STUDIES OF VASCULAR DNA SYNTHESIS DURING THE DEVELOPMENT OF HYPERTENSION.

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by

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Studies of vascular DNA synthesis during the development of hypertension. - Joanne S. Lymn.

Medial hypertrophy of arteries and resistance vessels is a consistent feature of hypertension although its relationship to the rise in blood pressure remains unclear.

Previous studies can generally be divided into morphological studies of resistance arteries, detecting only the end product of structural change, or biochemical studies of large conduit vessels which do not regulate peripheral vascular resistance. In this study I have monitored the changes in blood pressure, rate of DNA synthesis, protein content and DNA content of large vessels and small resistance arteries from rats with developing two-kidney, one-clip Goldblatt (2K, 1C) and genetic (SH) hypertension.

At three days post-2K, 1C a significant increase in [^{3}H]-thymidine incorporation was observed although blood pressure remained unchanged. By 28 days DNA synthesis had returned to control levels while blood pressure remained elevated. A significant rise in protein content was detected in the aorta and in the mesenteric arteries where DNA content was also elevated. No change in DNA/protein ratio was detected.

In contrast the rate of DNA synthesis of vessels from SH rats did not differ from control values at any age. At 18 weeks the aortic protein content was increased with a corresponding decrease in DNA/protein ratio. The protein and DNA content of the resistance vessels remained unchanged.

It would appear that the changes which lead to structural modifications in 2K, 1C hypertension begin before any significant rise in blood pressure, and that the medial hypertrophy can be attributed to cellular hypertrophy in the aorta and hyperplasia in the mesenteric arteries. However, structural modification in the SH rat appears to depend on mechanisms which do not involve amplification of DNA synthesis in postnatal life.

FOR MY HUSBAND AND FAMILY.

Love is a precious thing.

TABLE OF CONTENTS.

		Page no.
Acknowledgements.		
Chapter 1 -	**Introduction.**	
1.1	Hypertension - what do we mean?	1
1.2	Association with peripheral resistance.	2
1.3	Functional regulation of blood flow.	3
1.3.1	Autoregulation.	3
1.3.2	Rarefaction.	5
1.4	Structural regulation.	6
1.4.1	Early history.	6
1.4.1.1	Vasoconstriction.	7
1.4.1.2	Vascular structural change.	8
1.4.2	Direct morphological measurements.	9
1.4.3	Mechanisms of structural change.	11
1.4.3.1	Pressure - dependent.	11
1.4.3.2	Pressure - independent.	12
1.4.3.2.1	Flow related structural change.	13
1.4.3.2.2	Structural changes in a normotensive vascular bed.	14
1.4.3.2.3	Early structural changes in genetic hypertension.	16
1.4.3.2.4	Structural development in essential hypertension.	18
1.5	Relationship between structural changes and the rise in blood pressure.	19
1.5.1	Inositol lipid metabolism.	20

Page no.

1.5.1.1 Inositol trisphosphate and calcium mobilization. 21
1.5.1.2 Diacylglycerol. 22
1.5.2 Protein kinase C activation. 23
1.5.3 Na^+/H^+ exchanger and pH. 25
1.5.4 Proto-oncogene expression. 26
1.6 Signal transduction, growth and hypertension. 27
1.6.1 Measurement of DNA synthesis. 28
1.6.2 Growth kinetics, hypertrophy and hypertension. 30
1.6.2.1 Experimental hypertension. 30
1.6.2.2 Genetic hypertension. 33
1.7 Aims and objectives. 35

Chapter 2 - Materials and Methods.

2.1 Materials. 36
2.2 Equipment. 36
2.3 Animals. 36
2.3.1 Experimental hypertension. 36
2.3.2 Genetic hypertension. 37
2.3.3 Assessment of hypertension. 37
2.4 Preparation of tissues. 37
2.4.1 General preparation. 38
2.4.2 Vessel preparation. 38

		Page no.
2.5	Determination of DNA synthesis.	39
2.6	Analysis of protein content.	40
2.6.1	Quantitation by Lowry assay.	41
2.6.2	Quantitation by Bicinchoninic acid (BCA) method.	42
2.7	DNA solubilisation procedure	42
2.8	Ultrafiltration.	43
2.9	Analysis of DNA content.	44
2.9.1	Quantitation by Diphenylamine assay.	44
2.9.2	Quantitation by Hoechst 33258.	45
2.10	Electrophoretic analysis of DNA.	46
2.10.1	Protein digestion.	46
2.10.2	Electrophoresis.	47
2.11	Ethanol precipitation of DNA.	47
2.12	Radioimmunoassay for renin.	48
2.12.1	Preparation of nephrectomised rat plasma.	48
2.12.2	Radioimmunoassay.	48
2.13	Cell culture methods.	50
2.13.1	Routine cell maintenance.	50
2.13.2	Subculture.	50
2.14	Statistical analysis.	51

Chapter 3 - Experimental design.

3.1	Introduction.	52

		Page no.
3.2	Overview of techniques available for analysis of DNA.	52
3.3	Choice of DNA assay system.	54
3.4	Characterisation of DNA assay.	55
3.4.1	Fluorescence spectra.	55
3.4.2	Fluorescence enhancement as a function of DNA concentration.	57
3.4.3	Validation of assay system.	57
3.4.4	Comparison with Diphenylamine assay.	58
3.4.5	Dependence on DNA structure.	59
3.4.5.1	Double - stranded helix.	59
3.4.5.2	Polynucleotide structure.	60
3.5	Efficiency of DNA extraction.	60
3.5.1	Effect of increasing incubation time.	61
3.5.2	Effect of increasing incubation temperature.	62
3.5.3	Effect of increasing the concentration of extraction buffer.	62
3.5.4	Validation of improved DNA solubilisation.	64
3.5.4.1	Effect of dilution.	64
3.5.4.2	Electrophoresis of DNA.	64
3.6	Application to other vessels.	65
3.7	Manipulation of samples.	66
3.7.1	Concentration by ultrafiltration.	67
3.7.2	Effect of storage.	68

Page no.

3.8 Measurement of DNA synthesis. 69

3.8.1 General assay conditions. 70

3.8.2 Investigation of optimum incubation time. 70

3.8.3 Efficiency of hot acid extraction. 71

Chapter 4 - Experimental hypertension.

4.1 Choice of experimental model. 73

4.2 Incidence of hypertension. 73

4.3 Development of hypertension. 74

4.3.1 Blood pressure measurements. 74

4.3.2 Measurement of cardiac hypertrophy. 75

4.3.2.1 Heart weight / body weight ratio. 75

4.3.2.2 Heart weight / tibial length ratio. 76

4.4 Changes in plasma renin concentration. 77

4.5 Changes in DNA synthesis - Aorta. 77

4.5.1 Aortic uptake of [^{3}H]-thymidine 78

4.5.2 Aortic incorporation of [^{3}H]-thymidine. 78

4.5.3 Aortic protein content. 79

4.5.4 Aortic DNA content. 79

4.5.5 Aortic DNA / protein ratio. 79

4.6 Changes in DNA synthesis - Mesenteric Artery. 80

4.6.1 Mesenteric artery uptake of [^{3}H]-thymidine. 80

Page no.

4.6.2 Mesenteric artery incorporation of [^{3}H]-thymidine. 80

4.6.3 Mesenteric artery protein content. 81

4.6.4 Mesenteric artery DNA content. 81

4.6.5 Mesenteric artery DNA / protein ratio. 82

4.7 Changes in DNA synthesis - Subcutaneous vessels. 82

4.7.1 Subcutaneous vessel uptake of [^{3}H]-thymidine. 82

4.7.2 Subcutaneous vessel incorporation of [^{3}H]-thymidine. 82

4.7.3 Subcutaneous vessel protein content. 83

4.7.4 Subcutaneous vessel DNA content. 83

4.7.5 Subcutaneous vessel DNA / protein ratio. 83

4.8 Summary of results. 83

Chapter 5 - Genetic Hypertension.

5.1 Choice of genetic model. 85

5.2 Development of hypertension. 85

5.2.1 Blood pressure measurements. 85

5.2.2 Development of cardiac hypertrophy. 86

5.2.2.1 Heart weight / body weight ratio. 87

5.2.2.2 Heart weight / tibial length ratio. 87

5.3 Changes in DNA synthesis - Aorta. 88

5.3.1 Aortic uptake of [^{3}H]-thymidine. 88

5.3.2 Aortic incorporation of [^{3}H]-thymidine. 89

Page no.

5.3.3 Aortic protein content. 89

5.3.4 Aortic DNA content. 89

5.3.5 Aortic DNA / protein ratio. 90

5.4 Changes in DNA synthesis - Mesenteric Artery. 90

5.4.1 Mesenteric artery uptake of [^{3}H]-thymidine. 90

5.4.2 Mesenteric artery incorporation of [^{3}H]-thymidine. 91

5.4.3 Mesenteric artery protein content. 91

5.4.4 Mesenteric artery DNA content. 91

5.4.5 Mesenteric artery DNA / protein ratio. 92

5.5 Changes in DNA synthesis - Subcutaneous Vessels. 92

5.5.1 Subcutaneous vessel uptake of [^{3}H]-thymidine. 92

5.5.2 Subcutaneous vessel incorporation of [^{3}H]-thymidine. 93

5.5.3 Subcutaneous vessel protein content. 93

5.5.4 Subcutaneous vessel DNA content. 93

5.5.5 Subcutaneous vessel DNA / protein ratio. 93

5.6 Summary of results. 93

Chapter 6 - Discussion.

6.1 Introduction. 95

Page no.

6.2 Methodology. 96

6.3 Experimental hypertension. 97

6.4 Genetic hypertension. 106

Chapter 7 - Conclusions.

7.1 Experimental hypertension. 114

7.2 Genetic hypertension. 115

Appendices.

Appendix A 116

Appendix B 118

Appendix C 119

Appendix D 122

Appendix E 123

Bibliography. 125

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CHAPTER ONE.

INTRODUCTION.

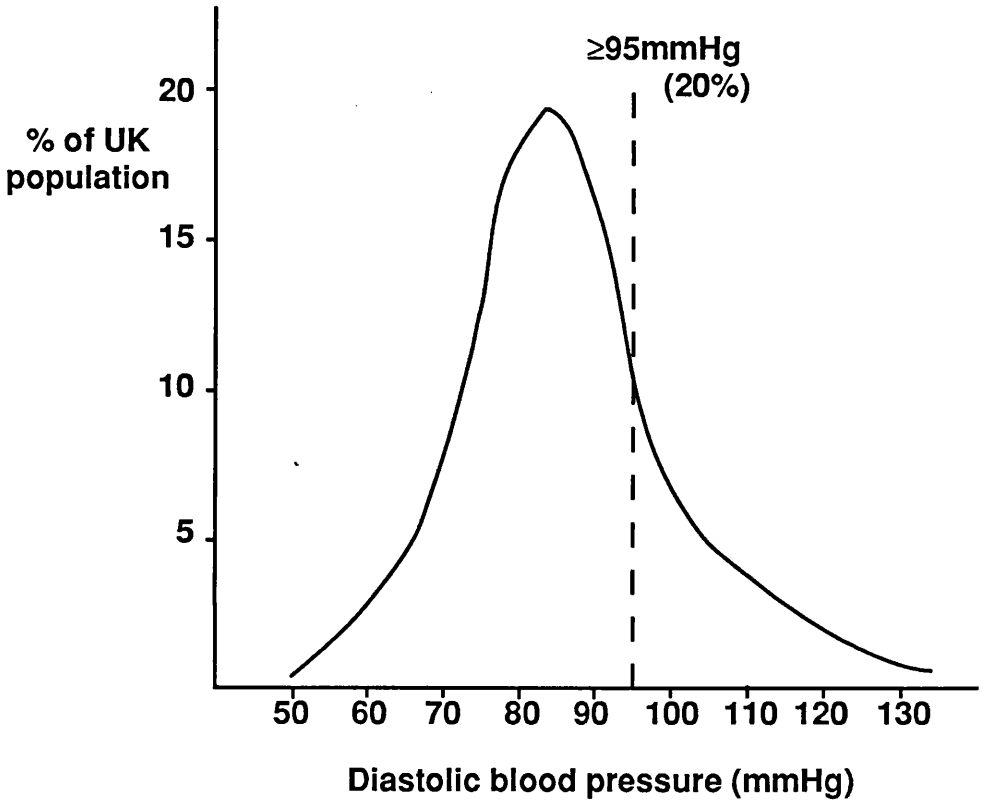
1.1 Hypertension - what do we mean?

Hypertension can be defined as persistently high arterial blood pressure (Dorlands Illustrated Medical Dictionary, 1974). In an unselected population blood pressure follows a normal pattern of distribution, and there is no obvious distinction between high and low blood pressure. The World Health organisation however, has defined hypertension as blood pressures of 160mmHg systolic and 95mmHg diastolic or above. Using this criteria approximately 20% of people would be classified as hypertensive (Figure 1), although repeated screening would reduce the incidence to around 10% of the population (WHO Tech Report Service, 1962).

Hypertension undoubtably increases a patients' susceptibility to major cardiovascular complications such as stroke and heart failure, with untreated severe, malignant hypertension having a mortality rate of 90% within one year. Fortunately this type of hypertension is rare and the vast majority of patients present with mild or borderline hypertension. Even so the life expectancy of hypertensive patients can be prolonged by effectively reducing blood pressure with anti-hypertensive therapy. Drug treatment is now recommended for all patients with a diastolic blood pressure of, or above, 100mmHg for a three month period (Swales et al, 1989).

The true heterogeneic nature of hypertension is now becoming increasingly apparent not only because of the massive variation in patient history and prognosis but also the responsiveness, or lack of it, to different anti-hypertensive

FIGURE 1.



Distribution of blood pressure levels in the general population.

On single screening approximately 20% of the population would have a diastolic blood pressure of over 95mmHg.

(Taken from; Thurston, 1991).

drugs. This must be correlated with a heterogenous pathophysiology of the disease. Currently, although the condition has been well documented our knowledge of its aetiology and course of development remains crude and limited. Research into the finer biological processes underlying the development of hypertension, aided by animal models and exciting new technology, is however both active and fervent.

1.2 Association with peripheral resistance.

The main determinants of arterial blood pressure are cardiac output and total peripheral resistance (Lund-Johansen, 1980) and can be crudely represented by;

Mean arterial pressure = peripheral resistance x cardiac output.

In the early stages of essential (Frohlich et al, 1970), genetic (Smith and Hutchins, 1979) and experimental (Ferrario et al, 1970) hypertension, the rise in arterial blood pressure is associated with an increased cardiac output coupled with normal peripheral resistance. However once hypertension is established cardiac output falls to normal levels and the overriding haemodynamic disturbance is a raised total peripheral vascular resistance (Ferrario et al, 1970; Frohlich et al, 1970 and 1971; Smith and Hutchins, 1979; Lund-Johansen, 1980).

