role for the renal medulla since uretero-caval anastomosis is associated with an intact and enlarging renal medulla, whilst ureteral ligation is followed by papillary necrosis (Muirhead, 1980a).

Transplants of fragments of whole kidney suspended in saline (autoexplantation) were found to protect against renoprival hypertension in the dog, even though the fragments were devoid of excretory function (Muirhead, Stirman & Jones, 1960). Moreover, explantation of the renal medulla protected against renoprival hypertension whilst cortical fragments did not; further evidence that a depressor factor resides in the renal medulla (Muirhead et al, 1960). The venous effluent from an unclipped, extracorporally perfused kidney of a rat with 2K1C hypertension lowered the BP of a recipient rat, suggesting a depressor agent is released from an acutely declipped kidney (Göthberg, Lundin & Folkow, 1982a).

A neutral lipid has been identified from renomedullary preparations and cultures of renomedullary interstitial cells (RIC), capable of lowering BP and distinct from prostaglandins which are also synthesised by the RIC. It has been named antihypertensive neutral renomedullary lipid (ANRL) and proposed to be the vasodepressor substance produced by the kidney (Muirhead, 1974). Vitride reduction of renal medulla extracts yielded two classes of antihypertensive lipid, one neutral and one polar. The neutral lipid is the natural product, whilst the polar renomedullary lipid (APRL) is semi-synthetic and probably does not occur in vivo (Muirhead, 1980a). Recently an antagonist to APRL has been used to gain information on the role of vasodepressor lipids in reversal of experimental renal hypertension (Masugi et al, 1984 and 1985), and this is discussed later (see section 3.2.3).

Elucidation of renal involvement in various forms of hypertension is greatly complicated by the heterogeneity and multifactorial nature of the disease (Page, 1949; Pickering, 1968; Folkow, 1982), but it is clear that the kidney may have a dual role in hypertension. The pressor and

depressor functions of the kidney, however, cannot completely explain renovascular hypertension in the rat or its surgical reversal (see Chapter 3), and therefore SNS activity was studied in these models.

CHAPTER 2

THE AUTONOMIC NERVOUS SYSTEM AND HYPERTENSION

2.1 Neurogenic Control of the Cardiovascular System

The autonomic nervous system (ANS) is a critical factor in cardiovascular homeostasis (Edis & Shepherd, 1970). Neurogenic control of BP requires several components: an afferent input via sensors and afferent pathways, integrative central control centres, and an efferent component linking the control centres to the effector organs (Rothe, 1976). The carotid sinuses, aortic arch and other large arteries are the major barosensory areas of the vascular system, activated by local distention of the vascular wall (Edis & Shepherd, 1970). A system of afferent fibres relays this information to vasomotor and cardiac modulator centres in the medulla oblongata (DeChamplain, 1976; 1977a; 1977b), which also receive afferent inpulses from higher centres located in the hypothalamus, limbic system and cortex, and from somatic afferent fibres. The integration of all these influences results in a continuous readjustment of peripheral cardiovascular tone via a network of efferent nerves (Kuchel, 1977).

2.1.1 Efferent Component of the ANS

The efferent component of ANS control of BP consists of the sympathetic and parasympathetic divisions, which are structurally and functionally distinct (Edis & Shepherd, 1970; Rothe, 1976). The parasympathetic division consists of a relatively minor network of cholinergic inhibitory fibres distributed mainly to the heart (Edis & Shepherd, 1970; DeChamplain, 1976; 1977a). In contrast the SNS consists of a dense network of excitatory efferent fibres distributed to the heart and all vascular beds, with the probable exception of the intracerebral vessels (Kuchel, 1977). Small arterioles, which are crucial to arterial

pressure homeostasis (Mellander & Johansson, 1968), are the most densely innervated segments of the vascular tree (Norberg, 1967; Burnstock, Gannon & Iwayama, 1970). Plasma noradrenaline (NA) has been shown to have a close positive correlation with arterial pressure, when the two variables were measured simultaneously over 24 hours in healthy volunteers. This is consistent with a role for the SNS in controlling the level of arterial pressure hour-by-hour over 24 hours (Richards, Nicholls, Espiner, Ikram, Cullens & Hinton, 1986).

2.2 Events at the Sympathetic Nerve Terminal

The most important events to consider at the sympathetic nerve terminal in order to gain an understanding of the SNS influence on the tone of the cardiovascular system are: biosynthesis, storage, release and metabolism of the neurotransmitter NA. These events also form the basis of several methods of assessing sympathetic neuronal activity in clinical and experimental hypertension (Kopin, 1977; Kuchel, 1977).

2.2.1 Biosynthesis and Storage of Catecholamines

Catecholamines are synthesised in the brain, peripheral sympathetic nerve endings and sites where chromaffin tissue occurs, including the adrenal medulla (Kuchel, 1983; Campese, 1983). The pathway for the synthesis of catecholamines is shown in Figure 2.1. The essential aromatic amino acid L-tyrosine enters the sympathetic nerve terminal from the circulation, where it is hydroxylated in the cytosol by tyrosine hydroxylase to form dihydroxyphenylalanine (DOPA). This reaction is the slowest step in the biosynthetic pathway and hence is rate-limiting in the formation of catecholamines (Levitt, Spector & Udenfriend, 1964; Spector, Sjoerdsma & Udenfriend, 1965). DOPA is

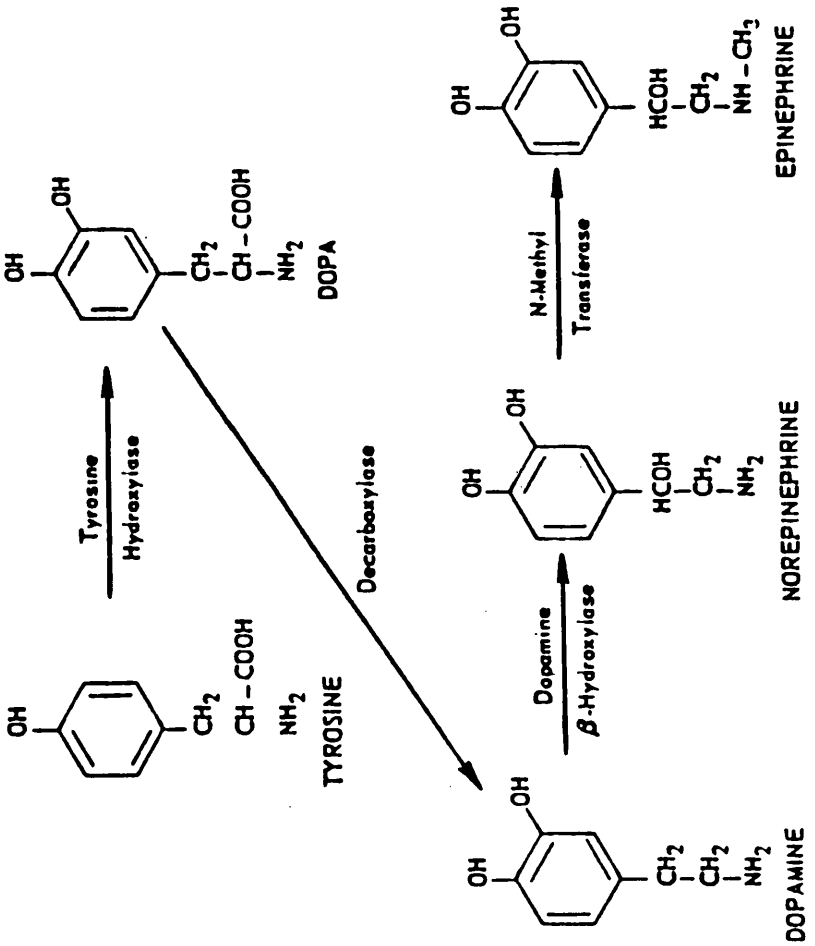


FIGURE 2.1 Pathway for the biosynthesis of catecholamines (reproduced from Kopin, 1977).

Norepineprine = noradrenaline; epinephrine = adrenaline.

converted to dihydroxyphenylethylamine, or dopamine (DA), by dopa decarboxylase in the cytosol. DA then enters the storage vesicles where it is transformed to NA by the action of intravesicular dopamine-β-hydroxylase (DBH). In the adrenal medulla and certain brain neurones NA is converted to adrenaline (ADR) by the enzyme phenylethanolamine-N-methyl transferase (PNMT) (Kuchel, 1983).

Newly synthesised NA is complexed with adenosine triphosphate (ATP) and magnesium, and stored in inactive form in specialised granulated vesicles within the neuron (Brody et al, 1983). The enzyme tyrosine hydroxylase is inhibited by DOPA and NA (Nagatsu, Levitt & Udenfriend, 1964), suggesting the synthesis of catecholamines is controlled by a negative feedback mechanism involving end-product inhibition (Kopin, 1977).

2.2.2 Release of Neurotransmitter into the Synapse

Nerve impulses pass distally via adrenergic neurones to the heart and blood vessels, where they cause the release of NA from the nerve endings (or varicosities) into the synapse (Kuchel, 1983). The synaptic cleft is a gap approximately 200-600Å wide which separates pre-synaptic from post-synaptic structures, across which nerve impulses must pass if the effector organ is to be stimulated. Chemical neurotransmission enables electrical signals arriving at the sympathetic nerve terminal to be translated into an effector response. NA is released from the pre-synaptic membrane, diffuses across the synaptic cleft and acts locally on specific adrenergic receptor sites on the effector cells of the heart, arterioles and venules (Kuchel, 1983). Stimulation of vascular alpha (α)-adrenoceptors (mainly α_1) will

produce vasoconstriction, whereas stimulation of cardiac beta (β)-adrenoceptors (β_1-adrenoceptors) will cause increased heart rate and myocardial contractility (Kuchel, 1983; Campese, 1983). In certain blood vessels NA may cause vascular β_2-adrenoceptor stimulation but, with the possible exception of coronary and facial vessels, usually not to the extent that vasodilatation ensues (Vanhoutte, Webb & Collis, 1980).

The arrival of a nerve impulse depolarises the pre-synaptic nerve terminal membrane and leads to an increased permeability to calcium ions (Burn & Gibbons, 1965). Calcium entry is proposed to initiate a contractile process which leads to the release of neurotransmitter into the synapse (Kopin, 1977), since colchicine, vinblastine and cytochalasin-B inhibit the stimulation induced release of NA (Wooten, Kopin & Axelrod, 1975). Prostaglandins of the E series depress the release of NA from sympathetic nerve terminals, and this is thought to be due to inhibition of calcium entry into the nerve ending (Johnson, Thoa, Weinshilboum, Axelrod & Kopin, 1971). Stimulation of sympathetic nerves leads to the release of DBH as well as NA (Gewirtz & Kopin, 1970), suggesting the vesicular and plasma membranes fuse extruding the contents of the vesicle into the synapse by exocytosis (Smith & Winkler, 1972; Kopin, 1977; Brody et al, 1983).

Various pre-synaptic receptors have been proposed to modulate the stimulation induced release of neurotransmitter (Vanhoutte, Webb & Collis, 1980). α and β adrenoceptors, DA, angiotensin, prostaglandin (E series) and other receptors have been identified on the pre-synaptic membrane (Langer, 1981; Kuchel, 1983). Stimulation of these receptors results in either inhibition or facilitation of NA release, and they may have a regulatory function (Brody et al, 1983). Pre-synaptic

β-adrenoceptors (β_2 receptors; Langer, 1981) are proposed to increase neurotransmitter release via a positive feedback mechanism, whilst pre-synaptic α-adrenoceptor (α_2 receptors; Campese, 1983) stimulation is inhibitory to NA release (Kopin, 1977; Vanhoutte et al, 1980; Langer, 1981). Furthermore, pre-synaptic β_2-adrenoceptors are thought to be more sensitive to agonists, so that during the initiation of release low concentrations of NA in the synapse accelerate the release process. When NA concentration reaches adequately high levels, the pre-synaptic α_2-adrenoceptors are stimulated and secretion is terminated (Kopin, 1977; FitzGerald & Dollery, 1979). The combined effects of positive and negative feedback mechanisms may result in a more sharply defined peak of neurotransmitter release (Kopin, 1977), although the physiological significance of pre-synaptic receptors is still the subject of controversy (Brody et al, 1983).

2.2.3 Inactivation of Released NA

The major route of inactivation of NA released into the synapse is re-uptake into the sympathetic neurone, termed "uptake 1" (Kopin, 1977; Brody et al, 1983; Kuchel, 1983), accounting for 75-80% of released NA (Campese, 1983). Inside the nerve terminal NA is either taken up into storage vesicles for re-release, or metabolised. Intraneuronal metabolism consists of an initial deamination by monoamine oxidase (MAO) (Kopin & Gordon, 1963), followed by the action of catechol-o-methyl transferase (COMT) to yield 3-methoxy-4-hydroxymandelic acid (vanillylmandelic acid or VMA) as the major end product (Kopin, 1977; Kuchel, 1983). Intraneuronal uptake constitutes an efficient means of rapidly terminating the response whilst conserving the released transmitter. "Uptake 1" is specific, saturable,

has a high affinity for NA and is sodium dependent. It is competitively inhibited by a variety of amines, cocaine and some drugs which interfere with sodium transport (Kopin, 1977).

The majority of neurotransmitter not returned to the neurone by "uptake 1" is removed from the vicinity of the receptors into extraneuronal tissue by "uptake 2", a high capacity, low affinity process (FitzGerald & Dollery, 1979). Extraneuronal uptake of NA is only apparent when the neurotransmitter is present in high concentration, or when intraneuronal uptake is blocked by cocaine or degeneration of the sympathetic nerve endings (Iversen, 1965). Inside extraneuronal tissues NA is chiefly O-methylated by COMT to yield normetanephrine (NMN) as the major metabolite. Extraneuronal metabolism also produces VMA and 3-methoxy-4-hydroxyphenylglycol (MHPG) to a lesser degree, resulting from the further degradation of NMN by MAO and aldehyde oxidase (AO) (Kuchel, 1983).

A small amount of free NA escapes from the synapse and enters the circulation. This is rapidly metabolised in the circulation and the liver, mainly by COMT to produce NMN. An alternative pathway for the inactivation of free NA, and other catecholamines in the circulation, is sulphoconjugation by phenolsulphotransferase (PST) in the platelets (Kuchel, 1983; Kuchel, Buu, Roy, Hamet, Larochelle & Genest, 1984). This pathway is poorly understood, but its existence is suggested by the high proportion of circulating catecholamine sulphates. PST is proposed to compete with COMT for the metabolism of circulating free NA; the NA which escapes both these processes may exert an action on extra-synaptic adrenergic receptors (Kuchel, 1983).

The ratio of the excretion of metanephrines (products of O-methylation) to that of VMA (product of deamination followed by O-methylation) grossly reflects the ratio of extraneuronal to intraneuronal metabolism of catecholamines. In humans this ratio is normally 1:10, indicating that intraneuronal degradation in cytoplasmic mitochondria is the predominant pathway (Kuchel, 1983). A minor proportion of catecholamines are metabolised extraneuronally and only a small amount of free NA, between 1-5% of released NA (Campese, 1983), is found unchanged in the urine (Kuchel, 1983).

A diagrammatic representation of the processes involved in sythesis, release, uptake and metabolism of the neurotransmitter NA is shown in Figure 2.2.

2.3 Assessment of SNS Activity in Experimental Hypertension

2.3.1 Direct Recording of Electrical Impulses in Sympathetic Nerves

Investigation of SNS activity in experimental hypertension has largely been approached indirectly, using techniques such as neural ablation and pharmacological intervention. Direct electrophysiological analysis of nerve traffic has been achieved in spontaneously hypertensive rats (SHR), where a 2-3 fold elevation in nerve discharge has been recorded along the sympathetic fibres of the greater splanchnic, renal and splenic nerves (Judy et al, 1976). The elevated sympathetic nerve activity appears to be localised to the nerves innervating the abdominal viscera, and may not apply to the vasomotor nerves innervating the skin and skeletal muscle (Judy et al, 1976). Direct measurement of nerve traffic along sympathetic nerves in other models

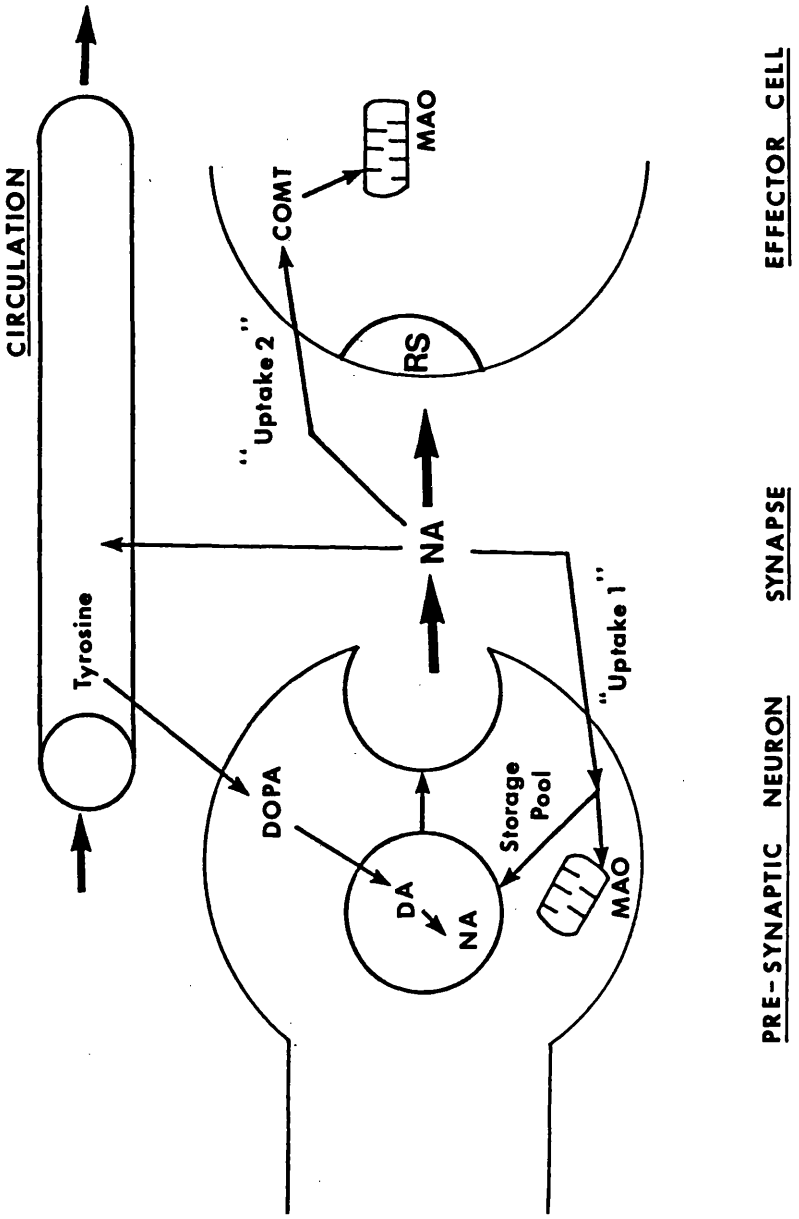


FIGURE 2.2 Synthesis and disposition of noradrenaline (NA) at the sympathetic nerve terminal. RS = receptor site; DA = dopamine; DOPA = dihydroxyphenylalanine; COMT = catechol-o-methyl transferase; MAO = monoamine oxidase.

of hypertension, including experimental renovascular hypertension in the rat, has not been carried out satisfactorily (Brody et al, 1983).

Electrophysiological measurement of sympathetic nerve activity is technically difficult and it is unclear which nerves would provide a representative estimate of the neurogenic contribution to elevated peripheral resistance in models of experimental hypertension. The visceral nerves innervating the organs which are integrally involved in cardiovascular regulation (e.g. renal nerves) would seem the most relevant.

2.3.2 Indirect Methods of Assessment

a) Measurement of Circulating NA levels

A small amount of NA released from peripheral sympathetic nerve terminals overflows into the circulation (Campese, 1983) and this is the chief source of plasma NA (Roizen, Moss, Henry & Kopin, 1974). The adrenal medulla releases catecholamines directly into the circulation, but less than 2% of the NA in plasma is thought to be derived from this source under normal conditions (Brown, Jenner, Allison & Dollery, 1981a). Measurement of plasma NA levels may therefore give a relative indication of the amount of physiologically active NA at receptor sites (DeChamplain, 1977a). An elevation in plasma NA would result from enhanced neurotransmitter release secondary to increased sympathetic neuronal activity (Dargie, Franklin & Reid, 1977a).

Circulating catecholamines are rapidly removed from the circulation, mainly by COMT and MAO in the liver (see section 2.2.3); the half-life

of intravenously infused NA being approximately 2 minutes (Campese, 1983; Chang, van der Krogt, Vermeij & van Brummelen, 1986). It is argued that this rapid turnover means that levels of NA in the plasma are rapidly responsive to changes in the rate of NA entry into the circulation; providing a much better index of SNS activity than can be obtained from urinary catecholamine levels (Kopin, 1977). The amount of neurotransmitter overflowing into the circulation depends on the width of the neuroeffector junction, as well as the rate of release. At narrow junctions the action of NA is efficiently terminated by "uptake 1" into the neurone, whilst at wider junctions a greater amount of NA will diffuse into the circulation (Campese, 1983). Based on the width of neuroeffector junctions, it has been calculated that sympathetic innervation to the smooth muscle in the walls of blood vessels may be the most important source of plasma NA (Campese, 1983).

Conditions known to activate the SNS have been shown to be associated with an elevation in circulating NA levels in normal human subjects. It is therefore proposed that under steady state standard conditions plasma NA levels provide a sensitive and useful index of overall sympathetic tone (Lake, Ziegler & Kopin, 1976; Goldstein, McCarty, Polinsky & Kopin, 1983b). Plasma NA is a better index of SNS activity than total catecholamine concentration, since the latter includes 20-37% of adrenaline, mainly of adrenomedullary origin (Kuchel, 1983).

However, the validity of the relationship between plasma NA levels and SNS activity has been criticised (Esler, 1982; Esler et al, 1985; Folkow et al, 1983; Mancia et al, 1983). Plasma NA is dependent not only on the rate of diffusion of the neurotransmitter from sympathetic nerve terminals into the plasma, but also the rate of removal of NA

from the circulation (Esler, 1982; Esler, Hasking, Willett, Leonard & Jennings, 1985; Chang et al, 1986). Overall NA 'spillover' into the plasma from sympathetic nerve terminals can be calculated, which avoids the confounding influence of plasma clearance of NA, but this still measures NA entry into the plasma rather than the amount of NA actually released into the synapse (Esler et al, 1985). Moreover, biochemical methods of assessing global SNS activity, such as plasma NA and overall NA spillover determinations, may be limited since SNS discharge is differentiated: sympathetic fibre activity can be increased to some target organs whilst it is unchanged or even decreased to others (Folkow, DiBona, Hjemdahl, Torén & Wallin, 1983; Esler et al, 1985). Consequently information on regional rates of NA release is required to fully characterise the pattern of SNS activity in clinical hypertension. Measurements of regional NA release into the plasma from individual organs have suggested a selective activation of the SNS, involving the kidneys and perhaps the heart, is present in essential hypertension, especially in young adults (Esler et al, 1985).

The site of blood sampling is an important consideration when measuring plasma NA. Venous plasma NA levels are more susceptible to regional differences in release and removal of the neurotransmitter by individual organs than arterial plasma NA levels (Esler et al, 1985). NA spillover rate determined from venous blood samples is reported to be overestimated by approximately 50% compared with that derived from arterial sampling, indicating there is a net release of NA into the circulation (Esler et al, 1985).

b) Urinary Catecholamine Levels

Urinary excretion of catecholamines has been used to assess SNS activity in clinical and experimental hypertension; based on the assumption that SNS activation would be associated with an increased release of NA into the blood, followed by an increase in its excretion and that of its metabolites (Kuchel, 1983). Urinary catecholamines represent the summation of total excretion of catecholamines from neural and non-neural tissues, in the periphery and the CNS, over the period of collection (DeChamplain, 1977b). There is no accurate way of determining what proportions of the catecholamines present in the urine are derived from NA released from the cardiovascular sympathetic fibres, the parenchymatous sympathetic fibres, the CNS, or the adrenal medulla (DeChamplain, 1977a; Kuchel, 1983). Furthermore, a change in activity of one portion of the SNS may lead to alterations in circulating catecholamine levels which are undetectable when diluted into the total pool of excreted NA (DeChamplain, 1977b).

Since very little NA appears unchanged in the urine, approximately 95-99% of catecholamines are metabolised (Campese, 1983), measurement of urinary catecholamine metabolites may provide a more reliable index of SNS activity (Kuchel, 1983). However, it is difficult to determine which, if any, of the urinary catecholamine metabolites would provide the best index of sympathetic neuronal tone in hypertension. Catecholamines and their metabolites in urine can at best only provide a crude average estimate of sympathetic events occurring during the period of collection (DeChamplain, 1977a; Kuchel, 1983). Excretion of NA and its metabolites is also affected by stress, exercise and certain drugs, and since catecholamines are present in certain foods urinary

catecholamine metabolites can be of dietary as well as endogenous origin (Kopin, 1977).

c) Other Indirect Methods of Assessment

Common methods of indirect assessment of SNS activity in experimental hypertension include: BP response to removal of SNS influence and measurement of circulating levels of dopamine-β-hydroxylase (DBH). Neither of these two methods provides a satisfactory index of SNS activity.

The major problem with the first is achieving a specific and complete sympathectomy (DeChamplain, 1976; Brody et al, 1983). Surgical sympathectomy is usually restricted to individual organs, as more generalised removal involves extensive and traumatic surgery. Immunosympathectomy by treatment with antiserum to nerve growth factor (anti-NGF) is restricted to small animals and is relatively incomplete, since it prevents the development of only those fibres which have not fully matured at birth (DeChamplain, 1976 and 1977a). Early attempts to produce chemical sympathectomy with NA depleting agents, such as reserpine (DeChamplain, 1976), and NA antagonists (DeChamplain, 1977b) were unsuccessful due to the lack of specificity of these compounds, which had actions unrelated to the SNS. The neurotoxins guanethidine and 6-hydroxydopamine (6-OHDA) appear to be more promising chemical agents (DeChamplain, 1977a), and the use of these compounds in renovascular hypertension in the rat is discussed in Chapter 4. Finally, failure of sympathectomy to alter BP does not necessarily mean the neurogenic mechanisms fail to affect BP when the SNS is intact; non-neural pressor mechanisms could compensate for the loss of neurogenic mechanisms in the sympathectomised state (Brody et al, 1983).

DBH is released into the synapse simultaneously with NA on nerve stimulation, diffusing almost entirely into the circulation (Gewirtz & Kopin, 1970; Weinshilboum et al, 1971a and 1971b). Plasma levels of DBH were therefore proposed to reflect SNS activity (Weinshilboum & Axelrod, 1971b; Axelrod, 1972). However, basal levels of DBH in the circulation vary widely in control subjects, and this is thought to be genetically controlled (Weinshilboum, 1979). Moreover, it is not always possible to demonstrate significant variations in plasma DBH under conditions known to activate the SNS, and there is no correlation between plasma DBH levels and either BP or plasma NA (Weinshilboum, 1979). The assumption that DBH in the systemic circulation is derived exclusively from the coupled release with NA from sympathetic nerves has also been questioned (Reid & Kopin, 1975). Circulating DBH concentrations were found to be an unreliable index of SNS function in 1K1C hypertension in rats (Dargie, Franklin & Reid, 1977a). Measurement of the neurotransmitter directly is more likely to be representative of SNS activity than measurement of DBH which is released in parallel with the neurotransmitter. Evaluation of sympathetic tone in clinical and experimental hypertension based on circulating levels of DBH should be interpreted with caution (DeChamplain, 1977a).

Other indirect methods of assessing SNS activity involve studying physiological parameters which are, at least in part, controlled by the SNS such as heart rate (Campese, 1983; Kuchel, 1983). Simultaneous heart rate and plasma NA measurements in healthy human volunteers over the course of 24 hours, show that these two variables are highly correlated (Richards et al, 1986). However, these physiological parameters on their own are less reliable than measurements of

circulating NA, since physiological parameters are affected by factors in addition to the SNS. In the absence of the SNS component other controlling mechanisms may increase their contribution to compensate, such that heart rate may not change following sympathectomy (Brody et al, 1983).

No single method is absolutely reliable for the assessment of SNS activity, which complicates any study to evaluate the neurogenic component in experimental hypertension. In the experiments reported in sections II and III, SNS activity in renovascular hypertension in rats was studied by measuring plasma NA levels, with parallel heart rate recordings being made as a second index to supplement this data. Plasma NA was determined from arterial rather than venous plasma samples, since the latter would be more affected by regional venous effluents from surrounding organs (Brown, Jenner, Allison & Dollery, 1981a; Chang et al, 1986).

2.4 Neurogenic Mechanisms in the Pathogenesis of Hypertension

Abnormalities in the neurogenic control of BP may be responsible for the pathogenesis of certain forms of clinical and experimental hypertension. These abnormalities may involve efferent pathways, adrenergic nerve terminals, the effector cells, afferent nerve pathways, or the CNS (Campese, 1983). Noradrenergic fibres have been demonstrated at various critical points along the cardiovascular reflex arc, at the level of the baroreceptors and at the level of the cardioregulator and vasomotor centres in the brain. Changes in the activity of adrenergic fibres at any of these sites could alter the efficiency of the neurogenic control of BP homeostasis and lead to hypertension (DeChamplain, 1977b).

2.4.1. Efferent SNS and Hypertension

Elevated SNS activity could lead to increased peripheral arteriolar resistance by the enhanced release of neurotransmitter into the synapse. Alternatively, enhanced sensitivity of the effector cells to vasoconstrictor agents could allow an increased peripheral resistance to be maintained at normal levels of SNS activity (Campese, 1983). A greater concentration of neurotransmitter at receptor sites could result from local defects in the mechanisms involved in synthesis, storage, release, uptake or metabolism of NA at the sympathetic nerve endings (DeChamplain, 1976).

Overactivity of the SNS could lead to a sustained elevation in arterial pressure through one, or a combination of the following mechanisms (Campese, 1983):

1) Increased sympathetic activity would lead directly to arteriolar vasoconstriction by stimulating the α_1-adrenergic receptors of arterioles.

2) Direct inotropic and chronotropic action on the heart. The resulting increase in cardiac output may ultimately stimulate autoregulatory vasoconstriction and/or vascular hypertrophy, causing increased vascular resistance.

3) Elevated SNS activity to the kidney may cause sodium retention via a direct action on the renal tubules, or via the enhanced release of renin. Renin release would be stimulated both by a direct action of neurotransmitter on intrarenal β -adrenoceptors

(Vandongen & Peart, 1974), and by an action of the SNS on renal afferent arteriolar tone and intrarenal baroreceptors (Davis & Freeman, 1976) (see section 1.3.1a). This would lead to vasoconstriction and increased peripheral resistance due to enhanced circulating levels of the pressor peptide angiotensin II.

4) There is evidence that adrenergic nerves may have a direct trophic effect on blood vessels. The level of SNS activity is suggested to influence the structural components as well as the function of the vasculature, particularly during the early stages of growth in neonatal rabbits and rats (Bevan, 1984). Increased SNS activity to resistance vessels may therefore lead to vascular hypertrophy, and hence increased peripheral resistance due to the increased wall:lumen ratio, in the absence of an elevation in BP. The resulting secondary increase in BP would then lead to further vascular hypertrophy of the resistance vessels (see section 3.2.4).

2.4.2. Central Neurogenic Mechanisms

The central nervous system (CNS) has been proposed to be involved in the pathogenesis of certain forms of hypertension (Zanchetti & Bartorelli, 1977). The brain is believed to be the principle site of action of several potent antihypertensive agents, for example clonidine and α-methyldopa (Kobinger, 1978). Furthermore, hypertension can be induced or prevented by chemical or surgical manipulations of various brain structures (Brody & Johnson, 1980; Buckley & Ferrario, 1981; Brody et al, 1983).

The hypothalamus integrates somatic, endocrine and autonomic functions in response to a wide variety of environmental and internal stimuli, and is the major structure controlling cardiovascular function (Brody et al, 1983). Pressor areas have been repeatedly localised in the posterior and lateral hypothalamic regions, whilst depressor areas have been found in the pre-optic and anterior regions of the hypothalamus (Brody et al, 1983). The periventricular tissue surrounding the anterior portion of the ventral third cerebral ventricle of the anterior hypothalamus (AV3V region), appears to be critical for the maintenance of body fluid homeostasis and for the development and maintenance of hypertension (Brody & Johnson, 1980). The primary site for termination of afferent nerve fibres from sinoaortic baroreceptors is the nucleus tractus solitarii (NTS), located in the dorsal part of the medulla oblongata; abnormalities in this region may lead to hypertension. Electrolytic destruction of the NTS in rats leads to lethal malignant hypertension, characterised by increased peripheral resistance and death within 6 hours (Reis, Doba, Snyder & Nathan, 1977).

Therefore, there is evidence that the CNS may be involved in certain forms of hypertension, although the nature of its role remains unclear (Brody et al, 1983).

2.4.3. Interaction Between SNS and Renin-Angiotensin System

Angiotensin II enhances the vasoconstrictor responses to SNS stimulation (Zimmerman, 1962; 1981; Malik & Nasjletti, 1976), and this interaction may be important in some forms of clinical and experimental hypertension characterised by elevated renin-angiotensin system

activity. The relationship between the two systems is bidirectional (Kuchel, 1977), since the SNS is a major influence on renin release (Davis & Freeman, 1976) (see section 1.3.1a). The mechanism by which angiotensin II enhances the pressor response to SNS stimulation could involve: increased neurotransmitter release into the synapse (Hughes & Roth, 1971); increased biosynthesis of NA (Roth, 1972), although this is not inextricably linked with the release of newly synthetised or endogenous NA; or inhibition of neuronal uptake of transmitter from the synapse (Khairallah, 1972).

Angiotensin II may also have a pressor effect through an action on the CNS. Infusion of angiotensin II into the brain via the vertebral arteries produces a pressor response (Lowe & Scroop, 1969), which is mediated by an increase in peripheral SNS activity (Buckley & Jandhyala, 1977; Zimmerman, 1981).

Elevated renin-angiotensin system activity could therefore be involved in certain forms of hypertension by facilitating the pressor response to SNS activity, either by an action on peripheral sympathetic nerve terminals or at the level of CNS (Zimmerman, 1981). However, it has been difficult to demonstrate the involvement of adrenergic facilitation by endogenous angiotensin II in pathophysiological conditions; leading to the suggestion that the direct effect of this peptide on vascular smooth muscle is of primary importance in renin-angiotensin system involvement in BP homeostasis (Zimmerman, Sybertz & Wong, 1984). Therefore, whilst an interaction between the SNS and renin-angiotensin system should not be dismissed, further work conducted under physiological conditions is necessary to delineate the nature of the interaction between these two systems in BP regulation and hypertension. The observation that the renin-angiotensin system is

an effective back-up mechanism for the maintenance of arterial BP when sympathetic function is interrupted, suggests that the renin-angiotensin system may have a substitutive rather than modulating influence on the SNS (Zimmerman et al, 1984).

CHAPTER 3

EXPERIMENTAL RENOVASCULAR HYPERTENSION IN THE RAT AND ITS SURGICAL REVERSAL

3.1 General Details

3.1.1 The Two Models

A constricting clip is applied to one renal artery to induce renal ischaemia whilst the contralateral kidney is either removed to produce one-kidney, one-clip hypertension (1K1C), or left *in situ* to produce two-kidney, one-clip hypertension (2K1C) (Page et al, 1979). The rat was the animal of choice in the present experiments both because of the ease with which hypertension can be produced in this species, and because both 1K1C (Byrom & Dodson, 1949) and 2K1C (Byrom & Wilson, 1938) models can be produced. This chapter is almost exclusively confined to observations in the rat, since considerable variation exists between species and it is dangerous to extrapolate findings from one species to another (Swales, Bing, Russell & Thurston, 1983).

3.1.2 Pattern of Blood Pressure Elevation

The pattern of BP elevation is similar for 1K1C and 2K1C hypertension in the rat, although hypertension usually develops more rapidly and is more severe in the 1K1C model (Swales, 1981a). Immediately after clipping there is a transient elevation in BP which is associated with a transient elevation in plasma renin (Miller, Samuels, Haber & Barger, 1975). In the 2K1C model, BP is elevated at 1-2 days post-clipping and remains at this level for up to 2 weeks. Thereafter there is a progressive rise in arterial pressure, reaching a plateau between 4-6 weeks post-clipping which is then maintained (Morton & Wallace, 1983). A similar pattern of BP elevation occurs in rats following the induction of 1K1C hypertension (Koletsky, Pavlicko & Rivera-Velez, 1971; Freeman, Davis, Watkins, Stephens & De Forrest, 1979). The degree of

hypertension produced in these models is dependent on the severity of the renal artery constriction (Leenen, Dejong & DeWied, 1973).

3.1.3 Surgical Reversal

Surgical reversal of experimental renovascular hypertension has been studied in an attempt to elucidate the mechanisms involved in the maintenance of elevated BP. However, there is no real evidence to justify the assumption that the fall in BP is wholly, or partly mediated by a reversal of the mechanisms which initiated the raised arterial pressure in the first place (Swales, Bing, Russell & Thurston, 1983). The effects of the anaesthetic a few hours after unclipping are also unknown, and therefore caution must be exercised when extrapolating from reversal studies to the maintenance of hypertension (Russell, Bing, Thurston & Swales, 1982a). Advantages of this approach include the fact that a definite time of onset cannot be identified for the development of hypertension, whilst the fall in BP is initiated by unclipping. Furthermore, reversal is usually concentrated into a short period of time, whereas the development of elevated arterial pressure may take several weeks. Studying hypertension of different durations should also give some insight into the influence of secondary processes, initiated by raised arterial pressure, in maintaining hypertension. Consequently, useful clues to the processes involved in the maintenance of hypertension may be obtained by studying its surgical reversal (Swales et al, 1983).

The effectiveness of surgical reversal may depend on the duration of hypertension and the procedure employed. Removal of the ischaemic kidney during the first few weeks of 2K1C hypertension in the rat causes

a rapid fall in BP to normal (Koletsky & Rivera-Velez, 1970; Gross, 1971; Thurson & Swales, 1974; Macdonald, Boyd & Peart, 1975; Thurston, Bing & Swales, 1980a). Most studies have demonstrated that extirpation of the ischaemic kidney does not completely normalise BP in long-standing 2K1C hypertension (Wilson & Byrom, 1941; Koletsky & Rivera-Velez, 1970; Thurston et al, 1974; 1980a), although Gross (1971) found that BP fell rapidly to normal following this procedure at this stage. Nephrectomy at any stage of 1K1C hypertension in the rat fails to lower BP, probably due to the development of the anephric state of volume expansion in these animals (Floyer, 1955; Gross, 1971).

Removal of the constricting clip from the renal artery during the first few weeks of 2K1C hypertension is followed by a prompt normalisation of BP within 24 hours (Thurston et al, 1980a; Bing et al, 1981a). In long-term 2K1C hypertension, unclipping has been found to be associated with both a rapid fall in BP to normal (Carretero & Gulati, 1978; Thurston et al 1980a; Bing et al, 1981a) and persistent hypertension (Floyer, 1955; Lundgren & Weiss, 1979). All animals in the latter two studies demonstrating persistent hypertension did, however, respond to unclipping with a significant fall in BP. Removal of the clip from the sole ischaemic kidney at any stage of 1K1C hypertension reduces BP to normal within 24 hours (Byrom & Dodson, 1949; Floyer, 1955; Gross, 1971).

Immediately after unclipping there is a major fall in BP in rats with 2K1C hypertension, of both a few weeks and four months duration (Russell, Bing, Thurston & Swales, 1982a). This also occurs in hypertensive rats following the sham operation and thus appears to be a non-specific response to anaesthesia and surgery. The BP completely recovers within 2-4 hours in the sham-operated group, whilst there is

only a partial recovery in unclipped rats followed by a decline in BP to normal over the next 6-12 hours (Russell et al, 1982a). A similar sequence of events occurs after declipping in the 1K1C model (Neubig & Hoobler, 1975).

3.1.4 Phases of 2K1C Hypertension

2K1C hypertension has frequently been divided arbitrarily into stages, but this has not been done in a consistent fashion. Some workers refer to any sustained hypertension as 'chronic' (e.g. Davis, 1977), whilst others reserve the term 'chronic' for hypertension of several months duration (Gavras, Brunner, Thurston & Laragh, 1975). Brown et al (1976) described three phases of 2K1C hypertension: I is the renin-mediated transient elevation in BP immediately after renal artery constriction; II is the early phase of sustained hypertension characterised by a prompt lowering of BP on unclipping; and phase III when unclipping does not reverse the hypertension. The demonstration of complete reversal of long-term 2K1C hypertension by declipping in the rat (Carretero & Gulati, 1978; Thurston et al, 1980a; Russell et al, 1982a), however, makes this classification difficult to apply. Morton & Wallace (1983) also separated 2K1C hypertension into three phases: an acute phase 1-2 days post-clipping associated with elevated plasma levels of renin and angiotensin II; an intermediate phase where BP rises whilst renin-angiotensin system activity is suppressed; and established hypertension associated with re-elevated plasma levels of renin and angiotensin II.

For the purpose of the experiments reported in this thesis, 2K1C hypertension has been divided into the three phases described by Thurston, Bing & Swales (1980a). The transient post-operative BP

elevation is phase I; thereafter until 6 weeks post-clipping is the 'early phase', and hypertension of greater than 16 weeks duration is 'chronic phase'.

3.2 Factors Involved in Experimental Renovascular Hypertension and its Reversal

Several mechanisms have been implicated in 1K1C and 2K1C hypertension in the rat, and the two models differ from each other in certain important respects (Godfrey, Kumar, Bing, Swales & Thurston, 1985). This section considers the factors postulated to have important roles, emphasising the differences between the two models and indicates that none of these factors can completely explain the elevated BP, or its surgical reversal, in either 1K1C or 2K1C hypertension.