That the control of peripheral resistance is the domain of the small arteries and arterioles is demonstrated by the

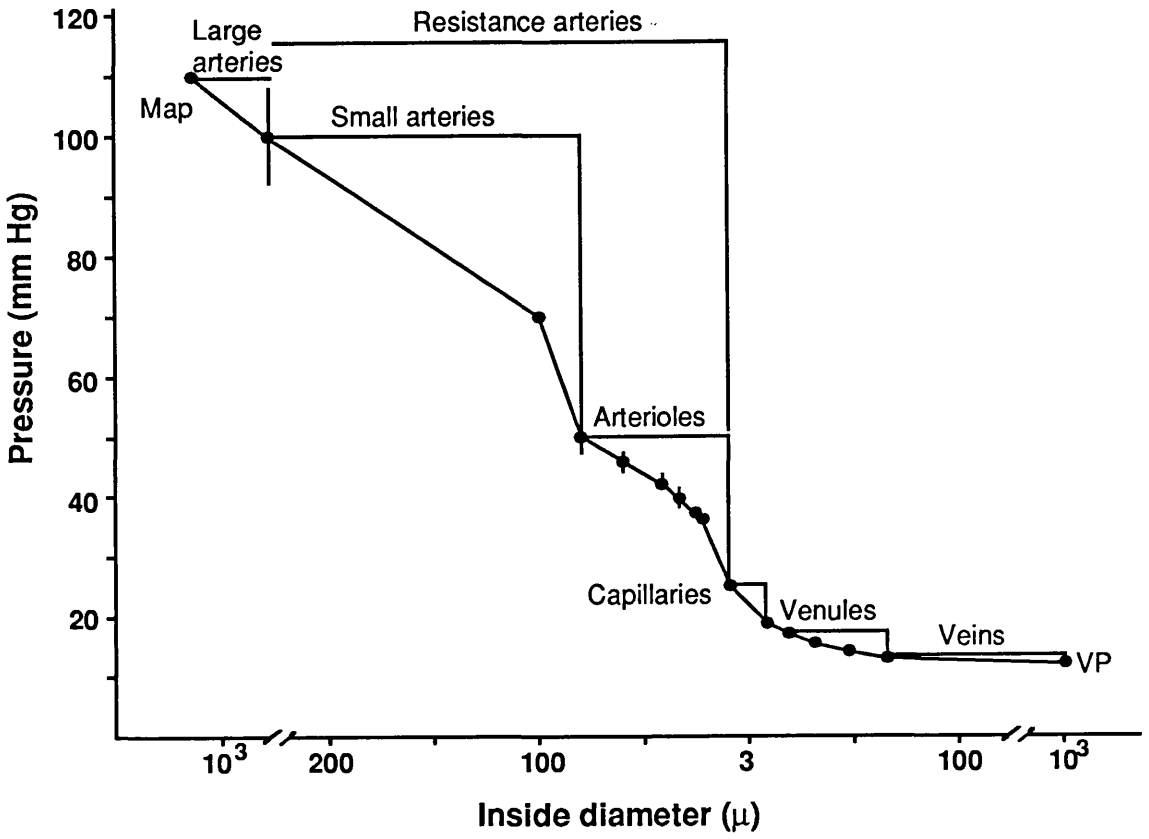
pressure profile shown in Figure 2. It is generally accepted that there is a uniform increase in this arteriolar resistance in established hypertension (London et al, 1984), but early studies suggested variations in resistance to flow in different vascular beds. Thus Brod et al (1962) demonstrated a decrease in renal and skin blood flow in essential hypertensives but forearm blood flow was increased. These differences coupled with the change in total peripheral resistance from early to established hypertension, led to the concept that blood flow in peripheral arterioles was regulated in response to changes in cardiac output (Ledingham and Cohen, 1962; Smith and Hutchins, 1979).

1.3 Functional regulation of blood flow.

1.3.1 Autoregulation.

It is well known that individual vascular beds are capable of regulating their blood flow and thereby the supply of oxygen to the tissues. Moreover, Granger and Guyton (1969) demonstrated that the phenomenon of autoregulation is not restricted to individual tissue responses and that whole-body autoregulation can occur. This concept is supported by studies showing a significant correlation between oxygen delivery and oxygen consumption, and, oxygen consumption and cardiac output in resting Wistar rats (Ledingham et al, 1984). Also these workers demonstrated that both cardiac output and oxygen delivery rose when blood volume was

FIGURE 2.



Pressure drop through hamster cheek pouch circulation.

MAP = mean arterial pressure.

VP = venous pressure.

(Taken from; Davis et al, 1986).

increased. In this situation, where oxygen delivery exceeds tissue demand, autoregulatory constriction of the blood vessels would be expected to occur. These findings are of enormous importance in the pathophysiology of hypertension since it has been proposed that autoregulation of blood flow in response to increased cardiac output is the initial determinant of the increased peripheral resistance (Ledingham, 1989).

Indirect evidence for this theory comes from studies of Spontaneously hypertensive (SH) rats. Between 32 and 41 days of age conscious SH rats have been shown to exhibit raised cardiac output and normal peripheral resistance compared to Wistar-kyoto (WKY) normotensive animals. However, between 80 and 120 days of age the cardiac output was normal and total peripheral resistance significantly increased (Smith and Hutchins, 1979). Moreover the small arterioles of the cremaster muscle of these animals have also been shown to exhibit an increased vasoconstrictor response to increasing oxygen concentrations (Lombard et al, 1984).

Direct evidence in favour of the importance of an autoregulatory response comes from studies of forearm blood flow in essential hypertension. Reduction in perfusion pressure leads to an autoregulatory vasodilation of the blood vessels (Blake et al, 1985) and similarly increasing the perfusion pressure leads to an autoregulatory vasoconstrictor response (Benjamin and Robinson, 1986). Interestingly the vasodilator response in these patients was incomplete compared to that seen in normotensive controls. This suggests

that an increased vessel wall thickness may augment arteriolar resistance at all levels of transmural pressure. The involvement of an autoregulatory response has also been effectively demonstrated in patients with coarctation of the aorta (Patterson et al, 1957) and in experimental animal models (Meininger et al, 1984; Stanek et al, 1987).
Although the evidence presented here makes the theory of autoregulatory vasoconstriction at first glance appear very convincing it is important to remember that the phenomenon does not occur universally. Thus, patients with severe labile hypertension of longstanding duration have been shown to exhibit a raised peripheral resistance in association with an increase in cardiac output (Ibrahim et al, 1975). Autoregulation therefore should be regarded only as an initial line of defense against sudden increases in blood flow through the tissues.

1.3.2 Rarefaction.

The ability of tissues to control local blood flow by autoregulatory constriction/dilation has already been discussed. It is possible however that functional regulation of blood flow extends to controlling the quantity as well as the diameter of small arterioles. Hutchins and Darnell (1974) demonstrated that the number of small arterioles in the cremaster muscle was significantly reduced in the SH compared to the WKY rat. This decrease in arteriolar density appeared to parallel the development of hypertension in the SH rat

passing through a state of temporary functional closure to a permanent anatomical obliteration with increasing age and duration of hypertension (Prewitt et al, 1982). Not suprisingly vascular rarefaction is not limited to the small arterioles but has also been shown to extend to the capillary network (Prewitt et al 1982). Similarly a decrease in open capillary density was demonstrated in young borderline hypertensives (Sullivan et al, 1983) although in this case the overall decrease in vascular density did not appear to involve arteriolar rarefaction. The reduction in filtering capacity of the capillary network would regulate oxygen delivery to the tissues in a manner similar to that achieved by autoregulatory constriction. Thus the two probably operate hand in hand giving an efficient and complete functional response to increased tissue blood flow.

1.4 Structural Regulation.

1.4.1 Early history.

It is interesting to note that vascular changes which characterise hypertension as a disease entity were detected long before Mahomed began routinely measuring arterial blood pressure in man (Folkow, 1978). Bright in 1836 first observed structural changes in the heart and blood vessels of patients with renal disease and speculated that these changes may be due to increased resistance. However, studies of these changes could only be made at autopsy and it was difficult to

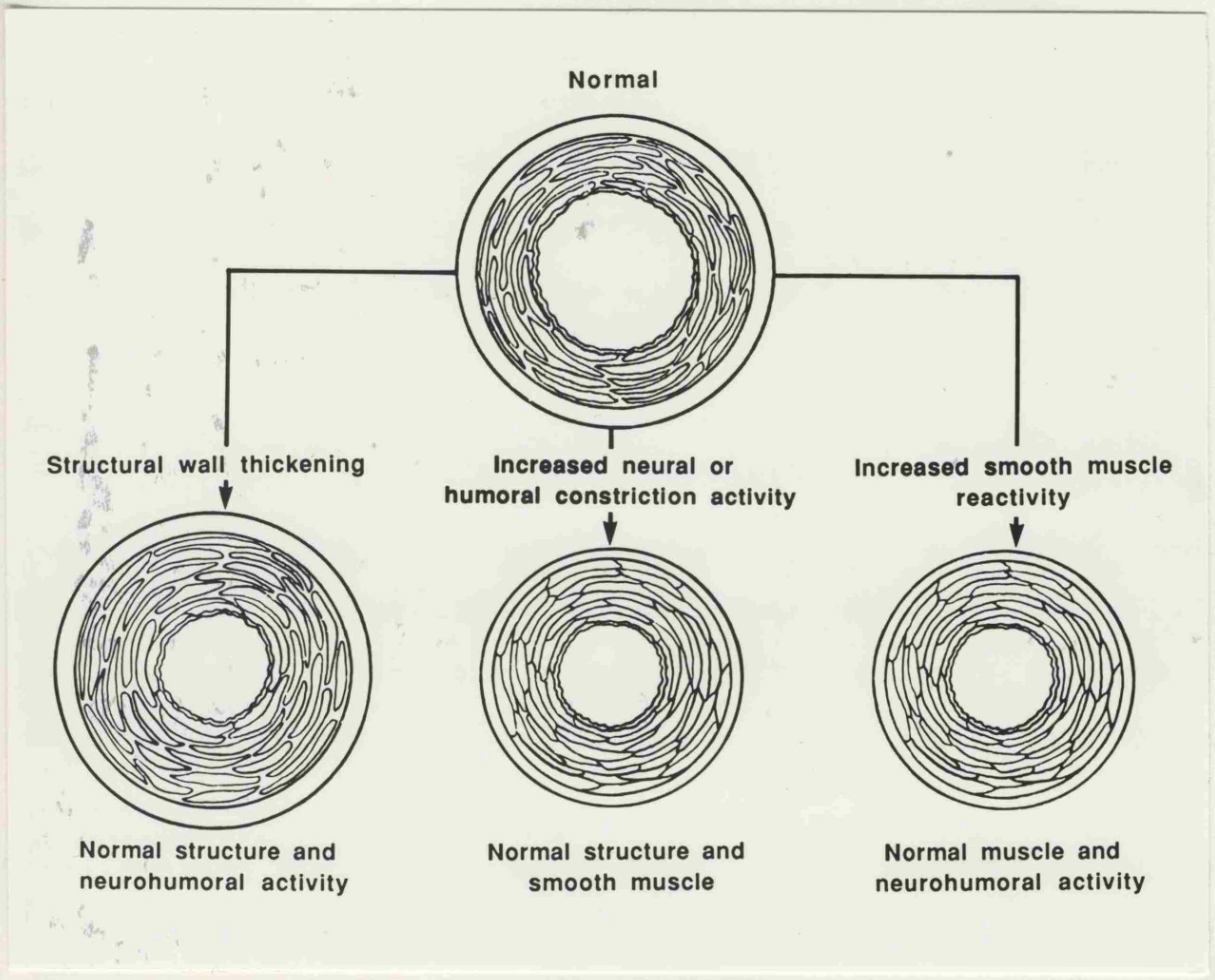
be certain whether the thickening of the vessel wall represented true structural changes or was an artefact of the fixation process.

The increase in peripheral vascular resistance associated with the narrowing of lumen diameter in small arteries could result from either an increase in the degree of vasoconstriction and/or structural changes (Figure 3).

1.4.1.1 Vasoconstriction.

The idea that an increase in the degree of vasoconstriction was responsible for the increased vascular resistance was supported by investigations of the connection between renal disease and hypertension. The observation by Tigerstedt and Bergman (1898) that an increase in blood pressure in normal rabbits could be induced by intravenous injection of supernatant from a rabbit kidney cortex homogenate, was one such instance. The pressor agent responsible was named renin and its discovery opened many new doors in the field of hypertension research. Some years later the work of Goldblatt et al (1934) in consistently producing hypertension in dogs by constriction of the renal arteries augmented this theory with Goldblatt speculating "there may be an accumulation or new formation of some substance.....which may effect a pressor action like that of a hormone." Thus inspired the search for pressor agents continued undaunted for many years.

FIGURE 3



Increased peripheral vascular resistance associated with the narrowing of lumen diameter could result from increases in the degree of vasoconstriction or from structural change.

1.4.1.2 Vascular structural change.

Evidence in favour of true vascular structural change initially came in 1936 when Pickering demonstrated that hypertensive subjects treated with vasodilators maintained a proportionally raised resistance compared to normotensive controls. This increased resting resistance could not be attributed to accentuated sympathetic activity, thereby implying some permanent vascular change. Despite these exciting results the hypothesis that structural change might be responsible for the raised peripheral resistance was largely ignored and attention focused on the more popular functional idea of humoral pressor agents. This lead Pickering to comment "the present impasse in hypertension is chiefly due to the fact that certain fundamentally important aspects of vascular behaviour are appreciated by contempory science either dimly or not at all."

This impasse would have been maintained but for the historic work of Bjorn Folkow. In 1956 Folkow, using forearm blood flow measurements reported that resistance to flow was increased in untreated hypertensive patients compared to normotensive controls, both at rest and at maximum vasodilation. However, there was no comparable change in the ratio between the resistances at rest and at maximum vasodilation. In contrast, normotensive patients, made acutely hypertensive by the infusion of noradrenaline showed an increase in resistance to flow at rest which was proportional to the mean arterial pressure, but the

resistance at maximal vasodilation was unchanged. In other studies Folkow demonstrated similar findings indicative of structural changes in genetic and experimental animal models of hypertension. Moreover, these changes appeared to be greater in the SH rat compared to rats with experimental hypertension for a given elevation of mean arterial pressure, suggesting genetic reinforcement of structural vascular change (Folkow, 1990).

Thus Folkow confirmed Pickering's hypothesis that the increased resistance to flow in hypertension must be attributable to a structural alteration of the vasculature. Folkow then went one step further by speculating that the adaptive vascular changes must in fact consist of growth of the vessel wall in such a way as to encroach on the lumen of the vessel producing an effective decrease in internal diameter, and increase in resistance even at complete relaxation.