3.2.1. Renin-Angiotensin System

The renin-angiotensin system is important to BP homeostasis through its combined ability to alter peripheral resistance and the volume and electrolyte content of the body fluids. It is also closely linked to adrenergic vascular control mechanisms, prostaglandins and the Kallikrein-Kinin system (Peach, 1977; Swales, 1979a).

The renin-angiotensin system is postulated to have an important role in the development of 2K1C hypertension in the rat, but to have no significant involvement in the 1K1C model (Swales, 1979a). Renal renin (Regoli, Brunner, Peters & Gross, 1962) and juxtaglomerular (JG) granulation (Heptinstall, 1965) are both normal or low in 1K1C hypertension. Although there is a transient post-clipping rise in

plasma renin activity in this model (Koletsky, Pavlicko & Rivera-Velez, 1971), this falls to normal within a few days and remains normal or low thereafter (Miksche, Miksche & Gross, 1970; Koletsky et al, 1971; Freeman et al, 1979).

Conversely, in the 2K1C model renal renin (Regoli, Brunner, Peters & Gross, 1962) and JG granulation (Tobian, Thompson, Twedt & Janecek, 1958; Heptinstall, 1965) are both increased in the ischaemic kidney, although greatly reduced in the contralateral kidney. Plasma renin concentration rises along with BP in the early stages of 2K1C hypertension (Swales, 1979a; Godfrey et al, 1985) and is certainly elevated by 3-4 weeks (Miksche, Miksche & Gross, 1970; Oates, Stokes & Storey, 1975), especially if hypertension is severe (Leenen, Dejong & DeWied, 1973; Möhring, Möhring, Näumann, Phillipi, Homsy, Orth, Dauda, Kazda & Gross, 1975). Moreover, after unclipping rats with 2K1C hypertension plasma renin rapidly falls to normal or subnormal levels (Thurston et al, 1980a), and the JG index falls to normal in the previously ischaemic kidney and rises to normal in the previously depleted contralateral kidney (Heptinstall, 1965). Removal of the ischaemic kidney also normalises plasma renin and JG granulation (Tobian et al, 1958) and renin content (Regoli et al, 1962) of the untouched kidney.

Even in 2K1C hypertension, however, the renin-angiotensin system is not solely responsible for the development and maintenance of elevated BP. The pattern of JG granulation before and after unclipping described above is also seen in rats with unilateral renal ischaemia which do not develop hypertension (Hepinstall, 1965). Plasma renin levels, elevated in the early phase of the 2K1C model, decline to normal as time

progresses despite the maintenance of elevated BP (Koletsky, Rivera-Velez, Marsh & Pritchard, 1967; Bing, Russell, Swales & Thurston, 1981a). Plasma renin concentration has been reported to be normal after a few weeks (Koletsky & Rivera-Velez; 1970; Carretero & Gulati, 1978) and after 34 weeks (Sen, Smeby, Bumpus & Turcotte, 1979) of hypertension. Although other workers have found plasma renin concentration to be elevated at 5 (Leenen, Dejong & DeWied, 1973), 6 (Oates, Stokes & Storey, 1975) and 10 (Miksche, Miksche & Gross, 1970) weeks and at 4 months (Thurston, Bing, Marks & Swales, 1980b) after clipping. JG granulation in the clipped kidney has returned to normal within 16 weeks of 2K1C hypertension (Latta, White, Osvaldo & Johnston, 1975). Furthermore, there is no consistent temporal relationship between the rise in BP and the rise in plasma renin concentration in 2K1C hypertension (Oates, Stokes & Storey, 1975; Morton & Wallace, 1983).

The competitive antagonist of angiotensin II, sarcosine^{1}-alanine^{8} angiotensin II (saralasin), has no effect on BP in 1K1C hypertension but does significantly reduce arterial pressure in rats with 2K1C hypertension of up to 6 weeks duration (Brunner, Kirshman, Sealey & Laragh, 1971). The saralasin response follows a similar pattern to plasma renin concentration, producing a significant BP fall in rats with 2K1C hypertension of a few weeks duration (Thurston & Swales, 1974; Carretero & Gulati, 1978), but having minimal effect after 3-4 months (Gavras, Brunner, Thurston & Laragh, 1975; Thurston et al, 1980b). Even in the early phase of 2K1C hypertension renin-angiotensin blockade with either saralasin (Macdonald, Boyd & Peart, 1975) or converting enzyme inhibition (Bing et al, 1981a; Russell et al, 1982a; Morton & Wallace, 1983) does not restore BP to normal. Removal of the constricting clip,

however, restores BP to normal within 24 hours (Thurston, Bing & Swales, 1980a).

Converting enzyme inhibition with captopril produces a significant fall in arterial pressure in chronic phase 2K1C hypertension, whilst saralasin has no significant effect at this stage (Bing et al, 1981a). The duration of captopril and saralasin infusion may be important, however, and this is discussed in more detail later (see p 52). Furthermore, captopril significantly lowers BP in rats with 2K1C hypertension previously treated with saralasin to inhibit the renin-angiotensin system (Thurston & Swales, 1978a); the former compound may therefore possess additional vasodepressor properties. Captopril has been proposed to potentiate vasodepressor kinins (Thurston & Swales, 1978a), although the different responses to the two inhibitors could also reflect the partial agonist properties of saralasin (Antonaccio & Cushman, 1981). Continuous infusion of converting enzyme inhibitor was found to prevent the development of 2K1C hypertension for the whole of a 12 day infusion period, whereas 1K1C hypertension developed 8 days after clipping despite maintaining converting enzyme inhibition for a further 4 days (Freeman et al, 1979). Continuous inhibition of converting enzyme with perindopril for periods of 6 weeks and greater, completely prevented the development of 2K1C hypertension in rats during the whole of the treatment period. Withdrawal of perindopril treatment was followed by a slow rise in BP (Doyle, Harrap & Torresi, 1986). The infusion of saralasin for 15 hours before unclipping and maintained for 24 hours afterwards, had no effect on the BP fall following unclipping in early or chronic 2K1C hypertension. Therefore changes in renin-angiotensin system activity alone are not entirely responsible for the reversal of 2K1C hypertension following unclipping (Russell et al, 1982a).

Protagonists of the renin hypothesis have proposed several factors which could allow renin-angiotensin system involvement in the later stages of 2K1C hypertension, when the acute pressor action of angiotensin II appears to have no role (Swales, 1979a). These include:

a) Sodium retention

It has been claimed that sodium retention supplements renin hypersecretion in 2K1C hypertension in the rat (Möhring et al, 1975; Leenen & Dejong, 1975). Thus the elevated BP is initially renin dependent, but subsequently becomes sodium and volume dependent in the chronic phase (Gavras et al, 1975). This was based on the restoration of BP response to saralasin after 15 weeks of 2K1C hypertension if rats were previously sodium depleted. However, this observation probably merely reflects the importance of the renin-angiotensin system for the maintenance of BP homeostasis in the face of sodium depletion (Swales, 1979a). Moreover, a combination of dietary sodium depletion and pharmacological blockade of the renin-angiotensin system failed to restore BP completely to normal in 2K1C hypertension (Swales & Thurston, 1977). There is no convincing evidence that sodium retention potentiates the effect of the renin-angiotensin system when plasma renin concentration is normal, as in chronic 2K1C hypertension (Swales, 1979a); or that sodium retention occurs in hypertension produced by continuous infusion of acutely sub-pressor doses of angiotensin II (Brown, Casals-Stenzel, Gofford, Lever & Morton, 1981b).

b) Slow pressor effect of angiotensin II

Continuous intravenous infusions of sub-pressor doses of angiotensin II have been shown to produce a gradual increase in systemic arterial

pressure over the course of several days in the rabbit (Dickinson & Lawrence, 1963), dog (McCubbin, De Moura, Page & Olmsted, 1965) and more recently in the rat (Brown et al, 1981b). Angiotensin II may thus have a slowly developing pressor effect not dependent on high circulating levels of the hormone (Brown et al, 1976 and 1979). A slowly developing pressor response was not seen with NA, and this phenomenon seems unlikely to be a generalised attribute of all vasoconstrictor substances (Casals-Stenzel et al, 1983). Hypertension produced by continuous infusion of low doses of angiotensin II has been reported to be prevented by dietary sodium restriction (Cowley & McCaa, 1976) and has therefore been attributed to sodium retention, although this is controversial (Brown et al, 1981b). The gradual rise in arterial pressure produced in rats by infusion of low levels of angiotensin II was not associated with sodium retention. Furthermore, the fall in BP following cessation of angiotensin II infusion was associated with sodium retention rather than loss in these rats (Brown et al, 1981b).

Patients with a renin-secreting tumour, and patients and animals with hypertension secondary to unilateral renal ischaemia characterised by elevated circulating angiotensin II levels; have blood pressures higher than can be induced by increasing plasma angiotensin II levels to a similar degree by infusion in normal individuals (Swales, 1979a). Continuous infusion of converting enzyme inhibitor from 2-5 weeks after the induction of 2K1C hypertension, when circulating renin and angiotensin II levels were found to be almost normal, prevented the usual increase in BP at this stage (Wallace & Morton, 1984). Furthermore, continuous infusion of saralasin or converting enzyme inhibitor produced a slow fall in BP to normal over 12 hours in rats with 2K1C hypertension of 6 weeks duration (Riegger, Millar, Lever, Morton & Slack, 1977). Rats with malignant and benign forms of both

1K1C and 2K1C hypertension, showed a rapid fall in BP in the first 24 hours and then an additional slow decline during the remaining 6 days of continuous converting enzyme inhibition (Bengis & Coleman, 1979). BP was reduced to normal in both forms of 2K1C hypertension, but was not normalised in either form of 1K1C hypertension. In all cases the rapid initial fall in arterial pressure was correlated with pre-infusion plasma renin concentration, and was supposed to reflect the direct effect of angiotensin II on vascular smooth muscle. The following prolonged fall in BP was associated with reversal of the slowly developing pressor effect of angiotensin II (Riegger et al, 1977; Bengis & Coleman, 1979).

However, these findings were not confirmed in a later study, where the BP fall produced by saralasin or captopril infusion was found to be maximal after 30 minutes; with no further fall over the rest of a 12 hour infusion period, in either early or chronic phase 2K1C hypertension (Bing, Russell, Swales & Thurston, 1981a). Furthermore, the BP response to saralasin was significantly correlated to the pre-infusion level of plasma renin in both phases of this model.

c) Enhanced sensitivity to angiotensin II

Vascular sensitivity to angiotensin II can be modified by specific or non-specific factors. A specific increase in pressor sensitivity involves an action on the binding of the angiotensin II molecule to its specific receptor; whereas a non-specific factor acts at the later chain of events which convert receptor binding into a mechanical event, and will affect the pressor response to other agents as well as angiotensin II (e.g. NA and SNS activation) (Swales, 1979a). A specific increase in pressor sensitivity to angiotensin II could be mediated by

an increase in the number of vascular angiotensin II receptors, although this has not been demonstrated satisfactorily in either experimental or clinical hypertension (Swales, 1979a). Receptor affinity for angiotensin II may be involved in normal BP homeostasis and in the pathogenesis of certain forms of hypertension (Brunner, Chang, Wallach, Sealey & Laragh, 1972). Based on the volume of specific antiserum required to block the pressor response to exogenous angiotensin II *in vivo*, sodium loading was proposed to enhance and sodium depletion reduce the affinity of specific receptors for angiotensin II. These observations, however, could equally be explained by changes in receptor occupancy by locally generated angiotensin II, at sites inaccessible to large antibody molecules (Swales, Tange & Thurston, 1975). Furthermore, it is also doubtful whether *in vivo* measurements using angiotensin antisera are a legitimate measure of receptor affinity (Swales, 1979a).

However, receptor occupancy by endogenous angiotensin II is an important determinant of the pressor response to exogenous angiotensin II (Thurston & Laragh, 1975). Increased circulating levels of angiotensin II in response to salt depletion increases the occupancy of receptor sites by endogenous hormone, leaving fewer sites available to bind exogenous angiotensin II; resulting in decreased pressor sensitivity to this agent. The pressor response to exogenous angiotensin II is depressed in both early and long-term 2K1C hypertension, but normal in 1K1C hypertension compared to age-matched normal rats (Aoki & Masson, 1969; Bing, Swales, Taverner, & Thurston, 1984). After unclipping, pressor responsiveness to angiotensin II was similar to age-matched controls in both models (Bing et al, 1984). Differences in the levels of renin-angiotensin system activity could explain these observations, whilst no hyperresponsiveness to angiotensin II was demonstrated, even in chronic phase 2K1C hypertension. Pressor responses to exogenous

angiotensin II were inversely related to plasma renin activity in normal and hypertensive rats (Bing et al, 1984).

If the BP response to pharmacological blockade of the renin-angiotensin system reflects the role of this system, it is unlikely that specific changes in responsiveness to normal or low levels of angiotensin II, by whatever mechanism, are important to the maintenance of an elevated BP in experimental renovascular hypertension (Swales, 1979a; Doyle, Harrap & Torresi, 1986), or contribute to the fall in BP on unclipping (Bing et al, 1984).

The major non-specific factor affecting the response to vasoactive agents is altered vascular geometry (Folkow, Hällback, Lundgren, Sivertsson & Weiss, 1973). However, in vivo pressor responses to angiotensin II and NA are not increased in 1K1C hypertension, or in the early and chronic phases of the 2K1C model, before or after unclipping (Bing et al, 1984). Interaction with cardiac output and baroreflexes may obscure pressor responses in the whole animal; and hypersensitivity to angiotensin II and NA has been reported in chronic phase (>16 weeks) 2K1C hypertension in an isolated blood perfused hindquarter preparation in the rat. No hypersensitivity to angiotensin II was seen in the early phase of this model (Mistry, Bing, Swales & Thurston, 1983). The possible influence of structural changes in the maintenance and reversal of renovascular hypertension is discussed in more general terms in section 3.2.4.

d) Vascular renin

Local generation of angiotensin II within the resistance vessel wall, rather than circulating angiotensin II, may play a role in 2K1C

hypertension in the rat (Thurston & Swales, 1974; Swales, 1979b). Renin-like activity (Gould, Skeggs & Kahn, 1964), converting enzyme activity (Aiken & Vane, 1972) and renin substrate (Desjardins-Giasson, Gutkowska, Garcia & Genest, 1981) are present in the vascular wall, and could allow the local generation of angiotensin II (Thurston, Swales, Bing, Hurst & Marks, 1979; Desjardins-Giasson et al, 1981).

Vascular renin-like activity could be of renal origin taken up from the plasma (Loudon, Bing, Thurston & Swales, 1983), or synthesised locally within the blood vessel wall (Ganten, Schelling, Vecsei & Ganten, 1976; Barrett, Eggena, Krall & Sambhi, 1981; Rosenthal et al, 1984). Theoretically vascular renin activity could maintain an elevated BP in the presence of normal or low plasma renin levels. However, Swales and co-workers showed that it was only possible to demonstrate a divergence between plasma and aortic renin levels for a few hours following bilateral nephrectomy in rats with 2K1C hypertension, when plasma renin was declining rapidly (Thurston et al, 1979 and 1981; Swales, 1979a). Following bilateral nephrectomy in rats with 2K1C hypertension of 4-6 weeks duration vascular renin levels fell slowly over 24 hours, thus having a much longer half-life than circulating renin after this procedure (Thurston et al, 1977 and 1978b; Swales, 1979b). Conversely, other workers have shown that aortic renin and plasma renin levels do not always show parallel changes (Barrett, Eggena & Sambhi, 1978). Furthermore, whilst one form of renin may be common to the kidney, plasma and aorta, at least one other renin-like enzyme within the aorta is not detectable in plasma and may be locally synthesised (Barrett et al, 1981; Rosenthal et al, 1984). Barrett et al (1981) also demonstrated that the concentration and relative proportions of renin-like enzymes within the aorta were not changed 30 hours after

bilateral nephrectomy in rats. It was therefore proposed that arterial and plasma renin systems are controlled separately.

The source of renin-like activity within the blood vessel wall therefore remains controversial. However, regardless of this, the close correlation between BP fall produced by saralasin and pre-infusion plasma renin concentration in 2K1C hypertension (Macdonald, Boyd & Peart, 1975), makes it unlikely that high levels of vascular renin activity maintain an elevated BP in the presence of normal or low plasma renin concentrations in this model (Swales, 1979a). Moreover, surgical reversal of 2K1C hypertension in the rat by unclipping is not entirely mediated by either the vascular or circulating renin-angiotensin systems (Brice, Russell, Bing, Swales & Thurston, 1983).

e) Effect of angiotensin II on the nervous system

Elevated levels of angiotensin II enhance the vasoconstrictor response to SNS stimulation, via an action at peripheral sympathetic nerve terminals (Malik & Nasjletti, 1976; Zimmerman, 1981). Angiotensin II may also have an action on the CNS, increasing peripheral SNS activity and hence BP (Buckley & Jandhyala, 1977; Zimmerman, 1981) (see section 2.4.3). Whilst the facilitatory actions of angiotensin II on the nervous system may contribute to the early stages of 2K1C hypertension, it is difficult to envisage a role for these mechanisms in long-term 2K1C hypertension when renin-angiotensin system activity is not elevated.

3.2.2 Sodium

The observed differences in plasma renin activity between 1K1C and 2K1C hypertension could be explained by differences in sodium balance. Renal ischaemia enhances proximal tubular sodium reabsorption (Stein, Abramson, Bercovitch & Levitt, 1965), so that in the absence of a contralateral kidney sodium is retained until a new equilibrium is produced by raised systemic arterial pressure, preventing further sodium retention. The untouched kidney in 2K1C hypertension can eliminate a saline load more rapidly than a normal kidney (Peters, Brunner & Gross, 1964), and is completely exposed to the raised arterial pressure which leads to a natriuresis and diuresis. Consequently, during development of 1K1C hypertension sodium balance is positive (Swales et al, 1972) and exchangeable sodium is increased (Tobian, Coffee & McCrea, 1969; McAreavey, Brown, Murray & Robertson, 1984) compared to controls. An increase in plasma and extracellular fluid volume and cardiac output have also been observed (Ledingham & Cohen, 1964). Conversely, sodium balance is either unchanged (McAreavey, Brown & Robertson, 1982) or negative (Swales et al, 1972) during the development of 2K1C hypertension. The early stages of this model have been reported to be associated with sodium retention (Möhring et al, 1975), a negative sodium balance only occurring in severe hypertension. A critical systolic BP of 180 mmHg was proposed, below which sodium is retained and above which sodium is lost (Möhring et al, 1975; Leenen & Dejong, 1975). Swales et al (1972) demonstrated no sodium retention in this model in the female Wistar rat, with a negative sodium balance developing within the first 14 days of 2K1C hypertension. Furthermore, 2K1C hypertension develops despite rigorous sodium restriction (Redleaf & Tobian, 1958; Swales & Tange, 1971; Thurston & Swales, 1976; Taquini, Gallo, Basso & Taquini, 1980).

The BP fall following unclipping in both phases of 2K1C hypertension is associated with sodium retention, rather than a natriuresis and diuresis (Thurston, Bing & Swales, 1980a). Cardiac output is reduced in both short-term (<6 weeks) and long-term (>16 weeks) 2K1C hypertension and is elevated to values similar to control following unclipping (Russell, Bing, Swales & Thurston, 1983). The fall in BP is therefore not mediated by a reduction in extracellular fluid (ECF) and plasma volume. However, it has been proposed that ECF and plasma volume expansion can occur in the face of net sodium and fluid loss from the kidneys, if there is a migration of fluid from intracellular to extracellular compartments (Möhring et al, 1975).

Sodium retention may contribute to 1K1C hypertension, although dietary sodium restriction does not affect the development and maintenance of elevated arterial pressure in this model (Redleaf & Tobian, 1958; Thurston & Swales, 1976). Acute sodium depletion by peritoneal dialysis was found to reduce BP in 1K1C rats, but not to normal levels (Swales & Tange, 1971). The fall in BP following unclipping is associated with a reduction in cardiac output (Ledingham & Cohen, 1962), natriuresis and diuresis (Liard & Peters, 1970), which suggest reversal of 1K1C hypertension is mediated by a loss of sodium and fluid. However, replacement of the sodium and fluid losses by intravenous infusion of saline to maintain a positive sodium balance (Neubig & Hoobler, 1975); or prevention of excretion by implanting the ureter of the sole ischaemic kidney into the inferior vena cava (uretero-caval anastomosis) (Floyer, 1955), do not prevent the fall in BP to normal after unclipping. Therefore, even in 1K1C hypertension sodium retention is not the only factor responsible for the elevated arterial pressure, and sodium and fluid loss alone cannot explain the fall in BP upon surgical reversal in this model (Muirhead & Brooks, 1980b; Godfrey et al, 1985).

The natriuresis and diuresis, which accompanies the fall in BP on unclipping rats with 1K1C hypertension, merely accelerates the antihypertensive action of the kidney and is not essential for its occurrence (Muirhead & Brooks, 1980b).

Sodium retention has been proposed to lead to hypertension via 'autoregulation' (Borst & Borst-de-Geus, 1963; Ledingham & Cohen, 1963; Guyton, 1977) (see section 1.3.1b). Although autoregulation can be demonstrated in isolated vessels, 'whole body autoregulation' has been questioned on the grounds that the response would be expected to be overriden by the many factors controlling the calibre of resistance vessels (Folkow & Neil, 1971). More direct evidence against this hypothesis is the observation of an initial rise in peripheral resistance, rather than cardiac output, in various forms of clinical (Kim, Onesti, Delguercio, Greco, Fernandes & Swartz, 1976) and experimental renal hypertension (Ledingham & Pelling, 1967; Fletcher, Korner, Angus & Oliver, 1976). It therefore appears that a rise in cardiac output is not necessary for BP elevation, and when it does occur in experimental hypertension it is a non-specific consequence of the surgical procedure (Fletcher et al, 1976; Swales, 1981a). However, it has been suggested that the renal control of sodium and water balance possesses overall dominance over other BP control mechanisms (Guyton, Coleman, Cowley, Scheel, Manning & Norman, 1972).

Sodium may have a direct effect on peripheral blood vessels in addition to causing ECF and plasma volume expansion. There are 3 main hypotheses for the mechanism of this action:

1) Sodium, and consequent fluid retention within the arteriolar wall could lead to an increased wall:lumen ratio and hence increased

peripheral resistance (Tobian, 1974). Increased intracellular sodium content has been demonstrated in the aortic wall of rats with 1K1C and 2K1C hypertension (Redleaf & Tobian, 1958). However, an increased arterial wall sodium and water content was only demonstrable in areas exposed to high pressure in dogs with experimental coarctation of the aorta (Hollander, Kramsch, Farmelant & Madoff, 1968). This suggests increased arterial or arteriolar wall sodium content is a consequence rather than a cause of hypertension.

2) Changes in smooth muscle cell membrane sodium transport may increase reactivity. Enhanced ionic turnover has been demonstrated in the aortic smooth muscle of spontaneously hypertensive rats (SHR), and the resulting partial depolarisation may enhance smooth muscle responsiveness to vasoconstrictor stimuli (Jones, 1973).

3) Alternatively, an interaction between intracellular sodium and calcium ions may alter smooth muscle contractility, and hence vascular tone and peripheral resistance (Blaustein, 1977). However, there is no evidence to implicate this type of mechanism in forms of hypertension where sodium retention does not occur, e.g. 2K1C hypertension in rats (Swales, 1981a). High resting calcium uptake has been demonstrated in aortic smooth muscle from spontaneously hypertensive and Dahl salt-sensitive models of hypertension in rats compared to controls (Rapp, Nghiem & Onwochei, 1986). Similar increases in aortic smooth muscle calcium uptake could also be demonstrated after the induction of hypertension in Dahl salt-resistant rats, by removing one kidney and imposing an 8% sodium chloride diet. It was therefore

proposed that changes in vascular smooth muscle cell calcium uptake represented a final common physiological pathway by which genetic and environmental factors influence BP (Rapp et al, 1986).

The sodium ion may be an important factor in some forms of clinical and experimental hypertension, although its mode of action has yet to be clarified (Swales, 1981a).

3.2.3 Vasodepressor Substances

The kidney has been proposed to have an endocrine antihypertensive function in addition to the control of sodium and water balance, and the evidence for this was discussed in section 1.3.2.

Selective damage to the renal papilla by 2-bromoethylamine hydrobromide (2-BEA) has been found to aggravate the elevated BP in 2K1C hypertension (Heptinstall, Salyer & Salyer, 1975). Other studies showed that renal medullectomy with 2-BEA had no effect on the development or maintenance of 2K1C hypertension, but prevented a complete fall in BP in these rats following unclipping (Bing, Russell, Swales, Thurston & Fletcher, 1981b; Taverner et al, 1984). When exposed to the venous effluent from an isolated, acutely declipped kidney of a rat with 2K1C hypertension in an extracorporal perfusion circuit, conscious normal rats responded with a marked fall in BP (Göthberg, Lundin & Folkow, 1982a). This suggests that powerful depressor agents are released from an acutely declipped kidney following 2K1C hypertension.

Three intrarenal vasodepressor systems have been implicated : prostaglandins, kallikrein-kinin system and renomedullary lipids, of

which the latter seem the most promising (Muirhead, 1980a). Two classes of antihypertensive lipid have been identified from renomedullary interstitial cells, one neutral (antihypertensive neutral renomedullary lipid, or ANRL; Muirhead, 1974) and one polar (antihypertensive polar renomedullary lipid, or APRL; Muirhead, 1980a) (see section 1.3.2). Continuous infusion of indomethacin, an inhibitor of prostaglandin synthesis, or aprotinin which suppresses kinin generation, both failed to have any effect on the maintenance or surgical reversal of 2K1C hypertension in rats (Russell, Bing, Swales & Thurston, 1982b). Indomethacin also had no effect on the reversal of 1K1C hypertension in the rabbit (Romero, Ott, Aguilo, Torres & Strong, 1975).

ANRL, on the other hand, has been derived from the renal venous effluent following unclipping rats with 1K1C hypertension (Muirhead, Byers, Desiderio, Pitcock, Brooks, Brown & Brosius, 1982). Acetyl glyceryl ether phosphorylcholine (AGEPC) has a strong antihypertensive action after intravenous injection in experimental animals, and is proposed to be an active component of APRL (Masugi, Ogihara, Saeki, Otsuka & Kumahara, 1985). 3-(N-n-octadecylcarbamoyloxy)-2-methoxypropyl-2-thiazolioethylphosphate (CV-3988) specifically inhibits the hypotensive effect of AGEPC, having no effect on other vasoactive hormones. Intravenous infusion of CV-3988 inhibits the rapid decrease in BP following unclipping in rats with 1K1C (Masugi et al, 1984) and 2K1C hypertension (Masugi et al, 1985). This suggests a humoral antihypertensive factor is released from an unclipped kidney, and an endogenous AGEPC may be one of the factors that lower BP in 1K1C and 2K1C hypertension following declipping (Masugi et al, 1985).

3.2.4 Structural Changes

The elevated BP in 2K1C hypertension is proposed to produce ischaemic vascular lesions in the contralateral kidney, causing the further release of renal pressor substance which aggravates the hypertension. A 'vicious circle' is thus initiated resulting in increasing BP and progressive renal damage (Wilson & Byrom, 1939). In support of this, persistent hypertension was observed in 66% of 2K1C rats following excision of the clamped kidney; the degree of residual hypertension being related to the extent of the vascular lesions within the untouched kidney (Wilson & Byrom, 1941). The clipped kidney is protected from the increased arterial pressure by the renal artery constriction (Levy, Light & Blalock, 1938) and has no vascular damage (Wilson & Byrom, 1939).

Resistance vessels adapt structurally to an increased pressure load with a thickening of the media layer of the vessel wall, leading to an increased wall:lumen ratio. Following vascular hypertrophy a raised peripheral resistance could be maintained at normal levels of smooth muscle activity (Folkow, Hällback, Lundgren, Sivertsson & Weiss, 1973). Left ventricular hypertrophy appears after 1 week of 2K1C hypertension in the rat, closely followed by adaptive structural changes in the resistance vessels which are complete within 3 weeks (Lundgren, 1974). Structural and functional processes may therefore co-operate, since structural cardiovascular adaptation is seen before the BP rise is complete. An increased sodium and water content of the arterial and arteriolar wall may also contribute to the increased peripheral resistance (Tobian, 1974). However, this "water-logging" effect only occurs in the later phases of 2K1C hypertension, and even then has far less haemodynamic significance than the structurally based increase in

wall thickness (Lundgren, 1974). Significantly increased amounts of collagen are present in the aortic wall in long-term 2K1C hypertension (>135 days), which will further increase the wall:lumen ratio at this stage (Lundgren, 1974).

Cardiovascular structural changes are, however, unlikely to be the only factors maintaining an elevated BP in the established phase of experimental renovascular hypertension (Swales, 1979a). Arterial pressure falls immediately on unclipping rats with both 1K1C (Byrom & Dodson, 1949) and 2K1C hypertension (Thurston et al, 1980a), whereas a slow fall would be expected if the mechanism of reversal was the regression of structural changes (Swales, Bing, Russell & Thurston, 1983). In both early (<6 weeks) and chronic (>16 weeks) 2K1C hypertension BP is reduced to normal within 24 hours of unclipping (Russell, Bing, Thurston & Swales, 1982a). However, cardiovascular structural changes take 3-4 weeks to reverse after short-term 2K1C hypertension (Lundgren, 1974) and do not regress completely after long-standing hypertension; probably due to the increased collagen and elastin component to the increased arterial wall thickness at this stage (Wolinsky, 1971; Lundgren & Weiss, 1979). The rapid reversal of BP elevation following unclipping despite the presence of vascular hypertrophy implies that the resistance vessels are in a state of subnormal tone (Hallbäck-Norlander, Noresson & Lundgren, 1979). After reversal of short-term 2K1C hypertension the complete regression of cardiovascular structural changes will eventually stabilise the normotensive state.

Structural changes in the resistance vessels may therefore contribute to the maintenance of long-term renovascular hypertension, but they are likely to develop as a consequence of raised arterial pressure rather than being important to its development (Mistry et al, 1983).

3.2.5 Other Factors

A number of additional pressor agents have been demonstrated and the best characterised of these are: nephrotensin, renopressin, sensitising factor and tonin. All of these remain to be fully characterised and their role in experimental renovascular hypertension is uncertain (Swales, 1981b).

3.3 Concluding Remarks

The mechanisms involved in the development, maintenance and reversal of 1K1C and 2K1C hypertension in the rat remain obscure. The two models have similar degrees of hypertension but different associated changes in sodium balance and renin, neither of which can completely explain the hypertension in either model (Godfrey et al, 1985). A vasodepressor hormone of renal origin may contribute to the BP fall on unclipping in both these models (Masugi et al, 1984; 1985). Cardiovascular structural changes may contribute to the later phases of 1K1C and 2K1C hypertension; but they are likely to develop as a consequence of elevated arterial pressure, and are unlikely to be the only factors involved in the maintenance of high BP in chronic renovascular hypertension (Swales, 1979a).

Neurogenic mechanisms mediated by the peripheral SNS may be involved, but studies on the SNS in experimental renovascular hypertension in the rat are sparse (see section II). The experiments reported in sections II and III were designed to investigate SNS activity in 1K1C and 2K1C hypertension in the rat, before and after removal of the clip from the renal artery.

SECTION II

PLASMA NORADRENALINE STUDIES:

Plasma noradrenaline concentration and heart rate in two models of renovascular hypertension in the rat, before and forty-eight hours after surgical reversal

CHAPTER 4

INTRODUCTION

INTRODUCTION

Early renal denervation experiments showed that completely stripping the renal pedicle of its extrinsic nerve supply had no effect on the development of Goldblatt renovascular hypertension in dogs (Page, 1935). This suggested neurogenic mechanisms played little or no role in the development and maintenance of renal hypertension. More recently, however, evidence in favour of a neurogenic component mediated by the SNS has accumulated, although this may depend on the model of renovascular hypertension studied. Thus, NA turnover in the heart and blood vessels has been found to be elevated in 1K1C, but unchanged in 2K1C hypertensive rats compared to controls (Brody et al, 1983). NA and adrenaline synthesis have also been demonstrated to be elevated in the adrenal glands of rats with 1K1C hypertension (Racz, Kuchel, Buu & Garcia, 1986).

Plasma NA has been reported to be significantly elevated at 7, 14 and 28 days after induction of 1K1C hypertension, whilst being unaltered at any stage in the 2K1C model, compared to corresponding loose-clip control rats (Reid, Dargie, Franklin & Fraser, 1976; Dargie, Franklin & Reid, 1977a and b). These results are consistent with a role for neurogenic mechanisms mediated by the peripheral SNS in the development and maintenance of 1K1C hypertension, but not in the 2K1C model. However, blood samples were collected from rats following decapitation, and studies on conscious rats have demonstrated elevated plasma NA levels in both 1K1C and 2K1C hypertension (Katholi, Winternitz & Oparil, 1982a; Katholi, Whitlow, Winternitz & Oparil, 1982b). In the latter two studies, an enhanced hypotensive response to ganglionic blockade with hexamethonium bromide was also reported in 1K1C and 2K1C hypertensive rats compared to controls. This was interpreted as evidence of SNS

activation in both models. 1K1C hypertension has also been shown to be associated with an increased urinary excretion of NA and adrenaline (Racz et al, 1986). By contrast, however, reduced plasma NA levels have recently been observed during the development and maintenance of 1K1C hypertension (Vlachakis, Ransom, Kogosov, Woodcock, Alexander & Maronde, 1984).

In the only study of changes after reversal of hypertension by unclipping, it was reported that elevated plasma NA levels fell to normal in both 1K1C and 2K1C hypertension (Katholi et al, 1982a and b). Direct recording of electrical impulses in efferent renal sympathetic nerves has provided further evidence that sympathetic nerve activity falls acutely after unclipping 2K1C hypertensive rats; this was attributed to the release of a renomedullary vasodepressor lipid (Göthberg, Lundin, Folkow & Thorén, 1982b; Göthberg & Thorén, 1984). Heart rate has been reported to be both increased (Hallbäck-Norlander, Noresson & Lundgren, 1979) and decreased (Russell, Bing, Swales & Thurston, 1983) in the early phase of 2K1C hypertension (<6 weeks duration), whilst being normal in the chronic phase of this model (Russell et al, 1983). Neither study, however, showed any change in heart rate after unclipping.

Elevated SNS activity in experimental renovascular hypertension may have a central component. Pre-treatment with intracisternal injections of 6-hydroxydopamine (6-OHDA), to deplete central noradrenergic neurons, prevents both the development of 1K1C hypertension in the rat and the rise in plasma NA which normally accompanies the elevated BP (Dargie, Franklin & Reid, 1977a). SNS activation in this model may therefore be, at least in part, centrally mediated. Furthermore, 1K1C hypertension has been found to be associated with an increased hypothalamic NA

content which falls to normal upon renal denervation, concomitant with a marked decrease in arterial pressure (Winternitz, Katholi & Oparil, 1982).

Conversely, treatment with intracisternal 6-OHDA prior to clipping had no effect on the BP elevation or plasma NA concentration in 2K1C hypertension (Dargie, Franklin & Reid, 1977b).

Renal denervation in rats with 1K1C hypertension produces a significant fall in BP, though not to normotensive levels (Katholi, Winternitz & Oparil, 1981), and a fall in plasma NA concentration to normal (Katholi et al, 1982a). Similar findings have been obtained following denervation of the clipped kidney in the 2K1C model, whilst denervation of the non-clipped kidney had no effect on BP or plasma NA (Katholi et al, 1982b). The antihypertensive effect of renal denervation of the clipped kidney was not associated with any changes in renin-angiotensin system activity or sodium balance in either model. The antihypertensive response to renal denervation was therefore proposed to be due to interruption of afferent rather than efferent renal nerves, which leads to attenuation of peripheral sympathetic tone mediated via a central component (Katholi, 1983). Further support for the hypothesis that it is afferent rather than efferent renal nerves which are important, comes from the suggestion that there is an attenuation of efferent sympathetic nervous control of renal vascular resistance in established renal hypertension (Fink & Brody, 1980). It was suggested that renal ischaemia on the other hand, caused increased activity in afferent renal nerves which stimulated cardiovascular regulator centres in the hypothalamus, and thus increased peripheral SNS tone (Katholi et al, 1982a; 1982b; 1983). In support of this, the development of 1-kidney and 2-kidney renovascular hypertension in rats can be prevented, and

established hypertension reduced to near normotensive levels, by a lesion of the periventricular tissue surrounding the anteroventral third ventricle (AV3V) region of the anterior hypothalamus (Brody & Johnson, 1980).

Recently, however, the importance of intact renal nerves in the development and maintenance of 1K1C hypertension has been disputed. Renal denervation performed at the time of 1K1C surgery, and repeated at 3-week intervals to prevent nerve regeneration, could only delay the onset of BP elevation by approximately 5 weeks leaving the final hypertensive level unaltered. Placing the rats on a high sodium intake prevented even this delay in the development of 1K1C hypertension in renal denervated rats (Norman, Murphy, Dzielak, Khraibi & Carroll, 1984). Moreover, Villarreal et al (1984) found that ablation of the renal nerves failed to have any effect on either the development or maintenance of 1K1C hypertension, regardless of the severity and duration of BP elevation.

The neurotoxins guanethidine and 6-OHDA produce a specific and extensive sympathectomy (DeChamplian, 1977a). These compounds may therefore permit an evaluation of the role of the SNS in BP regulation in animals with experimental hypertension (DeChamplain & Van Ameringen, 1972). 1K1C hypertension has been shown to develop in rats following complete destruction of the peripheral SNS by chronic guanethidine treatment and adrenal medullectomy (Douglas, Johnson, Heist, Marshall & Needleman, 1976). Similarly, prior sympathectomy with guanethidine failed to attenuate the development of 2K1C renal hypertension in the rat (Douglas et al, 1975), suggesting neurogenic mechanisms mediated by the SNS have no role in either of these models.

Following intravenous administration at low doses, 6-OHDA acts as a false neurotransmitter, but at high doses produces a rapid ablation of the peripheral SNS without affecting the adrenal medulla (Kostrzewa & Jacobwitz, 1974). Conflicting data have been obtained following peripheral sympathectomy with 6-OHDA in 2-kidney renal hypertensive rats. Chronic 6-OHDA treatment combined with adrenal medullectomy has been demonstrated to attenuate the development of 2K1C hypertension and to lower BP during established hypertension in this model (Antonaccio, Ferrone, Waugh, Harris & Rubin, 1980). The development and maintenance of 2K1C hypertension has, however, also been reported to be unaffected by treatment of neonatal rats with 6-OHDA prior to clipping the renal artery, despite achieving a high level of functional sympathectomy of the vascular system (Provoost, DeJong & Magnus, 1978). Similarly, repeated 6-OHDA treatment from birth, accompanied by adrenal medullectomy, had no effect on the development of 2K2C hypertension (Kurnjek, Mangiarua, Basso & Taquini, 1983); although a small degree of residual SNS activity was found to remain following this treatment.

The use of 6-OHDA, however, presents problems since the compound has additional effects besides peripheral sympathectomy (Brody et al, 1983). When applied to immature rats there is a high mortality and evidence of permanently impaired growth and exploratory behaviour. Furthermore, peripheral administration also causes alterations in central noradrenergic neurons, suggesting the compound can cross the blood/brain barrier. These additional effects confound the interpretation that prevention or attenuation of hypertension produced by treatment of immature rats with 6-OHDA is attributable to destruction of the peripheral SNS (Brody et al, 1983). There is also rapid nerve regeneration and experiments with 6-OHDA have not always included procedures to evaluate the functional innervation of the vasculature

(DeChamplain & Nadeau, 1971; Brody et al, 1983). The effectiveness of treatment also depends on the age of the animal, since 6-OHDA is most effective when given to immature rats (Brody et al, 1983).

Data concerning the involvement of the SNS in 1K1C and 2K1C hypertension in the rat are therefore contradictory, and little is known of the changes resulting from surgical reversal. Furthermore, it is not clear when SNS activation is demonstrated whether this is essential to the development of hypertension, or merely secondary to the elevated BP. Most evidence suggests SNS activity is increased in 1K1C hypertension, whilst evidence in the 2K1C model is more conflicting.

To assess SNS involvement in the established phase of renovascular hypertension and in the BP change after reversal of hypertension, both plasma NA and heart rate have been measured in conscious rats with 1K1C and 2K1C hypertension of 4-6 weeks duration, before and 48 hours after unclipping the renal artery. Stress is a major problem when measuring plasma NA concentration. Plasma NA levels recorded in blood samples collected from decapitated rats (Reid et al, 1976; Dargie et al, 1977a and 1977b) were several times (5-6 fold) greater than those obtained from conscious rats via an indwelling arterial cannula (Katholi et al, 1982a and 1982b). The experimental protocol employed here involved sampling from conscious, unrestrained rats with care being taken to ensure stress of the animal was minimal.

In addition, chronic 2K1C hypertension (>16 weeks after clipping) has been studied to assess the possible changes in SNS involvement with duration of hypertension in this model. There has been a suggestion that the contribution of neurogenic mechanisms to the elevated BP increases with duration of hypertension, possibly coinciding with the

fall in renin-angiotensin system activity which occurs in the chronic stages of 2K1C hypertension (Reed, Sapirstein, Southard & Ogden, 1944; Fiorentini, Bellini & Fernandes, 1981).

1K1C and 2K1C renovascular hypertension in the rat offer an opportunity to study SNS activity in models having similar degrees of hypertension but opposing changes in sodium balance and plasma renin (Godfrey et al, 1985).

CHAPTER 5

MATERIALS AND METHODS I :

ANIMAL PROCEDURES

5.1 The Animals

Female Wistar rats weighing 170-190g were used throughout. They were anaesthetised with ether (anaesthetic diethyl ether BP, May & Baker Ltd., Dagenham, UK) during all surgical procedures, which were carried out under aseptic conditions with sterilised instruments. Rats were housed 4-5 in a cage (510 x 330x 280 mm in size), fed conventional rat chow (Labsure Economy, K & K Greef Ltd., Croydon, UK) and allowed to drink tap water ad libitum. The ambient temperature was maintained between 20-23°C, humidity was 50% and there was a 12 hour light/dark cycle (dark between 2000 and 0800 hours daily).

5.1.1 Induction of Hypertension

Two models of renovascular hypertension were studied. A clip was applied to the left renal artery and the contralateral kidney was either removed to produce 1K1C hypertension (Byrom & Dodson, 1949), or left untouched to produce 2K1C hypertension (Byrom & Wilson, 1938).

Clipping the left renal artery

The left flank overlying the kidney was shaved, swabbed with ethyl alcohol and an incision made in the loin just below the lower border of the rib cage. The overlying muscle layers were cut and retractors inserted to open the wound. The kidney was cleared of perirenal fat and the adrenal gland, before being held clear of the area with a cotton swab slit to accommodate the renal pedicle. Careful dissection revealed the renal artery and vein. The renal artery was isolated close to the junction with the abdominal aorta, and a constricting clip (0.2mm internal diameter) placed around the main renal artery. Clips were made

from annealed silver ribbon (2.25mm x 0.13mm, Thessco Ltd., Sheffield, UK) cut into 15mm lengths and bent around a feeler gauge to obtain a precise internal diameter. The clip consisted of one short (approximately 5mm) and one long arm (approximately 10mm).

The kidney was then replaced in the abdomen, ensuring the renal pedicle was not twisted, and the muscle layers of the body wall closed with braided sterile silk sutures (4/0 Mersilk, Ethicon Ltd., Edinburgh, UK). The skin was closed with 3 metal surgical clips (12mm x 3mm Michel clips, Thackray Ltd., London, UK), which were removed after 3 weeks.

Right nephrectomy

In a rat undergoing unilateral nephrectomy the right flank was shaved and swabbed with ethyl alcohol, before making a loin incision and inserting retractors to open the wound. The kidney was cleared of perirenal fat and the right adrenal gland, exposing the renal pedicle which was clamped near the hilum with artery forceps. A silk ligature (4/0 Mersilk) was tied proximal to the clamp and the kidney separated from the pedicle. The artery forceps were then removed and the wound closed as described earlier.

Loose clip controls

Age-matched loose clip control rats were prepared at the same time for each group. In these rats a non-constricting silver clip (0.5mm internal diameter) was placed on the left renal artery, with or without contralateral nephrectomy as appropriate.

5.1.2 Experimental Groups

Three groups of hypertensive rats were prepared:

1. Early phase 1K1C hypertension, studied 4-6 weeks after clipping.
2. Early phase 2K1C hypertension, again used 4-6 weeks after clipping.
3. Chronic 2K1C hypertension, where hypertension was produced as described in section 5.1.1 but rats were studied more than 16 weeks post-clipping.

Parallel groups of age-matched loose clip control rats were prepared at the same time.

5.1.3 Indirect Blood Pressure

The systolic BP of all groups was monitored indirectly at weekly intervals from 3 to 6 weeks after clipping by light plethysmography, with the animals under light ether anaesthesia (Swales & Tange, 1970). Systolic BP recorded in this way has been found to correlate well with direct mean arterial pressure measurements (Swales & Tange, 1970; Borkowski & Quinn, 1983).

Hypertension was defined as an indirect BP greater than 140mmHg on two separate occasions, one week apart. Rats prepared for the chronic 2K1C hypertension study had indirect BP greater than 140mmHg recorded between 3-6 weeks, and then again after a period of time exceeding 16 weeks post-clipping. All loose clip control rats had a BP less than 140mmHg on all occasions.

Incidence of hypertension

74% of 1K1C rats became hypertensive by the end of the fourth week, and 14% of animals died within the first 4 weeks. 71% of 2K1C rats were hypertensive 4 weeks after clipping and 4% died within this time.

5.1.4 Cannulation of the Carotid Artery

At the time of study (either 4-6 weeks or >16 weeks post-clipping) each rat was weighed and the left common carotid artery cannulated.

Preparation of the cannula

A short length (150mm) of P25 polythene tubing (0.40mm internal diameter [ID] x 0.80mm external diameter [OD]; Portex Ltd., Hythe, UK) was bent into a U-shape in a beaker of boiling water, to prevent the cannula twisting when in position in the neck. The P25 tubing was then attached to a length (approximately 1000mm) of wider bore polythene tubing (P50) (0.58mm ID x 0.96mm OD; Portex Ltd., Hythe, UK) by a 15mm length of silastic tubing (0.75mm ID x 2.5mm OD; Sterilin Instruments, Hampshire, UK). The opposite end of the P50 tubing was heat flared to facilitate attachment to an adapter (Clay-Adams, New Jersey, USA), which in turn was connected to a plastic 3-way tap (Vygon, Cirencester, UK). A 10ml disposable plastic syringe (Monoject, St. Louis, USA) was used to fill the whole cannula with 0.9% saline containing 10 units/ml heparin (Weddel Pharmaceuticals Ltd., Wrexham, UK) as anticoagulant. The free end of the P25 polythene tubing was cut to an angle of 45° with a scalpel blade, to facilitate its introduction into the carotid artery.

Cannulation of the carotid artery

The rat was anaesthetised and a small incision made between the scapulae. The animal was then placed ventral side uppermost and a mid-line incision made on the anterior surface of the neck. The thyroid gland and surrounding adventitia was carefully teased apart with forceps and cotton swab sticks (Johnson & Johnson Ltd., Slough, UK), to reveal the left common carotid artery located in the plane of the sterno-hyoid and the sterno-clydomastoid group of muscles. The P25 portion of the cannula was threaded from the incision between the scapulae to the front of the neck through a subcutaneous tunnel. A 10-20mm length of artery was carefully cleared from the fascia of the carotid sheath by blunt dissection, the vagus nerve and sympathetic nerves were identified and carefully isolated from the carotid artery. Three silk ligatures (4/0 Mersilk) were placed around the artery. The cranial ligature was tied to occlude the blood supply superior to it, and the vessel held taught with this ligature using a pair of artery forceps as a weight. Tension was also applied to the proximal or thoracic ligature with artery forceps, temporarily occluding the artery.

A small incision was made in the carotid artery between the ligatures. The bevelled end of the cannula (P25) was then introduced into the lumen of the vessel, and advanced past the loosened proximal ligature to a pre-marked point on the catheter (25mm). This ensured that the tip was positioned in the aortic arch, and post-mortem examination at the end of the experiment confirmed this position. Patency was checked by observing the backflow of blood into the cannula. Tension was then re-applied to the proximal ligature with the artery forceps, whilst a middle ligature was tied around the cannula and the vessel. The cannula was finally secured by tying the proximal and distal ligatures firmly

around the artery, before closing the neck incision with silk sutures. The catheter was sutured to the skin at the point where it was exteriorised between the scapulae and a pair of light curved mosquito forceps with padded jaws used to occlude it. After removal of the P50 polythene tubing, the P25 tubing was cut so that approximately 35mm was exposed. A 15mm length of silastic tubing (0.75mm ID x 2.5mm OD) was placed on the end of the cannula and a pin (approximately 15mm in length with the head removed) used to seal it. The forceps were removed and a length of light flexible stainless steel spring (3mm ID and approximately 35mm in length) was placed over the exposed part of the cannula and taped to the back of the rat to protect the polythene tubing. Following this operation rats were housed individually in cages (400 x 280 x 210 mm in size) with free access to food and water for 48 hours to recover from the operation and anaesthetic.

5.1.5 Direct Blood Pressure, Heart Rate and Blood Sampling

Forty-eight hours after cannulation the tape and metal spring were carefully removed. A catheter, consisting of P50 polythene tubing (approximately 1000mm in length) attached at one end to a 3-way tap, filled with heparinised saline (10 units/ml heparin in 0.9% saline) and covered with stainless steel spring (400mm in length x 3.0mm ID), was used to attach the rat to the recording equipment as follows:

The rat was placed in a small box (150 x 130 x 120 mm) and light forceps with padded jaws were used to clamp the exposed part of its carotid cannula just above the neck. The pin was removed and the free end of the P50 catheter (described above) connected to the rat's arterial (P25) cannula via the silastic tubing joint. After removal of the clamp, the metal spring was positioned to cover the polythene tubing from its point

of emergence between the scapulae. This connection procedure was performed without anaesthetising the animal. The line was flushed with a maximum of 0.2mls heparinised saline and patency checked by observing the backflow of blood into the catheter. The catheter was subsequently closed to the air with the 3-way tap to prevent the backflow of blood. The rat was placed in an opaque container (cylindrical in shape, 160mm in diameter and height), with free access to food and water for a 2-3 hour acclimatisation period (Plate 5.1). The purpose of this container was two-fold: firstly it was small and therefore, although the rat was not physically restrained, excessive movement was restricted; and secondly, as it was opaque the rat was less susceptible to visual disturbances.

Rats were found to sit peacefully in these cages, ensuring blood samples were obtained from resting unstimulated animals. Wire mesh was secured over the top of the cage and the 3-way tap used to connect the rat's catheter to a Statham P23gb strain gauge transducer (Stagg Instruments, Henley on Thames, UK) and Grass polygraph recorder (Model 7D; Grass Instruments Co., Quincy, Massachusetts, USA) (See Plate 5.2). Calibration of the Grass recorder was effected, and checked regularly, using a standard mercury sphygmomanometer.

Direct blood pressure and heart rate

After the 2-3 hour acclimatisation period direct arterial pressure was recorded over 30-60 minutes on the Grass polygraph recorder, at a chart speed of 10mm/min. The mean direct BP (diastolic pressure plus one-third of the pulse pressure) was calculated as an average of measurements taken every 5 minutes from the resulting trace. Heart rate was then determined from a 60 second strip of trace, at a chart recorder

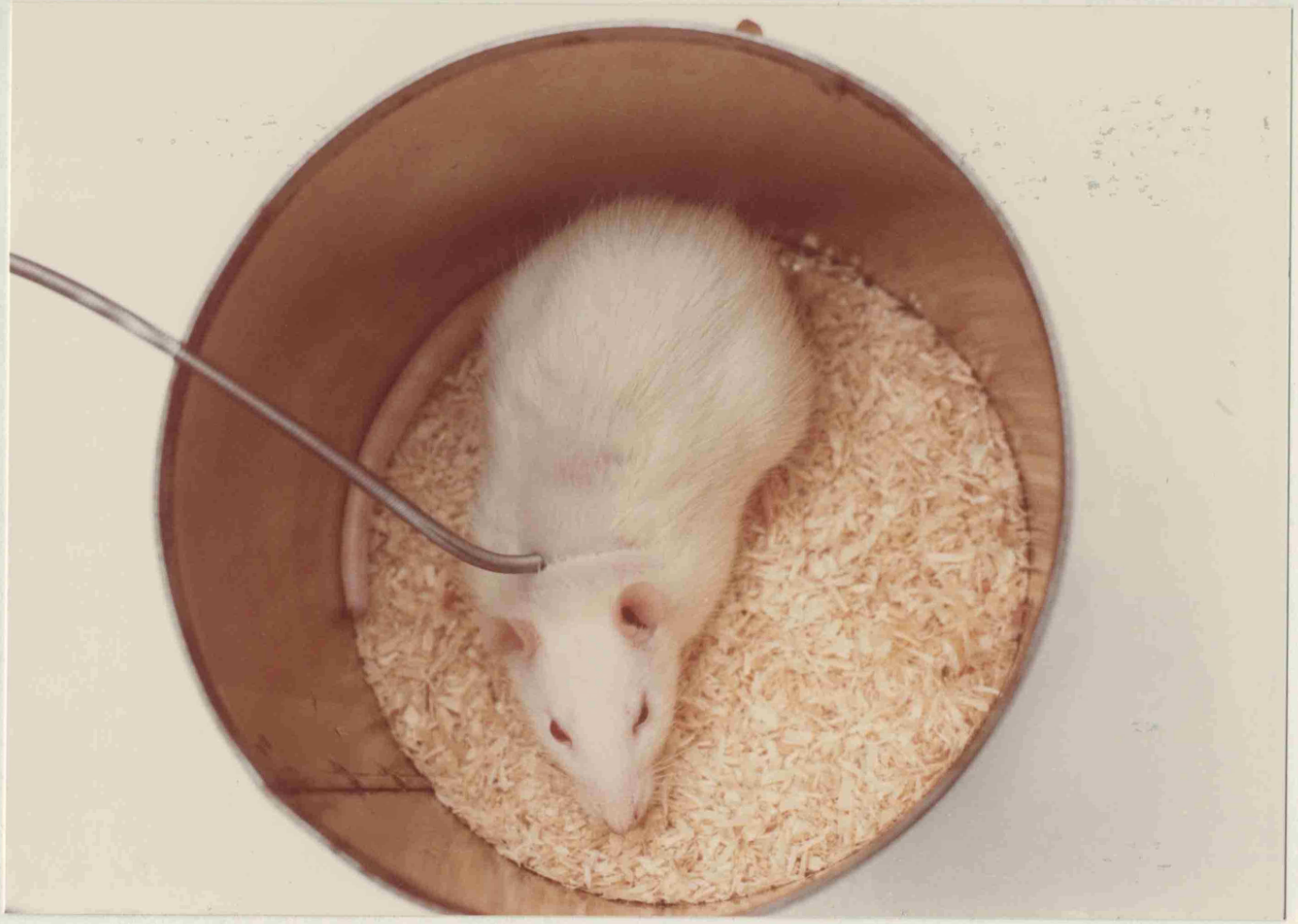


PLATE 5.1 Surface view of a rat in an opaque container, prior to blood pressure and heart rate recording and blood sampling.

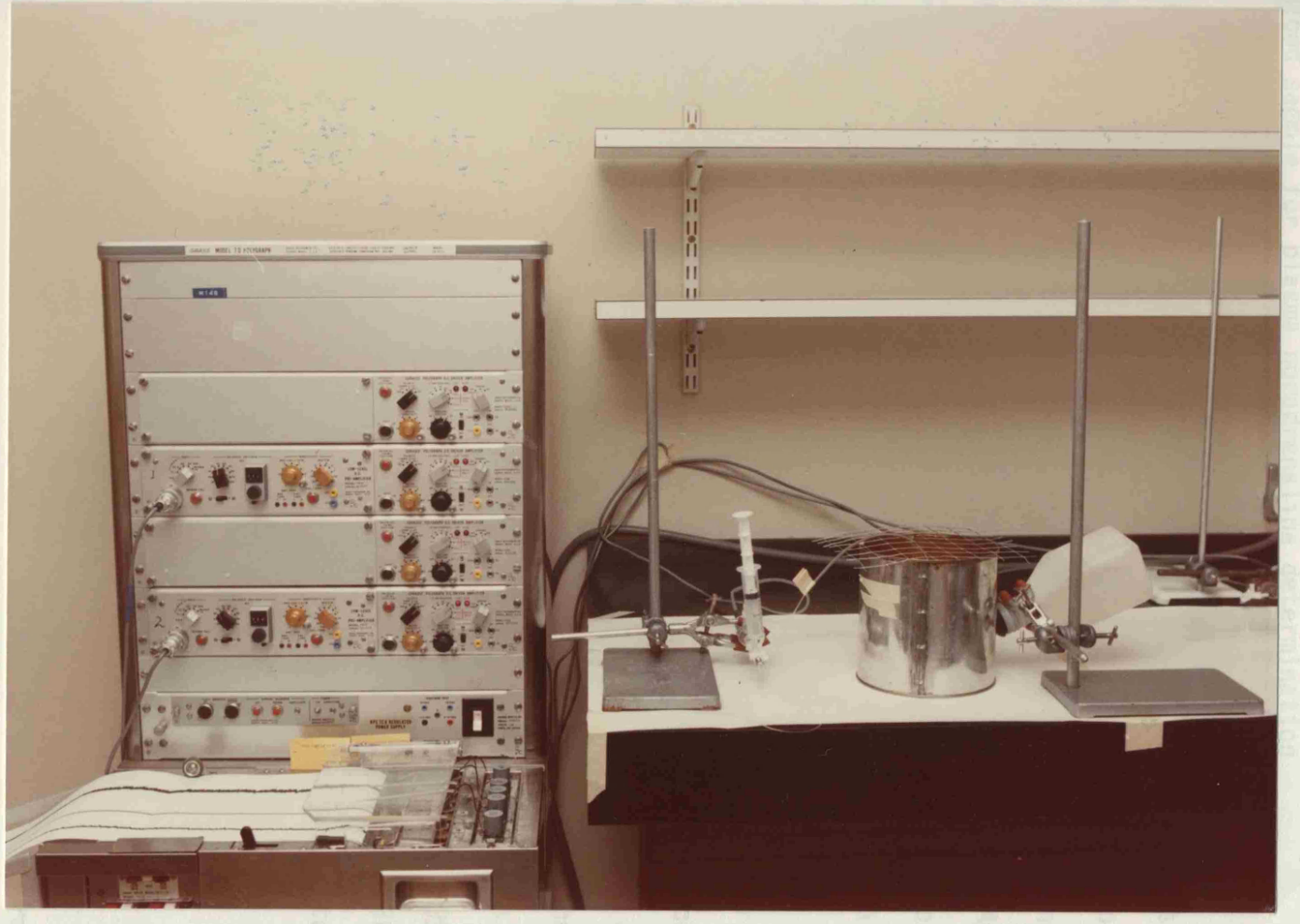


PLATE 5.2 Photograph illustrating a rat in a sampling container, attached to a Statham P23gb pressure transducer and Grass polygraph recorder.

speed of 10mm/sec. The heart rate recorded for each rat was an average of three values calculated from 10 second sections of trace at 10 second intervals.

Blood sample for plasma noradrenaline determination

Immediately after the mean direct BP and heart rate recordings had been made, the carotid cannula was cut at a point just over the top of the cage. This was to ensure that the dead space within the tubing was as small as possible to minimise the stress of blood sampling. Arterial blood was allowed to drip under its own pressure from the carotid cannula, the first few drops were discarded to ensure the heparinised saline was flushed from the line. Exactly 1.0ml of blood was collected into a chilled LP3 tube containing 0.1ml of 300 units/ml heparin in 0.9% saline (i.e. 30 units of heparin in isotonic saline) as anticoagulant. The volume of blood collected was replaced by slowly infusing an equal volume of heparinised saline into the rat, before rejoining the two ends of the cut cannula with silastic tubing. The blood sample was immediately spun at 4°C in a pre-cooled refrigerated centrifuge (MSE Chilspin, Fisons Scientific Apparatus, Loughborough, UK) at 2800 rpm (1420g) for 8 minutes. The plasma fraction was removed and stored at -70°C until assayed for noradrenaline (NA) content.

5.1.6 Removal of the Clip from the Renal Artery

After the pre-unclipping blood sample had been obtained, the rat was anaesthetised and its left flank was shaved and swabbed with ethyl alcohol. The original left loin incision was re-opened and retractors inserted into the wound. The kidney was carefully cleared of perirenal fat and held away from the area with a cotton swab around the renal

pedicle. A thin rough fibrous capsule invariably enveloped the clip and this had to be removed by blunt dissection to reveal the renal artery and silver clip. Fine forceps were used to prise apart the two arms of the clip, avoiding traction on the artery. The clip was then removed, care being taken to avoid tearing the renal artery or the renal vein.

Finally the kidney was replaced in the abdomen and the wound closed as described earlier (section 5.1.1). The carotid catheter was resealed (as in section 5.1.4) and the rat replaced in a standard size individual cage (400 x 280 x 210 mm) for a further 48 hours to recover from the anaesthetic.

5.1.7 Post-Unclipping Direct Blood Pressure, Heart Rate and Blood Sampling

Forty-eight hours after unclipping, BP and heart rate measurement and blood sampling were repeated as described earlier (section 5.1.5), again in conscious animals without further anaesthesia. "Dampening off" of the catheters was occasionally noted on the second sampling day (i.e. a reduction in pulse pressure). This was, however, infrequent and the effect on the mean arterial pressure calculated was minor and insignificant compared to the large changes in arterial pressure seen after unclipping. Immediately after the plasma NA sample had been obtained, a second 1.0ml blood sample was collected into a chilled LP3 tube containing 0.1ml of 10% potassium EDTA (ethylenediaminetetraacetic acid, dipotassium salt; Fisons Scientific Apparatus, Loughborough, UK) as anticoagulant. This sample was also immediately centrifuged at 4°C for 8 minutes (2800 rpm (1420g); MSE Chilspin), and the plasma fraction separated and stored at -20°C until assayed for creatinine concentration. Finally, rats were killed by injecting 60mg of

pentabarbitone sodium BP (May & Baker Ltd., Dagenham, UK) down their carotid lines. A post-mortem examination was carried out on each animal to confirm the position of the cannula tip in the aortic arch, and the absence of macroscopic evidence of renal infarction.

A diagrammatic representation of the experimental protocol employed to obtain plasma NA and heart rate measurements in rats with 1K1C and 2K1C hypertension and controls, before and 48 hours after unclipping, is shown in Figure 5.1 (Experimental Protocol 1).

5.1.8 Control Experiment for the Repeated Measurement of Plasma Noradrenaline in the Same Animal

In the experiments described above, two blood samples were taken from a rat to compare plasma NA before and after unclipping. It was therefore necessary to determine the inherent variation in plasma NA concentration between successive blood samples taken from the same rat 48 hours apart.

Normal rats (approximately 8 weeks post-weaning) were cannulated (see section 5.1.4), and the procedure of BP and heart rate recording and blood sampling described in sections 5.1.5 and 5.1.7 was carried out. A diagrammatic representation of the procedure is shown in Figure 5.2 (Experimental Protocol 2). Any effect the first blood sample may have on the plasma NA concentration of the second blood sample taken 48 hours later, in the absence of a laparotomy to unclip the renal artery or any changes in BP, could thus be elucidated.

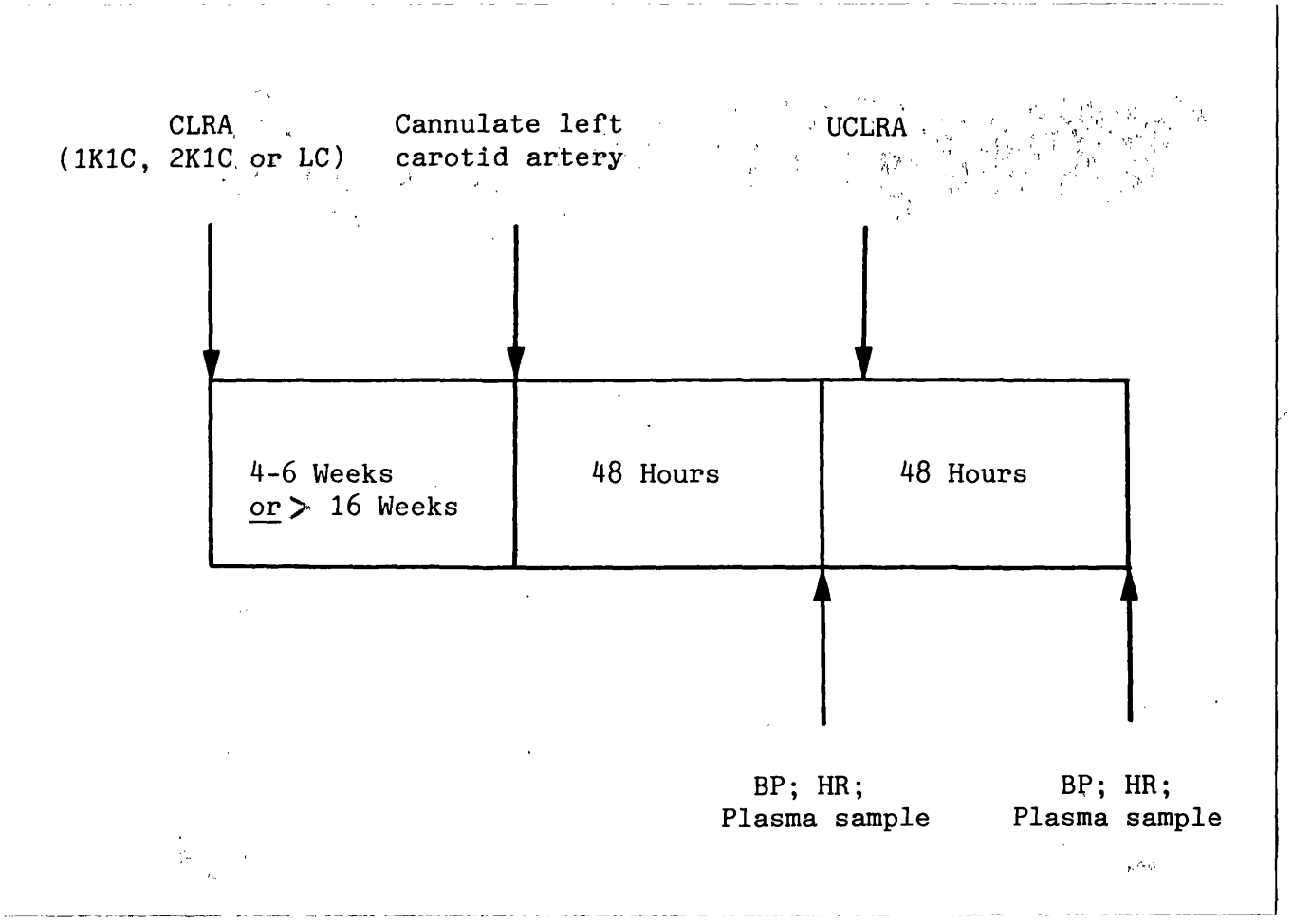


FIGURE 5.1 Experimental protocol 1: plasma noradrenaline and heart rate measurements in rats with 1K1C and 2K1C hypertension and corresponding loose clip (LC) controls, before and 48 hours after unclipping.
CLRA = clip left renal artery; UCLRA = unclip left renal artery.

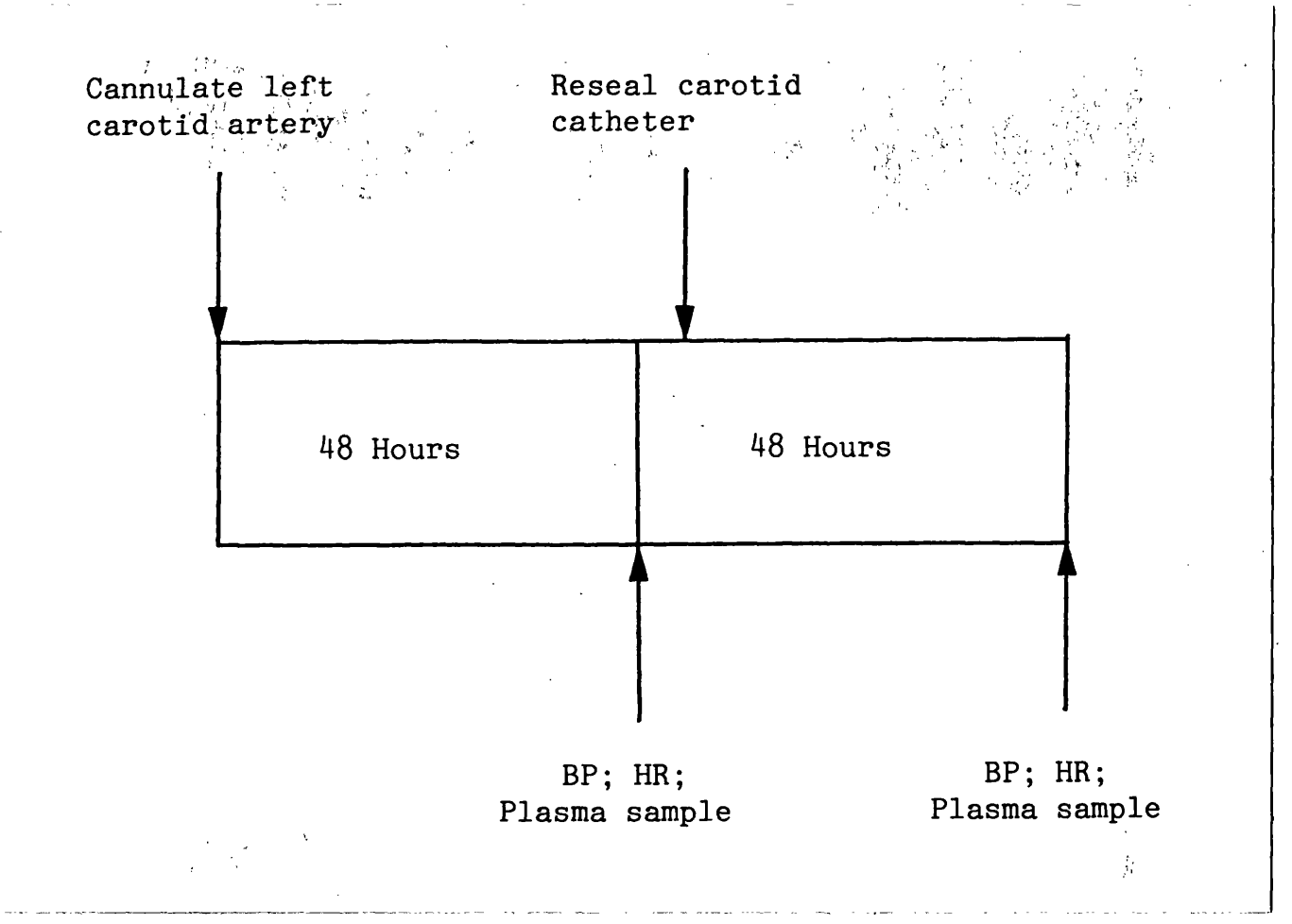


FIGURE 5.2 Experimental protocol 2: control experiment for the repeated measurement of plasma noradrenaline concentration, performed in normal rats.

5.2 Plasma Creatinine Concentration.

Plasma creatinine concentration was used to give a crude estimate of glomerular filtration in rats and hence some indication of the state of renal function (Selkurt, 1976). A colorimetric method of plasma creatinine determination (Jaffé method) without deproteinization was performed using a kit (Test-Combination Creatinine, Boehringer Mannheim GmbH Diagnostica). The rate of formation of a coloured complex of creatinine with picrate in alkaline medium was measured spectrophotometrically in 100µl of rat plasma (Bartels & Bohmer, 1971). The absorbance was measured at a wavelength of 490nm, in a cuvette with a 1cm light path mounted in a temperature controlled holder (25°C).

5.3 Statistics

Non-parametric statistical analysis was performed on the results, but for ease of presentation results are expressed as mean ± standard error of mean (SEM). Plasma NA and heart rate data were compared between groups using the Mann-Whitney U-test. Changes in these variables on unclipping within each group were analysed using the paired sign test. Non-parametric correlation and regression analysis was also performed. The Minitab statistics program (Pennsylvania State University) was employed for all statistical analyses, on a PDP/11 digital computer.

CHAPTER 6

MATERIALS AND METHODS II :

MEASUREMENT OF CATECHOLAMINES

6.1 Introduction

The measurement of the low levels of noradrenaline (NA) in the circulation: 2-4 nanomoles per litre (nmol/L) in humans (Hallman, Farnebo, Hamberger & Jonsson, 1978) requires a sensitive analysis system. The problem is accentuated when measuring NA in rat plasma due to the small volume of sample available. The crucial parameters for any catecholamine assay are selectivity, requiring either a specific detector or extensive sample extraction procedures, and very low detection limits.

A number of methods of measuring plasma catecholamines have been employed. Fluorimetric (Renzini, Brunori & Valori, 1970; Miura, Campese, De Quattro & Meijer, 1977), gas chromatographic (Jacob, Vogt, Knedel & Schwertfeger, 1978) and radioenzymatic (Peuler & Johnson, 1977) procedures have been reported. Fluorimetric techniques have largely fallen into disuse due to their lack of specificity and insensitivity. The derivatisation involved in gas chromatography often requires highly expensive equipment, especially if mass-spectrometric detection is used (Jacob et al, 1978). Radioenzymatic techniques have proven most popular (Engelman & Portnoy, 1970; Da Prada & Zürcher, 1976; Peuler & Johnson, 1977), but although the sensitivity of this approach is rarely in doubt the expense and time required is high (Hallman et al, 1978; Hjemdahl, 1984).

A method for measuring catecholamines in brain tissue samples based on high performance liquid chromatography with electrochemical detection (HPLC/ECD), and an analytical column containing strong cation exchange resin, has been described (Keller, Oke, Mefford & Adams, 1976). This

method was later adapted to measure catecholamines in human plasma (Hallman et al, 1978; Hjemdahl, Daleskog & Kahan, 1979). An HPLC/ECD method with reverse phase ion-pair separation (see section 6.1.1), rather than cation exchange chromatography, has also been described for the measurement of circulating catecholamines (Goldstein, Feuerstein, Izzo, Kopin & Keiser, 1981). Such a method has been employed to measure catecholamines in rat plasma and tissue samples after an alumina extraction procedure. Details of the HPLC/ECD system used are described later (section 6.3). Radioenzymatic and HPLC/ECD assays for the estimation of plasma catecholamines have been compared and have shown a close agreement (Hjemdahl et al, 1979; Goldstein et al, 1981; Falconer, Lake & Macdonald, 1982; Hjemdahl, 1984).

6.1.1 Ion-Pair Chromatography and Electrochemical Detection

Ion-pair chromatography is a modification of reverse phase liquid chromatography for separation of polar organic molecules, and is suitable for the separation of catecholamines (Holly & Makin, 1983). The technique involves a reverse phase chromatography column, and a mobile phase of an aqueous buffer containing a dilute solution of an ion-pair reagent, sodium octyl sulphonate in this case. This compound is a detergent which has a polar (hydrophilic) group at one end of the molecule and a non-polar (hydrophobic) group at the other. The exact mechanism by which it interacts with the reverse phase system is not clear. Originally it was thought that the ion-pair reagent molecules combined with the solute molecules, to form an ion-pair in the mobile phase prior to partition into the alkyl-bonded stationary phase. The analyte would thus interact with the column only when in the ion-pair form ("ion-pair mechanism"). More recently it has been proposed that sodium octyl sulphonate molecules partition to the stationary phase due

to the hydrophobic attraction of the alkyl groups, presenting the hydrophilic groups to the mobile phase. The stationary phase would then be physically (although not chemically) modified to give the appearance of an ion-exchange resin. Analyte ions are then postulated to partition into the stationary phase by an ion-exchange process ("dynamic ion exchanger theory"). Both views are probably partially correct and the mechanism of separation is probably a mixture of both processes (BAS LCEC Application Note No. 15, 1982).

Electrochemical detection is based on the principle that electroactive compounds oxidise at a certain potential, and thereby liberate electrons which create a measurable current. Catecholamines are readily oxidised to form quinones when a potential of +0.6-+0.7V is applied and are therefore suitable for this method of detection (see section 6.3.4).

6.2 Sample Preparation

Plasma samples were taken through an alumina extraction procedure (Anton & Sayre, 1962) prior to injection onto the HPLC/ECD system. This served both to concentrate the low levels of catecholamines and to purify the samples.

Alumina extraction procedure

All reagents were analytical grade and all compounds were obtained from Sigma Chemical Co., St. Louis, USA, unless otherwise stated. All standards were made up in 0.1M perchloric acid, which prevents oxidation of catecholamines.

After separation of the plasma fraction from a blood sample, the volume was accurately recorded. 250µl of a solution of dihydroxybenzylamine hydrobromide (DHB), 150nmol/L in 0.1M perchloric acid, was then added to each plasma sample as internal standard, prior to storage at -70°C. This enabled losses during storage and extraction to be accounted for, and in addition a low pH stabilises catecholamines within the sample against oxidation.

At the time of extraction plasma samples were allowed to thaw at room temperature, then immediately transferred to assay tubes placed on ice. With each batch of samples two standards for calibration and a reagent blank were also required. A standard solution containing 5pmol/100µl each of noradrenaline hydrochloride (NA), adrenaline (+) bitartrate (ADR) and dopamine hydrochloride (DA) was prepared from an aliquot of stock solution (1.5µmol/L of each compound in 0.1M perchloric acid, stored at -70°C) by dilution with 0.1M perchloric acid. Each assay standard consisted of 250µl of this 5 pmol/100µl solution of NA, ADR and DA (i.e. 12.5 pmol each of NA, ADR & DA), to which was added 250µl of internal standard solution (150nmol/L DHB in 0.1M perchloric acid), as described for the plasma samples. The reagent blank consisted of distilled water in place of sample or standard. All tubes were then taken through the extraction procedure described below:

The pH of each tube was adjusted to 7.8-8.2 with 1.0M Tris-buffer, pH 8.6 (containing 0.05M potassium EDTA, and stored at 4°C) and 20-30mg of acid washed alumina (BAS, Anachem Ltd., Luton, UK) added. Tubes were stoppered and mixed on a spiral mixer (Denly Spiramix, Sussex, UK) for 15 minutes; at this pH catecholamines are selectively adsorbed onto the alumina. After this period the alumina was allowed to settle, the supernatant was removed and discarded. Approximately 3.0mls of ice-cold

distilled water was added and the tubes mixed by manual invertion. After the alumina had settled the supernatant was removed and discarded. The latter two procedures were repeated to give three washes with ice-cold distilled water. On the final wash as much of the liquid as possible was removed, then 250µl of 0.1M perchloric acid was added. Sample tubes were placed on ice and mixed frequently by manual agitation for 10-15 minutes, to allow the catecholamines to be extracted from the alumina into the acid. Finally, after the alumina had settled, the supernatant was removed and placed in Eppindorf microcentrifuge tubes on ice. This extract was injected directly on to the HPLC/ECD system.

The average recovery of NA from the alumina extraction procedure was 70±1% (mean±SEM, n=20), which is in agreement with others (Hallman et al, 1978; Goldstein et al, 1981).

6.3 Details of the High Performance Liquid Chromatograph and Electrochemical Detector (HPLC/ECD)

The components of the HPLC/ECD system are shown in plate 6.1. A high pressure pump circulates eluent buffer around the closed system, mobile phase being recycled from the detector outlet back to the mobile phase reservoir. Samples are introduced into the stream of solvent at the top of the pre-column via an injection valve. Separation of the catecholamines is achieved on the pre-column and column, which contain the same packing material. The pre-column acts as a filter to prolong the life of the main column. Effluent from the column passes over a thin layer glassy-carbon working electrode, where catecholamines are oxidised due to the potential applied between the detector and reference electrodes; an auxiliary electrode completes the circuit. Oxidative currents are measured, amplified and converted to voltages by an

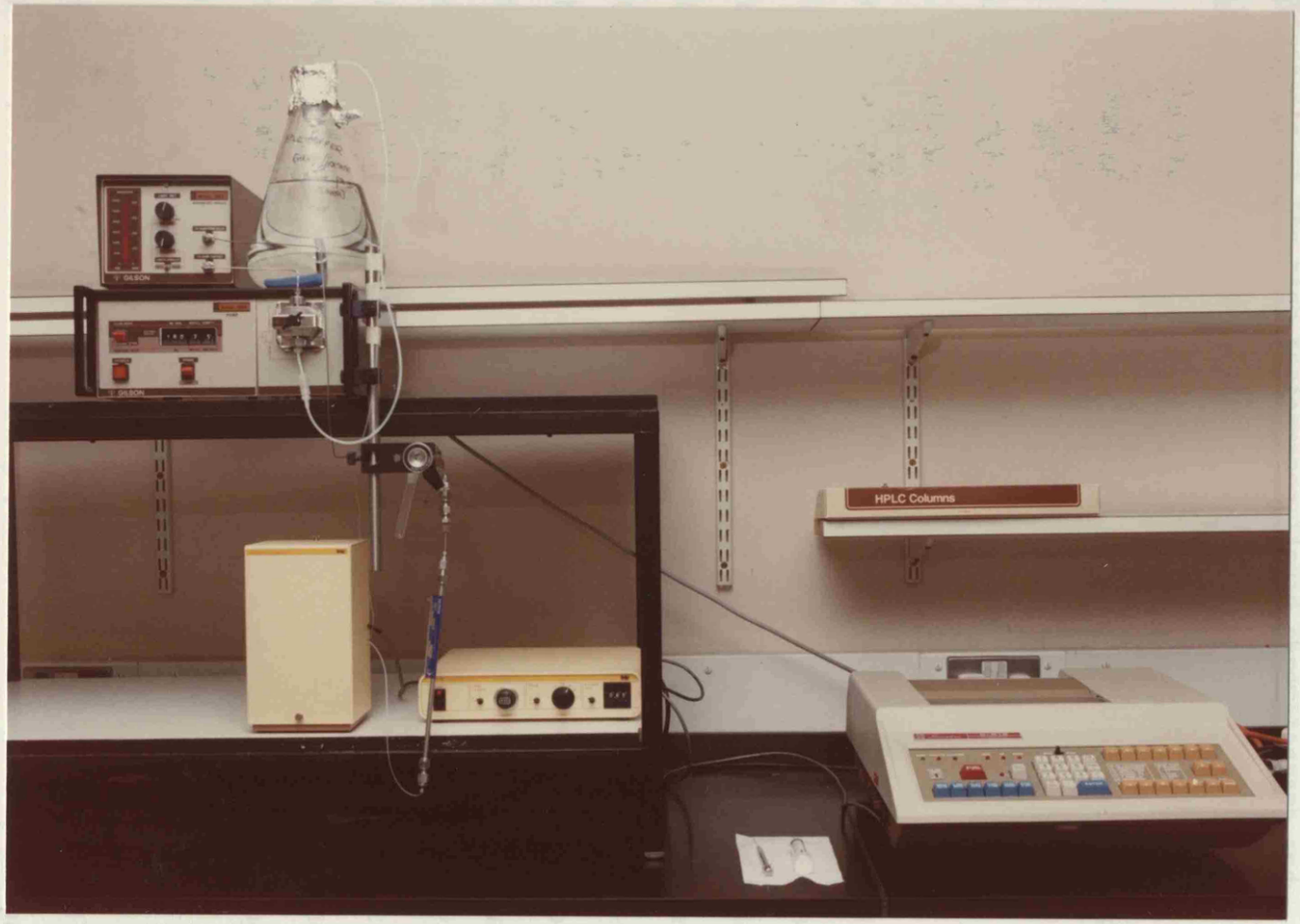


PLATE 6.1 The high performance liquid chromatograph and electrochemical detector (HPLC/ECD).

electronic controller, before being recorded as a series of peaks on a chart recorder or integrator. Full details of the components of this system are described below:

Components of the high performance liquid chromatograph and electrochemical detector

All components were supplied by Anachem Ltd., Luton, UK, unless stated.