1.4.2 Direct morphological measurements.

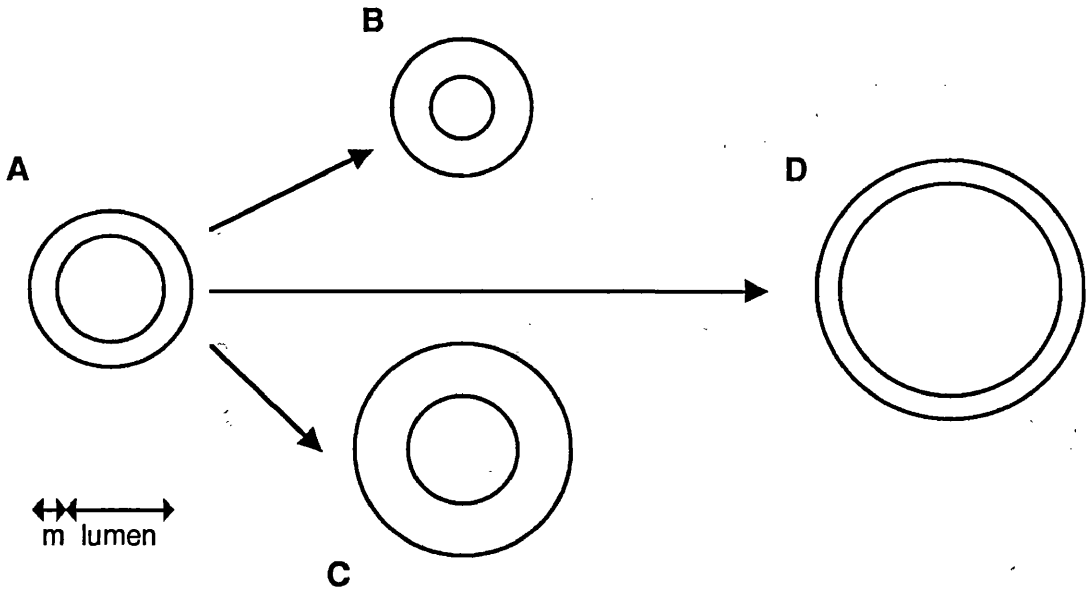
Although Folkow's conclusions were based on indirect studies, the predicted increase in the media to lumen ratio of blood vessels quickly became synonymous with vascular growth. Obviously confirmation of this theory by direct observations of vessel dimensions was essential particularly following the suggestion by Short (1966) that a reduced lumen and increased media to lumen ratio could be achieved without any change in

the amount of material in the vessel wall (Figure 4).

The next major breakthrough came with the work of Mulvany and Halpern (1976) who described the use of a myograph apparatus to determine the morphology and contractile properties of isolated small resistance vessels. Using this elegant new technique these authors demonstrated that mesenteric resistance vessels from SH rats exhibited an increased wall thickness, and an increased media/lumen ratio, compared to normotensive WKY animals (Mulvany and Halpern, 1977). Similarly an increased medial thickness has been observed in isolated resistance vessels from essential hypertensives (Aalkjaer et al, 1987) and from rats with experimental hypertension (Mulvany and Korsgaard, 1983; Vial and Boyd, 1989).

It has long been recognised that the pressor response of the vasculature to infused agonists is increased in essential (Duff, 1956), genetic (Holloway and Bohr, 1973) and experimental (Hinke, 1965; Beilin et al, 1970) hypertension, although the mechanism underlying the alteration in reactivity remained unclear. Folkow (1956) suggested that an increase in the media/lumen ratio of resistance vessels would effectively lead to vascular hyperreactivity without an alteration in sensitivity of the vascular smooth muscle cells. Mulvany et al (1978) confirmed this view by demonstrating a direct relationship between the increase in pressor response and the increase in media thickness of the vessel wall. Moreover when the pressor response to noradrenaline was expressed in terms of media volume there

FIGURE 4.



Different modes of structural changes of small arteries.

A - control situation showing media cross section.

B - media volume is unchanged, lumen diameter is reduced, media-to-lumen ratio is increased.

C - media volume is increased, lumen diameter is unchanged, media-to-lumen ratio is increased.

D - media volume is increased (as in C), lumen diameter is increased, media-to-lumen ratio is decreased.

(Taken from; Mulvany and Aalkjaer, 1990).

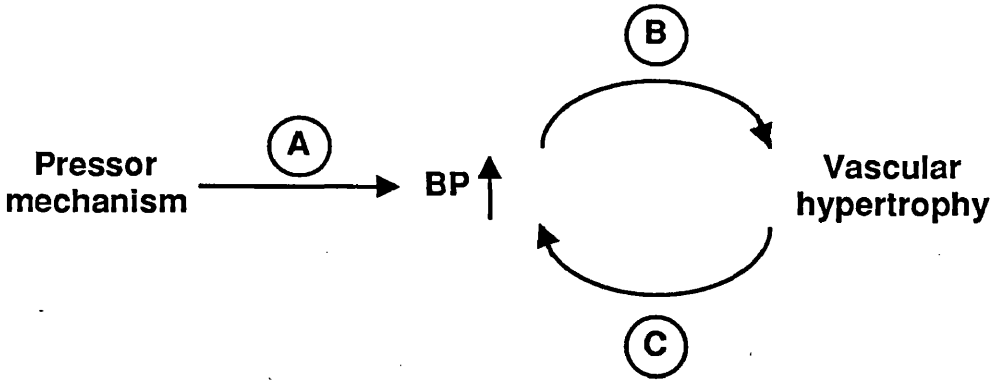
was no significant difference in dose responses between SH and WKY animals (Mulvany et al, 1980). Similarly Wright et al (1987) demonstrated that the conductance of resistance vessels was decreased in renovascular hypertensive rabbits in response to vasoconstrictor agents although the ED_{50} remained unchanged. The absence of a change in ED_{50} suggests that although contractility may be increased the threshold sensitivity of hypertrophied smooth muscle remains unchanged. Wright et al (1987) used a number of vasoconstrictor agents, acting via different types of membrane receptor, and obtained strikingly similar results indicating that the amplifier response is nonspecific. These observations taken in conjunction with those of Mulvany and his co-workers have lead to the concept that vascular hypertrophy acts as a pressor amplification system in hypertensive arteries, operating as a feedback mechanism similar to that originally proposed by Folkow (Figure 5).

1.4.3 Mechanisms of structural change.

1.4.3.1 Pressure-dependent.

The development of the Mulvany/Halpern myograph has provided the opportunity to study the time course for the development of vascular structural change. Warshaw et al (1979) compared the morphological and mechanical properties of both young (6 weeks) and old (50 weeks) SH and WKY rats and found that there was a significant increase in medial thickness in

FIGURE 5



Minor overactivity of a pressor mechanism ***A***, leads to a slight rise in blood pressure, initiating a positive feedback mechanism ***BCB***, and a progressive rise in blood pressure.

(Taken from; Lever, 1986).

mesenteric resistance arteries in the SH rat as early as at 6 weeks of age. Similarly Mulvany et al (1980) demonstrated that structural changes occur much earlier than had been previously accepted, but failed to detect significant changes at 6 weeks of age. The discrepancy between these two sets of results is probably due to the fact that the animals studied by Warshaw et al (1979) had significantly elevated blood pressures whereas those studied by Mulvany et al (1980) did not. Warshaw and his co-workers suggested that blood pressure and smooth muscle function are mutually related so that smooth muscle cell growth is blood pressure dependent and occurs as a response to the rise in pressure during postnatal life.

1.4.3.2 Pressure-independent.

Although Mulvany, Warshaw and fellow workers presented convincing evidence to suggest that structural change occurs as a direct consequence of the rise in blood pressure, others believe that this is an oversimplification of an intricate and complex situation (Frohlich and Tarazi, 1979). Furthermore, it has become clear that structural changes can develop in arteries of normotensive animals and in the veins of hypertensive animals, neither of which have been exposed to a raised blood pressure. The pressure-independent mechanisms which can induce structural change are described below.

1.4.3.2.1 Flow related structural change.

The hypothesis that blood flow may regulate vascular structure is not a new one, but had until recently received scant consideration. Support for this theory first began to accumulate when Kamiya and Togawa (1980) reported that increasing the blood flow through the carotid artery of dogs resulted in chronic adative dilation of the lumen of the vessel. Similarly decreasing the flow through blood vessels results in adaptive structural changes leading to a reduction in the lumen diameter of the vessel. These authors suggested that flow-oriented stress was the major cause of these adaptive changes with wall shear reacting in an autoregulatory manner. Guyton and Hartley (1985) demonstrated that a reduction in flow was correlated with a change in the cross-sectional area of the media of the vessel wall, indicating that flow-induced vascular change is structural rather than being functional in nature. Similar studies by Langille and O'Donnell (1986) demonstrated a reduction in the diameter of rabbit carotid arteries, following reduction in the rate of blood flow. This change could not be attenuated by the presence of smooth muscle relaxants lending further support to the idea of flow-induced structural vascular change. These results however, only indicate a correlation between flow and vascular structure and do not establish a causal mechanism.

The location of the endothelium exposes these cells directly to the blood flow whilst maintaining intimate contact with

the smooth muscle which effects changes in vessel diameter. It has been suggested that an interaction between these two cell types may control flow-induced vascular changes. Moreover an intact endothelial cell layer is neccesary for flow-induced vascular change to occur. Thus de-endothelialisation of arteries abolishes flow-induced structural changes in response to either increasing blood flow (Hull et al, 1984) or to decreasing blood flow (Langille and O'Donnell, 1984 and 1986) but has no effect on the contractile response.

1.4.3.2.2 Structural changes in a normotensive vascular bed.

The aortic coarctation model of hypertension is characterised by an elevated blood pressure above, and a normal arterial pressure below, the point of coarctation (Nolla-Panades, 1962). Thus this is a useful model for studying pressure independent vascular mechanisms involved in the hypertensive process. Studies of this model have shown that despite normal femoral artery pressures, the hindquarters of coarcted rats exhibit hyperreactivity to noradrenaline (Nolla-Panades, 1962) and a significantly increased resistance to flow (Bell and Overbeck, 1979). The resistance to blood flow decreased following maximal vasodilatation with sodium nitroprusside, but remained elevated compared to sham-coarcted animals, suggesting that structural changes have occured in the vessels of the hindquarters. In addition Liu et al (1988) demonstrated an increase in both media thickness and media

area of the abdominal aorta in coarcted rats. Similar structural changes have also been reported in small resistance arteries of the cremaster muscle (Plunkett and Overbeck, 1985) and in renal arterioles (Liu et al, 1988). These structural changes developed without a change in blood pressure which remained consistently normal in the hindquarters of coarcted rats even at eight weeks post-surgery (Overbeck and Magargal, 1989).

The role of sympathoadrenergic activity on the development of vascular structural change has been studied by Overbeck and co-workers in rats with aortic coarctation following sympathoadrenergic ablation. Sympathoadrenergic ablation attenuated but did not prevent cardiac hypertrophy (Overbeck, 1979), aortic medial hypertrophy proximal and distal to the point of coarctation (Overbeck, 1980) or structural changes in the arterioles of the cremaster muscle (Plunkett and Overbeck, 1988). Clearly these structural changes could not be attributed to a raised intravascular pressure or sympathoadrenergic influences, suggesting that humoral factors may be implicated. The time course of blood pressure elevation was similar to that produced by constricting a renal artery (Nolla-Panades, 1962) and plasma renin activity has been shown to be significantly elevated in coarcted rats (Overbeck et al, 1982), although the importance of angiotensin in the development of cardiovascular growth remains to be determined.

1.4.3.2.3 Early structural changes in genetic hypertension.

It has been generally accepted that vascular wall thickening in the SH rat, to a large extent, parallels the development of cardiac enlargement (Mulvany et al, 1980) and that both are adaptive responses to the increase in blood pressure. However, cardiac hypertrophy, and by implication vascular hypertrophy, has been found in the SH rat at thirty days of age when no significant difference in blood pressure could be detected (Yamori et al, 1979). Furthermore, Gray (1982) detected cardiac enlargement and an increase in aortic media thickness in SH rat pups at 12 to 24 hours after birth. These results suggest that cardiovascular structure may be genetically determined 'in utero' rather than being an adaptive response to the rise in blood pressure in post-natal life. Further support for this view comes from the studies of Eccleston-Joyner and Gray (1988) who have demonstrated an increased aortic medial thickness in 22 day old foetuses from SH rat dams. Although medial hypertrophy of the aorta does not contribute to the peripheral resistance it may be representative of the rest of the vascular tree and indicates that arterial pressure cannot be the sole determinant of hypertensive cardiovascular hypertrophy (Eccleston-Joyner and Gray, 1988).

On the other hand several authors have reported a significantly elevated blood pressure in the SH compared to the WKY rat in early neonatal life (Smeda et al, 1988; Morton et al, 1990) and even at birth (Gray, 1982). It is possible

that this difference in blood pressure, even if only transient, could cause the structural changes seen in the SH rat to develop before the phase of established hypertension. On the other hand it is possible that the foetal vasculature of the SH rat is affected 'in utero' by the elevated maternal blood pressure and the dams used by Eccleston-Joyner and Gray exhibited blood pressures of 105+5 mmHg in the WKY compared to 162+2 mmHg in the SH rat. Treatment of SH rat dams with hydralazine to normalise the blood pressure before and throughout the duration of pregnancy was performed by Smeda et al (1988). Hydralazine treatment was continued in the offspring of the dams up to 21 weeks of age and although blood pressure remained normal the development of structural change was almost identical to that observed in untreated hypertensive rats. The cross-sectional area of the media of the renal vessels in both the treated and the untreated SH groups was greater than that of either the treated or the untreated WKY group. These results lend further support to the idea that structural changes in the vasculature of the SH rat develop in the absence of an increased blood pressure. Similarly both Jesperson et al (1985) and Christensen et al (1989) treated SH and WKY rats with anti-hypertensive drugs from 4 weeks of age and monitored blood pressure and mesenteric vascular structure. Both groups found that antihypertensive treatment was able to normalise blood pressure but reported less success with media volume. Jesperson et al (1985) reported that treatment with hydralazine was unable to prevent an increase in wall

thickness. Christensen et al (1989) proved more successful using the angiotensin converting enzyme inhibitors perindopril and captopril. Perindopril reduced the media to lumen ratio by combined changes in media thickness and lumen diameter whereas captopril affected only the lumen diameter. Neither treatment however, significantly reduced media area. It would appear then that structural change can be neither prevented nor normalised by maintaining a normal blood pressure in these animals. Thus blood pressure is unlikely to be the sole determinant of hypertensive cardiovascular structural change.

1.4.3.2.4 Structural development in essential hypertension.

Cardiac hypertrophy has been demonstrated in the normotensive offspring of essential hypertensives (Savage et al, 1982). Moreover Takeshita et al (1982) demonstrated that the offspring of hypertensive patients exhibited an increased resistance to forearm blood flow compared to control groups matched for blood pressure. These results imply that there are structural vascular differences between the offspring of hypertensives and of normotensive controls and that the offspring of hypertensives may have inherited a genetic predisposition to hypertensive change. The idea of pressure-independent structural changes is further supported by the observations that the distensibility of forearm veins is considerably reduced in borderline hypertension (Takeshita and Mark, 1979). Since veins are not exposed to the increased

blood pressure any abnormalities in these vessels are presumably related to neural or humoral influences. The possibility that the decreased vein distensibility depends on adrenergic venoconstriction has been investigated using the administration of phentolamine. Interestingly although phentolamine caused an increase in the venous distensibilty, it did not reach those levels observed in normotensive controls. It would appear that these changes are non-pressure related and that adrenergic mechanisms account for only a small portion of the decreased venous distensibility.

1.5 Relationship between structural change and the rise in blood pressure.

Most of the studies described have investigated the temporal relationship between the development of vascular structural change and the rise in blood pressure using a morphological / histological approach, and this approach indicates that structural change occurs either as an adaptive response to the increase in wall tension (Mulvany et al, 1980) or develops in parallel with the increase in blood pressure (Lundgren et al 1974). Unfortunately these studies are of limited value in defining the relationship between structure and blood pressure since they are capable only of detecting the final stage of a series of cellular processes. Studies of the biochemical events which underly growth offer the promise of greater sensitivity because they are early markers of developing structural change.