6.3.1 Columns

An Altex Ultrasphere ion-pair (I.P.) column, 4.6mm internal diameter (ID) x 250mm in length, was used to separate catecholamines in injected samples. It consisted of closely packed microparticulate silica spheres (5µm in diameter), onto which are bonded octadecyl reverse phase groups (alkyl chains 18 carbon atoms long). This arrangement of the stationary phase allows a very large surface area of contact with the mobile phase, which greatly increases the efficiency of separation. Consequently, however, high pressures are required to propel the eluent buffer through the column. Addition of an ion-pair reagent such as sodium octyl sulphonate (see section 6.3.2) to the mobile phase modified the stationary phase to increase its affinity for the polar catecholamine molecules (see section 6.1.1).

Catecholamines are partitioned between the stationary and mobile phases by adsorption onto the stationary phase, which retards their passage through the column. The greater the affinity between the analyte and the stationary phase, the longer it takes to elute that analyte from the column. The components of the sample which had no affinity for the column were eluted quickly to produce the solvent front. Catecholamines

are separated from each other by virtue of their different affinities for the stationary phase, and emerge from the bottom of the column as a series of bands.

The pre-column (Altex Ultrasphere I.P., 4.6mm ID x 40mm in length) was present mainly to extend the life of the column by acting as a filter for particulate matter. In addition the pre-column contained exactly the same packing material as the main column and hence contributed to the separation of catecholamines.

Microparticulate reverse phase, ion-pair liquid chromatography is a significant improvement on the use of cation exchange chromatography, as used by Hallman et al (1978), for the assay of catecholamines in plasma; since the ionic strength of the column can be controlled by regulating the amount of ion-pair reagent in the mobile phase. This enables the chromatography to be finely tuned to optimise the separation of catecholamines (BAS LCEC Application Note No. 14, 1982; Hjemdahl, 1984).

6.3.2 Mobile Phase

All components of the mobile phase were analytical grade unless stated. The mobile phase consisted of 30mM citrate/80mM acetate aqueous buffer adjusted to pH 5.2 with glacial acetic acid, containing 0.7mM sodium octyl sulphonate as ion-pair reagent and 10% (v/v) HPLC grade methanol (Fisons Scientific Apparatus, Loughborough, UK). Sodium EDTA (0.75g/L) was also included to help reduce noise and large solvent peaks on the chromatogram. Two litres of buffer were prepared at a time and as this was recycled it was only necessary to remake it every 2 months. No increase in background current could be detected over this time, as the

level of catecholamines in plasma extracts was very small. However, the injection of large amounts of catecholamines (ng or µg quantities) was avoided.

The mobile phase was filtered through a Millipore filter (0.2-0.4µm) and required degassing before use. This was achieved by placing the buffer in a stoppered vacuum flask (2 litre) with a magnetic stirring bar, before the air was evacuated using a water pump for one hour.

Separation was finely tuned by careful adjustment of the mobile phase composition. Increasing the amount of sodium octyl sulphonate in the solvent increased the partition of catecholamines to the stationary phase, and hence increased their retention times. Conversely, increasing the amount of methanol reduced retention times. A balance between adequate separation and an efficient time to complete the chromatogram was required. The pH of the mobile phase (5.2) was also critical since the pH of the solvent affects the charge on the solutes and hence their affinity for the column. The response of the detector was also influenced by the ionic strength and pH of the mobile phase. Optimising the composition of the mobile phase enabled the separation of NA, ADR, DA and the internal standard DHB within 30 minutes. After equilibration each catecholamine was recorded after a specific and relatively constant elution time, which enabled peak identification.

6.3.3 High Pressure Pump

A single piston pump with a 5ml pump head, capable of generating pressures of up to 6,000 psi (Gilson 302 metering pump), was used to circulate mobile phase around the HPLC/ECD system. This model incorporated a pulse damper to prevent the baseline from oscillating

with the pump piston cycle. Electrochemical detection is sensitive to fluctuations in flow rate (Holme & Peck, 1983) and it is therefore necessary that a constant optimum flow rate is selected. In this assay the flow rate was set to 0.9ml/min (resulting in a pressure of 2000-3000 psi). During periods of inactivity the flow rate was reduced to 0.3ml/min (500-600 psi), the minimum rate at which the piston operated smoothly. This enabled mobile phase to be constantly circulated, without subjecting the HPLC to the high pressures required during sample analysis.

Air bubbles within the system can cause problems with detection by causing current surges and noisy baselines. Degassing or cavitation of the solvent was reduced by placing the mobile phase reservoir at a level higher than the pump inlet, to ensure a constant head of pressure on the mobile phase at pump level (Plate 6.1). The 'refill' setting (1-10) controlled the rate at which the pump piston drew back and filled with buffer. A balance between minimum refill time and prevention of cavitation was required.

6.3.4 Electrochemical Detector

The electrochemical detector consisted of a Bioanalytical Systems (BAS inc., West Lafayette, USA) LC-3A electronic controller and LC-17 transducer package. The LC-17 transducer (illustrated in Plate 6.2) contained the liquid contact parts, which consisted of a thin layer flow cell incorporating a glassy-carbon working electrode (BAS, TL-5A detector electrode cell) and a silver/silver chloride reference electrode (BAS, RE1). A Faraday cage surrounded the electrodes to reduce background electrical noise (Plate 6.1). This cage was grounded

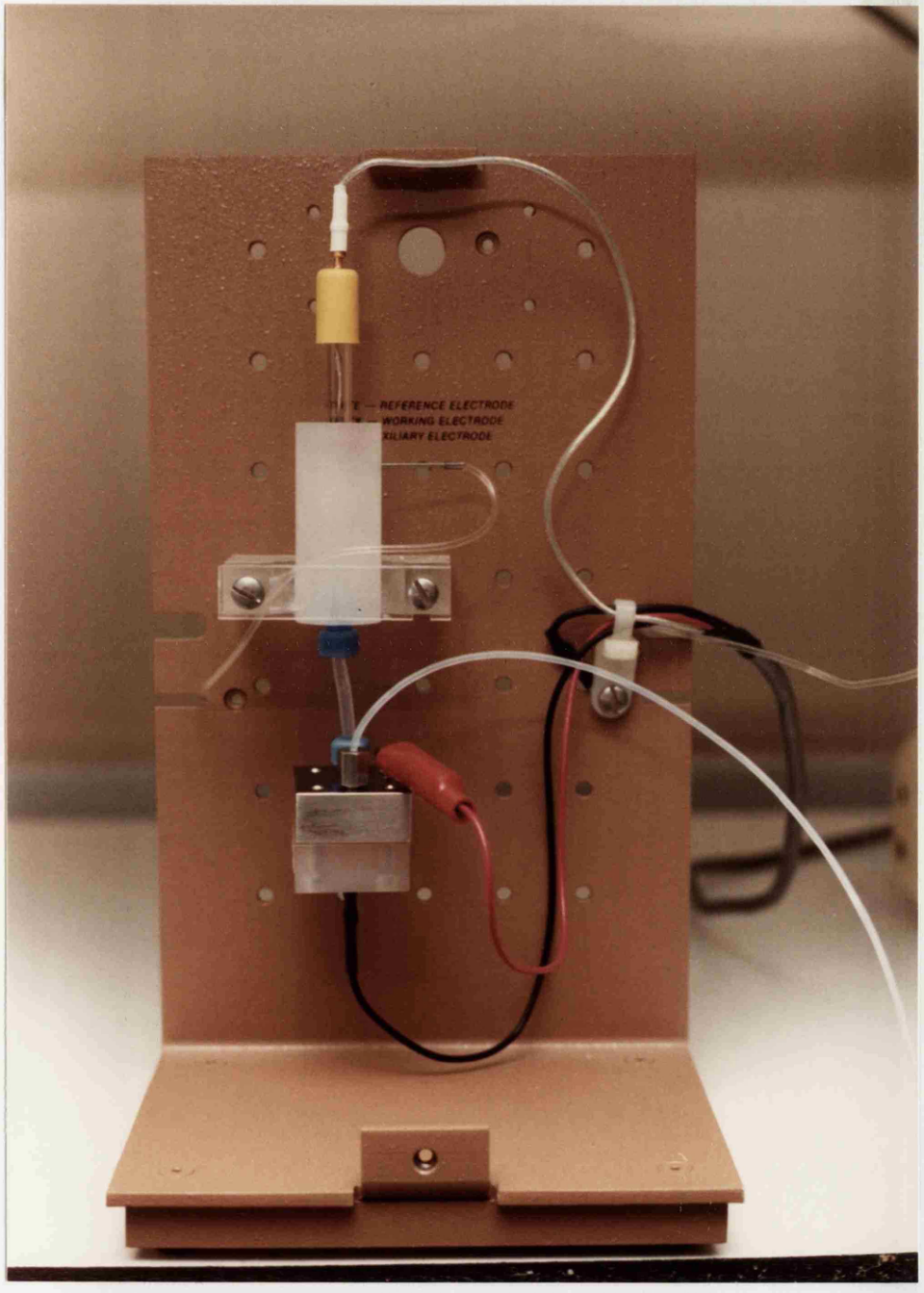


PLATE 6.2

The LC-17 transducer containing the working electrode flow-cell, silver/silver chloride reference electrode and auxiliary electrode. The front of the Faraday cage has been removed for the photograph.

via a single connection to earth, whilst the LC-3A controller was grounded to the cage.

The detector was operated in amperometric mode, which means that the working electrode was maintained at a constant operating potential, in this case +0.65V. The function of the electronic controller was to apply a potential (+0.65V) to the working electrode, using the potential of a silver/silver chloride electrode as reference point, under zero current conditions. A tubular steel auxiliary electrode was present to pass current and complete the circuit. This potential was sufficiently positive to cause the separated bands of catecholamines in the column effluent to be oxidised to the ortho-quinone as they passed over the electrode surface, producing 2 protons and 2 electrons (see Figure 6.1). Thus producing a nanoampere current at the working electrode. This was measured, amplified and converted to a voltage output by the electronic controller, before being recorded on a time base recorder/digital integrator (Shimadzu C-R1B Chromatopac integrator; Dyson Instruments, Tyne & Wear, UK) (Keller et al, 1976; BAS LCEC Application Note No. 15, 1982; Holly & Makin, 1983). The current detected by the controller was proportional to the concentration of the electroactive component, provided the flow rate, temperature and electrode area remained constant (Keller et al, 1976). As oxidisable bands of solute passed over the thin layer electrode the current increased and decreased as a function of time to yield the HPLC/ECD chromatogram (see Figures 6.2 and 6.3 later).

The sensitivity of the electrochemical detector has been reported to be 0.15pmol for NA (Goldstein et al, 1981). Catecholamines are more easily oxidised than many other compounds, therefore, the selectivity of detection can be increased by keeping the oxidising potential as low as

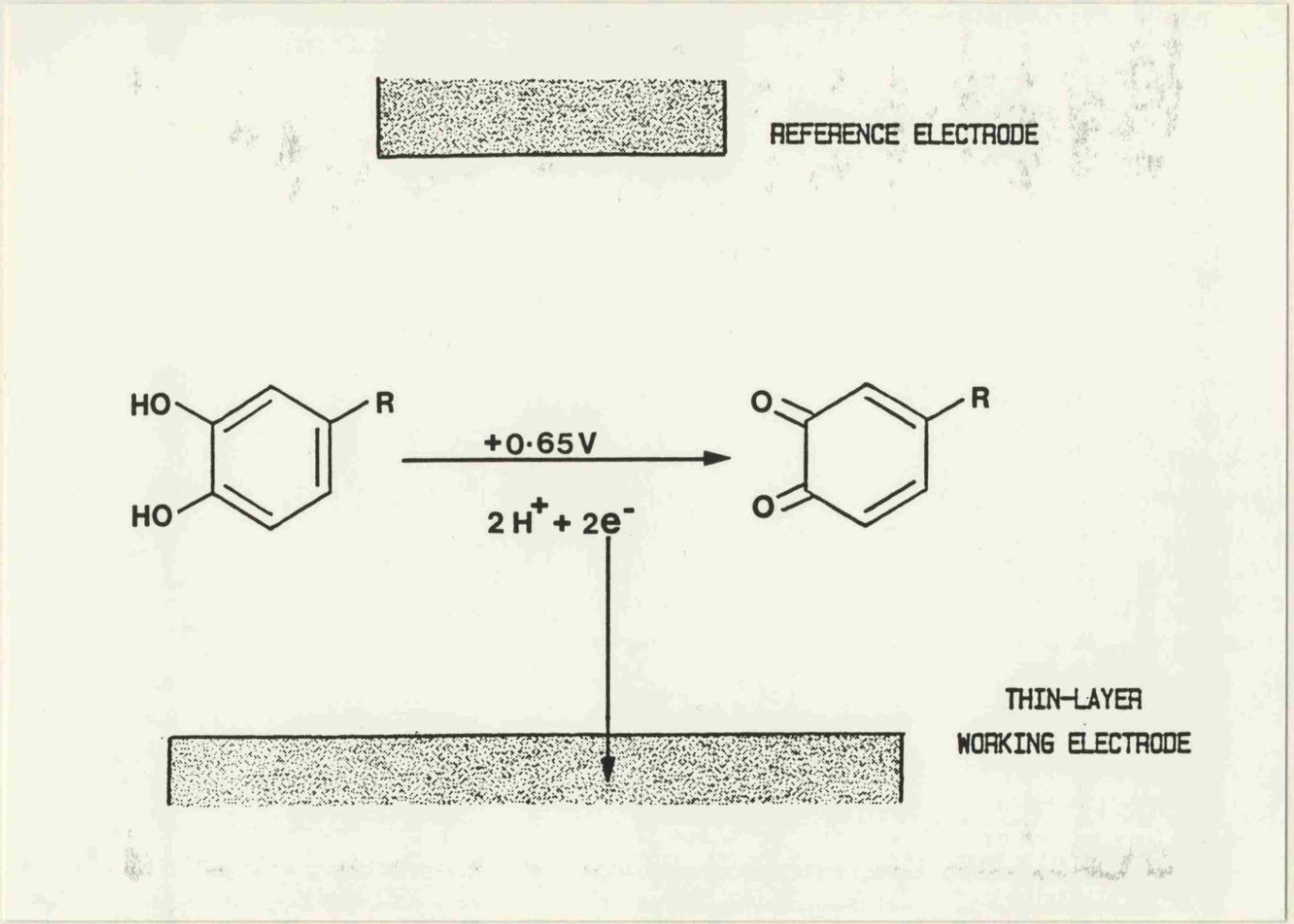


FIGURE 6.1

Diagram to illustrate the principle of electrochemical detection for the measurement of catecholamines.

possible (Hjemdahl, 1984). The operating potential used here (+0.65V) eliminated the current from compounds that oxidise at higher potentials, whilst maintaining maximum sensitivity for catecholamines (Keller at al, 1976; Hjemdahl, 1984). The best detection limits and lowest background noise are obtained with carbon-paste working electrodes, but these require relatively frequent re-packing (BAS LCEC Application Note No. 14, 1982). A glassy-carbon electrode was used here since although slightly less sensitive (Hjemdahl, 1984), it offers greater reliability and durability than a carbon paste electrode. Moreover, oxidation by-products can be removed from the surface of a glassy-carbon electrode by a simple polishing procedure, whereas graphite-paste electrodes have to be re-packed (BAS LCEC Application Note No. 14, 1982).

Amperometric electrochemical detection systems, as described above, oxidise less than 10% of analyte molecules as they flow over the surface of the working electrode. The alternative coulometric detection systems oxidise 100% of the catecholamine molecules in the column effluent, producing a higher current (Hjemdahl, 1984). However, the higher yield of the coulometric detector does not increase the sensitivity as compared to the amperometric detector; the noise level is increased in proportion to the increase in signal, resulting in a similar signal to noise ratio. BAS amperometric detectors with thin layer flow cells are at least as sensitive as any of the other types of detector cells available (Hjemdahl, 1984). The sensitivity of the present HPLC/ECD system was 0.1 pmol (20pg) for NA and 0.15 pmol (50pg) for ADR.

6.3.5 Injection Valve

Samples were introduced into the high pressure stream of mobile phase at the top of the pre-column via a syringe loading injection valve

(Rheodyne Model 7125). This six-port injection valve incorporated a precision 100µl loop, which ensured exactly 100µl of sample was introduced onto the column. The valve was loaded with a 250µl Hamilton syringe through a needle port in the valve rotor. Excess sample was void through a vent at the back of the valve and discarded. Mobile phase was then allowed to flow through the loop which washed the sample onto the top of the pre-column.

For maximum resolution it was essential that the internal volume of connecting tubing was kept to a minimum. Stainless steel tubing (0.25mm ID x 1.56mm OD) was used between the injection valve, pre-column and column, whilst PTFE tubing (0.3mm ID x 1.56mm OD) was used between the column and detector flow cell. Larger bore tubing (0.5-0.75mm ID) was used to connect the pump to the injection valve and for the drain from the detector flow cell, to minimise the resistance to flow in these less critical areas.

6.3.6 Maintenance and Trouble-Shooting

Considerable maintenance and trouble-shooting are necessary to maintain the high sensitivity of the HPLC/ECD system required for the measurement of catecholamines in plasma (Hjemdahl, 1984). A few of the most frequently occurring problems and their solutions are considered in this section.

Loss of sensitivity of the electrochemical detector, characterised by reduced peak heights and an increase in detection limit, is encountered relatively frequently. This usually results from a build up of oxidation by-products on the surface of the glassy-carbon electrode, reducing the surface area across which catecholamines can be oxidised.

A metallographic polishing technique is used to return the electrode to a high lustre, and this usually restores the sensitivity of the detector. The glassy-carbon electrode requires polishing every 2-3 months and the mobile phase is also replaced at the same time.

Another common problem is deterioration of the chromatogram where peaks broaden in width, develop a shoulder, or split into two; leading to less efficient separation and loss of sensitivity. This could be due to deterioration of the columns, which may be regenerated by disconnecting the column and pre-column from the detector and flushing them thoroughly with pure HPLC grade methanol. Alternatively, an obstruction in the pump or injection valve may be responsible, which can be cleared by passivating these components with 50% nitric acid.

If neither of the above procedures are successful, another cause of poor chromatography is a void or debris at the top of the pre-column. Voids are completely refilled with a suitable packing material, a 10µm ion-exchange resin (Partisil-10 SCX) was used here. Debris and contaminants are removed from the top of the pre-column and the packing replaced as above. If problems with the chromatogram persist, it is likely that dead space or channels have formed within the column, which must therefore be replaced with a new column (Molnar, Huhn & Lamer, 1984).

Maintaining high performance

The HPLC/ECD system can be operated at high levels of sensitivty with the minimum of operator intervention if the guidelines listed below are adhered to (BAS LCEC Application Note No. 14, 1982):

The instrument should be dedicated to the catecholamine assay on a 24 hour basis and mobile phase re-cycled, thus avoiding the necessity to make up new batches of solvent daily. The detector electrode should be switched off as little as possible, but always switched off before changing the voltage, disconnecting any wiring from the electrodes, or turning the power off. External electrical disturbances should be minimised by surrounding the electrodes with a Faraday cage and ensuring the equipment is well grounded. The current supply should also be stable, which may require the use of a mains filter (Scilabub Ltd., Coalville, UK). A chelating agent such as EDTA added to the solvent (0.75g per litre in this case) reduces background chemical noise derived from new steel or other components.

6.4 Recordings and Calibration

The HPLC/ECD chromatogram was recorded on an integrator (Shimadzu C-R1B Chromatopac integrator; Dyson Instruments, Tyne & Wear, UK), which was programmed to calculate the concentration of individual catecholamines present in a sample (pmol per 100µl injected). The calibration program used for recording and measuring catecholamine levels in plasma samples is listed in Appendix 1.1.

6.4.1 Calibration

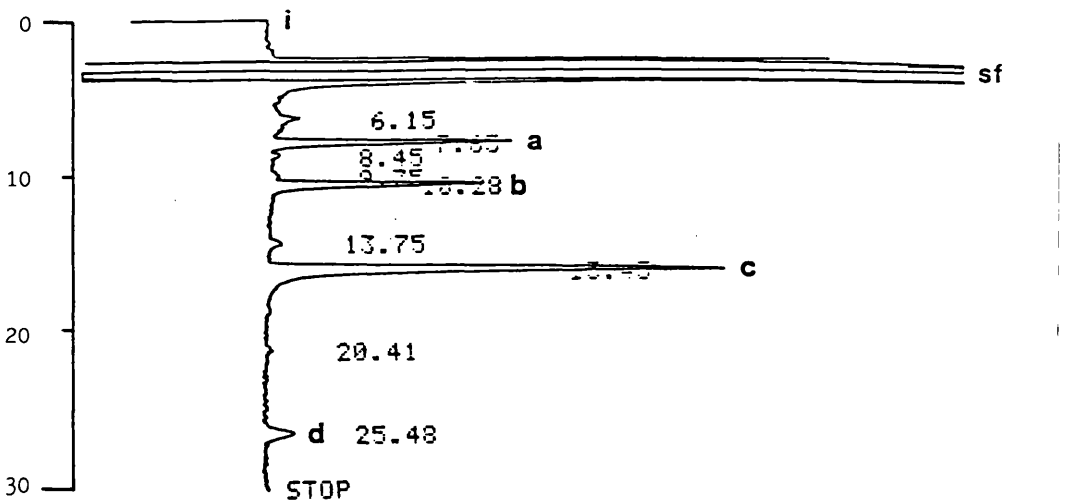
An internal standard calibration procedure was employed to enable inter-sample variations in recovery of catecholamines from the alumina extraction to be accounted for. An identical amount of the internal standard dihyroxybenzylamine (DHB) was added to samples and standards prior to extraction (see section 6.2). Calibration was achieved by comparing the ratio relating catecholamine (e.g. NA) and DHB peak

heights between unknown samples and standards of known concentration. Standard curves of peak height versus concentration, following injection of picogram (pg) quantities of catecholamines, have consistently demonstrated a linear relationship and consequently there seems no reason to utilise peak areas for calibration (Keller et al, 1976; Goldstein et al, 1981).

Two alumina extracted standards were injected to calibrate the HPLC/ECD. A chromatogram obtained following the injection of a standard is shown in Figure 6.2. The solvent front was recorded first, followed by NA (approximately 7.7 mins), ADR (approximately 10.3 mins), DHB (approximately 15.5 mins) and finally DA (approximately 25.5 mins). The elution times (mins) and peak heights (arbitrary units) were listed, and the integrator was programmed to calculate a calibration factor (F1) for each catecholamine (e.g. NA) as follows:

$$F1\ (NA) = \text{Concentration of NA in standards (5 pmol/100µl)} \times \frac{\text{DHB peak height (mean of 2 standards)}}{\text{NA peak height (mean of 2 standards)}}$$

Calibration factors for ADR and DA were calculated in the same way. An identification table was constructed to ensure the correct calibration factor was used for each catecholamine, utilising the mean absolute retention time from the 2 standards and a time window of 5% for each peak. Therefore peaks occurring within 5% of the standard retention times in the subsequently injected samples were identified accordingly. An example of an identification table from a plasma catecholamine assay is shown in Appendix 1.2. Sample extracts were then injected and the catecholamine concentrations calculated by the integrator as demonstrated below for NA:



NAME	TIME	CONCENTRATION	HEIGHT
NA (a)	7.65		18437
ADR (b)	10.28		16203
DHB (c)	15.45		35334
DA (d)	25.48		2121

FIGURE 6.2

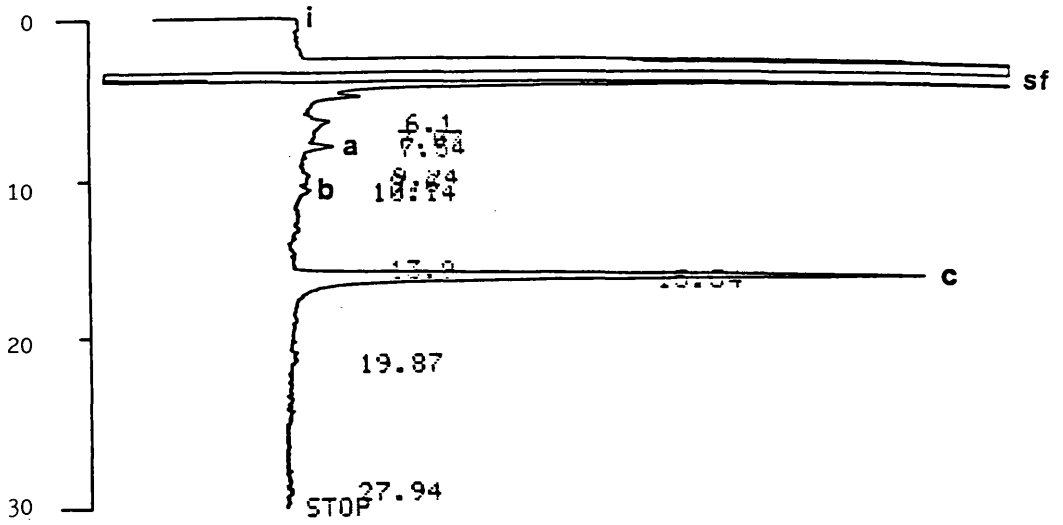
Chromatogram obtained following injection of an alumina extract of a standard solution containing 5pmol/100µl of noradrenaline (a), adrenaline (b), dopamine (d) and 15pmol/100µl dihydroxybenzylamine (c). The solvent front (sf) and point of injection (i) are also indicated.

$$\text{Concentration of NA (pmol/100µl)} = \frac{\text{NA peak height in sample}}{\text{DHB peak height in sample}} \times \text{Calibration factor for NA (F1[NA])}$$

A chromatogram obtained following the injection of a rat plasma sample is shown in Figure 6.3. Catecholamine peaks were labelled and the corresponding concentrations (pmol/100µl) calculated and printed. Dopamine (DA) was rarely detected in plasma samples. Adrenaline (ADR) was recorded inconsistently in rat plasma, although it was recorded in the example demonstrated in Figure 6.3. The glassy-carbon detector electrode used here was not sensitive enough to record ADR reliability in the very small volumes of sample available. Conversely, extremely consistent results were obtained for noradrenaline (NA). The sensitivity of the assay was 0.1 pmol (20pg) for NA and 0.15 pmol (50pg) for ADR in a 100µl injection. This equates to a limit of quantitation of 0.5 and 0.75 pmol/ml for NA and ADR respectively in rat plasma samples; the minimum plasma sample volume used was 0.5ml prior to alumina extraction.

6.4.2 Calculation of Results

The concentrations printed on the integrator were expressed in pmol/100µl, since 100µl of extract was injected onto the column. This figure was divided by the volume of plasma originally extracted and mutlipled by 2.5 (as 100µl of the 250µl perchloric acid extract was injected) to give concentrations in pmol/ml of plasma. Variations in recovery of catecholamines from the alumina extraction were accounted for automatically by the internal standard calibration. Differences in recovery between samples were assumed to affect catecholamine and internal standard peak heights within each sample equally, leaving the



NAME	TIME	CONCENTRATION	HEIGHT
NA (a)	7.54	0.3796	1942
ADR (b)	10.14	0.1789	809
DHB (c)	15.34		47450

FIGURE 6.3

Chromatogram obtained following injection of an alumina extract of a rat plasma sample onto the HPLC. Concentrations of noradrenaline (a) and adrenaline (b) are expressed in pmol/100µl.
i = point of injection; sf = solvent front; C = dihydroxybenzylamine (internal standard) peak.

ratio between the two unaffected; it was this ratio which was used to determine catecholamine concentrations in samples.

6.4.3 Linearity

Before the HPLC/ECD system was used to assay plasma samples, performance was characterised by demonstrating the linearity of standard curves relating peak height to NA concentration, both in standard solutions and in plasma. The intra- and inter-assay coefficients of variation were also determined for the NA assay.

Linearity of a standard curve of noradrenaline

A standard curve of NA in 0.1M perchloric acid was prepared by serial dilution of a solution containing 5 pmol/100µl. The peak height (arbitrary units) was plotted against pmol of NA injected (Figure 6.4), and excellent linearity was obtained over the concentration range 0.1-5.0 pmol of NA ($r = 0.999$). All measurements were made with the detector range set to 20 nanoamps full scale deflection, as for plasma catecholamine assays.

Linearity of a standard curve of noradrenaline in plasma

Known concentrations of NA in 0.1M perchloric acid were added to 1.0ml aliquots of a human plasma sample, taken from a forearm vein, to produce a standard curve of NA in plasma. The samples were then extracted (see section 6.2) and the concentration measured on the HPLC/ECD. Figure 6.5 shows a plot of NA concentration detected (pmol/ml) against NA concentration added to the plasma sample (pmol/ml), demonstrating the excellent linearity of the assay for standard curves of NA in plasma

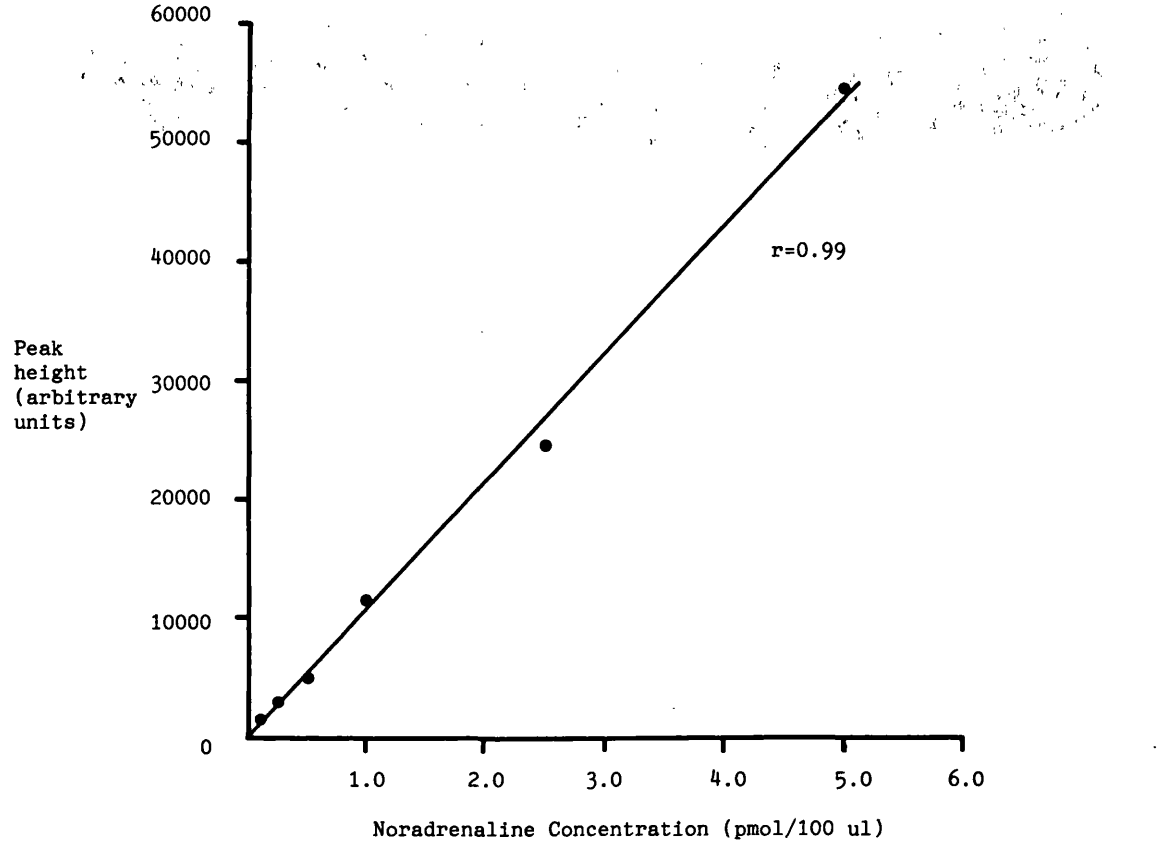


FIGURE 6.4 Linearity of the HPLC assay for the measurement of noradrenaline over the concentration range 0.1-5.0 pmol/100µl.

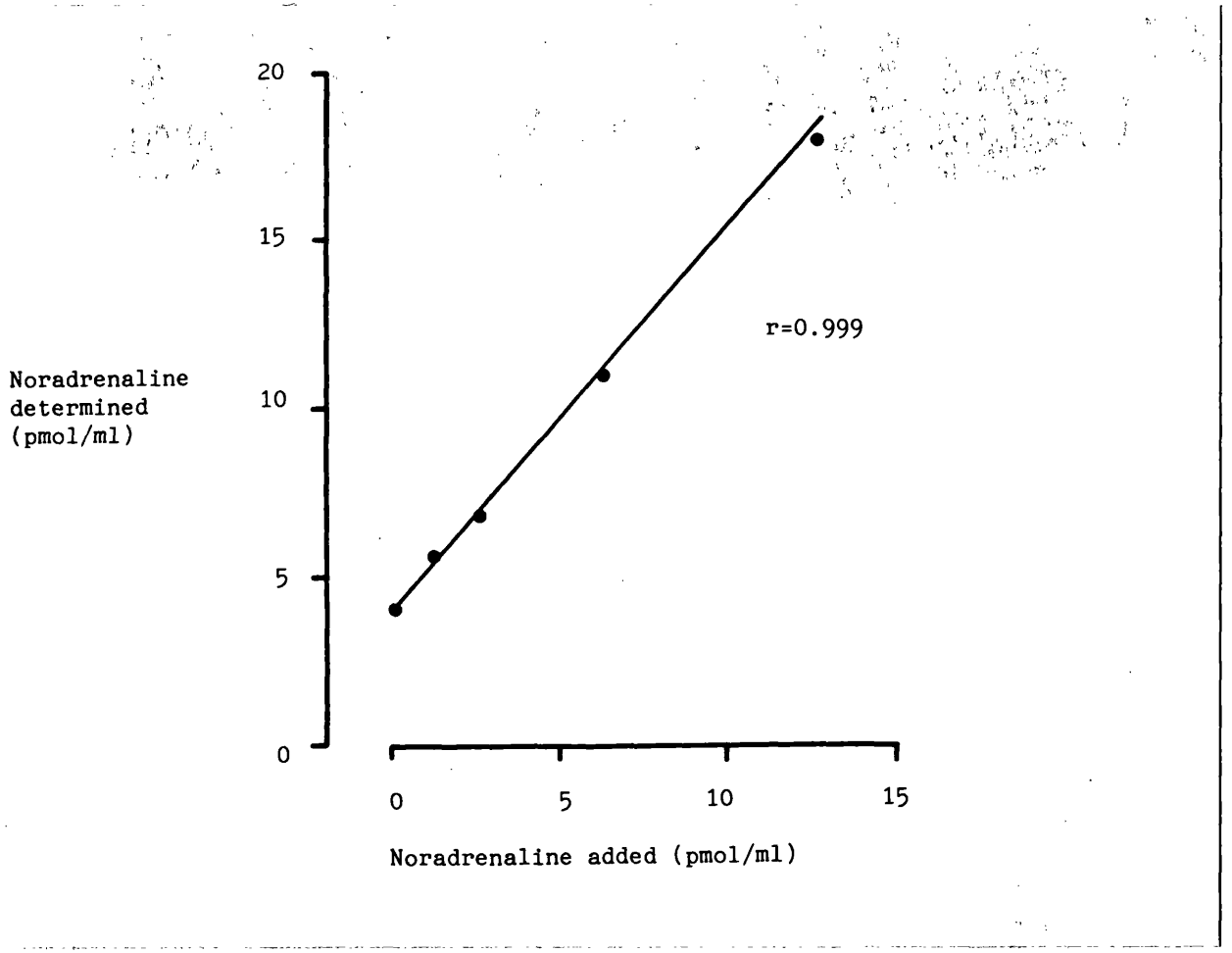


FIGURE 6.5 Noradrenaline concentration in human plasma after the addition of known amounts of noradrenaline. The noradrenaline concentration of the plasma before addition was 4.02 pmol/ml (mean of two determinations).

(r = 0.999; with a slope of 1.11). This is in agreement with the results of others (Hallman et al, 1978; Goldstein et al, 1981). The NA content of the plasma sample before addition was 4.02 pmol/ml (mean of 2 observations).

Intra- and inter-assay coefficients of variation

The intra-assay coefficient of variation was calculated by measuring the NA concentration in 10 aliquots of a standard solution of NA (50 pmol/ml in 0.1M perchloric acid) in one assay. To calculate the inter-assay coefficient of variation aliquots of the 50 pmol/ml solution of NA were assayed on separate occasions, over the course of several weeks. In each case the coefficient of variation was calculated as:

$$\frac{\text{Standard deviation}}{\text{mean}} \times 100\%, \text{ for the}$$

concentrations of NA recorded. The intra- and inter-assay coefficients of variation for the NA assay were thus 3% (n = 10) and 4% (n = 18) respectively.

In addition, the variability within plasma assays was investigated by assaying 6 human plasma samples in duplicate, and comparing the concentrations of NA recorded. The combined mean plasma NA concentration of all samples was 2.72 pmol/ml (n = 12), and the standard deviation of the difference between duplicates was 0.10 pmol/ml (n = 6 pairs). The variation between plasma assays was studied by separating plasma samples from 6 human volunteers into 2 aliquots, and measuring the NA concentration in these on separate occasions. This produced a combined mean plasma NA concentration of 3.19 pmol/ml (n = 12), and the

standard deviation of the difference between the two aliquots was 0.09 pmol/ml (n = 6 pairs). The mean difference between sample pairs, given by:

$$\frac{\text{Mean difference in NA concentration between sample pairs (pmol/ml)}}{\text{Combined mean NA concentration (pmol/ml)}} \times 100\%$$

was 5% (n = 6 pairs) both within and between plasma NA assays.

CHAPTER 7

RESULTS

7.1 General Details

Direct blood pressures (BP) are given as mean arterial pressures (diastolic pressure plus one-third pulse pressure). Although the high performance liquid chromatography (HPLC) assay (see Chapter 6) is designed to measure plasma catecholamines, data for plasma NA only is presented. The levels of adrenaline present in the very small volumes of rat plasma available were mostly below the limit of quantitation of the assay (i.e. below the level that can be measured accurately and reproducibly).

There were no significant differences in body weight or plasma creatinine concentration between hypertensive and control rats in any of the 3 models studied. BP in all hypertensive groups fell to normal after unclipping, whilst this procedure produced no change in the BP of loose clip controls (Table 7.1).

TABLE 7.1

Body weight, plasma creatinine concentration and mean arterial pressure (MAP) before and 48 hours after unclipping, for each group of rats studied. Values are mean±SEM (early phase is 4-6 weeks and chronic phase >16 weeks post-clipping).

*P<0.05 vs control ; $^{+}$P<0.05 for the change on unclipping

Group	Body weight (g)	MAP before unclipping (mmHg)	MAP after unclipping (mmHg)	Plasma creatinine concentration (µmol/L)
1K1C				
Hypertensive (n=18)	216±5	171±5*	103±2^{+}	50±3
Control (n=17)	209±3	109±3	106±3	42±2
2K1C : early phase				
Hypertensive (n=18)	218±4	161±4*	109±5^{+}	44±3
Control (n=12)	210±5	112±5	104±4	37±4
2K1C : chronic phase				
Hypertensive (n=13)	283±8	159±4*	121±7^{+}	54±3
Control (n=11)	277±6	117±3	103±4	49±3

7.2 1K1C Hypertension (4-6 weeks post-clipping)

Plasma NA and heart rate were significantly elevated in hypertensive rats compared to loose clip controls ($P<0.05$ and $P<0.01$ respectively; Table 7.2 and Figure 7.16). The individual changes in plasma NA and heart rate on unclipping 1K1C and control rats are shown in Figures 7.1 (plasma NA) and 7.2 (heart rate). Plasma NA and heart rate fell after unclipping 1K1C hypertensive rats (-0.30±0.31pmol/ml and -15±15 beats/min respectively, mean±SEM), but neither fall was significant when analysed within individual rats using a paired significance test ($P>0.2$ for plasma NA and heart rate). Heart rate remained significantly faster in the 1K1C group compared to controls ($P<0.05$), which showed no change in plasma NA or heart rate on unclipping. Plasma NA, however, was not significantly different in experimental and control animals after removal of the clip.

There was a weak positive correlation between BP and plasma NA ($r=0.49$, $P<0.02$; Figure 7.3) and a strong correlation between heart rate and plasma NA ($r=0.72$, $P<0.001$; Figure 7.4) in the 1K1C group before unclipping. Furthermore, the change in heart rate and the change in plasma NA on unclipping were significantly correlated in this group ($r=0.61$, $P<0.005$; Figure 7.5). There was, however, no correlation between the changes in BP and plasma NA on unclipping ($r=0.36$, $P>0.05$). Correlation and regression analyses were performed on the ranked data from all 1K1C rats studied, regardless of their mean arterial pressure. The same procedure was followed for regression analyses performed on data from the early and chronic phases of 2K1C hypertension (sections 7.3 and 7.4).

TABLE 7.2

Plasma noradrenaline concentration and heart rate before and 48 hours after unclipping for the 1K1C model. Values are mean±SEM.

*P<0.05 vs control

	1K1C Model	
	Hypertensive (n=18)	Control (n=17)
Plasma noradrenaline (pmol/ml)		
before unclipping	1.64±0.32*	1.05±0.13
after unclipping	1.34±0.10	1.15±0.13
Heart rate (beats/min)		
before unclipping	404±10*	359±10
after unclipping	389±11*	359±9

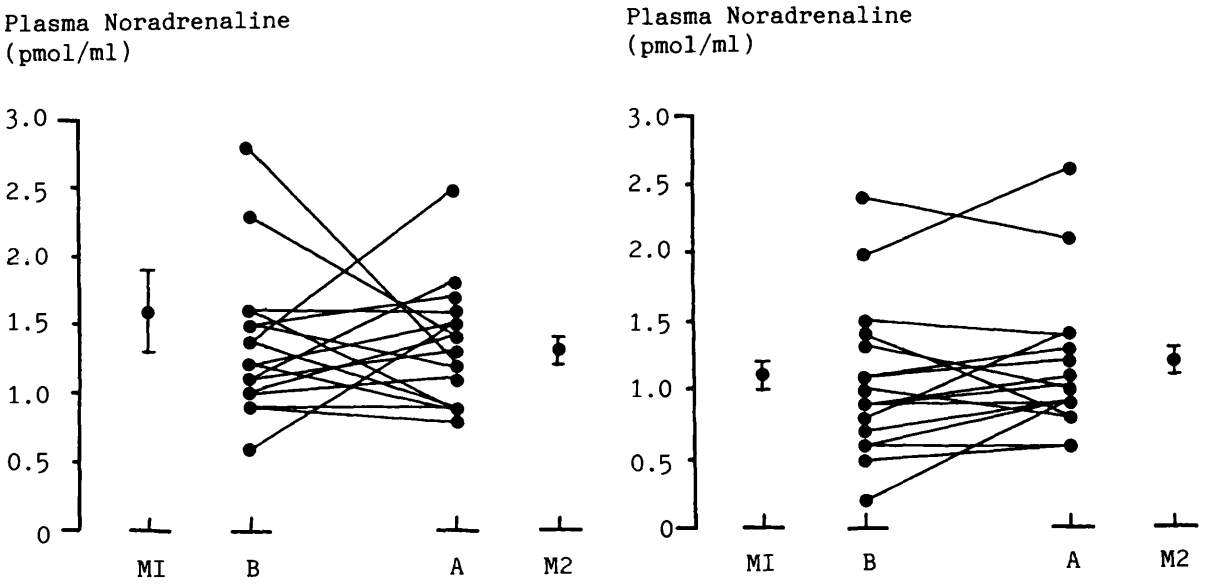


FIGURE 7.1 Individual plasma noradrenaline concentration before (B) and after (A) unclipping in (i) 1K1C hypertensive rats and (ii) 1-Kidney loose clip control animals. The mean values ± SEM before (M1) and after (M2) unclipping are also shown.

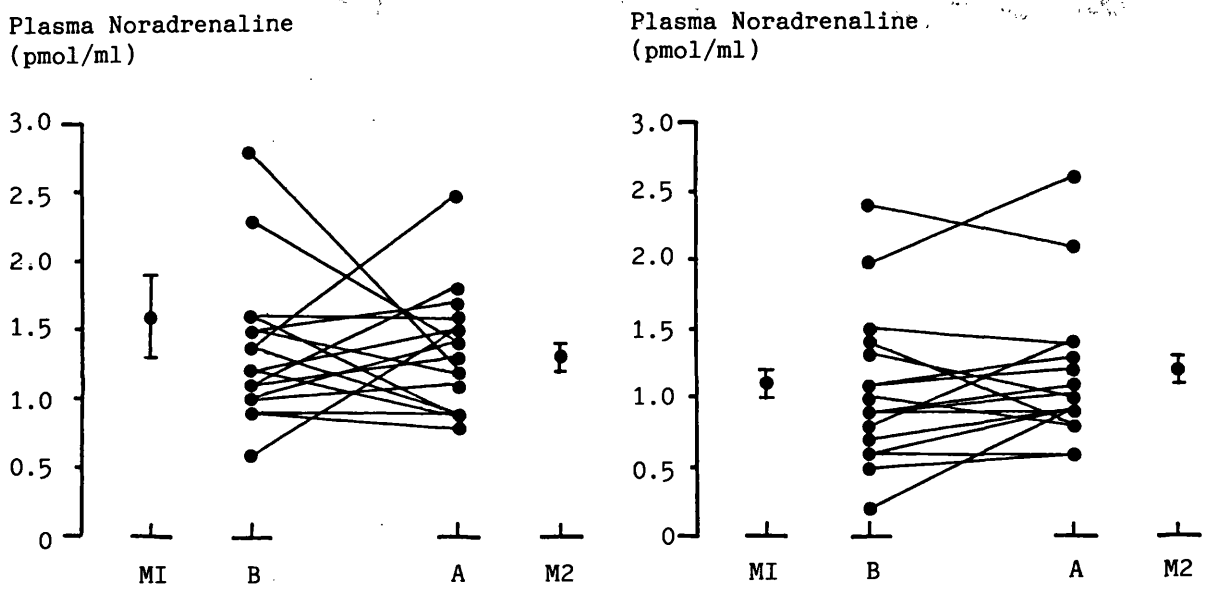


FIGURE 7.1 Individual plasma noradrenaline concentration before (B) and after (A) unclipping in (i) 1K1C hypertensive rats and (ii) 1-Kidney loose clip control animals. The mean values ± SEM before (M1) and after (M2) unclipping are also shown.

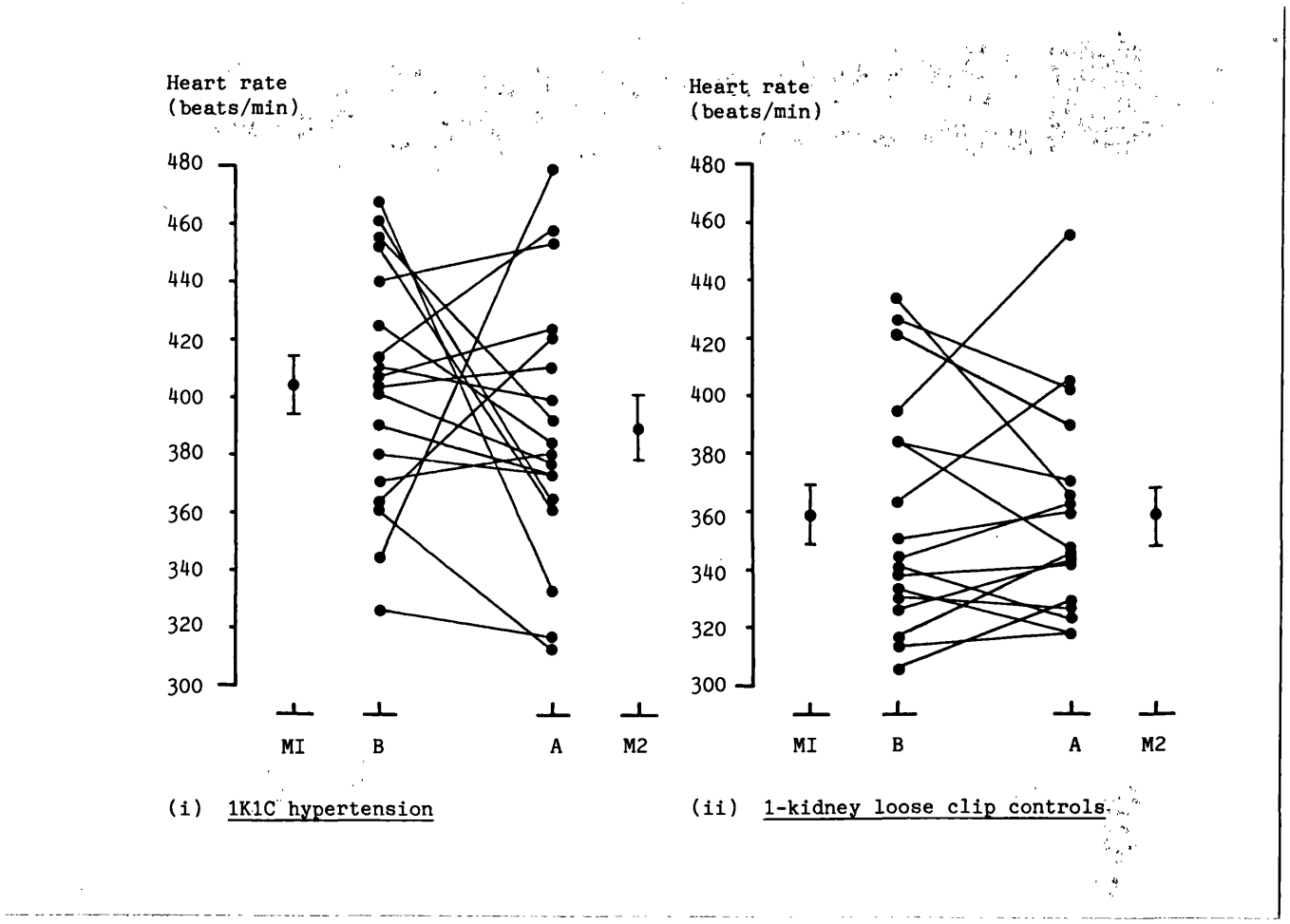


FIGURE 7.2

Individual heart rate before (B) and after (A) unclipping in (i) 1K1C hypertensive rats and (ii) 1-Kidney loose clip control animals. The mean values ± SEM before (M1) and after (M2) unclipping are also shown.

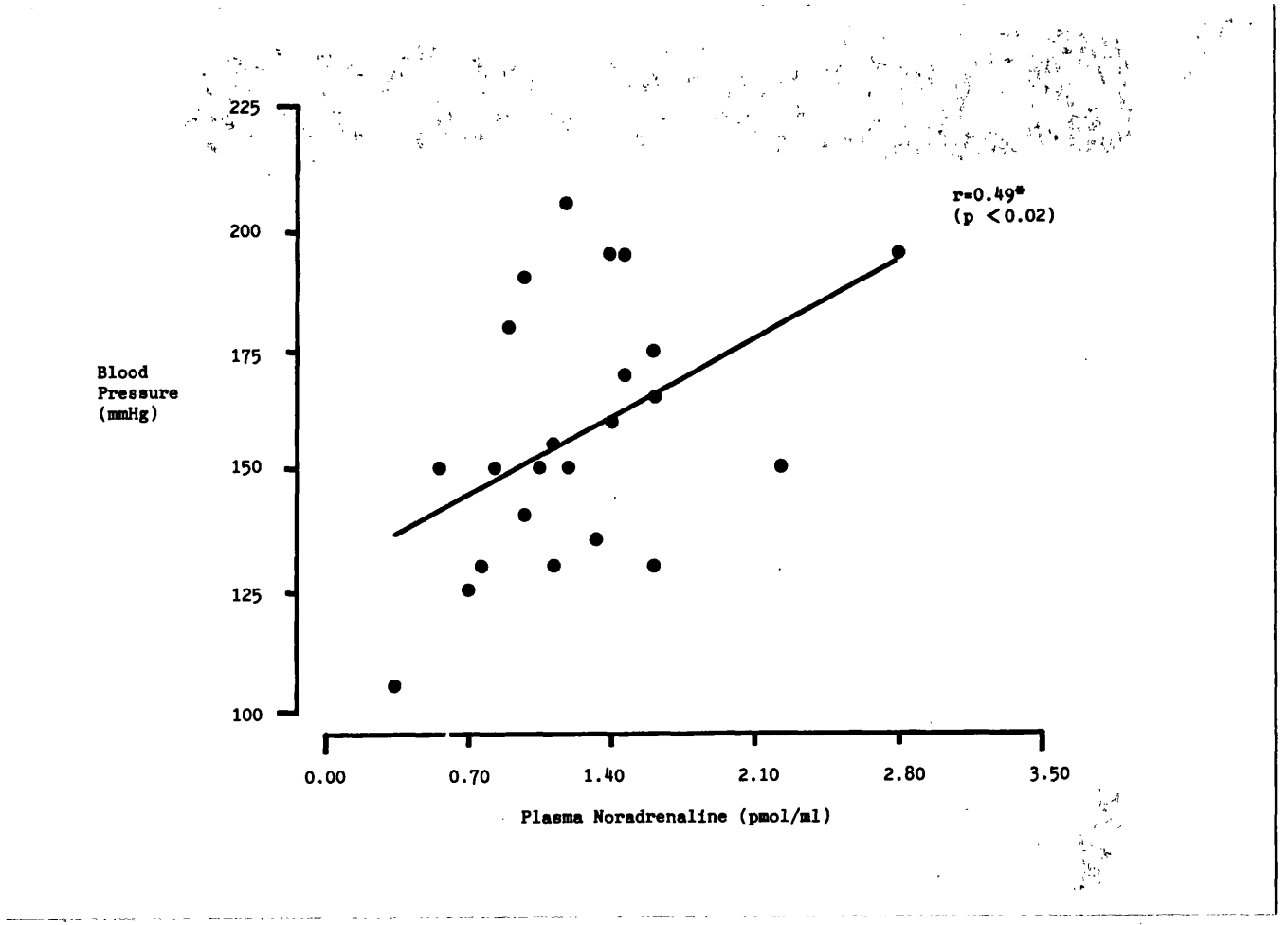


FIGURE 7.3

Plot of blood pressure against plasma noradrenaline concentration for 1K1C hypertension. * denotes a significant correlation between the two variables.

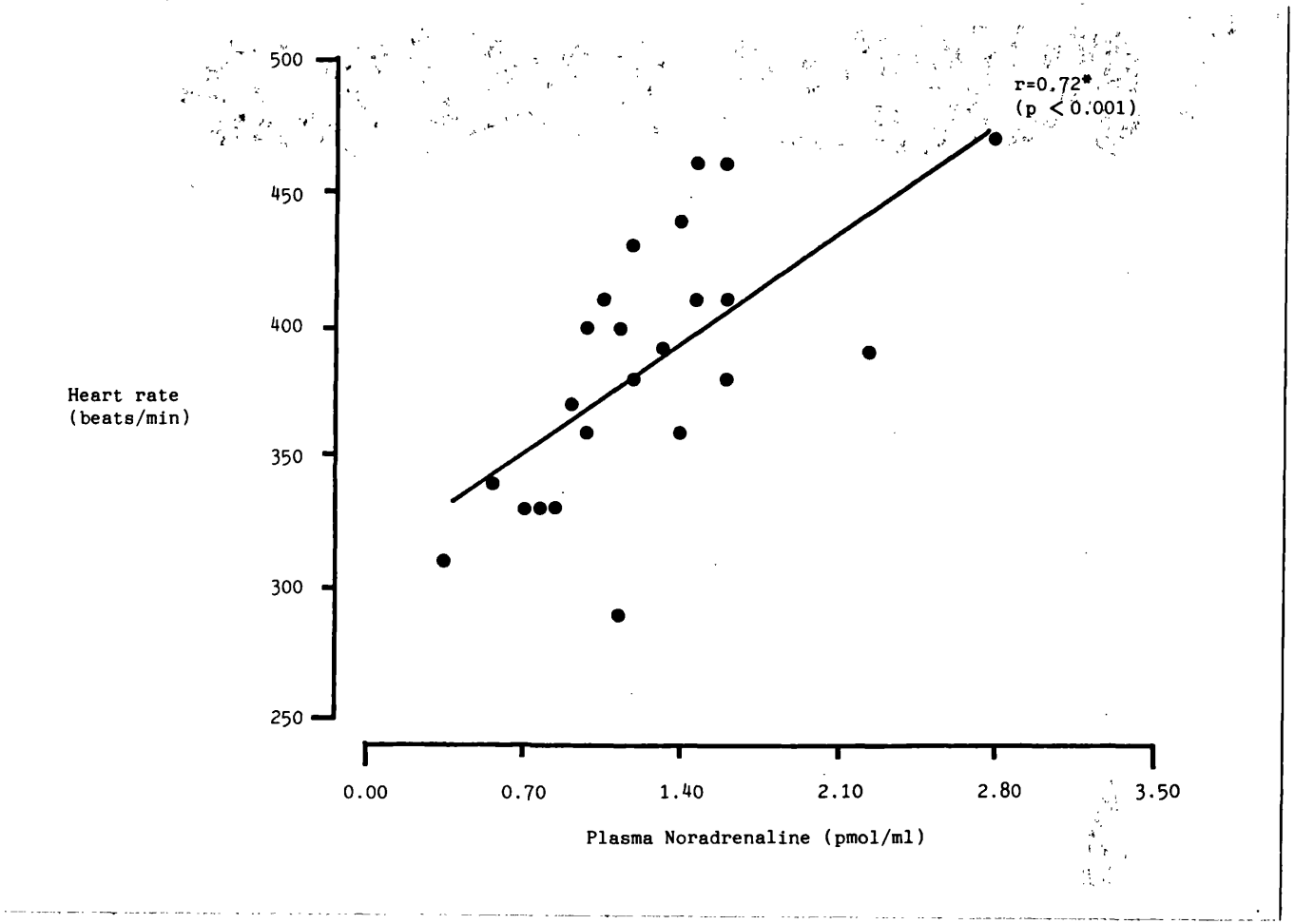


FIGURE 7.4

Plot of heart rate against plasma noradrenaline concentration for 1K1C hypertension. * denotes a significant correlation between the two variables.

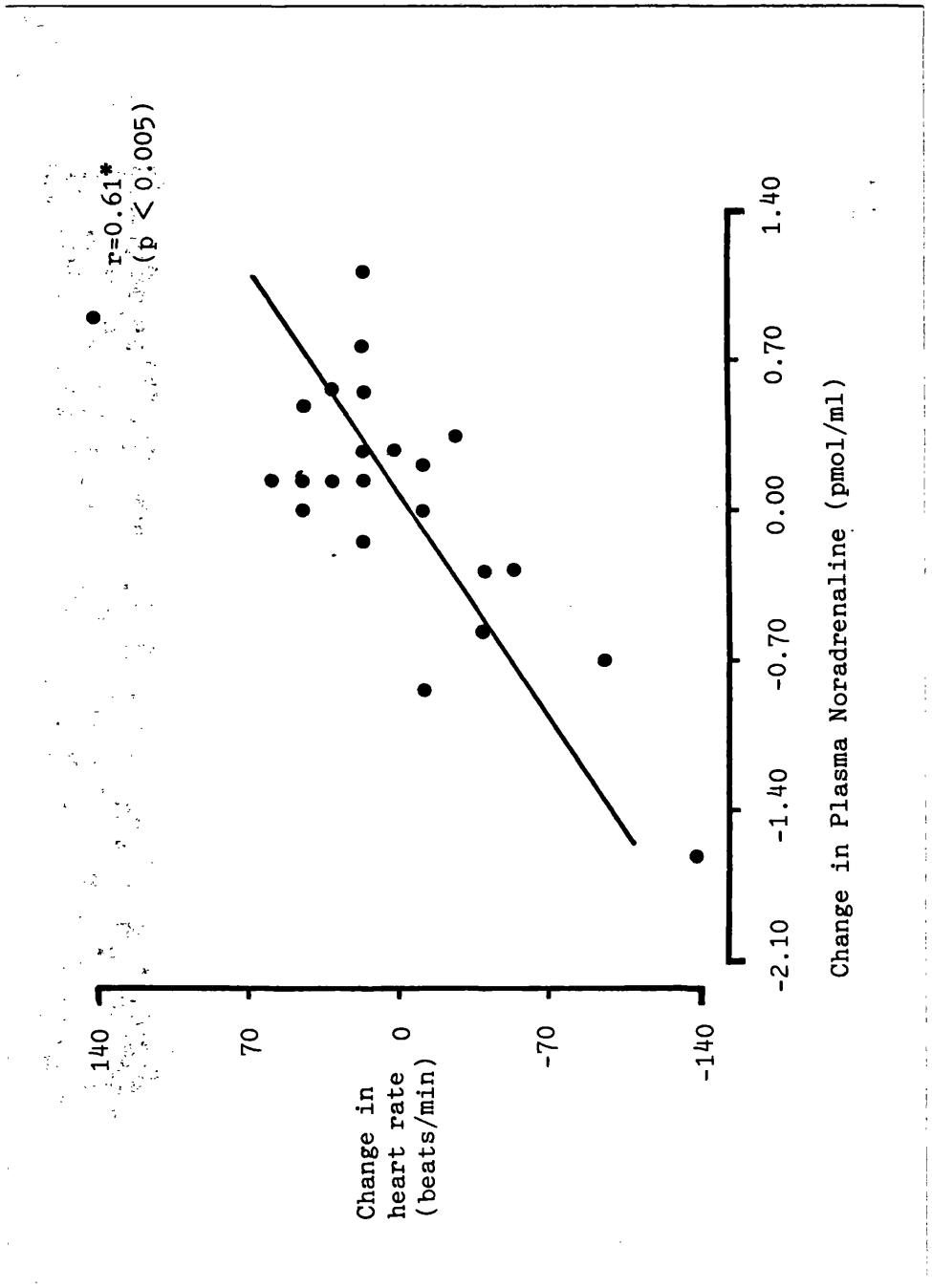


FIGURE 7.5

Change in heart rate plotted against change in plasma noradrenaline concentration on unclipping rats with 1K1C hypertension (4-6 weeks duration). * denotes a significant correlation between these two variables.

7.3 2K1C Hypertension : Early Phase (4-6 weeks post-clipping)

Plasma NA and heart rate were not different in hypertensive rats compared to controls, before or after unclipping (Table 7.3 and Figure 7.16). Both variables rose with unclipping in the two groups, and this was significant in the 2K1C hypertensive group ($P<0.05$ for both plasma NA and heart rate). However, statistical analysis of the changes in plasma NA (+0.35±0.14 and +0.18±0.33 pmol/ml in the hypertensive and control groups respectively, mean±SEM) and heart rate (+17±8 and +7±11 beats/min in hypertensive and control rats respectively, mean±SEM) on unclipping revealed no significant differences between the 2K1C and loose clip control groups ($P>0.3$ for the changes in both plasma NA and heart rate). Individual changes in plasma NA and heart rate following unclipping are illustrated in Figures 7.6 and 7.7 respectively.

There was no correlation between BP and plasma NA ($r=0.05$, $P>0.2$; Figure 7.8), or between heart rate and plasma NA ($r=0.31$, $p>0.1$; Figure 7.9) in rats in the early phase of 2K1C hypertension before unclipping. The changes in heart rate and plasma NA following unclipping were correlated in hypertensive rats ($r=0.45$, $P<0.05$; Figure 7.10), whilst the change in BP and the change in plasma NA were not correlated in this group ($r=0.30$, $P>0.1$).

TABLE 7.3

Plasma noradrenaline concentration and heart rate before and 48 hours after unclipping for the early phase (4-6 weeks post-clipping) of the 2K1C model. Values are mean±SEM.

*P<0.05 for the change on unclipping

	2K1C Model : Early Phase	
	Hypertensive (n=18)	Control (n=12)
Plasma noradrenaline (pmol/ml)		
before unclipping	2.15±0.17	2.17±0.26
after unclipping	2.49±0.15*	2.35±0.25
Heart rate (beats/min)		
before unclipping	371±11 (n=17)	372±11 (n=10)
after unclipping	388±8* (n=17)	379±10 (n=10)

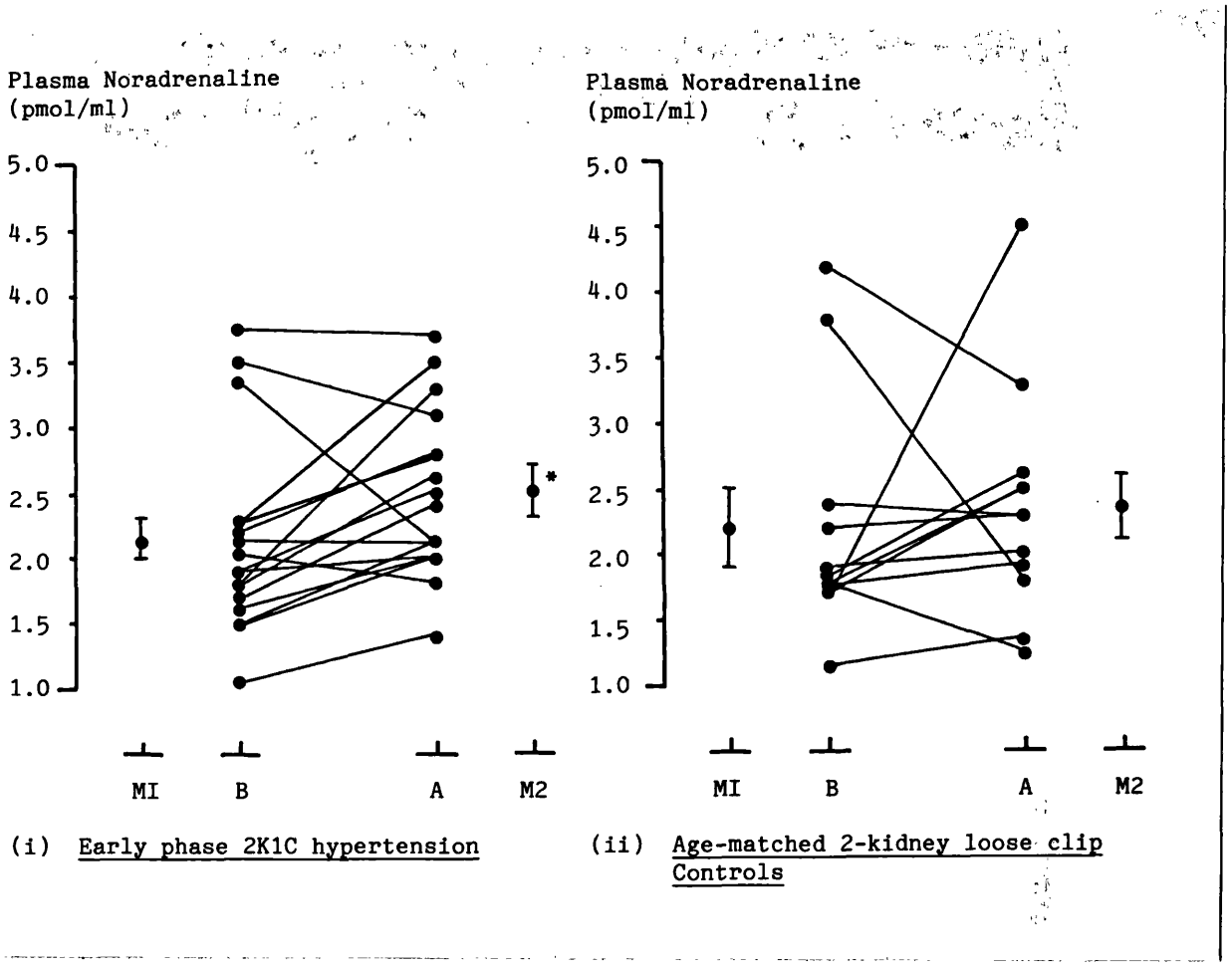


FIGURE 7.6 Individual plasma noradrenaline concentration before (B) and after (A) unclipping in (i) rats in the early phase (4-6 weeks post-clipping) of 2K1C hypertension, and (ii) age-matched 2-Kidney loose clip controls. The mean values ± SEM before (M1) and after (M2) unclipping are also shown. *P<0.05 for the change with unclipping.

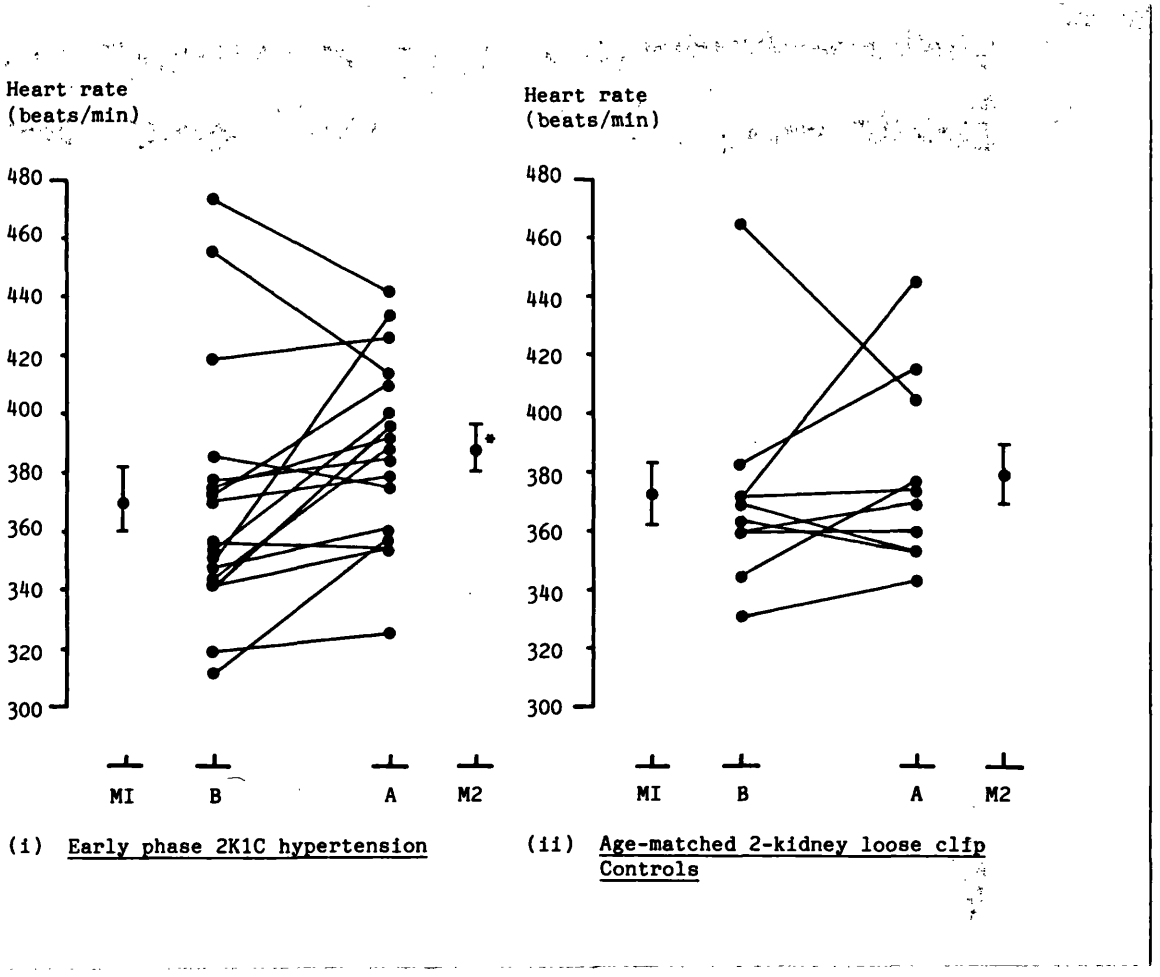


FIGURE 7.7 Individual heart rate before (B) and after (A) unclipping in (i) rats in the early phase (4-6 weeks post-clipping) of 2K1C hypertension, and (ii) age-matched 2-Kidney loose clip controls. The mean values ± SEM before (M1) and after (M2) unclipping are also shown.
*$P<0.05$ for the change with unclipping.

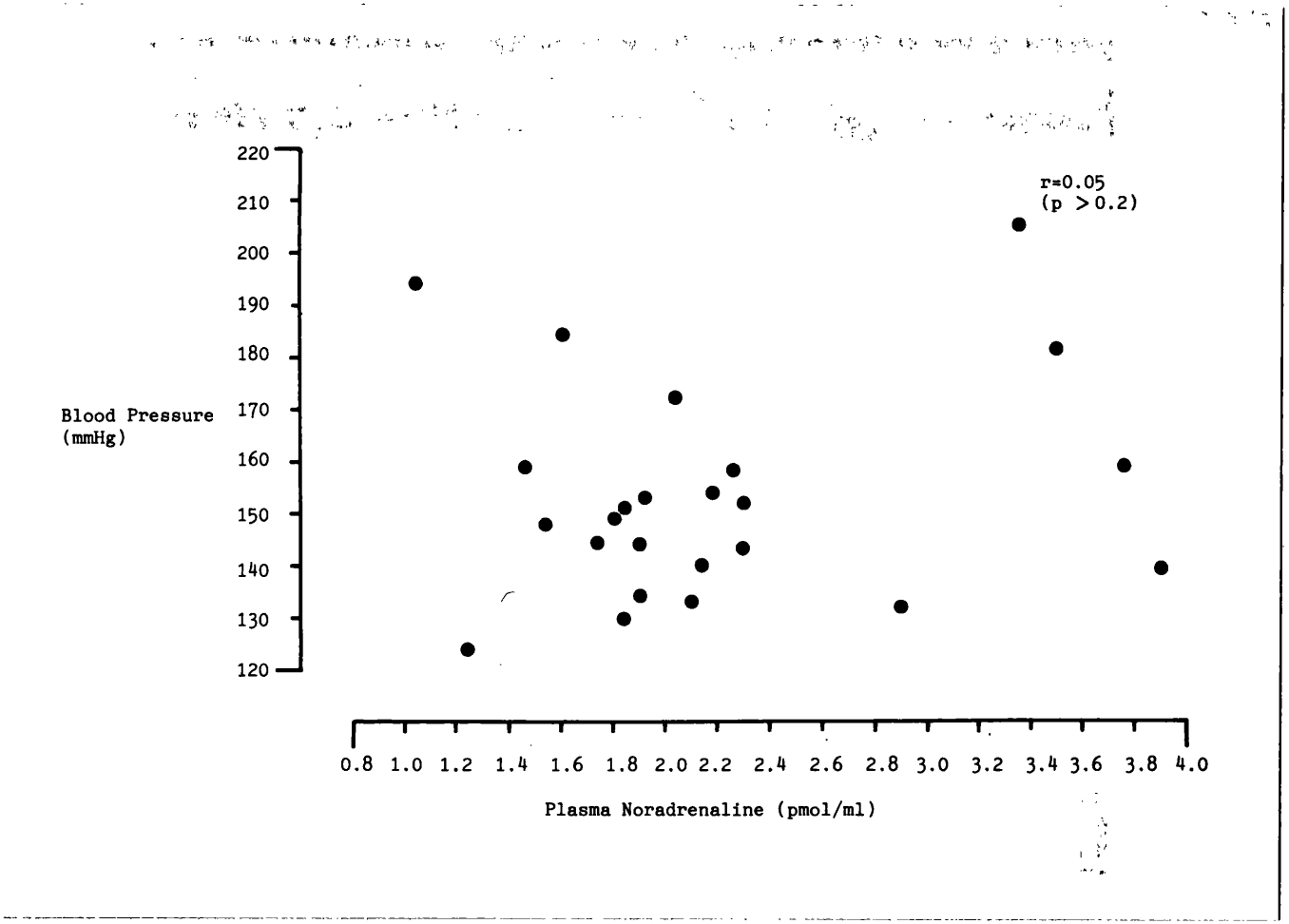


FIGURE 7.8 Plot of blood pressure versus plasma noradrenaline concentration for rats in the early phase of 2K1C hypertension (4-6 weeks post-clipping).

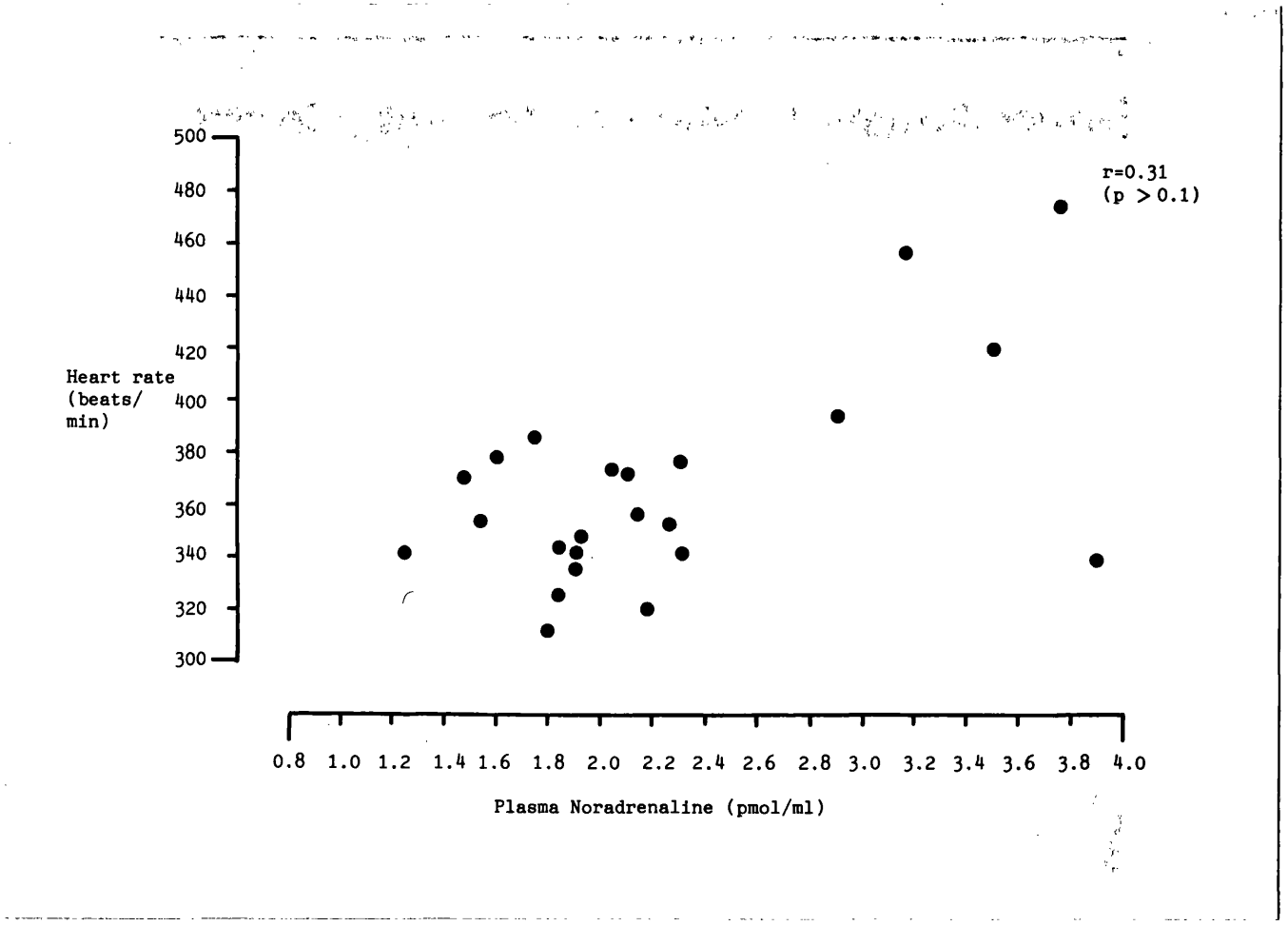


FIGURE 7.9 Plot of heart rate against plasma noradrenaline concentration for rats in the early phase of 2K1C hypertension (4-6 weeks post-clipping).

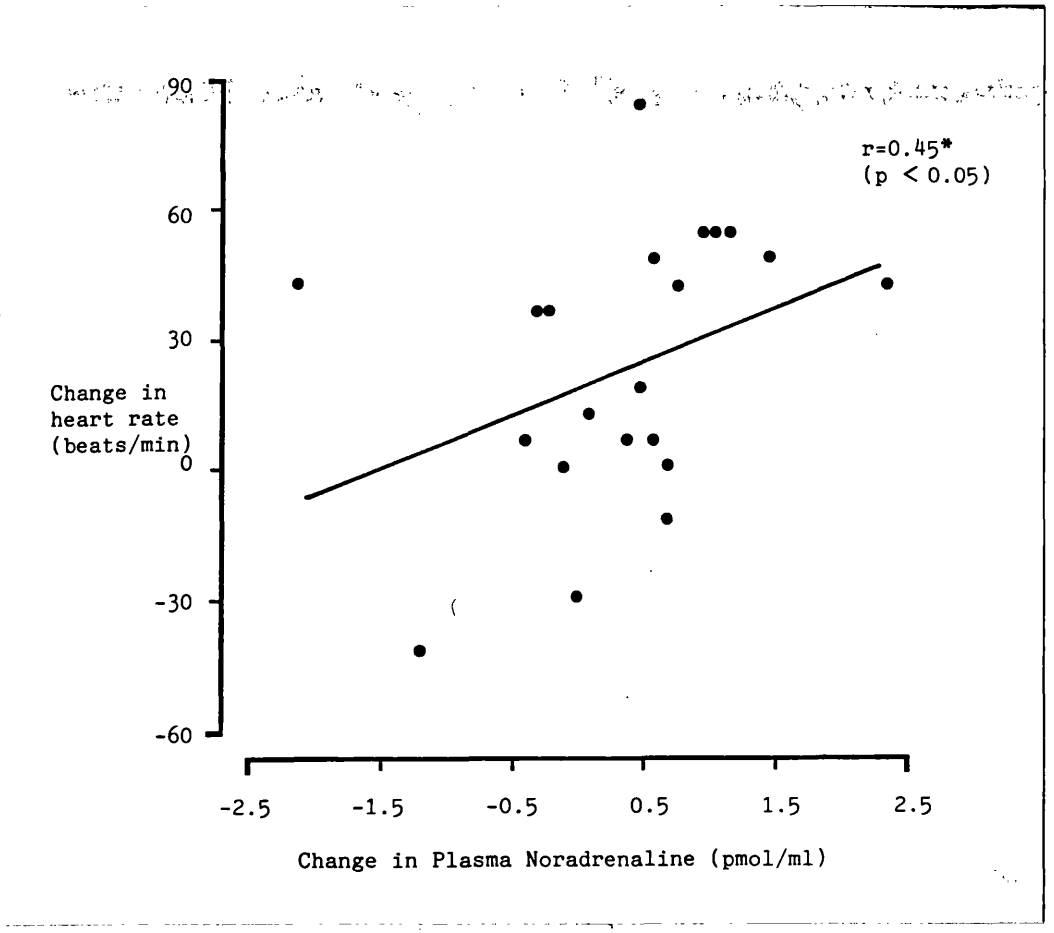


FIGURE 7.10 Change in heart rate plotted against change in plasma noradrenaline concentration following unclipping during the early phase of 2K1C hypertension (4-6 weeks post-clipping). * denotes a weak but significant correlation (P<0.05) between these two variables.