The binding of a mitogenic polypeptide to its plasma membrane specific receptor induces an ordered sequence of events which eventually lead to cell division. To date a number of stimulatary pathways have been identified and partially characterised (Figure 6). The pre-replicative phase, that is the time from receptor binding to the start of DNA synthesis, can last for more than 24 hours. By contrast the time lag between receptor binding and morphologically detectable vascular growth may be in the order of several days or a week. Thus detection of morphological change is a late phase comparable to that of icing in the process of baking a cake. Stimulated cells generally become committed to DNA synthesis in S phase. Although most of the steps that are obligatory for progression from receptor binding to S phase remain to be established in intact tissue, studies of cultured cells have defined several biochemical responses which occur rapidly following the addition of mitogenic substances and these will be discussed below.

1.5.1 Inositol Lipid Metabolism.

It is now thought that the initial response to receptor mediated binding of many growth factors involves the hydrolysis of a phosphorylated derivative of phosphtidylinositol (Vincentini and Villereal, 1986; Berridge, 1987). Phosphatidylinositol is converted by a two stage phosphorylation into phosphatidyl 4,5-bisphosphate (PIP_2) which is located in the inner leaflet of the plasma

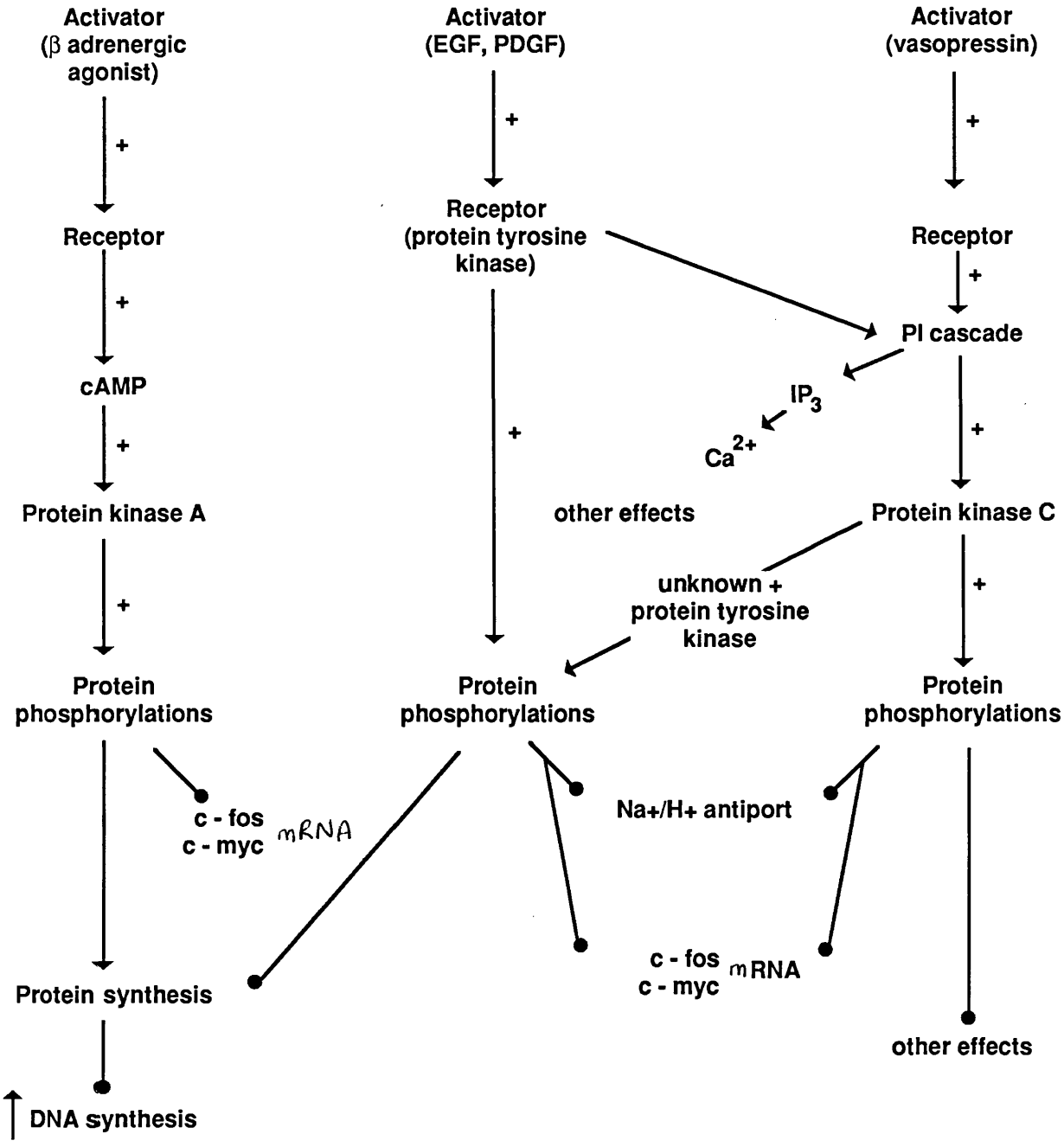
FIGURE 6

Cascade of biochemical events which may be initiated by receptor binding at the plasma membrane and eventually lead to a commitment to growth.

—● do not imply direct causal relationships.

(Taken from; Dumont et al, 1989).

FIGURE 6


Activator
(β adrenergic
agonist)
+
Receptor
+
cAMP
+
Protein kinase A
+
Protein
phosphorylations
c - fos
c - myc
mRNA
Protein synthesis
DNA synthesis
Activator
(EGF, PDGF)
+
Receptor
(protein tyrosine
kinase)
+
other effects
Protein
phosphorylations
Na+/H+ antiport
c - fos
c - myc
mRNA
Activator
(vasopressin)
+
Receptor
+
PI cascade
IP3
Ca2+
+
Protein kinase C
unknown +
protein tyrosine
kinase
+
Protein
phosphorylations
other effects

membrane and is believed to be the precursor used by the membrane receptor mechanisms (Combettes et al, 1987). Binding of an agonist to its receptor, causing conformational change of the inner surface of the receptor protein, activates the hydrolysis of PIP_2 by a membrane-bound phosphodiesterase (phospholipase C) resulting in the generation of the second messengers inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG). (Berridge and Irvine, 1984; Williamson, 1986).

The action of the enzyme phospholipase C is thought to be mediated not only by agonist / receptor binding but also by a guanosine 5'-triphosphate (GTP)-binding protein (Berridge, 1986 and 1987; Williamson, 1986). Evidence that a GTP-binding protein, as yet unidentified, functions as part of a calcium mobilizing signal transduction process came from reports that GTP analogues decreased the affinity of various receptors for binding to their agonists (Crane et al, 1982; Lynch et al, 1985). Its more specific involvement in inositol lipid metabolism was confirmed in 1985 by Sasagur et al who demonstrated that the addition of GTP analogues to vascular smooth muscle plasma membrane preparations caused an increase in both the breakdown of polyphosphoinositides and the formation of inositol phosphates.

1.5.1.1 Inositol trisphosphate and calcium mobilization.

Inositol trisphophate is hydrophilic and, on formation, is released into the cytoplasm where it acts to raise the

intracellular calcium ion concentration ($[Ca^{2+}]_i$). This rise in $[Ca^{2+}]_i$ is due to mobilization of Ca^{2+} from internal stores and does not depend on external Ca^{2+} levels (Joseph et al, 1985). More specifically it has been determined that the intracellular organelle responsible for IP_3-induced Ca^{2+} release is closely allied with endoplasmic reticulum structures (Dawson and Irvine, 1984; Muallem et al, 1985). The actual mechanism of the IP_3-induced calcium release remains to be identified or characterised but is presumably mediated by the binding of IP_3 to a specific protein in the endoplasmic reticulum membrane (Spat et al, 1986).

The importance of a rise in $[Ca^{2+}]_i$ as a signal for cell proliferation followed the observation that the Ca^{2+} ionophore A23187 stimulated DNA synthesis in quiescent pig (Maino et al, 1974) and human (Luckasen et al, 1974) lymphocytes. External calcium deprivation abrogated the response whereas deprivation of magnesium had little effect. These results coupled with the observations that IP_3 raises the intracellular calcium concentration by releasing Ca^{2+} from internal stores strongly support the hypothesis that an increased $[Ca^{2+}]_i$ is a vital, possibly obligatory, step in the signal transduction process.

1.5.1.2 Diacylglycerol

Diacylglycerol is hydrophobic and remains in the inner leaflet of the plasma membrane where it has a variety of physiological functions. A major action appears to be

promoting the binding of protein kinase C to the plasma membrane. DAG achieves protein kinase C activation by decreasing the Ca^{2+} requirement of the enzyme from mM to μM concentrations. DAG can be phosphorylated by diacylglycerol kinase to form phosphatidic acid which has been shown to trigger the phosphodiesteratic cleavage of inositol phospholipids and thereby mobilize Ca^{2+} from internal stores (Moolenaar et al, 1986). In addition DAG may be the substrate for a lipase which acts to release arachidonic acid which can then be converted into a whole variety of metabolites including prostaglandins and thromboxanes (Berridge, 1986). The relative amounts of DAG and IP_3 formed during the hormonal stimulation of cells appears to be important for expression of their separate signaling roles because of the different sensitivities of their effects. Thus a relatively mild agonist-induced stimulation of phospholipase C may be sufficient to elicit IP_3-induced mobilization of Ca^{2+} but not able to bring about full DAG-activation of protein kinase C (Bocckino et al, 1985). Conversely some tissues such as cardiac muscle, appear to lack the Ca^{2+} mobilization branch of the signaling system because of the absence or low sensitivity of the IP_3 receptor in the sarcoplasmic reticulum (Movsesian et al, 1985).

1.5.2 Protein Kinase C activation.

Protein kinase C (PKC) is a Ca^{2+} and phospholipid-dependent enzyme that is activated physiologically by DAG and in vitro

by various tumour-promoting agents such as phorbol myristate acetate. Protein kinase C probably does not exist as a single entity but appears to be a family of isoenzymes each consisting of a single polypeptide chain with a molecular weight of approximately 77,000 KDa (Nishizuka, 1984). At least three such isoenzymes have been isolated which may exhibit significant biochemical differences (Sekiguchi et al, 1987). Protein kinase C is composed of two functionally different domains which can be separated by Ca^{2+}-dependent thiol proteases (Kishimoto et al, 1983). One of these domains encompasses the regulatory and membrane-binding functions, whereas the other is hydrophilic and carries the catalytically active centre which is fully active without the need for Ca^{2+}, phospholipid and diacylglycerol (Nishizuka, 1984). In the resting state the enzyme is cytoplasmic and inactive. When stimulated however, the kinase is translocated to the membrane where it actively phosphorylates its substrates. Protein kinase C has a broad substrate specificity, phosphorylating seryl and threonyl residues, but not tyrosyl residues, of many endogenous proteins. Although protein kinase C activation is independent of calmodulin the enzyme can be profoundly inhibited by a large number of phospholipid-interacting drugs which are calmodulin inhibitors (Mori et al, 1980; Wise et al, 1982). The requirement of protein kinase C activation for divalent calcium ions appears to be virtually absolute, only strontium, of the divalent metal ions, will substitute for calcium but even so this is only 5% as potent an activator as

calcium (Takai et al, 1979).

It is more than likely that protein kinase C activation and calcium mobilisation act synergistically to elicit the full physiological growth-factor response of some cells including activation of the Na^+/H^+ exchanger and concomitant alkalinisation of the cytoplasm.

1.5.3 Na^+/H^+ Exchanger and pH.

The Na^+/H^+ exchanger is the best characterised exchange system mediating proton extrusion. Amiloride competitively inhibits the exchanger acting on the sodium binding site of the carrier (Frelin et al, 1983; Rothenberg et al, 1983; Paris and Pouyssegur, 1983). This amiloride-sensitive sodium uptake system has been demonstrated in many cell types including cultured vascular smooth muscle cells (Berk et al, 1987; Weissberg et al, 1987), and in intact tissues under resting conditions (Little et al, 1986; Ek and Deth, 1988). Diacylglycerol appears to activate the Na^+/H^+ exchanger via protein kinase C resulting in a rise in cytosolic pH which is one of the main signals conveying information from the cell surface to the nucleus (Combettes et al, 1987). Experimental evidence indicates that growth factor stimulation causes cellular alkalinisation by inducing a conformational change in the Na^+/H^+ exchanger which results in an increased affinity of the carrier for cytoplasmic protons (Moolenaar et al, 1983; Muldoon et al, 1985).

The importance of a rise in pH in the signal transduction

process is supported by studies where amiloride inhibition of the Na^+/H^+ antiporter prevented growth factor induced DNA synthesis (L'Allemain et al, 1984; Pouyssegur et al, 1985). However, Aalkjaer (1990) pointed out that most experiments have been carried out in bicarbonate-free media and that in the presence of bicarbonate amiloride or its analogues have no effect on growth hormone-induced DNA synthesis in vitro (Pouyssegur et al, 1985). Thus although a raised intracellular pH may play a vital role in the initiation of growth, activation of the Na^+/H^+ exchange in the presence of bicarbonate may play only a secondary role in this process. Mitogen stimulated cellular alkalinisation appears to be a secondary response to an elevation of the intracellular calcium concentration (Hesketh et al, 1984), and formation of a calcium/calmodulin complex (Vincentini and Villereal, 1986) and ultimately may act as a signal for protein synthesis (Winkler et al, 1980).

1.5.4 Proto-oncogene expression.

Proto- or c-oncogenes are expressed at low levels in most normal eukaryotic cells, differing from their transforming oncogene counterparts in that they are involved in the control of normal cell processes (Macara, 1985; Marban and Koretsune, 1990).

Two proto-oncogenes c-myc and c-fos are thought to encode for DNA-binding proteins that may influence cellular differentiation and/or proliferation (Marban and Koretsune,

1990). Their expression appears to be stimulated by such diverse mitogens as angiotensin II, vasopressin (Baudouin-Legros et al, 1989), concanavlin A (Moore et al, 1986), and interestingly by phorbol esters (Macara, 1985).

The idea that proto-oncogene expression occurs as part of the signal transduction process is supported by experiments demonstrating that the activation of c-fos and c-myc genes by the calcium ionophore A23187 was completely abolished by the addition of EGTA to the external medium (Moore et al, 1986). Morgan and Curran (1986) showed that induction of c-fos by nerve growth factor is calcium dependent. These authors also demonstrated that the addition of the 1,4-dihydropyridine calcium channel antagonist nisoldipine completely blocked the potassium-induced expression of c-fos mRNA. On the other hand the mitogens concanavlin A and PDGF bring about both c-fos and c-myc stimulation which is independent of calcium concentration (Moore et al, 1986; Frick et al, 1986). Thus different mitogens cause proto-oncogene expression via both the phosphinositide pathway and by other signal transduction pathways such as the cAMP and tyrosine kinase pathways (Dumont et al, 1989).

1.6 Signal transduction, growth and hypertension.

Although tempting, the use of intermediate products of the signal transduction pathway to indicate growth may have limitations since the biochemical processes described are not necessarily indicators of a committed growth response.