7.4 2K1C Hypertension : Chronic Phase (>16 weeks post-clipping)

There was no difference in plasma NA and heart rate, before or after unclipping, in hypertensive rats compared to controls (Table 7.4 and Figure 7.16). Individual changes in plasma NA and heart rate on unclipping are illustrated in Figures 7.11 and 7.12 respectively. There was a small, non-significant fall in both plasma NA and heart rate (-0.16±0.23 pmol/ml and -25±14 beats/min respectively, mean ± SEM) after unclipping in chronic 2K1C hypertension ($P>0.1$ for the changes in both variables, paired sign test).

There was no significant correlation between BP and plasma NA ($r=0.49$, $P>0.05$; Figure 7.13), whilst there was a weak positive correlation between heart rate and plasma NA ($r=0.55$, $P<0.05$; Figure 7.14) in chronic 2K1C hypertension before unclipping. As in the previous two models the change in heart rate and the change in plasma NA on unclipping were correlated in the hypertensive group ($r=0.62$, $P<0.01$; Figure 7.15), whilst the changes in BP and plasma NA were not ($r=0.32$, $P>0.2$).

TABLE 7.4

Plasma noradrenaline concentration and heart rate before and 48 hours after unclipping for the chronic phase (>16 weeks post-clipping) of the 2K1C model. Values are mean ± SEM.

	2K1C Model : Chronic Phase	
	Hypertensive (n=13)	Control (n=11)
Plasma noradrenaline (pmol/ml)		
before unclipping	2.23±0.27	2.11±0.34
after unclipping	2.07±0.13	1.91±0.16
Heart rate (beats/min)		
before unclipping	425±16	399±21
after unclipping	399±10	371±6

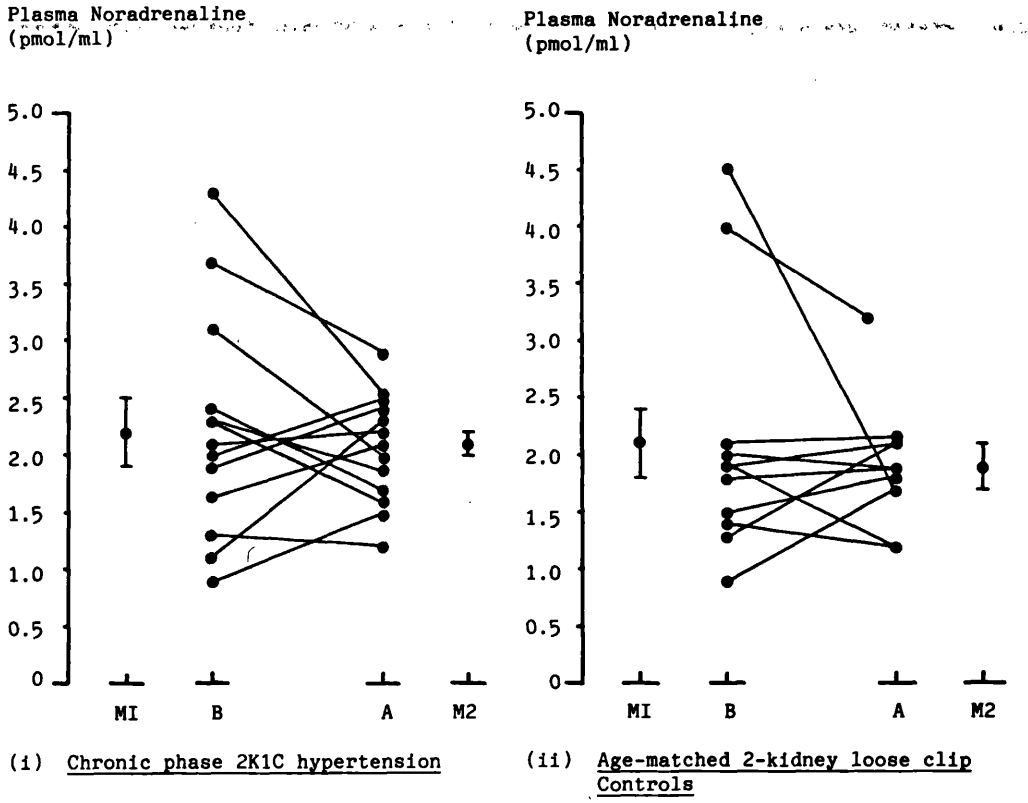


FIGURE 7.11 Individual plasma noradrenaline concentration before (B) and after (A) unclipping in (i) rats in the chronic phase (>16 weeks post-clipping) of 2K1C hypertension, and (ii) age-matched 2-Kidney loose clip controls. The mean values ± SEM before (M1) and after (M2) unclipping are also shown.

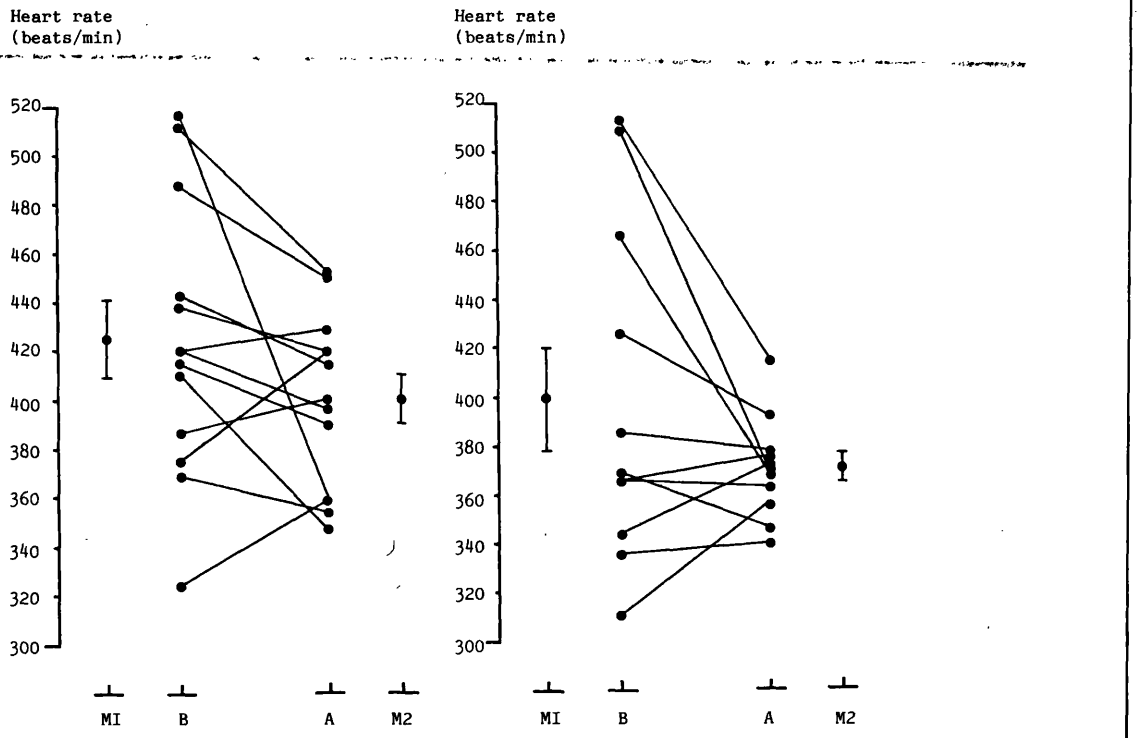


FIGURE 7.12 Individual heart rate before (B) and after (A) unclipping in (i) rats in the chronic phase (>16 weeks post-clipping) of 2K1C hypertension, and (ii) age-matched 2-Kidney loose clip controls. The mean values ± SEM before (M1) and after (M2) unclipping are also shown.

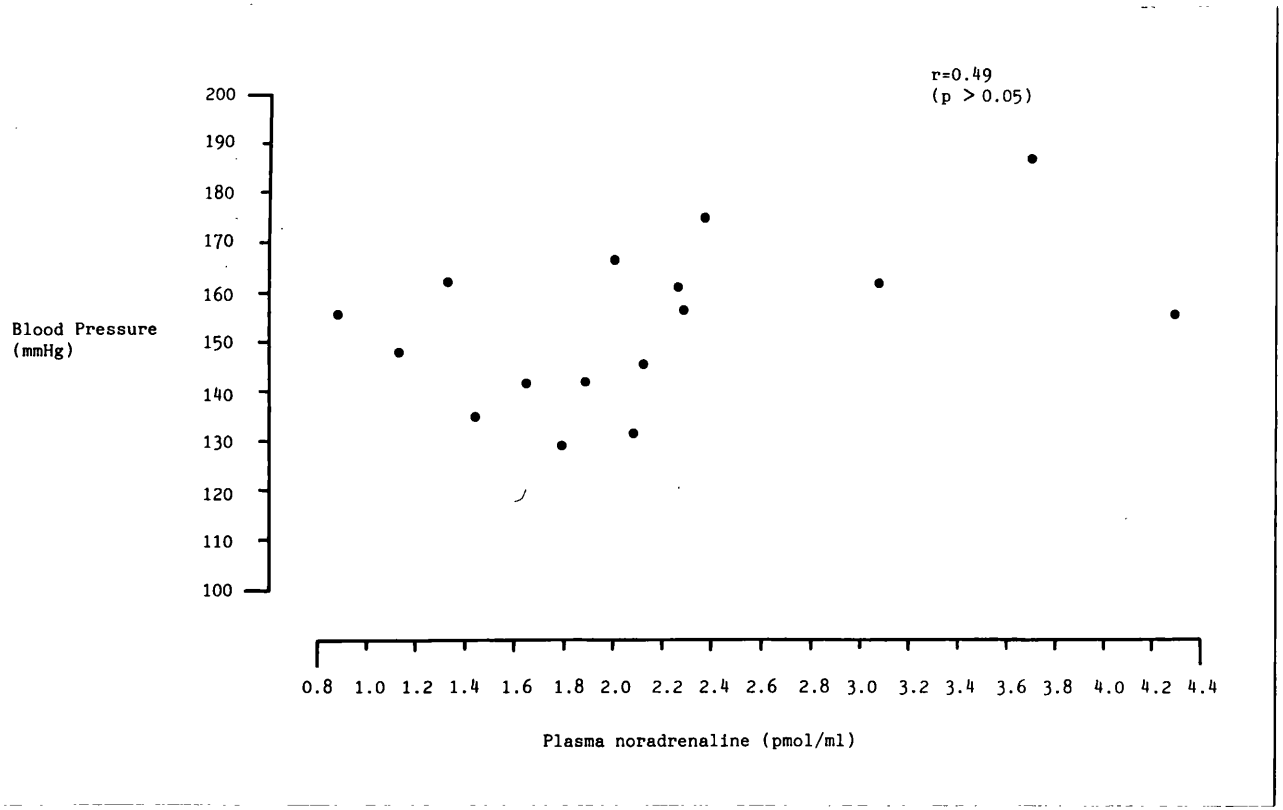


FIGURE 7.13

Graph showing a plot of blood pressure against plasma noradrenaline concentration for rats in the chronic phase (>16 weeks) of 2K1C hypertension.

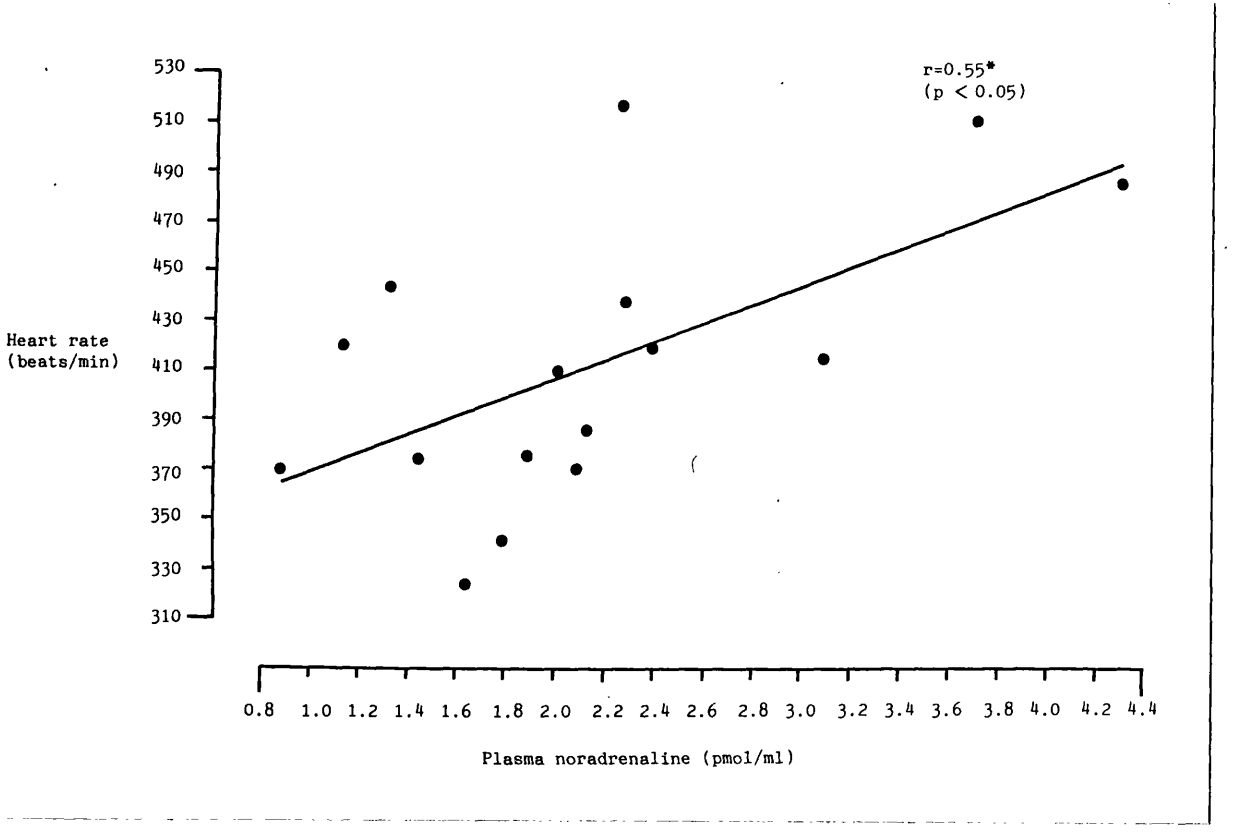


FIGURE 7.14

Graph showing a plot of heart rate versus plasma noradrenaline concentration for rats in the chronic phase (>16 weeks) of 2K1C hypertension. *denotes a significant correlation between these two variables.

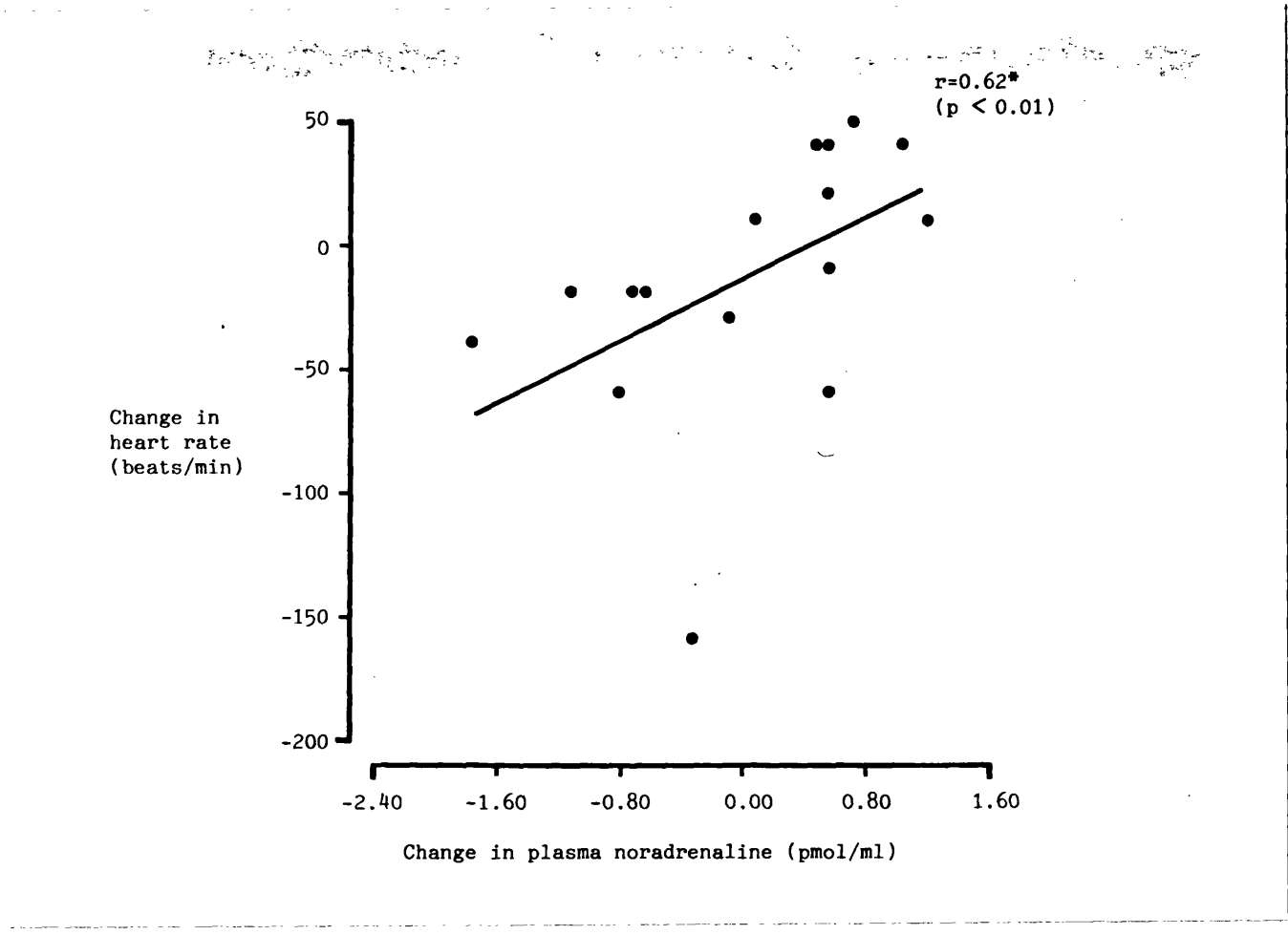


FIGURE 7.15 Change in heart rate plotted against change in plasma noradrenaline concentration following unclipping during the chronic phase of 2K1C hypertension (>16 weeks post-clipping). * denotes a significant correlation between these two variables.

7.5 Comparison of the Changes in Plasma Noradrenaline and Heart Rate on Unclipping

A summary diagram of mean plasma NA and heart rate values, before and 48 hours after unclipping, in all groups of rats studied is shown in Figure 7.16.

7.5.1 1K1C versus 2K1C Hypertension (both early phase)

Following surgical reversal of hypertension by unclipping, there was a non-significant fall in both plasma NA and heart rate in the 1K1C model; whilst there was a significant increase in both these variables in the early phase of 2K1C hypertension (see sections 7.2 and 7.3). This difference in the pattern of plasma NA and heart rate responses to unclipping between the early phases of 1K1C and 2K1C hypertension was significant ($P<0.05$ for plasma NA and heart rate), whereas there was no difference in the responses of the two control groups (see Table 7.5).

7.5.2 Early versus Chronic 2K1C Hypertension

There was a small fall in both plasma NA and heart rate after unclipping chronic 2K1C hypertensive rats, in contrast to the rise observed in the early phase of this model (see sections 7.3 and 7.4). The difference in the heart rate response to unclipping between the two phases of 2K1C hypertension was significant ($P=0.02$), whereas the difference in the plasma NA response was not ($P>0.05$). There was no significant difference between the two corresponding loose clip control groups (see Table 7.5).

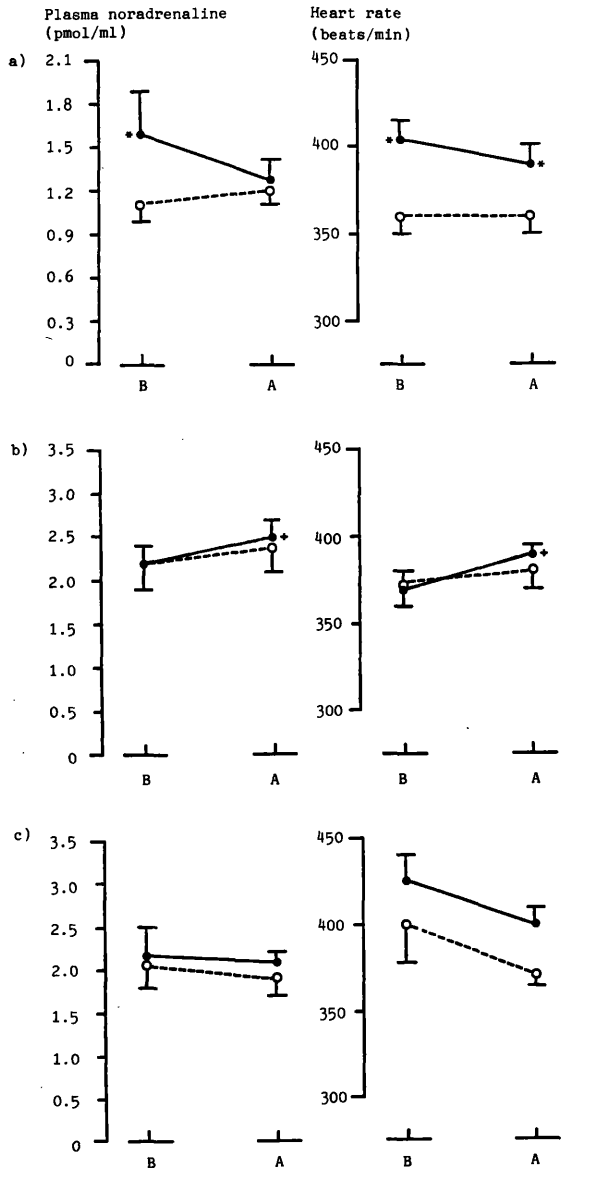


FIGURE 7.16

Summary diagram showing plasma noradrenaline concentration and heart rate before (B) and after (A) unclipping in hypertensive rats (●——●) and loose clip controls (o-----o). Values are mean and SEM.
a) Early phase of 1K1C model, b) Early phase of 2K1C model, c) Chronic phase of 2K1C model. *P<0.05 vs control; +P<0.05 for the change with unclipping.

TABLE 7.5

Comparison of the pattern of changes in plasma noradrenaline concentration and heart rate following unclipping between the experimental groups. The P value was obtained from a Mann-Whitney U-test.

	Change in plasma noradrenaline on unclipping (P value)	Change in heart rate on unclipping (P value)
1K1C vs 2K1C hypertension (both early phase)	0.04	0.04
1-kidney vs 2-kidney loose clip controls (both early phase)	0.74	0.55
Early vs chronic 2K1C hypertension	0.07	0.02
Early vs chronic 2-kidney loose clip controls	0.56	0.19

7.6 Control Experiment for the Repeated Measurement of Plasma Noradrenaline in the Same Animal

The aim of this control experiment was to validate the blood sampling procedure employed to obtain the results reported in sections 7.2-7.4 (see section 5.1.8 for details). There were no significant differences in plasma NA, heart rate or BP between values determined 48 and 96 hours after cannulation (sampling days 1 and 2 respectively) in normal rats (see below). The combined mean and the standard deviation of the difference between sample pairs were calculated to express the variability between the two sampling days. In addition the mean percentage difference in each variable, irrespective of sign, was calculated.

7.6.1 Variability Between Plasma Noradrenaline Samples

Individual plasma NA concentrations on sampling days 1 and 2 are illustrated in Figure 7.17. The mean values ± SEM were 1.70±0.17 and 1.76±0.16pmol/ml respectively (n=12). The combined mean was 1.73pmol/ml (n=24) and the standard deviation of the difference between samples from the same rat was 0.24pmol/ml (n=12). The mean difference between successive plasma NA concentrations was 15% (n=12).

7.6.2 Variability Between Successive Heart Rate and Blood Pressure Measurements

Individual heart rate values on the two sampling days are illustrated in Figure 7.17, the mean values ± SEM were 396±9 and 375±9 beats/min (n=12). The combined mean and the standard deviation of the difference between heart rate recordings were 386 (n=24) and 15 (n=12) beats/min

respectively. The mean difference between successive heart rate measurements was 9%. The mean difference between the two BP recordings was also 9% (mean±SEM, 106±3 and 102±3 mmHg on sampling days 1 and 2 respectively, n=12). The combined mean BP was 104mmHg (n=24), and the standard deviation of the difference between successive BP measurements was 7mmHg (n=12).

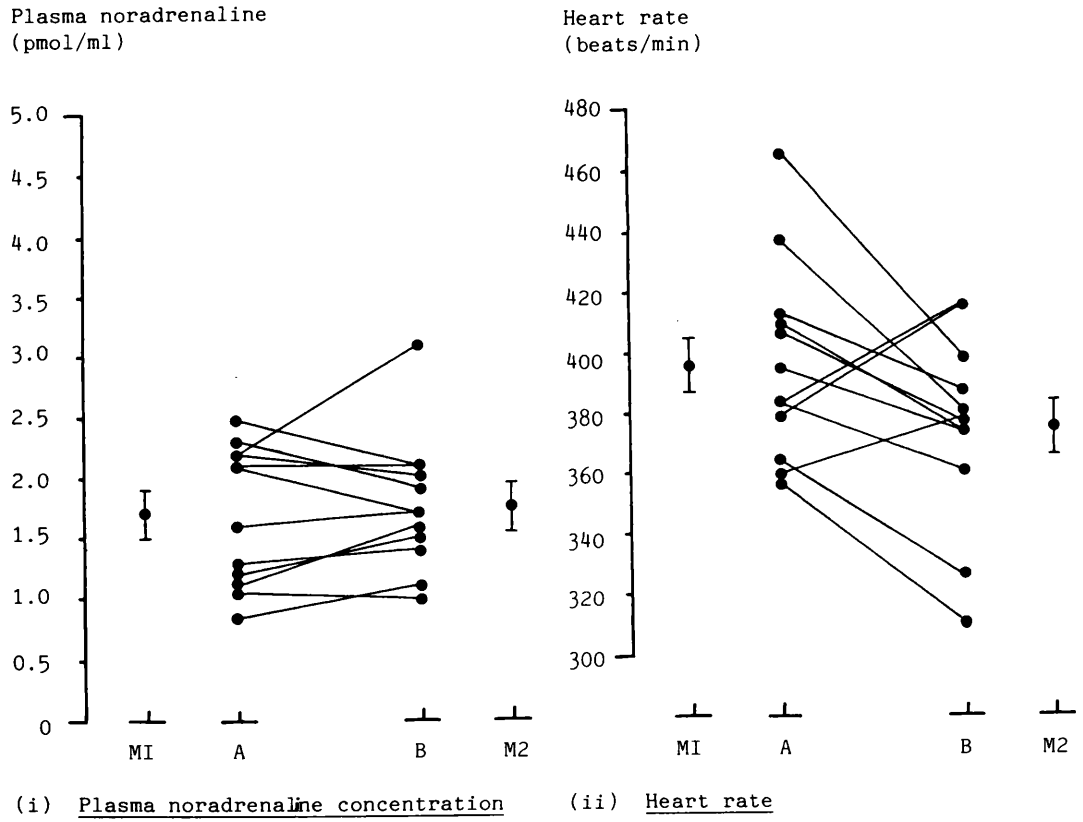


FIGURE 7.17 Individual plasma noradrenaline concentration and heart rate 48 hours (A) and 96 hours (B) after cannulation in normal rats. The mean values ± SEM on each occasion (M1 and M2 respectively) are also shown.

CHAPTER 8

DISCUSSION

DISCUSSION

The experiments reported in this section support a role for the SNS in 1K1C hypertension, but not in either the early or chronic phases of 2K1C hypertension. In the 1K1C model both plasma NA and heart rate were significantly elevated in hypertensive rats compared to loose-clip controls. Furthermore, both mean arterial pressure and heart rate were correlated with plasma NA in the hypertensive group. In contrast, there was no evidence of SNS involvement at any stage in 2K1C hypertension. Plasma NA and heart rate were similar in hypertensive and control rats in both the early and chronic phases of this model. Moreover, there was no correlation between BP and plasma NA in either phase.

The two models also differ in their responses to reversal of hypertension by unclipping. In 1K1C hypertension unclipping was associated with a small, though non-significant fall in both plasma NA and heart rate; heart rate remained significantly elevated compared with controls. Although plasma NA concentration was no longer significantly different from controls, it remained higher than control pre-operative values. These results suggest that the stimulus to increased SNS activity in 1K1C hypertension is not fully reversed at this stage, although baroreceptor mediated SNS activation secondary to the BP fall (McCubbin & Ferrario, 1977) may be maintaining the higher heart rate. The lack of correlation between change in BP and change in either heart rate or plasma NA is consistent with either of these, but does suggest that SNS activity and the BP fall are not directly related. This is not surprising in view of the multifactorial nature of blood pressure (BP) control and role of other systems (e.g. renin-angiotensin system) in determining BP responses.

In contrast, in early 2K1C hypertension unclipping was followed by a significant increase in both plasma NA and heart rate. The most likely explanation for these findings is SNS activation mediated by the baroreflex response to the BP fall (McCubbin & Ferrario, 1977); although as baroreflex responsiveness was not measured here this cannot be confirmed. A carotid baroreceptor mediated increase in efferent renal sympathetic nerve activity to the non-clipped kidney of 2K1C rats on unclipping, induced by the fall in BP, could also explain the sodium retention which occurs in this model following reversal of hypertension (Gottschalk, 1979; Thurston et al, 1980a). However, it has been shown that baroreceptor function (Salgado & Krieger, 1973) and sensitivity of the baroreflex arc (Floras & Jones, 1980) have adapted to the BP fall within 24 hours of unclipping in rats with renal hypertension of up to 8 weeks duration. Baroreceptors are known to adapt to increased BP with a reduction in baroreflex sensitivity (hence operating at higher pressure levels) within a few days of induction of experimental hypertension in rats (Krieger, 1970; Floras & Jones, 1979). In the present experiments SNS activity was measured in short term 2K1C hypertension 48 hours after unclipping, by which time the baroreceptors should have been "reset" to the lower, normotensive BP levels. Although plasma NA and heart rate were both significantly increased on unclipping early phase 2K1C rats, a tendency for these two variables to increase (although not significantly) was also seen in controls subjected to the same procedure. Consequently statistical analysis of the changes in plasma NA and heart rate on unclipping revealed no significant differences between hypertensive rats and the parallel control group. Furthermore, neither the change in plasma NA nor the change in heart rate were correlated with the BP fall on unclipping during the early phase of 2K1C hypertension.

The validity of the results obtained in the models of renovascular hypertension is strengthened by the results of the control experiment. This demonstrated low variability in successive plasma NA and heart rate values obtained from normal rats, when utilising the protocol for sampling and recording employed for experimental animals (section 7.6). The present results are in general agreement with the work of Reid et al (1976), which showed plasma NA to be significantly elevated in 1K1C hypertension, but to be unaltered in 2K1C hypertension, compared with corresponding loose clip controls. These samples were, however, obtained after decapitation, which would be expected to stimulate the release of catecholamines from the adrenal glands. In contrast, reduced plasma NA levels have been reported in 1K1C hypertension in the rat (Vlachakis et al, 1984). Also in disagreement with the present report, elevated plasma NA levels have been demonstrated to fall to normal along with BP on unclipping both 1K1C and 2K1C hypertensive rats (Katholi et al, 1982a and 1982b). Furthermore, heart rate has been found to be unchanged following unclipping in the early phase of 2K1C hypertension (Hallbäck-Norlander, Noresson & Lundgren, 1979; Russell, Bing, Swales & Thurston, 1983), although these measurements were not accompanied by parallel plasma NA determinations. Moreover, in the study of Hallbäck-Norlander et al (1979), rats were studied 2 hours after unclipping when the effects of the anaesthetic are unclear. The reasons for these different findings are unknown, although differences in the strain and sex of the rats used in the experiments may account for some of the variability. Renal denervation of the clipped kidney in 1K1C and 2K1C hypertension has been reported to reduce elevated plasma NA levels to normal, whilst BP was reduced but not normalised in both models (Katholi et al, 1982a and 1982b). This suggests that changes in the two variables are not directly related in either model, and is therefore consistent with the present results.

There is some debate about the validity of plasma NA measurements as an index of SNS activity (see section 2.3.2[a]). It is argued that SNS discharge follows a differentiated pattern which cannot be reflected accurately in mixed venous plasma NA concentration in humans (Brown, Jenner, Allison & Dollery, 1981a; Folkow, DiBona, Hjemdahl, Torén & Wallin, 1983; Esler, Hasking, Willett, Leonard & Jennings, 1985). It is also proposed that the haemodynamically unimportant skeletal muscle mass is the main contributor to plasma NA concentration (Folkow et al, 1983). However, although SNS responses to environmental and internal stimuli do follow a differentiated pattern, this does not necessarily mean that plasma NA concentration has no relation to overall sympathetic tone at rest (Goldstein, McCarty, Polinsky & Kopin, 1983b). The importance of the skeletal muscle contribution to plasma NA at rest has also been disputed (Goldstein et al, 1983b). Studies in human volunteers have revealed that under normal conditions the actions of NA are attributable entirely to its sympathetic neurotransmitter function. It is only under conditions of stress that NA may also have a minor hormonal role (Silverberg, Shah, Haymond & Cryer, 1978). However, Izzo (1983) contends that at physiological concentrations NA in the circulation should be considered to behave as a cardiovascular hormone, as well as an index of SNS activity.

In the present experiments care was taken to ensure samples were obtained under baseline steady state conditions. Arterial blood was sampled, rather than mixed venous blood which would be expected to be more affected by regional variations in NA clearance from the blood stream (Brown et al, 1981a). The findings here are strengthened by the parallel measurement of heart rate as a second index of SNS activity. Changes in plasma NA and heart rate closely paralleled each other in all three hypertensive groups of rats, as would be expected if both reflect

SNS activity. Catecholamines are filtered by the glomerulus and undergo tubular secretion in rats. There is also proposed to be significant renal metabolism of catecholamines, with O-methylation to metanephrines being the major metabolic route (Silva, Landsberg & Besarab, 1979). Consequently reduced urinary excretion of NA in 1K1C hypertension might explain the elevated plasma levels of the neurotransmitter. However, the increase in heart rate and close relationship of this with plasma NA concentration are against such a likelihood. Furthermore, a recent publication has shown that rats with 1K1C hypertension have an increased urinary excretion of NA and adrenaline rather than the reverse (Racz, Kuchel, Buu & Garcia, 1986). There was no evidence of renal impairment in 1K1C rats after unclipping in the present study, based on plasma creatinine concentrations and post-mortem examinations revealing an absence of macroscopic evidence of renal infarction; although specific assessments of renal function, and renal NA clearance, were not performed.

Several mechanisms have been postulated to explain the SNS activation in 1K1C hypertension. It may be mediated by a central component (Dargie, Franklin & Reid, 1977a; Petty & Reid, 1979; Winternitz, Katholi & Oparil, 1982). The afferent renal nerves have been proposed to be involved, producing a reflex increase in peripheral SNS tone via an effect on the hypothalamus (Katholi et al, 1981 and 1982a; Winternitz et al, 1982). However, other renal denervation studies demonstrated that intact renal nerves were not necessary for the development or maintenance of 1K1C hypertension in the rat (Norman, Murphy, Dzielak, Khraibi & Carroll, 1984; Villarreal, Freeman, Davis, Garoutte & Sweet, 1984). Alternatively, enhanced circulating levels of adrenaline may enhance NA release from sympathetic nerve terminals. It has been suggested that following an increase in circulating levels of

adrenaline, the hormone is accumulated in sympathetic nerve terminals. Adrenaline is then "co-released" with NA on nerve stimulation, in the vicinity of the pre-synaptic β_2-adrenoceptors upon which adrenaline acts to enhance NA release by positive feedback. (Majewski & Rand, 1986; see section 2.2.2). This mechanism is proposed to play a role in the development and maintenance of elevated arterial pressure in spontaneously hypertensive rats (SHR), and may result from a chronic elevation in plasma adrenaline levels (Majewski & Rand, 1986). However, this has not been investigated in experimental renovascular hypertension to date, and no evidence is available from the present experiments. The high performance liquid chromatographic catecholamine assay employed (see Chapter 6) was not sensitive enough to measure reliably the low levels of adrenaline present in the small volumes of rat plasma available.

The experiments presented here do not support the idea of a progressive increase in the contribution of the SNS to 2K1C hypertension, as the elevated level of renin-angiotensin system activity recedes to normal in the chronic phase (Reed, Sapirstein, Southard & Ogden, 1944; Fiorentini, Bellini & Fernandes, 1981). Plasma NA and heart rate were similar in hypertensive rats and corresponding controls in both the early and chronic phases of 2K1C hypertension. Hypertensive rats in the two phases did, however, show a different response to unclipping. Reversal of early phase 2K1C hypertension was associated with an increase in plasma NA and heart rate, whilst reversal of chronic 2K1C hypertension produced a small fall in both these variables. The cardiovascular system of rats in the two phases of the 2K1C model therefore responds differently to the fall in BP on unclipping. The reason for this is unknown. One possible explanation is that structural changes (notably an increased "stiffness") within baroreceptor walls, caused by

longstanding hypertension, reduce their elasticity and hence their ability to respond to changes in BP (Angell-James, 1973). Histological lesions in the arterial walls and receptors, demonstrated in chronic renovascular hypertension, may also affect baroreflex sensitivity (Angell-James, 1973).

Plasma NA concentrations were higher in 2-kidney rats than 1-kidney rats, and this was true of hypertensives and controls (see Tables 7.2-7.4, Chapter 7). The reason for this is unknown, but could reflect an important input of the kidneys to plasma NA; although further evidence presented later (see Chapters 11 and 12) contradicts such a hypothesis. If this was the case, however, then 1-kidney rats would be expected to have lower levels of plasma NA than 2-kidney rats. In humans the contribution of the kidneys to circulating NA has been estimated to be as low as 12% (Brown et al, 1981a). A later study (Esler et al, 1985) has calculated that whilst the kidneys are a major source of plasma NA, they contribute no more than 20-27% of total NA release to plasma in human subjects. However, this proportion was reported to be increased in patients with essential hypertension (Esler et al, 1985). On the basis of these observations it seems unlikely that differences in renal mass can totally explain the difference in plasma NA concentration between 1-kidney and 2-kidney rats obtained here. It may be explained by a 'period effect', since studies on the different models of hypertension were not performed in parallel, but were separated in time. The reason for the observed difference in circulating NA levels between the two models does not, however, affect the conclusions from the present study. Plasma NA concentrations were only compared between the hypertensive group and a parallel group of control rats within each model. No attempt was made to compare the absolute values between the three groups of hypertensive rats, only the

pattern of change on unclipping was compared between groups. Discussion on this point is continued later, in the light of further evidence obtained from the experiments reported in section III (see Chapter 12).

Models of renovascular hypertension could be potentially useful for the study of any interrelationship between the sympathetic and renin-angiotensin systems in the development and maintenance of hypertension; although this question was not addressed here. To avoid problems with excessive blood loss plasma renin concentration was not measured, nor were renin-angiotensin antagonists given as these might have influenced SNS activity. However, there is no evidence that increases in renin-angiotensin system activity are directly related to increases in SNS activity. There was no evidence of SNS activation in the early phase of 2K1C hypertension, when elevated levels of renin and angiotensin II are known to be present in the plasma in this model (Miksche et al, 1970; Oates et al, 1975). Elevated plasma NA concentration and heart rate were found in rats with 1K1C hypertension, at a time when renin-angiotensin system activity has been demonstrated to be normal or low (Swales, 1979a; Godfrey, Kumar, Bing, Swales & Thurston, 1985; see section 3.2.1).

In conclusion, the major findings of the experiments reported in this section using plasma NA and heart rate measurements as indices of SNS activity, are the clear differences demonstrated between rats with 1K1C and 2K1C hypertension; both during the established phase of hypertension and upon surgical reversal. There is evidence of SNS activation in the early phase of 1K1C hypertension, but not in either the early or chronic phases of the 2K1C model. The small reduction in indices of SNS activity observed after unclipping rats with short term 1K1C hypertension could implicate the SNS to a minor degree in reversal; but

since SNS tone is not reduced to the levels seen in controls this cannot be the only factor. Conversely, in the early phase of 2K1C hypertension SNS activity is increased after unclipping, probably stimulated by the fall in BP. No evidence was found to support a progressive increase in the contribution of the SNS to elevated arterial pressure in long term 2K1C hypertension.

SECTION III

TISSUE NORADRENALINE STUDIES:

Renal and cardiac noradrenaline levels in one-kidney and two-kidney, one-clip renovascular hypertension in the rat

CHAPTER 9

INTRODUCTION

INTRODUCTION

The experiments reported in section II demonstrated that plasma NA levels are increased in 1K1C but normal in 2K1C hypertension, despite similar degrees of hypertension in the two models. These studies were therefore extended to investigate whether the different plasma NA levels in the two models of hypertension were reflected in tissue levels of the neurotransmitter; particularly in the kidney which has been proposed to be a major source of plasma NA (Esler, Hasking, Willett, Leonard & Jennings, 1985; Goldstein, McCarty, Polinsky & Kopin, 1983b). Tissue NA measurements (Wegmann et al, 1962; Lefer & Ayers, 1969; Mogil et al, 1969; Barajas et al, 1976), catecholamine histochemical fluorescence techniques (Ljungqvist & Ungerstedt, 1972; Ljungqvist, 1974; Barajas et al, 1976) and electron microscopy (Barajas et al, 1976) have been used to study the level of sympathetic innervation to the kidney in animals with renovascular hypertension. However, most previous studies have been performed in dogs rather than rats. Furthermore, renal wrap models of renovascular hypertension have largely been employed, since these models avoid the need to manipulate the renal artery (Fink & Brody, 1978 and 1980).

Reduced catecholamine (Wegmann, Kako & Bing, 1962) and noradrenaline (NA) (Lefer & Ayers, 1969) content have been demonstrated in the clipped kidney of dogs with both 1K1C and 2K1C hypertension. Markedly reduced renal cortical NA content was also demonstrated in 1-kidney renal wrap hypertension, even though the renal artery is not manipulated in this model (Mogil, Itskovitz, Russell & Murphy, 1969). It has been proposed that adrenergic innervation to the ischaemic kidney is reduced or abolished in 2K1C hypertension in rats. Evidence for this is based on

measurements of renal catecholamine histofluorescence (Ljungqvist & Ungerstedt, 1972; Ljungqvist, 1974). However, the catecholamine content of the contralateral, untouched kidney has been reported to be reduced (Wegmann et al, 1962; Fink & Brody, 1978 and 1980) and normal (Ljungqvist & Ungerstedt, 1972; Ljungqvist, 1974; Barajas, Wang, Bennett & Wilburn, 1976) in the same model. Most investigators have found only modest changes in the NA content of the heart and other tissues in renal hypertension (Wegmann et al, 1962; Lefer & Ayers, 1969). The NA concentration in heart muscle is significantly lower in rats with both 1K1C and 2K1C hypertension, than in corresponding control animals (Robertson, Hodge, Laverty & Smirk, 1968). However, in this study the heart weight and heart weight/body weight ratios were increased in the hypertensive animals; thus the total NA content did not differ by a significant amount between hypertensive and control rats. The lower cardiac NA concentration therefore, could be explained by a "dilution effect" on a relatively constant amount of neurotransmitter by an increase in muscle mass (Robertson et al, 1968). Consequently in renal hypertension it is the kidney which appears to be most affected with regard to tissue NA content (Fink & Brody, 1978).

The only study of renal catecholamine content in rats with 2K1C hypertension showed reduced catecholamine content and histofluorescence in the ischaemic kidney, whilst both parameters were normal in the contralateral kidney (Barajas et al, 1976). A recent study in rats with 1K1C hypertension has shown that both renal and cardiac NA contents were significantly reduced, compared to sham controls (Racz, Kuchel, Buu & Garcia, 1986).

It has been suggested that reduced renal NA content in renovascular hypertension reflects a depletion of NA secondary to increased renal SNS

activity, and hence increased release or turnover of the neurotransmitter (Ljungqvist & Ungerstedt; 1972, Ljungqvist, 1974). A defect in storage and retention of NA within the specialised granules of the sympathetic nerves might result in an alteration in the amount of physiologically active NA available to the receptors (DeChamplain, Krakoff & Axelrod, 1967 and 1969; Krakoff, DeChamplain & Axelrod, 1967). Such a mechanism may explain the NA depletion found in several tissues, including kidney and heart, in 1-kidney desoxycorticosterone acetate (DOCA)/salt hypertension in rats. In these studies uptake of NA across the neuronal membrane into the cytoplasm was normal, but a defect in storage resulted in a depletion of tissue NA. It was further proposed that the state of sodium balance of the animal could have a role in control of NA storage. Changes in the ionic environment within the sympathetic nerve terminals, as a result of gross changes in sodium balance, might influence the binding and release characteristics of storage granules (DeChamplain et al, 1967 and 1969; Krakoff et al, 1967). However, in 1K1C hypertension reduced renal NA content has been localised to the soluble cytoplasm rather than storage granules and there was no reduction of endogenous NA in the granular fraction of any tissue studied; which argues against any defect in the ability of storage granules within sympathetic nerves to bind NA in this model (Lefer & Ayers,1969).

Alternatively, reduced renal NA content in experimental renovascular hypertension may reflect decreased renal sympathetic nerve activity; either because of reduced sympathetic innervation to the kidney, or decreased renal NA synthesis (Wegmann et al, 1962; Lefer & Ayers, 1969; Barajas et al, 1976; Fink & Brody, 1978). In support of this hypothesis the renal vasoconstrictor response to direct electrical stimulation of the renal nerves was significantly attenuated in the single kidney in the 1-kidney model, and the untouched kidney in the 2-kidney model, of

renal wrap hypertension in rats (Fink & Brody, 1978 and 1980). This could reflect diminished sympathetic neural influence on renal vasoconstrictor responses, since in both models the vessels themselves responded normally to intra-arterial infusions of NA. Furthermore, renal vascular responsiveness to nerve stimulation was reduced in parallel to the disappearance of catecholamine histofluorescence in the kidney (Fink & Brody, 1978).

Ultrastructural examination of the integrity of the renal sympathetic nerves in rats with 2K1C hypertension has yielded conflicting results. The reduced catecholamine histofluorescence observed in the ischaemic kidney has been associated with complete integrity of the renal sympathetic nerves (Ljungqvist, 1974). It was shown that catecholamine histofluorescence reappeared in the ischaemic kidney on incubation of specimens with α-methyl-noradrenaline; suggesting that the nerve terminals were not destroyed, since they were capable of neurotransmitter uptake (Ljungqvist, 1974). This has been disputed by Barajas et al (1976), who demonstrated that reduced catecholamine nerve histofluorescence and catecholamine content in the ischaemic kidney in 2K1C hypertension was associated with a marked loss of nerve terminals. Electron microscopy revealed that the nerves innervating the glomerular arterioles suffered the greatest interruption.

Neuropeptide Y (NPY) is a potent vasoconstrictor peptide consisting of 36 amino acids, which may be associated with noradrenergic nerves (Lundberg, Terenius, Hökfelt, Martling, Tatemoto, Mutt, Polak, Bloom & Goldstein, 1982). Within the kidney NPY has been demonstrated to be localised to the sympathetic nerves innervating the juxtaglomerular apparatus in several species (Ballesta, Polak, Allen & Bloom, 1984). Recently, NPY content has been shown to be significantly reduced in the

clipped kidney of rats with both 1K1C and 2K1C hypertension of 4-6 weeks duration. NPY content was also reduced in the contralateral (non-ischaemic) kidney in the 2K1C model, but to a lesser extent (Allen et al, 1986).

Thus there is little data on the NA content of tissues and organs in 1K1C and 2K1C renovascular hypertension in rats. Furthermore, in the studies that have been performed, tissue NA determinations have not been accompanied with parallel plasma NA measurements. In the experiments reported in this section parallel measurements of plasma, renal and cardiac NA were made in rats with both 1K1C and 2K1C hypertension of 4-6 weeks duration and parallel control groups. It was of particular interest to study the NA content of the contralateral kidney in comparison with that of the clipped kidney in 2K1C hypertension. Sympathetic nerves to the kidney run alongside the renal artery in the superficial adventitial layers (Barajas, 1978). In contrast to the induction of renal wrap models of renovascular hypertension, the positioning of a clip around the renal artery to induce Goldblatt models of renovascular hypertension may damage these nerves (Allen, Godfrey, Yeats, Bing & Bloom, 1986). This could confound the interpretation of any change in renal NA in these models (Fink & Brody, 1978 and 1980). In order to study the effect of any renal denervation during the application of the clip around the renal artery, two control groups were prepared for each model of hypertension; a loose clip group of rats as control for the elevated BP, and a sham-operated group as control for the manipulation of the renal artery involved in the procedure of clipping.

In view of the suggestion that NPY may co-exist with NA within sympathetic nerve terminals (Ballesta et al, 1984), it was of interest

to investigate whether renal and cardiac NA levels followed a similar pattern to those of NPY in 1K1C and 2K1C hypertension (Allen et al, 1986).

CHAPTER 10

MATERIALS AND METHODS

10.1 Animal Procedures

Most of the procedures have previously been described in detail in Chapter 5. Where this is the case the appropriate section is referenced. 1K1C and 2K1C hypertension were produced in female Wistar rats (section 5.1.1). For each model parallel groups of loose clip and sham-operated controls were prepared at the same time. Sham-operation consisted of a laparotomy and mobilisation of the left kidney only, the renal artery was not cleaned in order to leave renal nerves intact; with or without contralateral nephrectomy as appropriate. The indirect BP of all rats was monitored as before, and hypertensive and control animals were selected for study using the criteria described (see section 5.1.3). Four to six weeks after clipping or sham-operation, selected rats were cannulated (section 5.1.4) and allowed to recover from the operation for 48 hours. At the end of this period mean direct BP, heart rate and a 1ml blood sample for plasma NA determination were obtained, following exactly the same procedure as described earlier (section 5.1.5). Immediately after the blood sample for plasma NA concentration had been taken, a second 1ml blood sample was collected from the carotid cannula for plasma creatinine assay (see section 5.1.7). Two mls of heparinised saline was slowly infused to replace the volume of blood removed. Blood samples were immediately centrifuged and the plasma fractions separated and stored appropriately (sections 5.1.5 and 5.1.7) until assayed for NA or creatinine. The plasma creatinine assay is described in section 5.2. Rats were then killed by stunning and the kidneys and hearts harvested for subsequent NA determination as described below. The experimental protocol employed is illustrated in Figure 10.1.

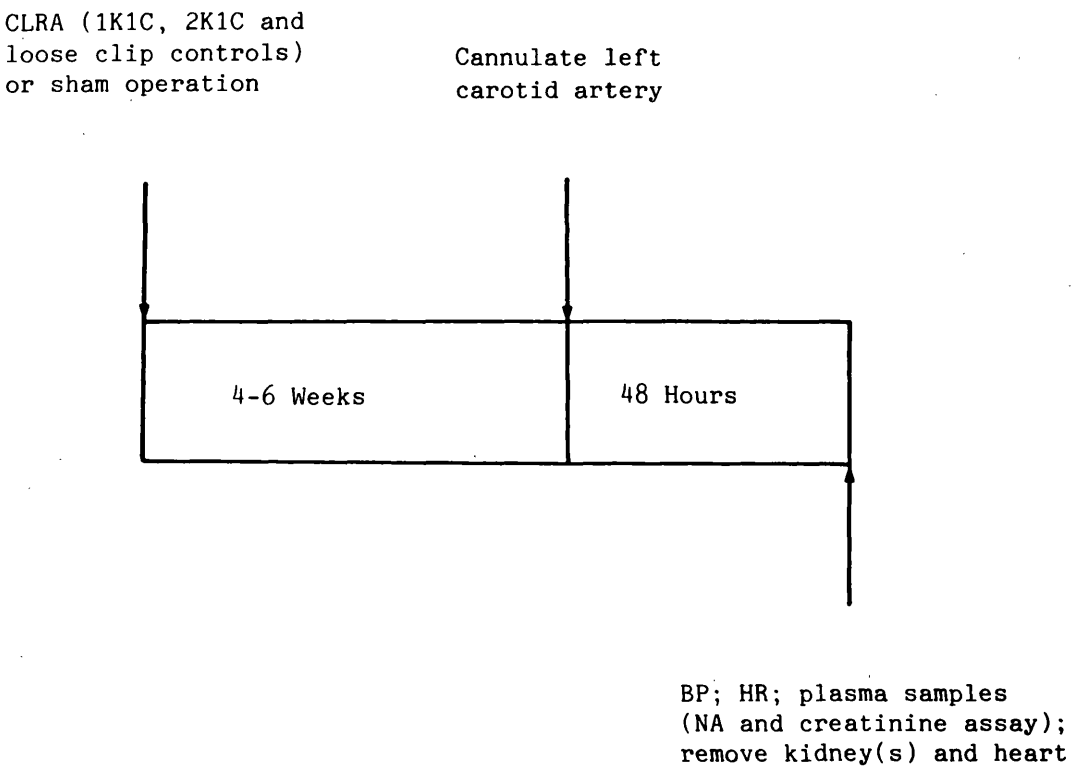


FIGURE 10.1

Experimental protocol 3: plasma, renal and cardiac noradrenaline (NA) measurements in rats with 1K1C and 2K1C hypertension and controls. CLRA = clip left renal artery.

10.1.1 Harvest and Storage of Organs

Immediately after killing, the kidney(s) and heart were carefully removed and cleaned of adhering fat and connective tissue by blunt dissection. The organs were then rinsed thoroughly with distilled water, gently blotted dry and placed in pre-weighed containers on ice. The wet weight of each organ was accurately recorded immediately. Once weighed each tissue sample was placed in a 10ml measuring cylinder and ice-cold distilled water added so that the volume of tissue plus distilled water was 8mls. The tissue was chopped into small pieces in the water with scissors and transferred to a 30ml capacity teflon (PTFE) on glass homogenisation tube (Jencon Scientific Ltd., Leighton Buzzard, UK). The tissue was then homogenised thoroughly in the water, and the resulting homogenate transferred to a 20ml Sterilin screw-top container. Dihydroxybenzylamine hydrobromide (1.5nmol DHB) in 1ml of 0.1M perchloric acid was added as internal standard and then 1ml of 1.0M perchloric acid. The homogenate was thoroughly mixed on a vortex mixer, before being stored at -70°C until assayed for NA. Therefore, tissues for NA assay were stored in 0.1M perchloric acid with a total volume of 10mls, including an internal standard to ensure that any loss of NA from the sample during storage or alumina extraction (see section 10.2 below) was accounted for automatically. The acidic conditions stabilised NA against oxidation.

10.2 Measurement of Noradrenaline in Plasma and Tissue Samples

Plasma samples were taken through an alumina extraction procedure (section 6.2) prior to injection on to the HPLC (section 6.3), and concentrations of NA were recorded and calculated as described in section 6.4.

10.2.1 Measurement of Tissue Noradrenaline Levels

Tissue NA levels were determined by a modification of the HPLC methods of Keller, Oke, Mefford & Adams (1976), and Sinaiko, Cooper & Mirkin (1980).

a) Sample preparation

After thawing to 4°C two 1ml aliquots from each homogenate were removed and placed in separate Eppindorf microcentrifuge tubes, and centrifuged at 11,600g for 10 minutes (Micro Centaur, MSE Scientific Instruments, Crawley, UK). Aliquots (250µl) of the resulting supernatants were then taken through an alumina extraction procedure similar to the one described for plasma samples in section 6.2. The standards and blank for the tissue extraction were prepared exactly as described in section 6.2; since the concentration of internal standard added to the homogenate prior to storage was calculated to give 37.5 pmol of DHB in each 250µl aliquot. This was a similar amount to that in the standard solution (250µl of 150nmol/L DHB). Batches consisting of samples, 2 standards and a reagent blank were taken through the following extraction procedure (Anton & Sayre, 1962; Keller et al, 1976; Sinaiko et al, 1980) (see section 6.2).

The pH of each tube was adjusted to 7.8-8.2 with 1M Tris buffer, pH 8.6, and 20-30mg of acid-washed alumina added. Tubes were then mixed on a spiral blood mixer for 15 minutes (Denly Spiramix, Sussex, UK). The alumina was allowed to settle and the supernatant removed and discarded. The contents of each tube were washed 5 times with ice-cold distilled water. On the final wash as much of the liquid as possible was removed, then 250µl of 0.1M perchloric acid was added. Each tube was placed on

ice and mixed frequently by manual agitation for 10-15 minutes. Finally the alumina was allowed to settle and the supernatant was removed and stored on ice. This extract was injected directly on to the HPLC.

b) Calculation of results

Tissue sample preparation and extraction was designed so that the HPLC system described in section 6.3 could be used to determine NA levels in tissue as well as plasma sample extracts. The electrochemical detector sensitivity was set to 20 nanoamperes full scale deflection in both cases. The amount of NA (nmol) in the original tissue homogenate was calculated : 0.1 x integrator result for 100µl of extract (pmol), and expressed both as nmol/g wet weight of tissue and nmol/whole organ. Tissue samples were assayed in duplicate and if the duplicates differed from each other by more than 10% the sample was reassayed.

c) Variability of the tissue noradrenaline assay

The variability within tissue NA assays was assessed by assaying 12 rat kidney homogenates in duplicate, and comparing the NA concentrations recorded. The combined mean NA concentration (nmol/g wet weight of tissue) was 1.38 (n=24), and the standard deviation of the difference between duplicates was 0.04 (n=12). The mean difference between duplicates (mean difference/combined mean x 100%) was 3%.

The variability between tissue NA assays was studied by measuring the NA concentrations in aliquots of rat kidney extracts on two separate occasions. The combined mean renal NA concentration and the standard deviation of the difference between aliquots were 2.03 (n=20) and 0.09 (n=10) nmol/g wet weight respectively. The mean difference between aliquots measured on separate occasions was 6%.

10.3 Statistics

Results are expressed as mean ± standard error of mean (SEM). Plasma, renal and cardiac NA levels were compared between the 3 groups in each model (hypertensive, loose clip and sham) using one-way analysis of variance in the first instance. If a significant F value was obtained from one-way analysis of variance, the variable was then compared between successive pairs of groups using the 'two sample test'. The two sample test is a parametric significance test which does not assume equal variance in the two populations under test. Comparisons of renal weights and renal NA levels within 2-kidney rats were made using the paired 'sign test'. Parametric correlation and regression analysis was performed where appropriate. All statistical analyses were performed using the Minitab statistics software package (see section 5.3).

CHAPTER 11

RESULTS

11.1 1K1C Hypertension

11.1.1 General Details (Table 11.1)

Blood pressure and body weight were significantly greater in rats with 1K1C hypertension compared to the two normotensive control groups ($P<0.01$ and $P<0.05$ respectively). Plasma creatinine concentrations were similar in all three groups of 1-kidney rats. Plasma noradrenaline (NA) and heart rate were increased in 1K1C hypertension, as reported previously (section II). However, in this case the increase in these variables failed to achieve statistical significance compared to the control groups ($P>0.05$). Plasma NA was lower in the sham-operated group compared to loose clip rats, but this difference was not significant ($P>0.05$). In agreement with the results reported in the previous experiments (section II), plasma NA concentration and heart rate were significantly correlated in 1K1C hypertension ($r = +0.58$, $P<0.02$; performed on ranked data).

11.1.2 Organ Weights (Table 11.2)

The wet weight of the kidney was greater in 1K1C hypertensive rats than in the normotensive controls ($P<0.05$, one-way analysis of variance); this difference was statistically significant compared to the loose clip group ($P<0.02$), but not compared to sham-operated rats ($P>0.2$). There was a significant increase in the wet weight of the heart in the hypertensive group, compared to both loose clip and sham-operated rats ($P<0.001$).

TABLE 11.1

Body weight, mean arterial pressure (MAP), heart rate, plasma noradrenaline and plasma creatinine concentrations in rats with 1K1C hypertension and the parallel loose clip and sham-operated control groups. Values are mean ± SEM.
NS = not significant (P>0.05)

Group	Body weight (g)	MAP (mmHg)	Plasma noradrenaline (pmol/ml)	Heart rate (beats/min)	Plasma creatinine (µmol/L)
1K1C hypertension (n=15)	214±3	171±5	1.88±0.20	384±12	47±3
1-kidney loose clip controls (n=14)	204±3	108±3	1.70±0.12	371±11	44±4
1-kidney sham-operated controls (n=14)	207±3	106±3	1.56±0.11	372±11	43±4
One-way analysis of variance	P<0.05	P<0.01	NS	NS	NS

TABLE 11.2

Wet weight of the kidney and heart in rats with 1K1C hypertension and the parallel loose clip and sham-operated control groups. Values are mean ± SEM.

* P<0.01 vs sham-operated group; + P<0.02 vs loose clip group.

Group	Organ wet weight (g)	
	Left kidney	Heart
1K1C hypertension (n=15)	0.88±0.03^{+}	1.06±0.03^{*+}
1-kidney loose clip controls (n=14)	0.79±0.02	0.70±0.02
1-kidney sham-operated controls (n=14)	0.83±0.02	0.71±0.01
One-way analysis of variance	P<0.05	P<0.01

11.1.3 Renal and Cardiac Noradrenaline Levels

Renal and cardiac NA levels have been calculated in nmol and expressed as both the concentration (nmol/g wet weight of tissue) and total content (nmol/whole organ).

a) Renal and cardiac noradrenaline concentration (nmol/g wet weight of tissue) (Table 11.3 and Figure 11.1)

One-way analysis of variance on the NA concentrations in both the kidney and heart revealed significant differences between the groups ($P<0.01$). Subsequent analysis using the two-sample test showed that renal NA concentration was significantly reduced in 1K1C hypertension compared to sham-operated controls ($P<0.001$). Renal NA concentration was also significantly reduced in 1-kidney loose clip rats ($P<0.001$ vs sham), but remained higher than in hypertensive rats ($P<0.02$). The NA concentration in the heart was significantly reduced in 1K1C hypertension ($P<0.001$ vs both normotensive control groups), whilst being similar in loose clip and sham-operated rats ($P>0.9$).

Renal and cardiac NA concentrations were significantly correlated in the loose clip ($r = +0.72$, $P<0.005$) and sham-operated ($r = +0.64$, $P<0.02$) control groups, and in 1K1C hypertension ($r = +0.62$, $P<0.01$).

b) Total renal and cardiac noradrenaline content (nmol/whole organ) (Table 11.3 and Figure 11.2)

A similar pattern to that described above for tissue NA concentration was observed for total renal and cardiac NA contents. However, owing to differences in organ weights between the groups, some of the statistical

significances were lost. As described for NA concentration, there was a graded response in renal NA content in the three groups (i.e. 1K1C < 1-kidney loose clip < 1-kidney sham). Total renal NA content was significantly reduced in both the 1K1C and 1-kidney loose clip groups, compared to sham-operated rats ($P<0.001$). However, although the NA content of the kidney in 1K1C hypertension was lower than in loose clip controls, this difference failed to achieve statistical significance ($P=0.07$).

There was a modest reduction in the NA content of the heart in 1K1C hypertensive rats compared to normotensive controls; but one-way analysis of variance showed that there was no significant difference between any of the three groups ($P>0.05$). The modest change in total cardiac NA content in 1K1C hypertension is in contrast to the significant reduction in NA concentration in the heart. This difference probably reflects the significant cardiac hypertrophy found in hypertensive rats (section 11.1.2). The reduction in total NA content in 1K1C hypertension was therefore more pronounced in the kidney than in the heart.

There was a significant positive correlation between the total NA contents of the kidney and heart in normotensive rats ($r = +0.67$, $P<0.01$ and $r = +0.74$, $P<0.01$ for the loose clip and sham-operated groups respectively). However, this significant correlation was lost in rats with 1K1C hypertension ($r = +0.43$, $P>0.05$).

TABLE 11.3

Concentration (nmol/g wet weight of tissue) and total content (nmol/whole organ) of noradrenaline in the kidney and heart of rats with 1K1C hypertension and the parallel loose clip and sham-operated control groups. Values are mean ± SEM. NS = not significant (P>0.05).

*P<0.001 vs sham-operated rats; +P<0.02 vs loose clip rats.

Group	Left Kidney		Heart	
	noradrenaline concentration (nmol/g)	noradrenaline content (nmol/kidney)	noradrenaline concentration (nmol/g)	noradrenaline content (nmol/heart)
1K1C hypertension (n=15)	1.05±0.09*+	0.91±0.07*	5.53±0.40*+	5.79±0.38
1-kidney loose clip controls (n=14)	1.59±0.19*	1.28±0.18*	10.24±0.64	7.23±0.54
1-kidney sham-operated controls (n=14)	2.67±0.16	2.23±0.13	10.35±0.96	7.29±0.68
One-way analysis of variance	P<0.01	P<0.01	P<0.01	NS

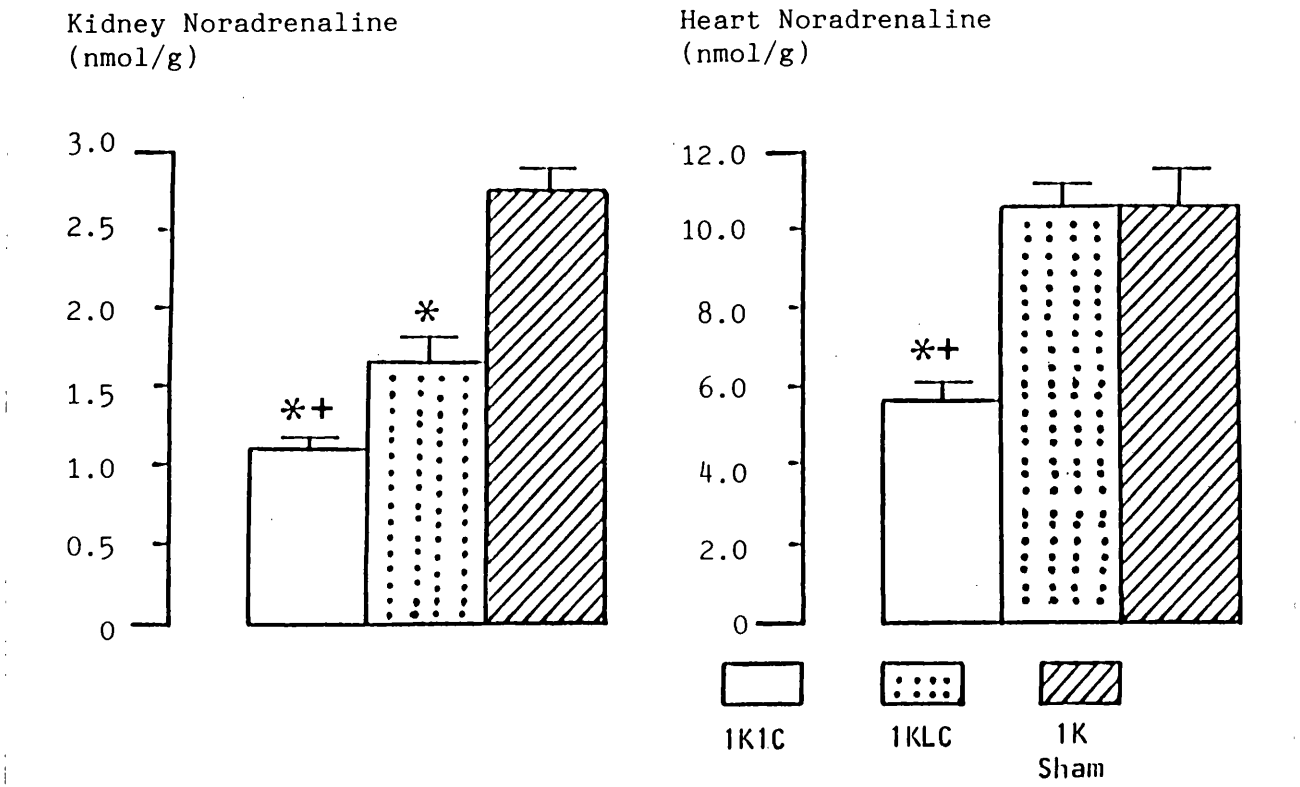


FIGURE 11.1

Renal and cardiac noradrenaline concentration (nmol/g wet weight of tissue) in rats with 1K1C hypertension (1K1C, n=15), and in loose clip (1KLC, n=14) and sham-operated (1K Sham, n=14) control groups. Values are mean and SEM. *P<0.001 vs sham-operated group; + P<0.02 vs loose clip group.

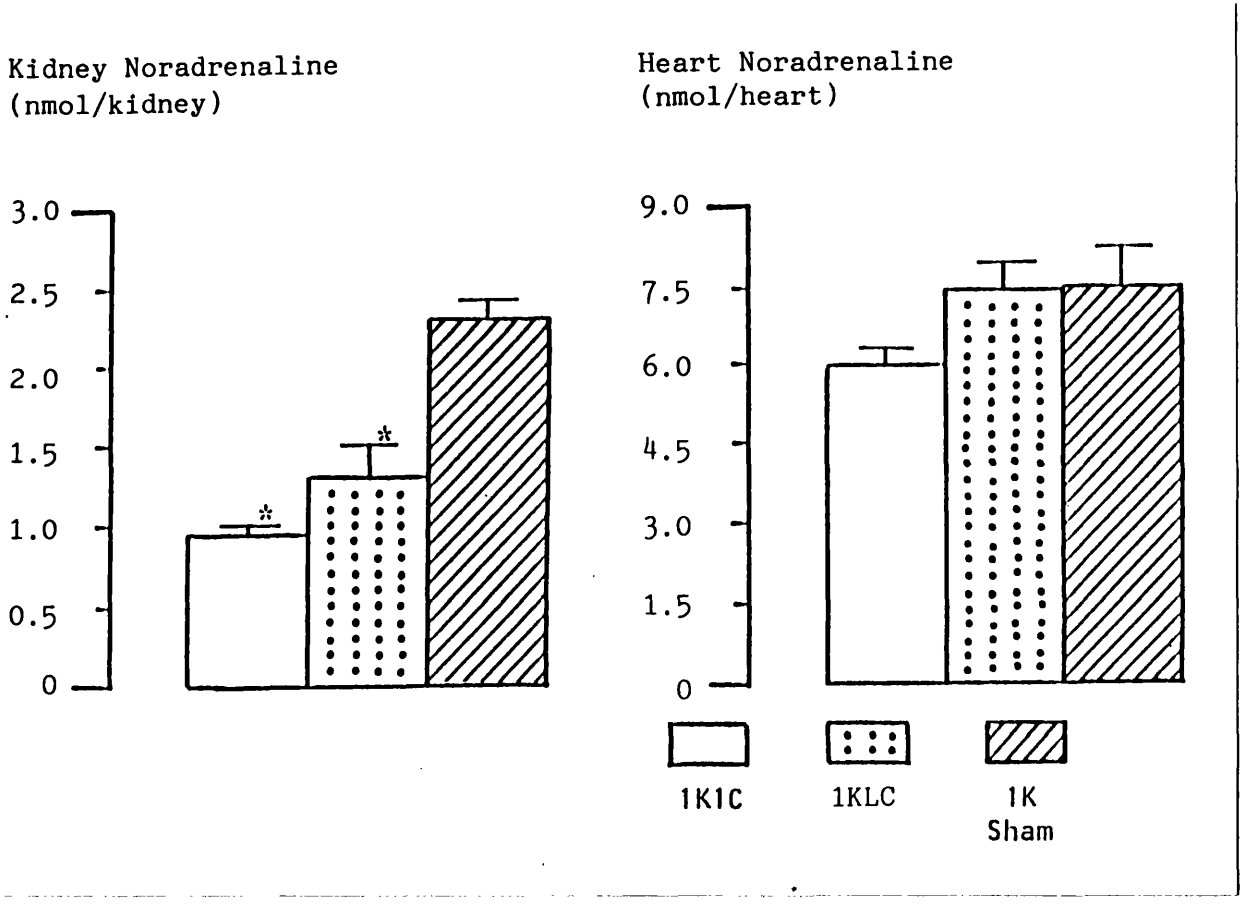


FIGURE 11.2

Total renal and cardiac noradrenaline content (nmol/whole organ) in rats with 1K1C hypertension (1K1C, n=15), and in loose clip (1KLC, n=14) and sham-operated (1K Sham, n=14) control groups. Values are mean and SEM. *P<0.001 vs sham-operated group.

11.2 2K1C Hypertension

11.2.1 General Details (Table 11.4)

Blood pressure (BP) was significantly elevated in 2K1C rats ($P<0.01$), but there were no differences in body weight or plasma creatinine concentration between any of the groups studied ($P>0.05$). Plasma NA concentration was similar in 2K1C hypertension compared to corresponding loose clip controls, confirming the results reported in section II. Furthermore, there was no correlation between heart rate and plasma NA in 2K1C rats ($r = 0.31$, $P>0.2$; performed on ranked data).

11.2.2 Organ Weights (Table 11.5)

The wet weight of the left kidney in 2K1C hypertensive rats was significantly decreased ($P<0.05$), whilst the wet weight of the contralateral kidney and the heart were both significantly increased ($P<0.001$), compared to either loose clip or sham-operated control groups. There was no difference in the wet weight of any of these organs between loose clip and sham-operated rats. The right kidney was heavier than the left kidney in all 2-kidney rats studied ($P<0.01$ for all 3 groups, paired sign test).

TABLE 11.4

Body weight, mean arterial pressure (MAP), heart rate, plasma noradrenaline and plasma creatinine concentrations in rats with 2K1C hypertension and the parallel loose clip and sham-operated control groups. Values are mean ± SEM.
NS = not significant (P>0.05).

Group	Body weight (g)	MAP (mmHg)	Plasma noradrenaline (pmol/ml)	Heart rate (beats/min)	Plasma creatinine (μmol/L)
2K1C hypertension (n=12)	210±5	171±5	1.31±0.10	417±15	43±5
2-kidney loose clip controls (n=13)	209±3	106±4	1.33±0.12	368±8	43±3
2-kidney sham-operated controls (n=12)	213±2	106±3	1.06±0.13	353±9	45±4
One-way analysis of variance	NS	P<0.01	NS	P<0.01	NS

TABLE 11.5

Wet weight of the kidneys and heart in rats with 2K1C hypertension and the parallel loose clip and sham-operated control groups. Values are mean ± SEM.

* P<0.05 vs sham-operated group; + P<0.02 vs loose clip group; 0 P<0.01 vs the contralateral untouched kidney.

	Organ wet weight (g)		
Group	Left kidney	Right kidney	Heart
2K1C hypertension (n=12)	0.57±0.03^{*+o}	0.95±0.03^{*+}	1.04±0.02^{*+}
2-kidney loose clip controls (n=13)	0.65±0.02^{o}	0.71±0.02	0.78±0.02
2-kidney sham-operated controls (n=12)	0.64±0.01^{o}	0.71±0.01	0.75±0.02
One-way analysis of variance	P<0.05	P<0.01	P<0.01

11.2.3 Renal Noradrenaline Levels

a) Renal noradrenaline concentration (nmol/g wet weight of tissue)
(Table 11.6 and Figure 11.3)

One-way analysis of variance showed there was a significant difference between the groups in both left and right kidney NA concentration in 2-kidney rats ($P<0.01$).

NA concentration (nmol/g wet weight of tissue) in the left (clipped) kidney was significantly reduced in 2K1C hypertension compared to sham-operated rats ($P<0.001$). Clipped kidney NA concentration was also reduced in 2-kidney loose clip rats ($P<0.02$ vs sham), but remained significantly higher than in the hypertensive group ($P<0.05$). There was a significant reduction in the NA concentration of the contralateral untouched kidney in 2K1C hypertension, compared to both normotensive control groups ($P<0.001$). Left kidney NA concentration was slightly higher than that of the right kidney in 2K1C rats, but this difference failed to achieve statistical significance ($P>0.05$). Right kidney NA concentration was similar in loose clip and sham-operated rats ($P>0.9$). Consequently, renal NA concentration was significantly lower on the left compared to the right side in the 2-kidney loose clip group ($P<0.01$). Conversely, NA concentration in the left kidney was significantly higher than in the right kidney in sham-operated rats ($P<0.01$).

In the loose clip and sham-operated control groups, there was a strong correlation between the NA concentrations of the left and right kidneys ($r = +0.93$, $P<0.001$ for both groups). There was, however, no significant correlation between left and right renal NA concentrations in 2K1C hypertension ($r = +0.33$, $P>0.2$).

b) Renal noradrenaline content (nmol/kidney)

(Table 11.6 and Figure 11.4)

An almost identical pattern to the one described above for NA concentration was observed when renal NA levels were expressed in nmol/kidney. One-way analysis of variance showed a significant difference between the groups in both left and right kidney NA contents ($P<0.01$). Total NA content in the left (clipped) kidney was significantly reduced in 2K1C hypertension compared to both sham-operated ($P<0.001$) and loose clip ($P<0.01$) controls. Left kidney NA content was also significantly reduced in loose clip compared to sham-operated rats ($P<0.05$); demonstrating a graded level of NA content in the left kidney in the 3 groups, exactly as reported for NA concentration previously (i.e. 2K1C hypertension < 2-kidney loose clip < 2-kidney sham). Total NA content in the right (untouched) kidney was significantly lower in 2K1C hypertensive rats, compared to both control groups ($P<0.001$). There was no significant difference between the NA contents of the two kidneys within 2K1C rats ($P>0.05$); indicating that the depletion in renal NA content was to a similar extent in the clipped and contralateral kidneys in this group. Right kidney NA content was similar in loose clip and sham-operated rats ($P>0.8$). The NA content of the clipped kidney was significantly lower than that of the contralateral kidney within 2-kidney loose clip rats ($P<0.01$). There was no significant difference in total NA content between the two kidneys in sham-operated rats ($P>0.2$).

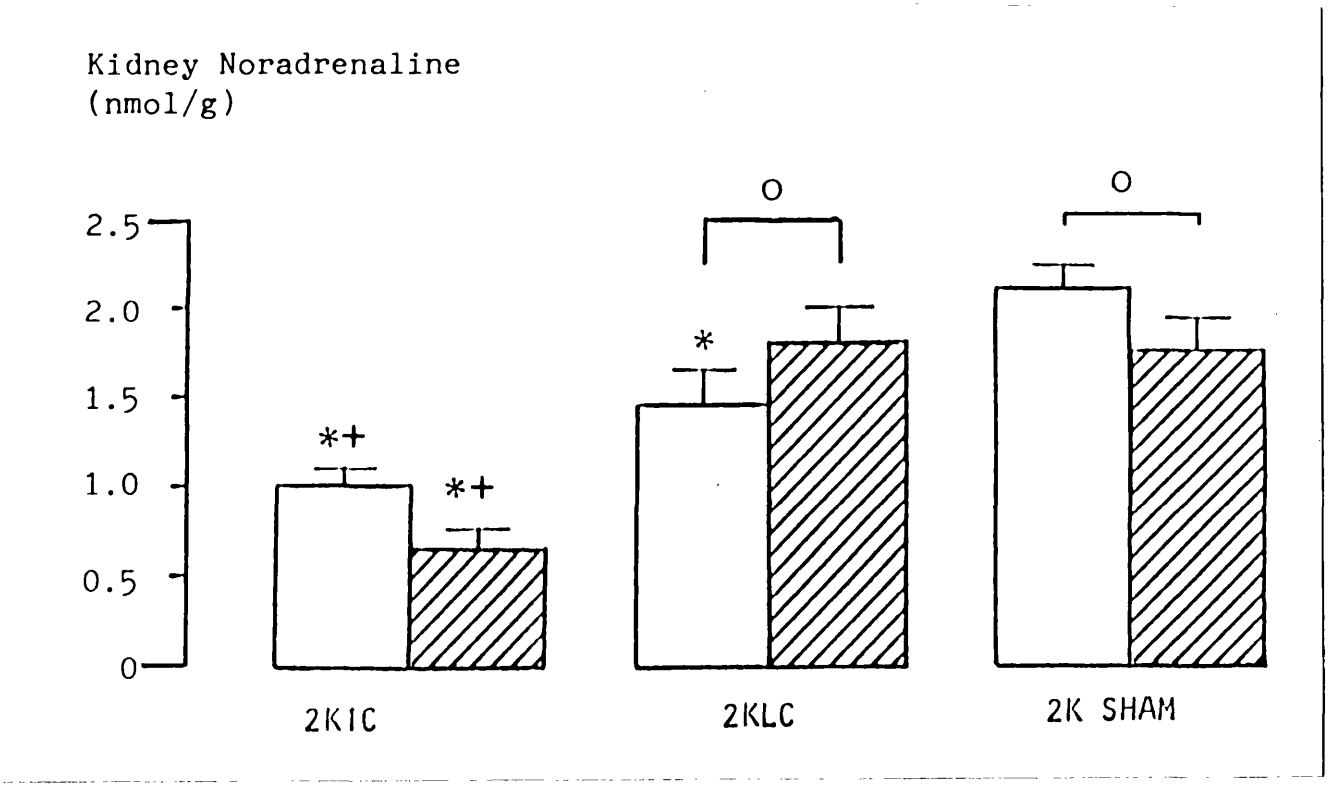
As observed for renal NA concentration, total NA contents in the left and right kidneys were strongly correlated in both loose clip and sham-operated groups ($r = +0.93$, $P<0.001$ for both groups). There was, however, no significant correlation between the total NA contents of the kidneys in 2K1C hypertension ($r = +0.50$, $P>0.05$).

TABLE 11.6

Concentration (nmol/g wet weight of tissue) and total content (nmol/kidney) of noradrenaline in the kidneys of rats with 2K1C hypertension and the parallel loose clip and sham-operated control groups. Values are mean ± SEM.

*P<0.05 vs sham-operated rats; $^{+}$P<0.05 vs loose clip rats; ^{o}P<0.01 vs the contralateral untouched kidney.

Group	Left Kidney (clipped/sham)		Right Kidney	
	noradrenaline concentration (nmol/g)	noradrenaline content (nmol/kidney)	noradrenaline concentration (nmol/g)	noradrenaline content (nmol/kidney)
2K1C hypertension (n=12)	0.97±0.11^{*+}	0.54±0.05^{*+}	0.64±0.08^{*+}	0.59±0.06^{*+}
2-kidney loose clip controls (n=13)	1.41±0.18^{*o}	0.93±0.13^{*o}	1.75±0.19	1.26±0.16
2-kidney sham-operated controls (n=12)	2.02±0.18^{o}	1.28±0.11	1.72±0.16	1.23±0.13
One-way analysis of variance	P<0.01	P<0.01	P<0.01	P<0.01



FIGUR E 11.3

Renal noradrenaline concentration (nmol/g wet weight of tissue) in the left (clipped/sham) kidney (clear bars) and right (untouched) kidney (hatched bars) in rats with 2K1C hypertension (2K1C, n=12), and in loose clip (2KLC, n=13) and sham-operated (2K Sham, n=12) control groups. Values are mean and SEM.

*P<0.02 vs sham-operated group; + P<0.05 vs loose clip group; o P<0.01 for the difference between the kidneys.