Moreover, proto-oncogene expression in thymocytes has been shown to be stimulated by sub-mitogenic doses of various mitogens (Moore *et al*, 1986). In addition many of the mitogens used in tissue culture work are well established vasoconstrictor substances *e.g.* Angiotensin II, endothelin, phorbol esters. Thus these agonists induce a similar signal transduction pathway but are capable of producing different tissue responses. In fact the formation of IP_3 by hydrolysis of the inositol phospholipids has been shown to be a major biochemical event associated with the activation of smooth muscle contraction (Miller-Hance *et al*, 1988), as has the release of calcium from intracellular stores (Somlyo *et al*, 1985).

One explanation for these different responses depends on vascular smooth muscle cells existing in either a contractile or synthetic phenotype (Schwartz, 1983) and different receptors may be exhibited by the two phenotypes (Demolle *et al*, 1989). Unfortunately the two phenotypes can be distinguished only by their expression of a stimulatory signal. Thus the only certain way of determining growth is the measurement of a committed growth response such as DNA synthesis.

1.6.1 Measurement of DNA synthesis.

The rate of DNA synthesis can be assessed easily using [^{3}H]-thymidine, a radio-labelled isotope of the pyrimidine base specific for DNA, *in vivo* in animal models or *in vitro* in

isolated tissues and cell culture. This technique for monitoring growth rates has been applied to hypertension research since the 1960's. Crane and Dutta (1963) used intraperitoneal injection of [^{3}H]-thymidine into rats, followed by autoradiography of processed tissue sections in order to determine alterations of cell growth dynamics in steroid-induced hypertension. Also similar methods have been used to investigate growth rates in aortic constriction (Fernandez and Crane, 1969), renovascular (Rorive et al, 1980) and genetic (Yang et al, 1989) hypertension. The autoradiographic method has a number of advantages, it allows nuclei which are synthesising DNA to be visualised and their relative position to be observed. Clusters of labelled nuclei provide a qualitative index of mitosis, as well as providing a quantitative measure of DNA synthesis in the form of a calculated labelling index.

An alternative approach depends upon measuring the quantity of [^{3}H]-thymidine present in the perchloric acid soluble fraction of tissue homogenates by scintillation counting. This method generally involves in vitro incubation of freshly isolated tissues with the [^{3}H]-thymidine in a physiological buffer solution or the addition of [^{3}H]-thymidine to the media of cells in culture. The method has been applied with a great deal of success using isolated aortae from rats with both renovascular (Rorive et al, 1980; Carlier et al, 1983; Loeb et al, 1986; Loeb and Bean, 1986) and genetic (Loeb and Bean, 1986) hypertension, providing clearly defined quantitative values for DNA synthesis without making

assumptions about polyploidy.

Bromodeoxyuridine incorporation has also been used as an alternative to [^{3}H]-thymidine for the measurement of DNA synthesis. This base analogue, as a mutagenic agent, will be incorporated into DNA in place of thymidine and will allow normal DNA replication to occur, although it will cause point mutations in the base sequence to occur. This analogue relies on histological processing of tissue samples.

1.6.2 Growth kinetics, hypertrophy and hypertension.

1.6.2.1 Experimental hypertension.

Medial hypertrophy of arteries is a consistent feature of experimental hypertension. If this hypertrophy represents a true structural change it may be that some disturbance in cell growth rates resulting in cellular hyperplasia forms the basis of the increase in tissue volume. Thus the relationship between the rate of cell turnover, as measured by [^{3}H]-thymidine incorporation, and the rise in blood pressure may provide a useful insight into the development of hypertension.

In 1963 Crane and Dutta demonstrated that incorporation of [^{3}H]-thymidine was increased in tissues from both young and hypertensive animals. These results suggest that young rats in the active growth phase of their life cycle have a greater capacity for DNA synthesis than adult rats, and moreover that adult rats can regain this synthetic ability when

hypertensive. The authors believed that these studies provided substantial evidence to support the idea that arterial smooth muscle hyperplasia is associated with hypertension. Similarly Fernandez and Crane (1969) reported that new cell formation was increased in rats with an aortic constriction. However systematic blood pressure measurements were not made and as a consequence the relationship between growth rates and hypertension is somewhat obscure.

Studies of one-kidney, one-clip (1K, 1C) renal hypertension in rats (Rorive et al, 1980) clearly show that medial hypertrophy had occurred by the end of the first week following renal artery clipping with an increase in both the rate of DNA synthesis and the total DNA content by that stage. The issue becomes less clear with regard to the relationship between these changes and the onset of hypertension with the authors observing only that 'the blood pressure of the rats with renal artery stenosis rapidly increased and reached maximum levels within three weeks.' More light was shed on the situation by Carlier et al (1983) who demonstrated that the blood pressure was significantly elevated just two days after renal artery clipping and also that the rate of DNA synthesis was significantly raised at four days. They concluded that the increase in DNA synthesis was strongly correlated to the increase in blood pressure with the rate of [^{3}H]-thymidine incorporation reaching a maximum as blood pressure rose and returning to control levels when blood pressure achieved a plateau. Unfortunately this study provided no information about rates of DNA

synthesis in control and hypertensive rats before four days after renal artery constriction. Thus it is possible that a significant increase in the rate of cell turnover may have occured within the first four days and may even have preceded the rise in blood pressure. Indeed this was exactly the situation found in rats with two-kidney, one-clip (2K, 1C) hypertension where although the blood pressure remained unchanged five days after renal artery clipping DNA synthesis was significantly increased (Loeb et al, 1986).

Clearly, medial hypertrophy of blood vessels in experimental hypertension appears to be associated with increased cell growth but whether this cell growth takes the form of true hyperplasia is still unclear and may depend on the experimental hypertensive model and the type of vessel studied. Carlier et al (1983) reported an increase in total DNA content and using an autoradiographic technique demonstrated radioactive nuclei next to each other in vessel sections suggesting mitosis and true cellular hyperplasia. Loeb et al (1986) on the other hand failed to demonstrate an increase in total DNA content despite finding elevated DNA synthesis rates up to 21 days post-renal artery clipping. They did however, record a decrease in the DNA/protein ratio of hypertensive vessels indicating cellular hypertrophy. This would be in keeping with observations of an increase in the frequency of polyploid smooth muscle cells from the aortae of rats with 2K, 1C hypertension. (Owens and Schwartz, 1983). However the increase in polyploidy did not achieve significance until one month after renal artery clipping but

was increased by 200% by five months, at which stage the mean smooth muscle cell protein content of the 2K, 1C animals was twice that of age-matched normotensive controls. Similar results have been reported in rats with aortic coarctation (Owens and Reidy, 1985).

1.6.2.2 Genetic Hypertension.

In genetic hypertension the relatioship between the rate of cell turnover and the development of medial hypertrophy is less certain. Loeb and Bean (1986) demonstrated similar rates of aortic DNA synthesis in four week old SH and WKY rats when there was no significant difference in blood pressure. Interestingly, there was an increased rate of aortic [^{3}H]-thymidine incorporation in 17 week old SH rats with established hypertension compared to the WKY however, there was no difference at 21 weeks of age. This led the authors to speculate that aortic DNA synthesis depends on the rate of blood pressure rise and by 21 weeks the blood pressure of the SH rat had become stable thus depressing the drive for DNA synthesis.

The increase in DNA synthesis detected in the SH rat appears to lead to a greater frequency of polyploid cells. Owens et al (1981) using an image analysis system demonstrated a significant increase in protein and DNA content of aortic smooth muscle cells from SH compared to WKY rats. These results suggest that the cellular response is hypertrophy

coupled with polyploidy, similar to that seen in the experimental hypertension. However this study was unsatisfactory because the authors provided no details of the blood pressure or the age of the animals used in their experiments. Later studies showed that although the frequency of polyploid cells increased with age in both SH and WKY rats the incidence was significantly greater in the 3 month old SH rat but plateaued when blood pressure and medial hypertrophy were fully established (Owens and Schwartz, 1982; Owens, 1985). The increase in polyploidy in the SH rat was confirmed by Rosen et al (1986) and these observations indicated that the rate of DNA synthesis must be increased in the SH rat at some time during the development of hypertension. Owens (1985) went on to show that anti-hypertensive drug treatment can prevent the onset of medial hypertrophy and the development of polyploidy in rats between 8 and 20 weeks of age.

All of these studies utilised the aorta, the largest conduit vessel which is easy to identify, isolate and manipulate. Blood flow is however controlled by contraction of much smaller arteries and the biological response of large arteries may not reflect the changes occuring in the small resistance arteries. Unfortunately few studies of the growth kinetics of these small arteries have been performed but Yang et al (1989) have demonstrated a significantly increased labelling index in the large mesenteric arteries of one week old SHR's but not in older rats. Moreover, a number of studies have investigated but failed to detect an increase in

the frequency of polyploidy in the mesenteric arteries of SH compared to WKY rats at any age (Black et al, 1988; Owens et al, 1988; Lombardi et al, 1989).

1.7 Aims and objectives.

The aim of this thesis was twofold, firstly to provide a clearer understanding of the relationship between the development of medial hypertrophy (as measured in terms of rates of DNA synthesis) and the rise in blood pressure, and secondly to elucidate the cellular nature of arterial hypertrophy.

Since the course of development of experimental and genetic hypertension are quite different it follows that the signals inducing growth may not be the same. Therefore the relationship between medial hypertrophy and the rise in blood pressure may also be different in the two models. In order to investigate this relationship it was essential that blood pressure and growth were accurately measured from early in development through to the established phase of hypertension in both hypertensive models. Moreover the investigation involved studies of both large conduit vessels and the small resistance arterioles which regulate peripheral resistance.

Hopefully the series of carefully planned investigations detailed within have achieved at least some of their objectives and provided a new and useful insight into the aetiology of arterial hypertrophy.

CHAPTER TWO.

MATERIALS AND METHODS.

2.1 Materials.

Materials and suppliers are listed in appendix A.

2.2 Equipment.

Equipment and manufacturers are listed in appendix B.

2.3 Animals.

Female Wistar, Spontaneously Hypertensive (SH) and Wistar-Kyoto normotensive (WKY) rats were obtained from the Biomedical Services Unit (BMSU) at Leicester University. All animals had free access to food and water and were maintained on a 12 hour light/dark cycle up to the time of sacrifice.

2.3.1 Experimental Hypertension.

Female Wistar rats weighing between 170 and 190 grams were used for the induction of two-kidney, one-clip Goldblatt hypertension. Animals were anaesthetized with ether and a small loin incision made. A silver clip, internal diameter 0.2mm, was applied to the renal artery, the contralateral kidney being undisturbed. Control animals were subject to a similar operation with the silver clip being placed next to, but not constricting, the renal artery. Animals were studied at 3, 7, 14 and 28 days post-renal artery constriction.

2.3.2 Genetic Hypertension.

The study of the development of genetic hypertension was performed using the Okamoto-Aoki strain of the spontaneously hypertensive rat, with age and sex matched Wistar Kyoto normotensive controls. Both strains of rat were obtained from colonies bred on site by the Biomedical Services Unit. Animals were studied at 5, 12 and 18 weeks of age.

2.3.3 Assessment of Hypertension.

Rats were lightly anaesthetized with ether and their systolic blood pressure measured using a photoelectric tail-cuff method (Swales and Tange 1970). Blood pressure measurements were made at appropriate times during the development of renovascular hypertension, at weekly intervals in the genetic hypertensive rats and routinely 24 hours prior to sacrifice for all animals.

2.4 Preparation of Tissues.

Tissues from female Wistar, SH and WKY rats were all prepared in the same general manner. Animals were weighed and then culled by a single blow to the head followed by cervical dislocation.

2.4.1 General preparation.

Immediately following sacrifice the animals chest cavity was opened and a blood sample obtained from the heart. Blood samples were centrifuged at $4^{o}C$, 1500 x g for 15 minutes. The resulting plasma was carefully removed and stored at $-20^{o}C$ prior to assaying for plasma renin concentration. The heart, aorta (from the heart to the bifurcation), skin, small intestine and hindlimbs were all excised and transferred to a beaker of physiological salt solution, PSS (see Appendix C), on ice.

Hearts were rinsed in PSS, blotted dry and weighed, a heart weight/body weight ratio was then determined as a measure of cardiac hypertrophy. The muscular tissue was stripped from the hindlimbs to obtain the tibia which were then dried overnight at room temperature and their dry lengths measured. A heart weight/tibial length ratio was then calculated as a further measure of cardiac hypertrophy (Yin et al, 1982).

2.4.2 Vessel preparation.

Aortae were cleaned of all overlying fat and connective tissue, blotted and their length and weight recorded. The small intestine was pinned out to display the bed of blood vessels and, with the aid of a dissecting microscope, four branches (second or third order) of the superior mesenteric artery were carefully cleaned, measured and dissected. In addition two resistance arterioles, supplied by the

subclavian artery, were dissected from just under the skin, approximately 1cm (centimetre) from the midline incision. These vessels were accordingly cleaned and their length measured using a vernier caliper.

2.5 Determination of DNA Synthesis.

The procedure for determining DNA synthesis in isolated whole vessels is based on the method described by Loeb et al (1986), and has been modified for use with both large and small vessels.

Cleaned and dissected vessels were placed in bijou bottles containing 2ml of ice-cold PSS. Samples were then pre-incubated in a 37^{o}C shaking water bath with an atmosphere of 95% Oxygen 5% Carbon dioxide, for 1 hour. Subsequent to this 184KBq [methyl-^{3}H]-thymidine / ml PSS, was added to each sample and the incubation continued for a further 18 hours.

At the end of the incubation period the vessels were removed from the bijou bottles and rinsed three times in ice-cold NTE (Appendix C), to ensure removal of surface radioactivity. The vessels were then homogenised in 1ml NTE using glass-on-glass hand-held homogenisers. Two 100µl aliquots of the homogenates were removed for deoxyribonucleic acid (DNA) and protein determination, the remainder being adjusted to a concentration of 0.2N Perchloric acid (PCA) by the addition of 800µl of 0.4N PCA. Samples were then centrifuged at 1600 x g for 15 minutes. Aliquots (3 x 200µl) of the supernatant were removed for counting to establish the amount of acid

soluble radioactivity. The remaining s upernatant was discarded and the pellet washed once in 2ml 0.2N PCA to remove any residual acid soluble radioactivity. The pellet was then resuspended in 2ml 0.5N PCA and heated at 100^{o}C for 30 minutes in order to hydrolyse and extract the DNA. Samples were then rapidly cooled on ice and re-centrifuged at 1600 x g for 15 minutes. Aliquots (3 x 200µl) of the resulting supernatant were removed to determine the incorporation of [methyl-^{3}H]-thymidine into the hydrolysate. All traces of supernatant were carefully removed from the pellet using a Gilson p20 pipette, and the pellets were resuspended in 1ml 1.0N HCl. These suspensions were used to determine the incorporation of [methyl-^{3}H]-thymidine into the non-extracted DNA, and the efficiency of the hot PCA extraction. All samples were counted in a Packard liquid scintillation analyzer with Optiphase-X scintillation fluid.

2.6 Analysis of Protein Content.

Two methods for measuring protein content have been utilised in these studies. Both methods are based on the ability to monitor cuprous ion production in the reaction between protein and alkaline Cu^{2+} (Biuret reaction). The Lowry protein assay was the most widely used, but the Bicinchoninic Acid (BCA) method for the analysis of protein was used for the small resistance vessels from the SH and WKY rats.

2.6.1 Quantitation by Lowry Assay.

This method is based on the assay system described by Lowry et al (1951). Duplicate standards, over the range 3µg - 50µg, were made up to a volume of 100µl using a stock solution of bovine serum albumin (BSA) at 1mg/ml and deionised water. Several different volumes of samples were used to ensure that at least one fell within the limits of the assay, all samples were diluted to a final volume of 100µl with deionised water. Protein was dissolved by the addition of 20µl of 5M NaOH to both standards and samples.

Freshly made solution C (2% Na_2CO_3 in 0.1M NaOH with 1/100 volume of both 1% $CuSO_4$ and 2% NaKTartrate) at a volume of 500µl was then added to each tube. All tubes were vortexed and left to stand for 10 minutes at room temperature. Following this 100µl of a mixture of Folin Ciocalteu's phenol reagent and deionised water (in a ratio of 1:1), was added and each tube was immediately vortexed. Tubes were then allowed to stand for a further 30 minutes at room temperature.

The optical density of both standards and samples was measured at a wavelength of 750 nanometres (nm) and a standard curve of absorbance at 750nm versus µg of protein was plotted. The protein content of the samples was then determined by reading their values off this curve.

2.6.2 Quantitation by Bicinchoninic Acid (BCA) Method.

This assay system is based on the method described by Smith et al (1985). A stock solution of BSA (100µg/ml) was diluted in deionised water to provide a range of protein standards. Duplicate standards, over the range 500ng - 15.0µg were used routinely. Samples and standards were made up to a volume of 500µl with deionised water. A fresh solution of working reagent, prepared from reagents A and C (Appendix C) in a ratio of 1:1, was prepared. An equal volume (500µl) of this working reagent was added to each tube. Tubes were vortexed and incubated at 60°C for a period of 1 hour. Following incubation the absorbance of all tubes was measured at 562nm and a standard curve of absorbance versus µg protein was plotted. The protein content of the samples was then determined by reading values from this calibration curve.

2.7 DNA Solubilisation Procedure.

The extraction of DNA from crude tissue homogenates was based on a detergent solubilisation method originally described by Fiszer-Szafarz et al (1981) and subsequently adapted by Downs and Wilfinger (1983).

DNA solubilisation was initiated by the addition of 30µl of extraction buffer (10N Ammonium hydroxide and 2% Triton X-100 in NTE) to each 100µl sample (aliquots of homogenates obtained as described in 2.5). The tubes were quickly capped, gently mixed and incubated at 37°C for 10 minutes. After

incubation each sample was diluted with filtered NTE to give a final concentration of 0.025N NH_4OH and 0.005% Triton X-100 and centrifuged at 1600 x g for 30 minutes. The supernatant was removed carefully and stored at -20°C prior to concentration by ultrafiltration. Pellets were carefully dried and stored at -20°C prior to electrophoretic analysis to determine the efficiency of DNA extraction.

2.8 Ultrafiltration.

Extracted, stored DNA samples were concentrated by means of an Amicon stirred cell ultrafiltration system using diaflo YM30 membranes which have a molecular weight cut-off point of 30,000. Membranes were rinsed in deionised water for a period of 1 hour, with at least three changes of water, prior to use in the ultrafiltration system.

Concentration of DNA was acheived by forcing the NH_4OH/Triton X-100 buffer through the membrane to waste, under the pressure of Nitrogen gas. Samples were concentrated to a volume of approximately 500µl and were carefully removed from the membrane to an eppendorf tube. The membrane was washed, at least once, with 200µl of buffer, and the washes transferred to the eppendorf tube. Samples were pulsed in an MSE microcentaur and an accurate measurement of their volume obtained. Samples were then stored at -20°C prior to DNA determination.

2.9 Analysis of DNA Content.

There are a variety of methods available for the measurement of DNA. The analysis of DNA in this work entails the use of both a colorimetric and a fluorescent assay technique.

2.9.1 Quantitation by Diphenylamine Assay.

This spectrophotometric assay system is based on that described by Burton (1955). Calf thymus DNA, from a stock solution of 0.1mg/ml, was used to give standard DNA measurements. Duplicate standards, over the range 1µg - 30µg were used routinely. Standards were made up to a volume of 400µl with deionised water and adjusted to a concentration of 0.5M PCA by the addition of 100µl of 2.5M PCA.

Samples were adjusted to a concentration of 0.25M PCA and 100µg/ml BSA added to each, this acts as a co-precipitant but does not interfere in the extraction or analysis procedures. Samples were vortexed and allowed to stand on ice for 45 minutes. Following this samples were spun at 11600 x g for 10 minutes. The resulting supernatant was discarded and the pellet washed once with 0.25M PCA to remove any soluble nucleotides before being resuspended in 0.5M PCA.

DNA was hydrolysed by heating at 70^{o}C for 1 hour after which the solutions were cooled rapidly on ice. Samples were then centrifuged and the supernatant used for DNA estimation.

Two volumes of Diphenylamine working reagent (appendix C) were added to both the standards and the samples. All tubes

were briefly vortexed and incubated at 37°C for 18 hours. Following incubation the absorbance of both the standards and the samples was measured at 600nm and a standard curve of absorbance versus µg DNA was plotted. The DNA content of the samples were then read from this curve.

2.9.2 Quantitation by Hoechst 33258.

Hoechst 33258 is a fluorochrome which, on combination with DNA, gives a significant enhancement of fluorescence (Brunk et al; 1979). The assay system utilises this enhancement and is based on that described originally by Labarca and Paigen (1980), with further modifications by Downs and Wilfinger (1983).

Highly polymerised calf thymus DNA, from a stock solution of 50µg/ml, was used to give standard DNA measurements. Stock DNA was diluted with assay buffer (0.025N NH_4OH, 0.005% Triton X-100 in NTE) and the resulting standards, over the range 5ng-150ng, were stored on ice.

Hoechst 33258 dye was dissolved in filtered NTE at a concentration of 25µg/ml. Immediately prior to assay an aliquot of dye was diluted to a final concentration of 100ng/ml and stored, foil wrapped, on ice.

Hoechst dye solution at a volume of 1.5ml was pipetted into labelled tubes along with 50µl of each DNA standard, 50µl of assay buffer was used as a blank solution, and their fluorescence measured, (excitation wavelength 350nm, emission wavelength 455nm, slit widths 10nm). Concentrated DNA samples

were assayed, in triplicate, in the same manner. Three standard curves were measured routinely and the mean of these curves was plotted (fluorescence-blank value versus ng DNA). The DNA content of the concentrated samples were then determined by reading from this curve.

2.10 Electrophoretic Analysis of DNA.

Electrophoretic analysis of DNA is important as it provides the only visual means of determining the efficiency of DNA solubilisation. The method was used routinely as a qualitative measure of non-extracted DNA but was also occasionally used in a semi-quantitative fashion.

2.10.1 Protein Digestion.

The method of protein digestion is based on that described by Clegg et al (1986), and has been used here as the essential first step in the electrophoretic analysis of DNA. Contaminating proteins were digested from pellets obtained in section 2.7 in the following manner. Pellets were allowed to thaw slowly at room temperature prior to the addition of 34µl of TE buffer (10mM Tris, 1mM EDTA), 4µl of 10% SDS in H_2O and 2µl 5% protease. Tubes were capped and incubated overnight at 37^{o}C. Following incubation the digested pellets were transferred to labelled eppendorf tubes and stored at -20^{o}C before being run on agarose gels.

2.10.2 Electrophoresis

Digested pellets were thawed slowly on ice and 8µl of a solution of 0.5mg/ml bromophenol blue in 50% sucrose was added as a tracking dye. Electrophoresis was performed using 0.8% agarose gels with TBE (90mM Tris, 90mM Borate, 2.5mM EDTA, pH 8.3) as the running buffer. Gels were electrophoresed at 100 volts until the tracking dye had travelled $^{3}/_{4}$ of the length of the gel. DNA was stained using ethidium bromide at a concentration of 1µg/ml in both the gel and the running buffer. DNA was visualised on an ultra-violet flat bed transilluminator at 254nm and photographed using a polaroid camera.

2.11 Ethanol Precipitation of DNA.

Stored digested pellets (Section 2.10.1) were thawed slowly on ice and resuspended in 500µl of NTE buffer prior to the addition of 1/10 volume of 3M sodium acetate and 2 volumes 100% ethanol. Tubes were capped, vortexed and the DNA allowed to precipitate at -70^{o}C for 2-3 hours, or -20^{o}C overnight. Samples were then centrifuged at 11600 x g for 15 minutes. The ethanol supernatant was removed to a fresh tube and the ethanol blown off under nitrogen gas, any radioactivity remaining in this tube was resuspended in 500µl of NTE buffer and counted. The remaining DNA pellet was also resuspended in NTE buffer and counted.

2.12 Radioimmunoassay for Renin

Renin is a proteolytic enzyme which converts angiotensinogen (renin-substrate) into angiotensin I, which is, in turn, converted to the octapeptide angiotensin II. The determination of plasma renin concentration is a useful index of the activity of the renin-angiotensin-aldosterone cascade system and can be acheived by the radioimmunoassay of angiotensin I, provided that there is an excess of available renin substrate. This was performed using a Renin MAIA kit purchased from Serono Diagnostics.

2.12.1 Preparation of nephrectomised rat plasma.

Wistar rats (approximately 250g) were anaesthetized with diethyl ether and a bilateral nephrectomy performed. The animals were given access to food and water until sacrifice 24 hours later. Rats were again anaesthetized with ether, the body cavity was opened and the rats were bled from the aortic bifurcation. All animals were then culled by cervical dislocation. Rat blood was collected in EDTA coated tubes on ice followed by centrifugation at 1500 x g for 25 minutes at 4^{o}C. The plasma, which is renin-free and rich in renin substrate, was removed and stored at -20^{o}C until use.

2.12.2 Radioimmunoassay.

Plasma samples were allowed to thaw slowly on ice. Rat heart

plasma was then diluted to a final volume of 1ml with nephrectomised rat plasma and mixed thoroughly prior to the addition of 10µl phenylmethylsulphonyl fluoride protease inhibitor followed by 100µl of generation buffer (pH6.0). Samples were then vortexed. Aliquots of 100µl from each sample mix were pipetted into four tubes. Two tubes were labelled '37' and were incubated in a 37^{o}C waterbath for 90 minutes. The remaining two tubes were marked '4' and were incubated in an ice bath for the corresponding period of time. Duplicate standards, over the range 0.2ng/ml-25ng/ml were used routinely. Following incubation all tubes were transferred to an ice bath (including tubes for total radioactivity, non-specific binding and total binding). 100µl of ^{125}I-Angiotensin I was added to all tubes followed by 100µl of Angiotensin I antiserum (except to total radioactivity and non-specific binding tubes). Tubes were vortexed and incubated in an ice bath at 4^{o}C for 18 hours. After incubation, 1ml of magnetic second antibody was added (except to total radioactivity), tubes were then vortexed and allowed to stand at room temperature for 10 minutes. Following this the tubes were placed in a magnetic separator and allowed to sediment for 10 minutes before being decanted and counted for 1 minute in a Packard autogamma.

A standard curve of percentage binding versus concentration of renin was plotted, sample estimates were then derived directly from this graph. Plasma renin concentration of samples was then calculated using the following equation.

$$\text{Plasma renin concentration} = \frac{(\text{ng/ml'37'} - \text{ng/ml'4'})}{1.5} \times \text{dilution}$$

2.13 Cell Culture methods.

The technique of growing isolated, dispersed cells *in vitro* has been used in this study to produce confluent cell cultures of Chick embryo fibroblast cells. This cell line was chosen as it is easily maintained and chick cells exhibit a DNA content per cell which is well documented (Puzas and Goodman, 1979; Fiszer-Szafaraz *et al*, 1981).

2.13.1 Routine cell maintenance.

Chick embryo fibroblast (CEF) cells were grown as a monolayer culture on 60mm plastic tissue culture dishes. The culture environment was routinely maintained at 37°C with 5% CO_2 in the gas phase. Culture medium (see appendix C) was changed every two or three days.

2.13.2 Subculture.

Confluent cell plates underwent routine trypsinisation for the purposes of subculture or obtaining isolated cells for DNA analysis. The culture medium on the confluent plate was removed to a sterile universal, and a solution of warmed 0.25% trypsin in versene ($^1/_{10}$ media volume) was added to

each plate. Plates were incubated at 37°C for 15 minutes to allow cell detachment to occur, following this the old culture medium was returned to the plates (the serum in this medium inhibits further trypsin activity). Cell suspensions were then transferred to the universal and the cell plates washed with 5ml warm Hank's balanced salt solution (HBSS), this wash was then added to the cell suspension and centrifuged for 5 minutes at 1000 x g. The cell pellet was washed twice more with 5ml warm HBSS and finally resuspended in 1ml of CEF complete medium. The cells were counted in a haemocytometer and either plated out at a concentration of 200,000/plate or washed and resuspended in NTE prior to DNA determination.

2.14 Statistical Analysis.

Data are expressed in the text as mean ± standard error of the mean. Statistical analysis of normally distributed data was performed using either a Students t-test for unpaired samples or the Scheffe test for analysis of variance (Scheffe 1953). Non-parametric data on the other hand were analysed using the Wilcoxon rank sum test. A 'P' value of ≤ 0.05 was considered significant.

CHAPTER THREE.

EXPERIMENTAL DESIGN.

3.1 Introduction.

Measurement of tissue DNA content yields important information concerning cell cycles, cell ploidy and cell numbers. These studies relate to vascular smooth muscle DNA synthesis and thus required the accurate measurement of DNA in both large conduit vessels (aorta) and small resistance arterioles (mesenteric arteries and subcutaneous vessels). This neccessitates the use of a method which is capable of detecting small quantities of DNA and handling large numbers of samples. Thus it was important to develop an assay system which is sensitive down to nanogram levels of DNA. This chapter gives an overview of the currently available DNA assay techniques and describes the adaptation and characterisation of the methods used in these studies.

3.2 Overview of techniques available for analysis of DNA.

A large number of methods are currently available for the quantification of DNA and they can be divided into three groups; spectrophotometric, 3,5-diaminobenzoic acid dihydrochloride (DABA) and fluorescent dye methods.

The oldest and most well established method of quantifying DNA depends on the colourimetric reaction between DNA and diphenylamine in a mixture of acetic and sulphuric acids (Dische, 1930). Burton (1956) modified this method by adding acetaldehyde to produce a method of DNA analysis that was

both reliable and less susceptible to chemical interference. This basic assay system is still widely used today although it can be modified to increase the stability of the reagents (Richards, 1974) or to decrease the neccessary incubation time (Gendimenico et al, 1988). The major drawback of this assay system lies in the relative lack of sensitivity, being reliable only to a level of 1-3μg. Thus although the method is simple and accurate it is suitable only for large tissue samples.

In the 1970's the need arose for more sophisticated methods of DNA analysis and attention was focused on the use of fluorescent techniques. One such method utilised the reaction of DABA with the deoxyribose of purine deoxyribonucleotides (Puzas and Goodman, 1979). More recently this has been developed into a rapid assay system sensitive to nanogram levels of DNA (Fiszer-Szafarz et al, 1981; Johnson-Wint and Hollis, 1982). However the DABA reagent does not store well and its purification can be both time-consuming and tedious.