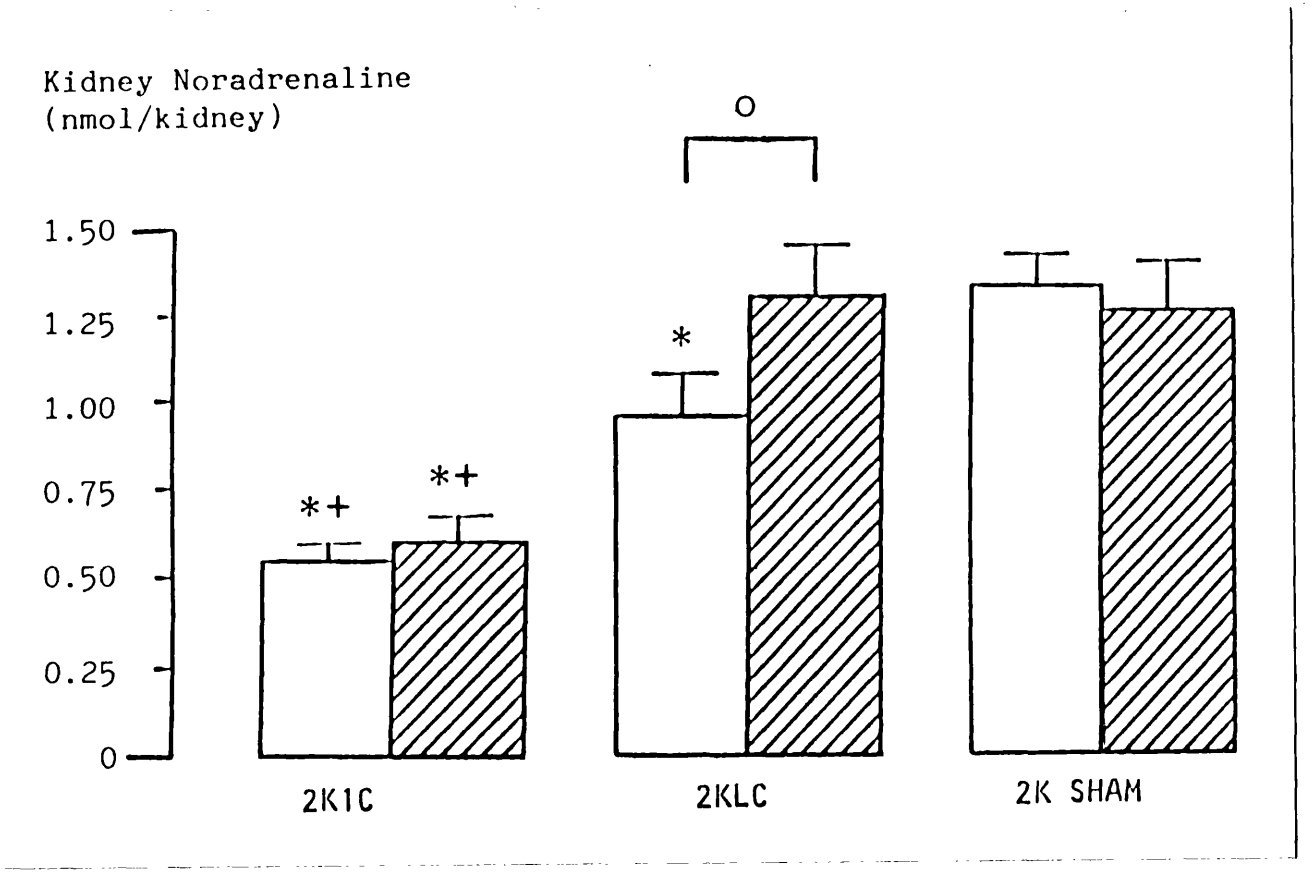


FIGURE 11.4

Total renal noradrenaline content (nmol/kidney) in the left (clipped/sham) kidney (clear bars) and right (untouched) kidney (hatched bars) in rats with 2K1C hypertension (2K1C, n=12), and in loose clip (2KLC, n=13) and sham-operated (2K Sham, n=12) control groups. Values are mean and SEM.

*P<0.05 vs sham-operated group; + P<0.01 vs loose clip group; o P<0.01 for the difference between the kidneys.

11.2.4 Cardiac Noradrenaline Levels (Table 11.7, Figures 11.5 and 11.6)

One-way analysis of variance showed there was a difference between the groups in both concentration and total content of NA in the heart ($P<0.01$ and $P<0.05$ respectively).

Cardiac NA concentration (nmol/g wet weight of tissue) was significantly reduced in 2K1C hypertension compared to both control groups ($P<0.001$). Cardiac NA concentration was similar in loose clip and sham-operated rats ($P>0.7$). In the two normotensive groups there was a strong correlation between the NA concentrations in the left kidney and heart, and between the NA concentrations in the right kidney and heart ($r > +0.8$, $P<0.001$). In 2K1C hypertension this relationship between renal and cardiac NA concentrations was less obvious (left kidney vs heart: $r = +0.52$, $P<0.05$; right kidney vs heart: $r = +0.29$, $P>0.2$).

Similar trends were observed when results were expressed as total cardiac NA content (nmol/heart). There was a modest reduction in cardiac NA content in 2K1C hypertension; this was statistically significant compared to loose clip ($P<0.01$) but not sham-operated rats ($P=0.07$). There was, however, no significant difference in this variable between the two normotensive control groups ($P>0.5$). Therefore, as in the 1K1C model, depletion of total NA content was more marked in the kidneys (both ipsilateral and contralateral) than in the heart in 2K1C hypertension. The modest reduction in cardiac NA content in 2K1C rats is in contrast to the significant reduction in cardiac NA concentration compared to controls. The significant hypertrophy of the heart which occurs in hypertensive rats (section 11.2.2) accounts for this difference. Left kidney and right kidney NA contents were correlated with cardiac NA content in both loose clip ($r > +0.8$, $P<0.001$)

and sham-operated rats ($r > +0.77$, $P < 0.005$). Neither clipped kidney nor contralateral kidney NA content was correlated with heart NA content in 2K1C hypertension ($r = +0.18$ and $+0.33$ respectively, $P > 0.2$).

TABLE 11.7

Concentration (nmol/g wet weight of tissue) and total content (nmol/heart) of noradrenaline in the heart of rats with 2K1C hypertension and the parallel loose clip and sham-operated control groups. Values are mean ± SEM.

*P<0.01 vs sham-operated rats; $^{+}$P<0.01 vs loose clip rats.

Group	Heart	
	noradrenaline concentration (nmol/g wet weight)	noradrenaline content (nmol/heart)
2K1C hypertension (n=12)	3.44±0.25^{*+}	3.58±0.29^{+}
2-kidney loose clip controls (n=13)	6.38±0.59	4.90±0.36
2-kidney sham-operated controls (n=12)	6.10±0.60	4.56±0.42
One-way analysis of variance	P<0.01	P<0.05

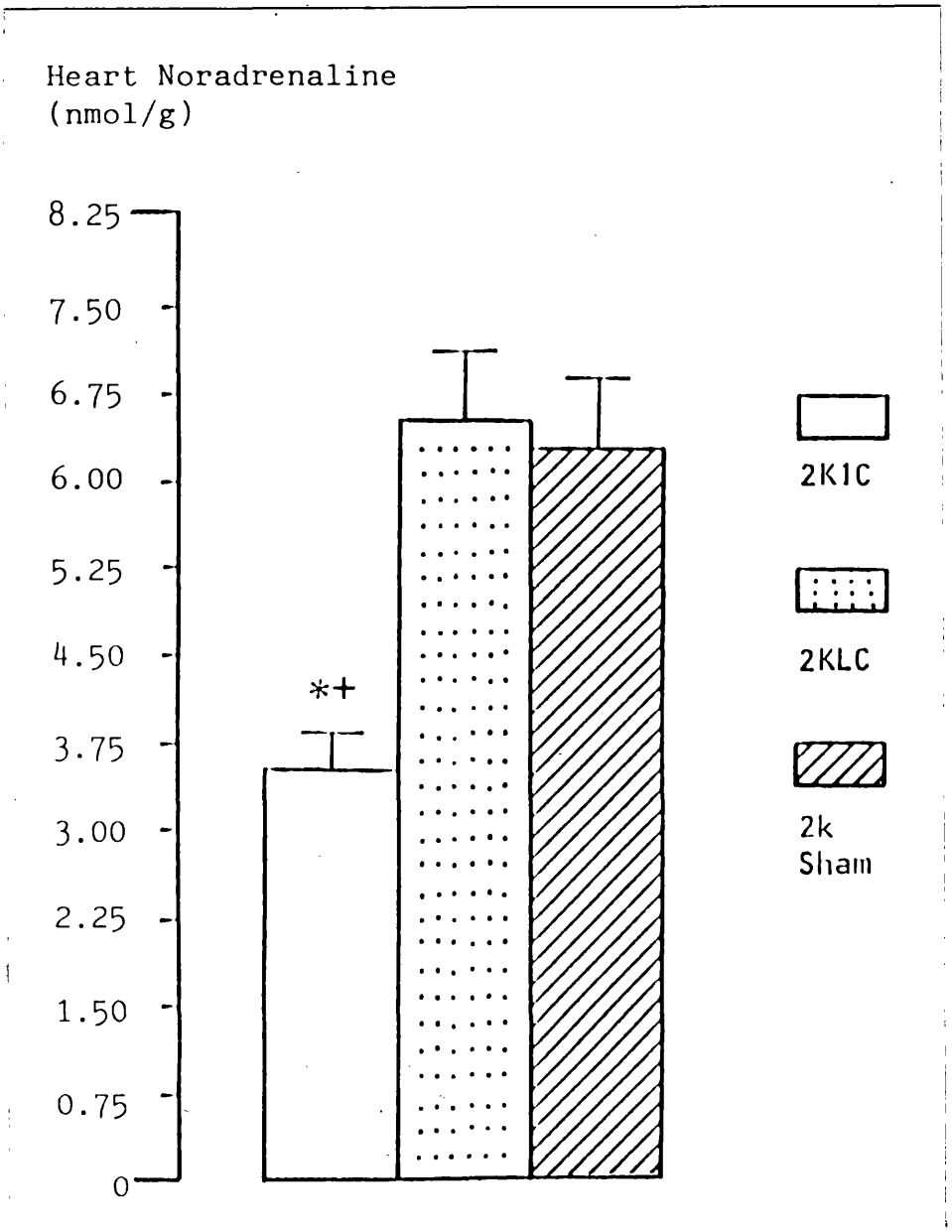


FIGURE 11.5

Cardiac noradrenaline concentration (nmol/g wet weight of tissue) in rats with 2K1C hypertension (2K1C, n=12), and in loose clip (2KLC, n=13) and sham-operated (2K Sham, n=12) control rats. Values are mean and SEM.

*P<0.001 vs sham-operated group; $^{+}$P<0.001 vs loose clip group.

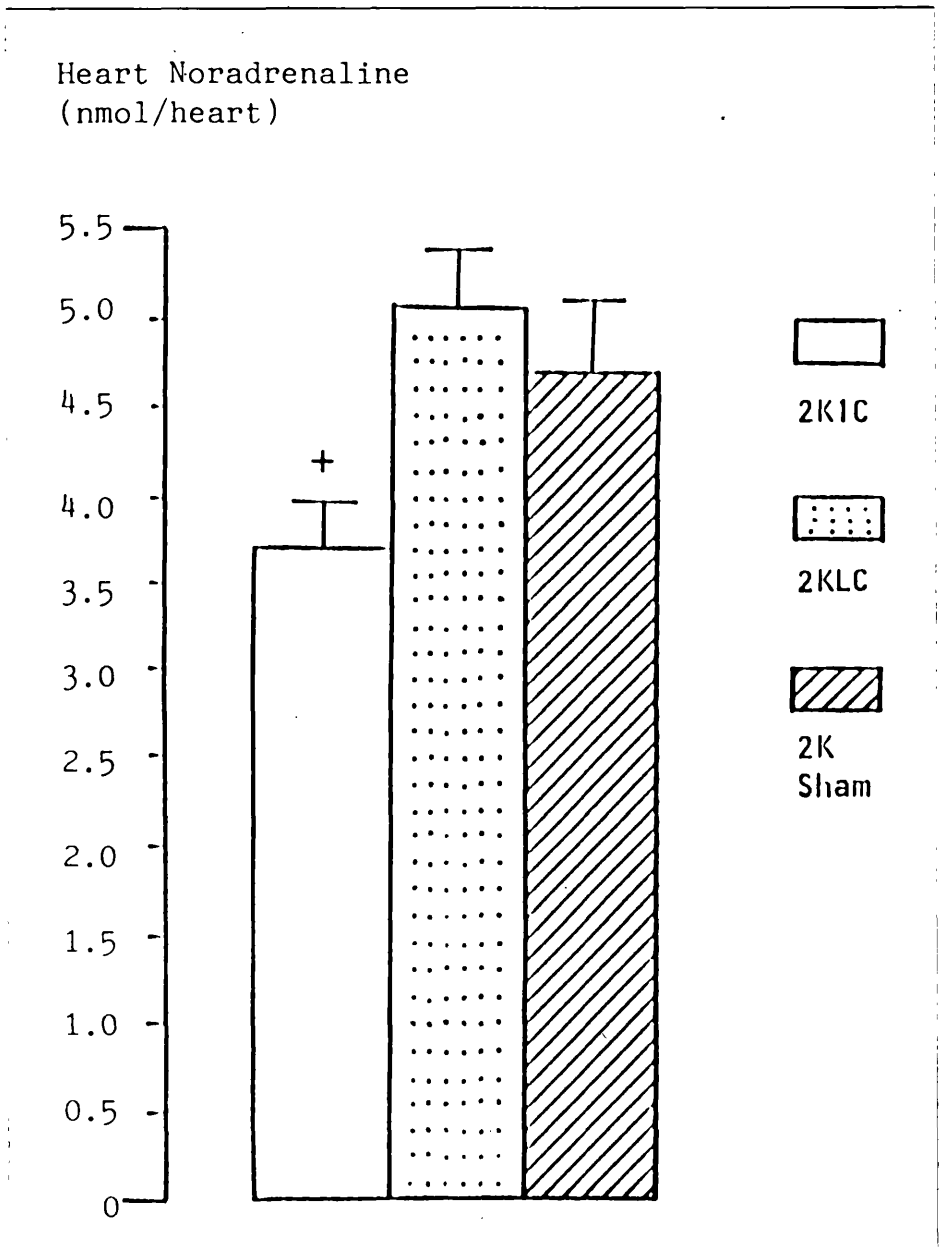


FIGURE 11.6

Total cardiac noradrenaline content (nmol/heart) in rats with 2K1C hypertension (2K1C, n=12), and in loose clip (2KLC, n=13) and sham-operated (2K Sham, n=12) control rats. Values are mean and SEM.

$^{+}P<0.01$ vs loose clip group.

CHAPTER 12

DISCUSSION

DISCUSSION

The application of a loose clip to the renal artery in uninephrectomised rats produced a significant reduction in renal NA concentration, with no effect on cardiac NA concentration, compared to sham-operated controls. 1K1C hypertension was associated with a further reduction in renal NA concentration and a significant reduction in the NA concentration of the heart. Similarly, the procedure of loose clipping one renal artery in 2-kidney rats produced a reduction in ipsilateral renal NA concentration compared to sham-operated rats. This was not associated with any effect on the NA concentrations in the contralateral kidney or the heart in loose clip rats. 2K1C hypertension, on the other hand, resulted in a further reduction in clipped kidney NA concentration, a similar reduction in contralateral kidney NA concentration, and a significant reduction in cardiac NA concentration. There was therefore, a definite graded response in left (clipped or sham) kidney NA concentration in the three groups in both models: hypertensive < loose clip < sham. This may indicate that the procedure of clipping and hypertension (and/or renal ischaemia) have independent, additive effects on ipsilateral renal NA concentration. Hypertension, however, has a more generalised effect which includes contralateral renal and cardiac NA concentration.

The pattern of renal and cardiac NA contents (nmol/whole organ) in 1K1C, 2K1C and control rats was similar to that described above for the concentration of NA. However, owing to differences in organ weight between hypertensive and control rats, some of the statistical significances were lost (see Chapter 11). This is particularly so for cardiac NA, NA concentration was significantly reduced in both 1K1C and 2K1C hypertension compared to corresponding control groups. However, both models were associated with significant hypertrophy of the heart

and the reduction in total NA content in the heart was much less pronounced. Therefore, the lower cardiac NA concentration in 1K1C and 2K1C hypertension could be explained, at least in part, by a "dilution effect" on a relatively constant amount of neurotransmitter by the increased muscle mass (Robertson et al, 1968). Consequently, in terms of total NA content, tissue NA depletion was more pronounced in the kidney than the heart in 1K1C and 2K1C hypertension. This is in agreement with the results of others (Wegmann et al, 1962; Lefer & Ayers, 1969).

In both models there was a strong relationship between renal and cardiac NA concentration in the normotensive control groups, whilst this was less obvious in hypertensive animals. The disparity between normotensive and hypertensive animals was more pronounced when results were expressed as total NA contents. These results suggest hypertension may have a differential effect on NA handling by the kidney(s) and heart in both the 1K1C and 2K1C models.

The wet weight of the right kidney was significantly greater than that of the left kidney in all 2-kidney groups. This finding was expected in 2K1C hypertension where the clipped kidney was visibly reduced in size, whilst there was hypertrophy of the contralateral kidney. It was, however, unexpected in the sham-operated group where the difference in renal weight could not be due to denervation, since the renal artery was not manipulated. In previous experiments the same tendency for the right kidney to be heavier than the left has been observed in female Wistar rats, of similar age to the ones used in the present study. It seems likely, therefore, that this is a characteristic of the strain of rat. This phenomenon may, however, influence the interpretation of renal NA levels in 2-kidney sham-operated rats. Renal NA concentration

was significantly higher in the left compared to the right kidney, whilst there was no difference in total NA content between the kidneys in this group.

The reduction in ipsilateral renal NA concentration which occurs in 1-kidney and 2-kidney loose clip rats, compared to corresponding sham controls, is consistent with an interruption of the efferent renal nerves during the procedure of placing a clip around the renal artery. However, the contralateral depletion of renal NA concentration associated with 2K1C hypertension, cannot be explained on the grounds of mechanical denervation. In 2-kidney loose clip rats NA concentration and content were both significantly reduced in the left compared to the right kidney. However, neither of these variables were significantly different between the kidneys in 2K1C hypertension.

The results obtained for 1K1C hypertension are in general agreement with previous work. A recent study (Racz et al, 1986) demonstrated reduced renal and cardiac NA contents in rats with 1K1C hypertension. Earlier experiments in dogs suggested 1K1C hypertension was associated with a depletion of renal, but not cardiac NA content (Wegmann et al, 1962; Lefer & Ayers, 1969). The data for 2K1C hypertension are more conflicting. Depletion of catecholamines has been reported to occur in both kidneys, even when only one renal artery is clipped (Wegmann et al, 1962). However, previous studies in rats have shown that tissue NA content, and catecholamine nerve histofluorescence, were significantly reduced in the ischaemic kidney; whilst both factors were normal in the non-ischaemic kidney (Barajas et al, 1976; Ljungqvist, 1974). The degree of hypertension in 2K1C rats in the present study (mean arterial pressure, 171±5mmHg) was greater than that reported by Barajas et al (1976) (systolic BP, 157±4mmHg). This may explain, at least in part,

why the NA depletion in the untouched kidney of 2K1C hypertensive rats observed here was not seen by Barajas et al (1976).

Rats with 2K1C hypertension were studied 4-6 weeks after clipping in the present study. It is possible that renal NA depletion may reverse in the chronic stages of this model (Ljungqvist & Ungerstedt, 1972). However, in 2-kidney renal wrap hypertension the reduction in renal catecholamine histofluorescence was maintained in rats with chronic hypertension (Fink & Brody, 1978). The pattern of renal and cardiac NA levels on unclipping rats with 1K1C and 2K1C hypertension is also unknown and warrants investigation.

Comparison of loose clip and sham-operated groups in each model enabled the denervating effect of clipping to be studied in the absence of any elevation in BP. The application of a clip around the renal artery appears to produce only a partial denervation of the ipsilateral kidney. This is supported by the observation that whilst the NA content of the clipped kidney was reduced in 1K1C hypertension, surgical denervation (achieved by stripping the renal artery and painting with 20% phenol) produced a significantly greater decrease in NA content (Villarreal et al, 1984). Ultrastructural examination has led to conflicting results regarding the integrity of the renal sympathetic nerves to the clipped kidney in rats with 2K1C hypertension. Thus reduced adrenergic nerve histofluorescence in the clipped kidney has been reported to be associated with complete integrity of the renal sympathetic nerve terminals (Ljungqvist, 1974). Conversely, Barajas et al (1976) demonstrated a dramatic reduction in the number of sympathetic nerve terminals associated with the ischaemic kidney in rats with 2K1C hypertension (see Chapter 9).

The reduction in endogenous renal NA in 1K1C and 2K1C hypertension could reflect a NA-depleting effect of locally enhanced release and turnover of the neurotransmitter. This would indicate that increased renal sympathetic nerve activity may be important to the development of renovascular hypertension (Ljungqvist & Ungerstedt, 1972; Ljungqvist, 1974). Reduced renal NA concentration could, however, equally reflect reduced renal sympathetic nerve activity; with renal hypertensive animals releasing less NA per impulse compared with controls. This would probably occur secondary to a reduction in renal NA stores, resulting in less neurotransmitter being available for release (Wegmann et al, 1962; Lefer & Ayers, 1969; Barajas et al, 1976; Fink & Brody, 1978 and 1980). The results from the present study are consistent with either decreased synthesis, or more rapid turnover of NA in the kidney(s) in 1K1C and 2K1C hypertension. It is not possible to differentiate between these two possibilities, since measurement of renal NA (both concentration and total content) is not selective for active neurotransmitter; NA stored in sympathetic nerve terminals in the kidney will also be measured. Whatever the explanation for the renal NA depletion in Goldblatt models of renovascular hypertension, it is unlikely that increased renal sympathetic nerve function is important to the aetiology of the raised BP. Both 1K1C (Villarreal et al, 1984; Norman et al, 1984) and 2K1C hypertension (Ljungqvist, 1974) have been demonstrated to develop in rats, despite complete denervation of the kidney subject to renal artery constriction.

Facilitation of NA release from sympathetic nerve terminals by angiotensin II liberated following clipping (Zimmerman, 1981), could enhance sympathetic drive to, and hence lead to catecholamine depletion in, the ischaemic kidney in 2K1C hypertension (Ljungqvist & Ungerstedt, 1972). In support of this, Ljungqvist & Ungerstedt (1972) reported that

the reduced adrenergic nerve histofluorescence pattern in the ischaemic kidney returned to normal in the chronic stages of 2K1C hypertension; in parallel with the fall in plasma renin concentration (Koletsky et al, 1967; Bing et al, 1981a). However, although adrenergic nerve facilitation by angiotensin II may contribute to the renal NA depletion in 2K1C hypertension, it cannot explain the similar pattern of renal NA depletion in rats with 1K1C hypertension. The 1K1C model is associated with normal, or sub-normal circulating levels of renin and angiotensin II (Miksche et al, 1970; Koletsky et al, 1971). Moreover, the hypothesis of Ljungqvist & Ungerstedt (1972) proposed that the greatest reduction in renal NA content would occur in the ischaemic kidney, where the level of angiotensin II is particularly high. The similar reduction of renal NA content in the contralateral kidney in 2K1C rats argues against such an idea. Renal renin and angiotensin II levels are both greatly reduced in the non-ischaemic kidney in 2K1C hypertension (Regoli et al, 1962).

Neuropeptide Y (NPY) content has been demonstrated to be reduced in the ischaemic kidney, and in the left ventricle and septum of the heart, in rats with 1K1C and 2K1C hypertension. Contralateral kidney NPY content was also reduced in 2K1C hypertension, although to a lesser extent than that of the ipsilateral kidney (Allen et al, 1986). These changes in renal and cardiac NPY content are in agreement with those of NA content reported here. This would support the view that NA and NPY co-exist within sympathetic nerve terminals (Ballesta et al, 1984). In both 1K1C and 2K1C hypertension there was no change in the concentration of NPY in the major arteries and veins, including the renal artery, or in the adrenal glands and brain stem. This indicates that hypertension does not initiate a generalised reduction in tissue NPY concentration. NPY is thought to lie within the terminals of sympathetic nerve fibres

(Ballesta et al, 1984) which reach the kidney along the renal artery (Barajas, 1978). In view of this, it is interesting to note that the reduction in NPY content of the clipped kidney was not associated with any depletion of NPY concentration in the renal artery distal to the clip, in either the 1K1C or 2K1C models (Allen et al, 1986). This would argue against damage to the renal sympathetic nerves, which lie in the superficial adventitial layers of the renal artery, being responsible for the reduction in ipsilateral renal NPY (and NA) content following clipping.

Plasma NA concentration in rats studied in this section followed a similar pattern to that described earlier (section II). Plasma NA concentration and heart rate were elevated in rats with 1K1C hypertension; although in this case the difference in these variables was not statistically significant compared to the normotensive control groups. Plasma NA and heart rate were, however, positively correlated in 1K1C rats. Plasma NA concentrations were not different in 2K1C hypertensive rats and corresponding normotensive controls. The source of the elevated plasma NA in 1K1C hypertension remains unclear. It is most likely derived from increased neurotransmitter release, and consequent increased spillover into the circulation, from sympathetic nerves innervating the vasculature (Dargie et al, 1977a). Alternatively, reduced renal and cardiac NA content in 1K1C hypertension may reflect an increased release of NA into the plasma by these organs. This appears unlikely, however, since a similar pattern of renal and cardiac NA depletion was found in 2K1C hypertension; despite the fact that there was no elevation in plasma NA concentration associated with this model.

There was a tendency for plasma NA concentration to be reduced in sham-operated rats, compared with hypertensive and loose clip rats. This was true for both the 1-kidney and 2-kidney models, although none of the differences were statistically significant. It is possible that the application of a clip to a single renal artery may produce slightly elevated levels of plasma NA, regardless of whether the rats develop hypertension and independent of the presence or absence of a contralateral normal kidney. It is doubtful, however, whether any biological significance can be attributed to this observation.

In the previous experiments (section II) plasma NA concentration was higher in 2-kidney than 1-kidney rats, regardless of the level of BP. One possible explanation forwarded for this finding was that the kidney is a major contribution to plasma NA. However, in subsequent experiments (section III) the situation was reversed. Plasma NA concentration was highest in 1-kidney rats, again independent of the level of BP. The latter results contradict the idea of a direct relationship between the amount of renal tissue and the level of plasma NA. A 'period effect' could be responsible, since in both sets of experiments the two models of hypertension (together with corresponding control groups) were studied several months apart. However, the major importance of these results is that they serve to emphasise the need for parallel control groups when studying plasma NA levels in experimental hypertension. In the present experiments (sections II and III), comparisons of plasma NA concentration were only made between hypertensive animals and parallel control groups. No attempt was made to compare absolute values between the models, only the pattern of change after unclipping (see section II).

In conclusion, the major findings from the present study of renal and cardiac NA levels in 1K1C and 2K1C hypertension were as follows:

The procedure of applying a loose clip to the renal artery produced a fall in ipsilateral renal NA concentration. This is consistent with mechanical denervation of the kidney during placement of the clip around the artery. Hypertension resulted in a further reduction in ipsilateral renal NA and a significant reduction in cardiac and contralateral renal (2K1C) NA concentration. The NA depletion in the contralateral kidney of 2K1C hypertensive rats cannot be due to the mechanical effects of clipping the renal artery. A similar pattern was observed when results were expressed as total organ contents of NA. The reduction in total cardiac NA content (nmol/heart) was much less pronounced than that of cardiac NA concentration (nmol/g wet weight of tissue) in both models. The significant cardiac hypertrophy found in hypertensive animals is the most likely explanation for this. Therefore, in terms of NA content, the kidney was more affected by NA depletion than the heart in 1K1C and 2K1C hypertension.

The most striking conclusion from these experiments is that despite differences in the level of renin-angiotensin system activity, sodium balance and plasma NA concentration, renal and cardiac NA levels show a remarkably similar pattern in 1K1C and 2K1C hypertension. The similar changes in renal and cardiac NA concentrations (and contents) in 1K1C and 2K1C hypertension suggests they occur in association with the sustained elevation in BP in both models. However, a similar depletion in the NA content of the untouched kidney has been reported in 2-kidney renal wrap rats that remained normotensive. These rats also showed a decreased renal vasoconstriction in response to renal nerve stimulation

in both kidneys, four weeks after wrapping a single kidney (Fink & Brody, 1980). It has therefore been suggested that manipulations involved in producing renal models of hypertension, generate a common factor responsible for both increased arterial pressure and renal NA depletion. Furthermore, either effect may be produced independently of the other (Fink & Brody, 1978 and 1980). A strong relationship was observed between renal and cardiac NA levels in 1-kidney and 2-kidney normotensive control rats. This relationship was less obvious in 1K1C and 2K1C hypertension, especially when results were expressed in nmol/whole organ, and suggests hypertension may alter NA handling in the kidney(s) and heart differentially in both models.

It is possible that the renal NA depletion demonstrated in renovascular hypertension may reflect a decreased neurogenic influence on the kidney, resulting from a reduction in the amount of NA available for release. Moreover, this could be a compensatory physiological mechanism to oppose an overt hypertensive stimulus, regardless of the nature of the stimulus; so that the rise in BP is not as great as it otherwise might be (Fink & Brody, 1980). This hypothesis cannot be confirmed or disproved from the present results.

CHAPTER 13

FINAL SUMMARY AND CONCLUSIONS

FINAL SUMMARY AND CONCLUSIONS

The experiments performed in this thesis were designed to study SNS activity in two models of renovascular hypertension in the rat. In these models unilateral renal ischaemia was induced in rats by placing a constricting clip on the renal artery. The opposite kidney was either removed to produce 1K1C hypertension (Byrom & Dodson, 1949), or left *in situ* undisturbed to produce the 2K1C model (Byrom & Wilson, 1938). The degree of BP elevation is similar in the two models, but the fundamental mechanisms underlying the hypertension are different. The factors postulated to be involved in the pathogenesis, and surgical reversal, of 1K1C and 2K1C hypertension were discussed in detail in the last part of section I (Chapter 3). None of the factors proposed to date can completely explain the BP elevation, or BP fall after surgical reversal, in either model.

The present work was divided into two series of experiments, and these were reported and discussed separately. In the first set of experiments (section II), plasma noradrenaline (NA) concentration and heart rate were measured as indices of SNS activity in rats with 1K1C and 2K1C hypertension, before and after surgical reversal by unclipping. Rats were studied in the early phase of 1K1C hypertension (4-6 weeks post-clipping) and in the early and chronic (>16 weeks post-clipping) phases of the 2K1C model. Plasma samples were obtained from conscious, unstimulated rats via indwelling carotid artery catheters, 48 hours after cannulation and 48 hours after unclipping. These studies were then extended to investigate whether the patterns of plasma NA concentration associated with 1K1C and 2K1C hypertension, were reflected in renal and cardiac levels of the neurotransmitter (section III).

Parallel measurements of plasma, renal and cardiac NA were made in rats in the early stages of both models (4-6 weeks post-clipping). The major findings were as follows:

In the first series of experiments (section II) clear differences were observed between 1K1C and 2K1C hypertension, both during established hypertension and after surgical reversal by unclipping. Heart rate was measured to supplement plasma NA data and there was a strong relationship between plasma NA concentration and heart rate in 1K1C hypertensive rats, where there was evidence of SNS activation. Furthermore, changes in heart rate on unclipping followed a similar pattern to changes in plasma NA: changes in the two variables on reversal were significantly correlated in all three models of hypertension, as would be expected if both reflect SNS activity.

There was evidence of SNS activation in 1K1C hypertension, since plasma NA concentration and heart rate were significantly elevated compared to the parallel loose clip control group. Furthermore, BP was positively correlated with plasma NA in 1K1C rats. Conversely, there was no evidence of SNS involvement at any stage in the 2K1C model. Plasma NA and heart rate were similar in hypertensive and age-matched control rats, in both early and chronic phase 2K1C hypertension. Moreover, there was no correlation between BP and plasma NA concentration in either phase of this model.

There was a small fall in indices of SNS activity after unclipping rats with 1K1C hypertension, indicating that reduced SNS activity may play a minor role in reversal. However, SNS tone was not reduced to the level seen in control animals and therefore cannot be the only factor involved. This could suggest that the stimulus to increased SNS

activity in 1K1C hypertension is not completely reversed at this stage. It is possible, however, that a baroreceptor mediated SNS activation, secondary to the BP fall, may be maintaining the elevated heart rate seen in 1K1C rats after unclipping. The lack of correlation between change in BP and change in either plasma NA or heart rate on unclipping, provides further evidence that SNS activity and the BP fall are not directly related in this model. Removal of the clip from the renal artery of rats after 4-6 weeks of 2K1C hypertension, on the other hand, was followed by a significant increase in both plasma NA concentration and heart rate. This may be due to a baroreceptor mediated SNS activation in response to the fall in BP. However, SNS activity was measured 48 hours after unclipping and baroreceptors have been shown to 'reset' to the lower, normotensive BP by this time (Salgado & Krieger, 1973; Floras & Jones, 1980). Furthermore, the pattern of change in plasma NA concentration and heart rate on unclipping was not significantly different between rats with 2K1C hypertension and parallel loose clip controls.

There was no evidence of a progressive increase in the contribution of the SNS with duration of hypertension in the 2K1C model. The cardiovascular system of rats in the two phases of 2K1C hypertension did, however, respond differently to unclipping. In contrast to the rise seen in the early phase, reversal of long-term 2K1C hypertension was associated with a small fall in indices of SNS activity. One possible explanation for this is that structural changes within baroreceptor walls, caused by long-standing hypertension, reduced their ability to respond to changes in BP (Angell-James, 1973). This must remain purely speculative since baroreceptor function was not assessed in the present experiments.

The measurement of plasma NA concentration as an index of SNS activity has been criticised (as discussed in section 2.3.2 and Chapter 8), and it must be accepted that it is a fairly crude index. However, all the other methods of assessing sympathetic nervous function in experimental animals currently available also have major drawbacks (see section 2.3). Arterial plasma NA levels will give an indication of overall sympathetic tone at rest (Goldstein et al, 1983b), and as such provide important clues to SNS involvement in experimental renovascular hypertension. It is essential, however, that plasma NA concentrations in hypertensive animals are only compared with those of parallel control groups, as reported here.

Despite differences in plasma NA concentration, renal and cardiac NA levels in 1K1C and 2K1C hypertension showed a remarkably similar pattern (section III). In this second series of experiments two control groups were prepared for each model; a loose clip group as control for the elevation in BP, and a sham-operated group as control for the procedure of clipping. In both models there was a similar descending order of left kidney NA concentration (nmol/g wet weight of tissue) in sham-operated, loose clip and hypertensive rats. Left (clipped) kidney NA concentration was significantly reduced in 1K1C and 2K1C hypertension compared to corresponding loose clip rats, and in turn significantly reduced in loose clip compared to sham-operated rats. This graded response is consistent with a partial denervation of the ipsilateral kidney during the procedure of placing a clip around the renal artery; with an additional depletion of NA in this kidney following the induction of renal ischaemia, and subsequently hypertension. Contralateral kidney NA concentration was also significantly reduced in 2K1C hypertension, such that there was no difference in NA concentration between the two kidneys in these rats. There was no difference in right

kidney NA concentration between 2-kidney loose clip and sham-operated rats. Thus, in 2-kidney loose clip rats renal NA depletion was confined to the clipped kidney and is likely to be the result of damaging the renal sympathetic nerves during clipping. The contralateral depletion in renal NA concentration in 2K1C hypertension cannot be explained by denervation. Similar patterns of renal NA were observed when results were expressed as total NA (nmol/kidney).

1K1C and 2K1C hypertension were both associated with a marked reduction in cardiac NA concentration compared to the normotensive control groups. Cardiac NA concentration was similar in loose clip and sham-operated rats in both models. The elevated BP, however, produced significant hypertrophy of the heart; consequently, a 'dilution effect' on the neurotransmitter by the increased tissue mass probably accounts for a large proportion of the decrease in cardiac NA concentration in 1K1C and 2K1C hypertension. Total cardiac NA content (nmol/heart) was slightly reduced in both models compared to normotensive controls, but this was much less pronounced than the reduction in NA concentration. Changes in renal and cardiac NA contents in rats with 1K1C and 2K1C hypertension were similar to those described for Neuropeptide Y (NPY) (Allen et al, 1986). This supports the idea that NA and NPY co-exist within sympathetic nerve terminals (Ballesta et al, 1984).

The reduction in endogenous renal NA concentration (and content) in 1K1C and 2K1C hypertension, could reflect a NA-depleting effect of chronically enhanced renal sympathetic nerve activity in these models. Alternatively, renal NA depletion may reflect decreased renal sympathetic nerve activity in animals with renovascular hypertension, secondary to reduced NA synthesis. Such a reduction in the neurogenic control of renal function could be a compensatory mechanism to oppose a

rise in arterial pressure, regardless of the nature of the hypertensive stimulus. It is not possible from the present results to distinguish between these possibilities.

The similar changes in renal and cardiac NA levels in rats with 1K1C and 2K1C hypertension - despite differences in plasma NA concentration, and well documented differences in renin-angiotensin system activity and sodium balance - suggests these changes may occur secondary to the elevated BP in both models. Alternatively, altered NA handling in the kidney(s) and heart may be contributing to the maintenance of raised BP in both 1K1C and 2K1C hypertension. The strong correlations between renal and cardiac NA levels in normotensive rats were less obvious in 1K1C and 2K1C hypertension, and this may suggest a differential effect on NA handling in the kidney(s) and heart in rats with renovascular hypertension. Further work is required to clarify this. Finally, the pattern of renal and cardiac NA after reversal of 1K1C and 2K1C hypertension by unclipping is not known, but data on this experimental situation could provide more useful information.

APPENDIX 1

Calibration program and example of an identification table for the high performance liquid chromatographic assay for catecholamines

APPENDIX 1.1

Internal standard calibration program for the catecholamine assay:

Width	:	20
Slope	:	200
Drift	:	500
Minimum area	:	500
Doubling time	:	0
Lock	:	5.5
Stop time	:	30
Attenuation	:	6
Speed	:	2.5
Method	:	1043
Sample weight	:	100
I.S. weight	:	1

APPENDIX 1.2

An example of an identification and calibration table from a plasma catecholamine assay:

Name	Time (mins)	Window (%)	Calibration Factor (F1)	Concentration Factor
DHB	15.45	5	1	1
NA	7.65		9.272	5
ADR	10.28		10.49	5
DA	25.48		41.74	5

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PUBLICATIONS AND PRESENTATIONS

The following publications have been produced from work described in this thesis:

1. WALKER, S.M., BING, R.F., SWALES, J.D. & THURSTON, H. (1986) Plasma noradrenaline in Goldblatt models of renovascular hypertension in the rat, before and after surgical reversal. Clin. Sci. 71 : 199-204

2. WALKER, S.M., BING, R.F., SWALES, J.D. & THURSTON, H. (1985) Plasma noradrenaline in two models of renovascular hypertension before and after surgical reversal. Clin. Sci. 68 (Suppl. 11) : 41p Medical Research Society, January, 1985.

3. WALKER, S.M., BING, R.F., SWALES, J.D. & THURSTON, H. (1985) To evaluate the role of the sympathetic nervous system before and after surgical reversal of one kidney and two kidney, one clip hypertension. Second European Meeting on Hypertension, Milan, Italy. June, 1985.

4. WALKER, S.M., BING, R.F., SWALES, J.D. & THURSTON, H. (1986) Plasma, renal and cardiac noradrenaline in one- and two-kidney renovascular hypertension. Clin. Sci. 70 (Suppl. 13) : 43p Medical Research Society, December, 1985.

TITLE: STUDIES ON THE SYMPATHETIC NERVOUS SYSTEM IN EXPERIMENTAL RENOVASCULAR HYPERTENSION

AUTHOR: STEPHEN M. WALKER (1987)

ABSTRACT

1. In these studies the role of the sympathetic nervous system (SNS) has been investigated in two models of renovascular hypertension in the rat and after surgical reversal.

2. Plasma noradrenaline (NA), heart rate and blood pressure (BP) were measured simultaneously in conscious rats with one-kidney, one-clip (1K1C) (4-6 weeks) and two-kidney, one-clip (2K1C) (4-6 and >16 weeks) hypertension, and parallel loose clip controls, before and 48 hours after unclipping. BP in all hypertensive groups fell to normal after unclipping.

3. Plasma NA was elevated in 1K1C hypertension and fell on unclipping. Conversely, in early 2K1C hypertension plasma NA was unaltered before and rose after unclipping. Plasma NA did not change with unclipping in chronic 2K1C hypertension and was not different from controls. Heart rate showed a similar pattern. Unclipping loose clip control rats produced no change in BP, plasma NA or heart rate.

4. Heart rate was correlated with plasma NA in 1K1C hypertension, and changes in these variables on unclipping were correlated in all three models. BP was only correlated with plasma NA in 1K1C hypertensive rats.

5. In contrast, renal and cardiac NA levels showed a remarkably similar pattern in 1K1C and 2K1C hypertension of 4-6 weeks duration. Ipsilateral renal NA was reduced in loose clip rats compared to sham-operated controls. This is most likely due to renal denervation during clipping. Hypertension produced a further reduction in ipsilateral renal NA and a reduction in cardiac and contralateral renal (2K1C) NA.

6. It is concluded that the SNS may have a minor role in 1K1C, but not in 2K1C, renovascular hypertension. However, changes in SNS activity upon reversal of hypertension do not explain the BP fall in either model. Changes in renal and cardiac levels of NA in renovascular hypertension are primarily secondary to sustained elevation of BP.