Evolving in parallel with the DABA techniques were methods utilising fluorescent dyes such as 4',6-diamidino-2-phenylindole.2HCl (Kapuscinski and Skoczylas, 1977) and bisbenzimidazole Hoechst 33258 (Cesarone et al, 1979) which combine with DNA to give an enhanced fluorescence. Both of these fluorescent dyes have been used for the measurement of DNA and these DNA assays are sensitive down to nanogram levels of DNA and may also be used with crude tissue homogenates (Labarca and Paigen, 1980). More recently the assay of DNA by Hoechst 33258 has been modified for use with

specific tissues e.g Cartilage explants (Kim et al, 1988).

3.3 Choice of DNA assay system.

Originally it was decided to make DNA content measurements of crude tissue homogenates thereby negating the need for prior extraction procedures. Consequently a number of crude aortic homogenates were prepared and their DNA content determined using Hoechst 33258 fluorescent dye in the manner described by Brunk et al (1979). Although fluorescence increased linearly with the sequential addition of calf thymus DNA standards it was not always possible to obtain a linear response to the sequential addition of crude homogenate. As a consequence there were large variations in measurements of the same tissue. Much of this problem was due to the relative inhomogeneity of the tissue samples which could be overcome by brief sonication (approximately 15 seconds) immediately prior to assay. However, the inclusion of a sonication step although resulting in more visibly homogenous samples gave fluorescence values for each sample which tended to decrease with time (data not shown), probably due to reaggregation of the particulate matter. Brief sonication was therefore of limited value and increasing the length of sonication time to 1 - 2 minutes did not overcome this problem. No further increase in sonication time was attempted because of the possibility of shearing the native DNA structure with a resulting decrease in fluorescence (Kapuscinski and Skoczylas, 1977). Since much of the material comprising the

crude homogenate will be protein increased homogeniety of tissue samples may be achieved by digestion with proteases. However the use of a protease enzyme and SDS (Clegg et al, 1986) is also associated with instability of fluorescence values, possibly due to the concentration of SDS in the final assay solution (Cesarone et al, 1979). Having had relatively little success quantifying DNA from crude homogenates it was thought preferential to extract the DNA from the samples allowing it to be assayed in a relatively uncontaminated form.

3.4 Characterisation of DNA assay.

A fluorometric system using Hoechst 33258 (H33258) was chosen because it combined maximum sensitivity in a simple and rapid assay. The DNA extraction from crude homogenate was based on the detergent solubilisation method originally described by Fiszer-Szarfarz et al (1981) and adapted for use with fluorescent dyes by both Downs and Wilfinger (1983) and Sorger and Germinaro (1983). The experiments in this section were performed in order to define the nature and characteristics of this chosen assay system

3.4.1 Fluorescence Spectra.

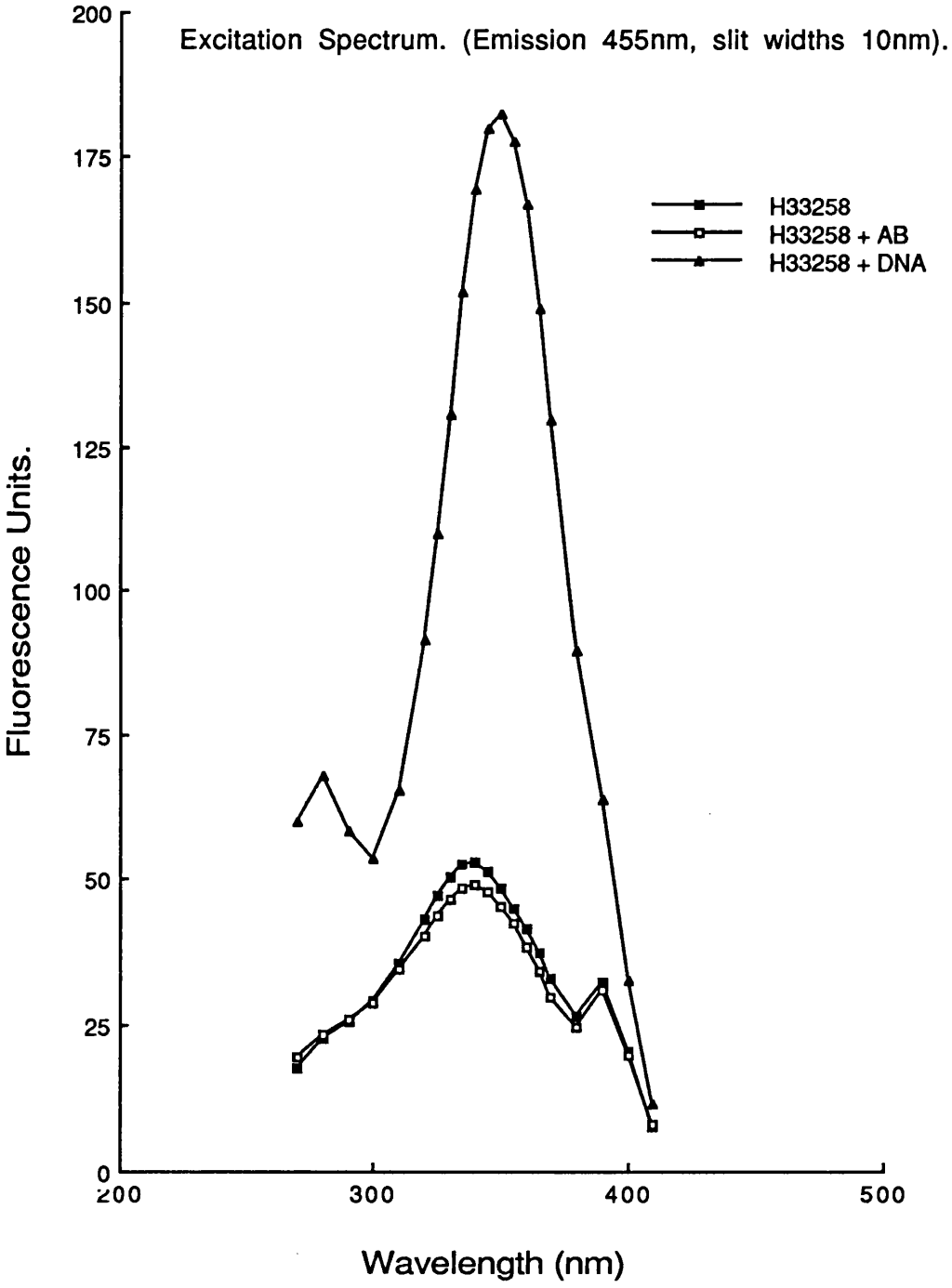
A profile of the excitation spectrum of H33258 dye solution in NTE was defined by measuring the fluorescence over a range of excitation wavelengths (270 - 410nm), maximum fluorescence

being observed at 340nm. Similar excitation profiles were obtained for both H33258 dye solution plus assay buffer (0.025N NH_4OH, 0.005% Triton X-100 in NTE) and for H33258 dye solution plus 150ng DNA made up in assay buffer (Figure 7). However the wavelength at which maximum excitation fluorescence was obtained shifted to 350nm after the addition of calf thymus DNA.

After fixing the excitation wavelength at 350nm fluorescence was measured over a range of emission wavelengths (380 - 510nm). The emission spectrum of H33258 alone shows a broad band of fluorescence over the wavelengths 440nm to 470nm (Figure 8) although this band was more precisely defined (450 - 460nm) with the addition of calf thymus DNA.

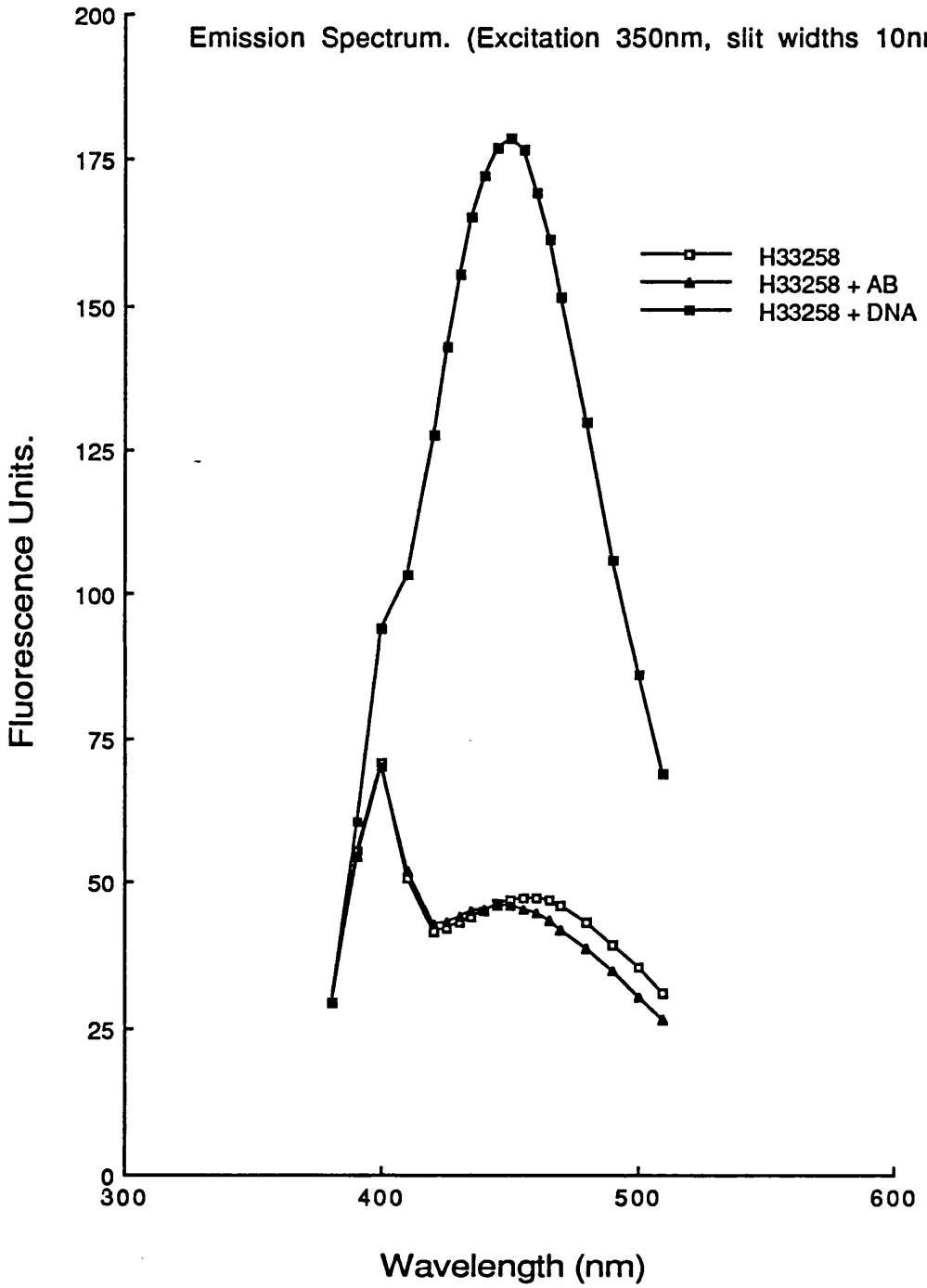
The intrinsic fluorescence of the H33258 dye solution alone appeared to be somewhat quenched by the addition of assay buffer however the magnitude of this quenching was small being only 7% of the peak fluorescence for the excitation spectrum and 3% for the emission spectrum. A similar level of quenching was reported by Downs and Wilfinger (1983) who also determined that the sensitivity of the assay system would be unaffected. In fact the quenching was negligible compared to the enhancement of fluorescence which occurs with the addition of calf thymus DNA. The addition of 150ng DNA gave a 3.8 fold increase in the fluorescence detectable in both the excitation and the emission spectra and was similar to the level of enhancement obtained previously by other workers (Cesarone et al, 1979; Kim et al, 1988).

FIGURE 7.



Fluoresence profile, over a range of excitation wavelengths, of H33258 dye alone and in combination with both assay buffer (AB) and 150ng calf thymus DNA.

FIGURE 8.



Fluoresence profile, over a range of emission wavelengths, of H33258 dye alone and in combination with both assay buffer (AB) and 150ng calf thymus DNA.

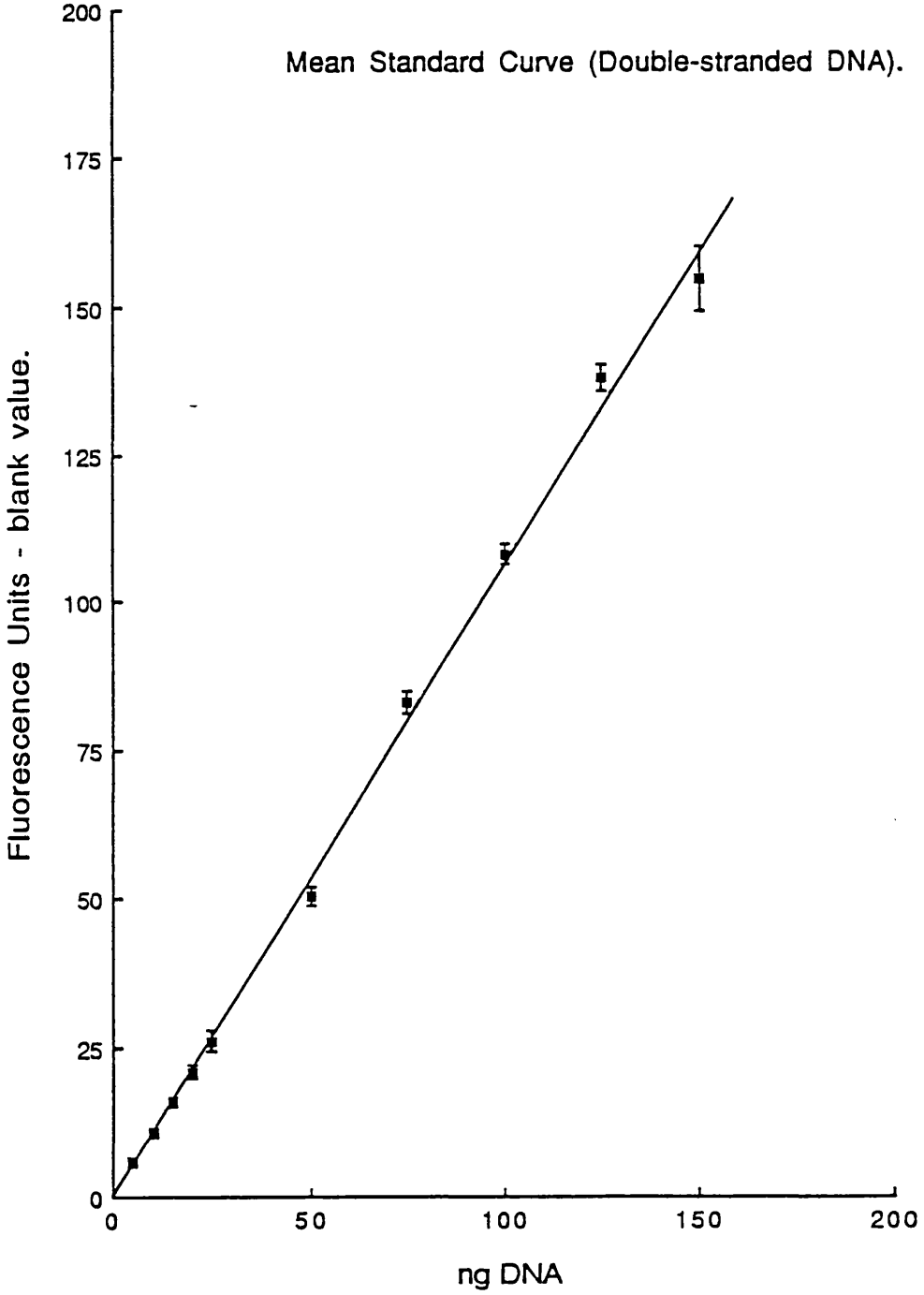
3.4.2 Fluorescence enhancement as a function of DNA concentration.

The addition of increasing concentrations of DNA to the H33258 dye solution resulted in a linear increase in fluorescence enhancement, indicating that the assay system can indeed be used as a measure of the DNA content of unknown samples. A mean calibration curve was calculated from a number (n = 12) of individual curves and is shown in Figure 9. The coefficient of variation among the calculated slope values was 3.2% with the mean slope value being 1.106. As can be seen from Figure 9 the linear response was maintained over the range 5ng to 150ng calf thymus DNA making the assay system more sensitive than many of the methods employing H33258 previously described (Cesarone et al, 1979; West et al, 1985).

3.4.3 Validation of assay system.

Validation of the assay system was performed using chick cells which have a well documented DNA content (Puzas and Goodman, 1978; Fiszer-Szafarz et al, 1981; Johnson-Wint and Hollis, 1982). Confluent chick embryo fibroblast cell cultures (2^{o} - 5^{o} passage) were harvested and counted in a haemocytometer. The DNA was then solubilised from the cells and measured in the fluorometric assay system. The addition of chick cell lysate gave an increase in fluorescence in response to increasing numbers of cells assayed. The mean DNA

FIGURE 9.



Effect of increasing concentrations of double stranded calf thymus DNA on the fluoresence of H33258 dye.

value obtained by this method was 2.98pg/cell which correlates well with other previously recorded values for chick cells (Table 1).

3.4.4 Comparison with Diphenylamine assay.

Six 12 week old female WKY animals weighing 189g with a mean blood pressure of 93mmHg were used. After culling the aorta was excised, dissected free of fat and connective tissue, weighed (total = 127.5 mg) and their lengths measured (total = 254.3 mm). Vessels were homogenised in NTE and aliquots of this homogenate divided into two groups (A and B). Group A aliquots were extracted with hot PCA and assayed using the method described by Burton (1945). Group B aliquots were solubilised using NH_4OH/Triton X-100 and assayed by the H33258 method. The coefficient of variation obtained by the Burton assay was 4.3% whilst that obtained by the fluorometric assay was 6.5%. These results indicate that both methods of DNA estimation are reliable and reproducible. Although both methods produced similar results the values obtained by the Diphenylamine assay were higher than by the fluorometric assay (135.9µg DNA vs 112.5µg DNA). The discrepancy between these results can be explained by the fact that the fluorometric assay system, with its greater sensitivity, was designed for use with small quantities of DNA. Hence NH_4OH/Triton X-100 is probably less efficient at solubilising the large quantities of DNA used in this experiment. It must also be remembered that the final volume

TABLE 1.

DNA content (pg/cell) of chick embryo fibroblasts obtained using the H33258 assay system.

Previous documented literature values.

Puzas and Goodman	(1978)	2.76 pg/cell
Fiszer-Szafarz et al	(1981)	3.37 pg/cell
Johnson-Wint and Hollis	(1982)	2.89 pg/cell

TABLE 1.

Assay Number	Total DNA (ng)	No. cells assayed.	pgDNA/cell
1	1280	330,000	3.88
2	800	330,000	2.42
3	720	330,000	2.18
4	1000	330,000	3.03
5	37	11,956	3.09
6	48	15,940	3.01
7	63	19,925	3.16
8	29	9,025	3.21
9	38	12,636	3.01
10	45	16,247	2.77
		Mean	2.98

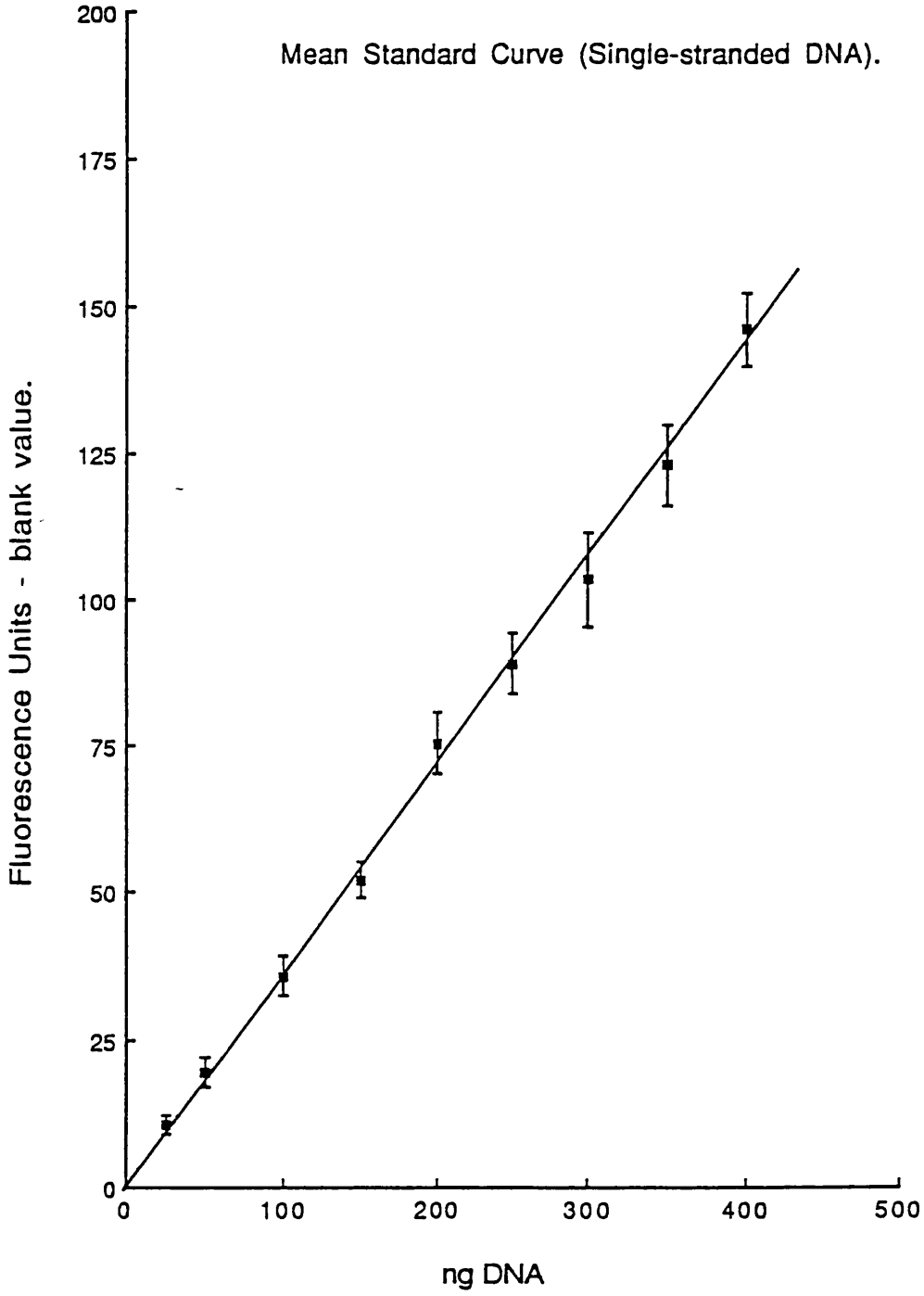
of DNA extract assayed in the Diphenylamine assay was 1/12 of the original homogenate volume whereas only 1/2400 of the original volume was required for assay in the fluorometric system. This may introduce multiplication errors into the results obtained from the use of the fluorometric assay system in this situation.

3.4.5 Dependence on DNA structure.

3.4.5.1 Double-stranded helix.

The fluorometric DNA assay does not require DNA to be in the double-stranded helical form, it can in fact be used to quantify single-stranded DNA e.g. as obtained by alkaline elution method (Parodi et al, 1978). Single-stranded DNA can be prepared from calf thymus DNA by heating at 100°C for 30 minutes to dissociate the DNA strands, followed by rapid cooling on ice, which prevents strand re-association. Single-stranded DNA prepared in this way was used to produce a number of individual calibration curves (n = 6) with a mean curve being determined from these (Figure 10). The fluorescence enhancement of H33258 with single-stranded DNA was decreased by approximately two thirds compared to that found with native double-stranded DNA. The slope value of the single-stranded curve was 0.363 compared to 1.106 for double-stranded DNA which closely agrees with that reported by Brunk et al (1979). In addition, although the overall sensitivity of the single-stranded assay was decreased compared to the

FIGURE 10.



Effect of increasing concentrations of single stranded calf thymus DNA on the fluoresence of H33258 dye.

double-stranded assay it was linear down to 25ng DNA indicating a greater sensitivity than other assays (Stout and Becker, 1982). In further experiments the single-stranded DNA assay was validated using chick embryo fibroblasts. Chick embryo fibroblast cell cultures were harvested, the DNA solubilised, and converted by heating to single-stranded DNA which was measured by using H33258. The mean DNA content was 2.92pg/cell (n = 6) which compares favourably to that obtained in the double-stranded assay (2.98pg/cell) and with the documented values for these cells (Puzas and Goodman, 1978; Fiszer-Szafarz et al, 1981; Johnson-Wint and Hollis, 1982).

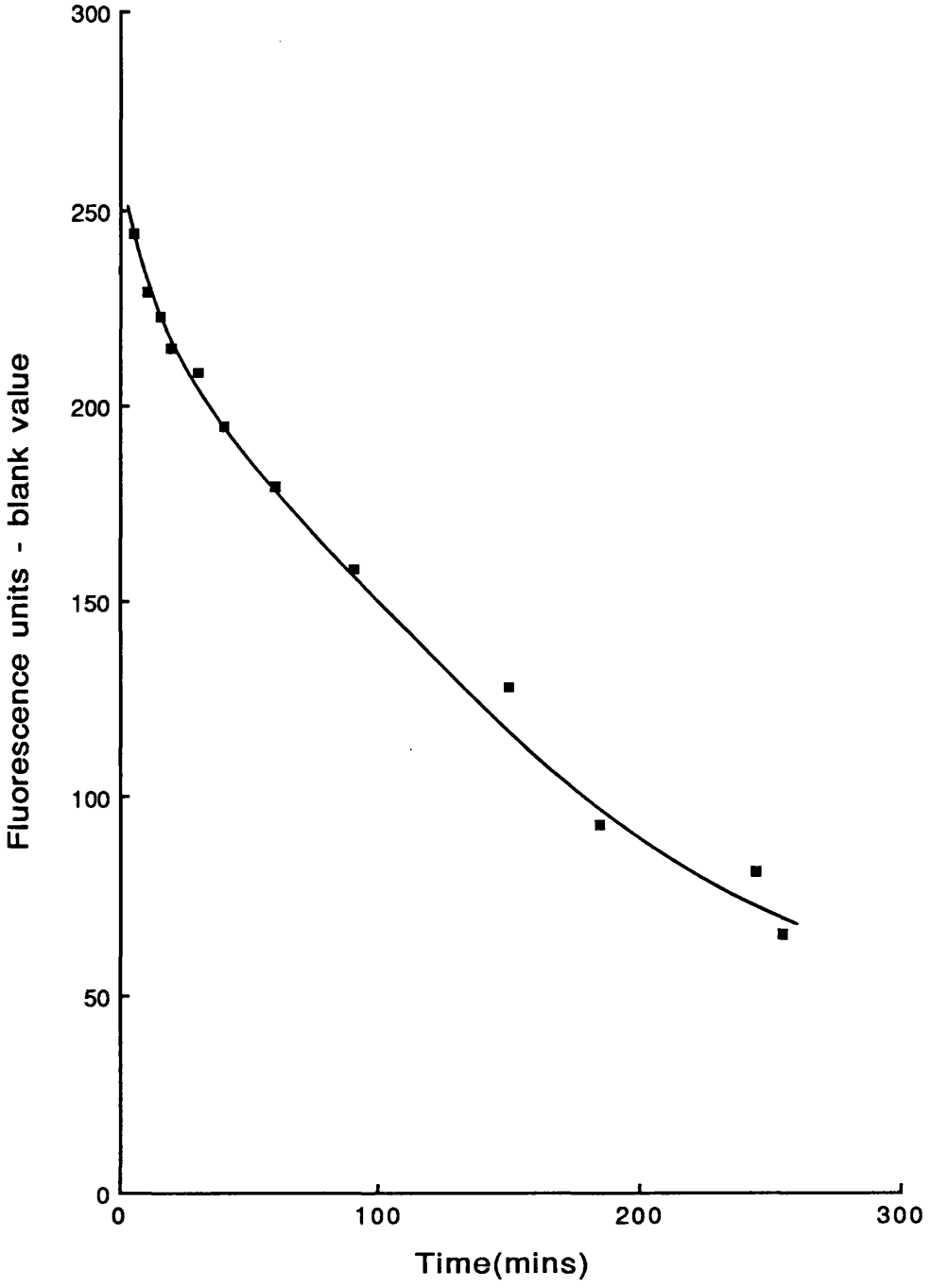
3.4.5.2 Polynucleotide structure.

Although the fluorometric assay can be used with both double and single stranded DNA, the presence of an intact polynucleotide structure is essential. The enzyme DNAse degrades intact DNA by cutting it transversely to produce shorter and shorter chain lengths, which is associated with a loss in acid precipitability (Labarca and Paigen, 1980). When calf thymus DNA was incubated with this enzyme at 37^{o}C there was a progressive reduction in fluorescence activity of H33258 with increasing incubation time (Figure 11).

3.5 Efficiency of DNA extraction.

The most important thing to remember about the assay of DNA

FIGURE 11.



Effect of incubating double stranded calf thymus DNA with DNase for increasing lengths of time on the flouresence obtained with H33258 dye.

content of tissue samples is that any assay system can only measure DNA which has been successfully extracted . It was important therefore to investigate the efficiency of the DNA solubilisation procedure originally described by Downs and Wilfinger (1983). This method results in the production of a supernatant which is assayed by H33258. The efficiency of this extraction was investigated by determining the DNA remaining in the pellet. The residual pellet was dried carefully and the protein content digested with proteinase K in SDS. The resulting suspension was electrophoresed in agarose with ethidium bromide and was visualised under ultra-violet light. The presence of a quantity of DNA in the gel indicated that the efficiency of the DNA solubilisation could be improved (gel not shown).

3.5.1 Effect of increasing incubation time.

In order to try and improve the efficiency of the extraction procedure the effect of increasing the incubation time was investigated. Three female WKY animals (12 weeks old) were weighed (mean = 170g) and their blood pressure measured (mean = 106mmHg) prior to culling. The aortae were excised, cleaned, homogenised in NTE and a series of aliquots prepared. The aliquots were extracted with 10µl of 10N NH_4OH/2% Triton X-100 extraction buffer and incubated at 37^{o}C for 10, 30, 60 or 90 minutes. Following incubation all samples were diluted with NTE, centrifuged and the DNA content of the supernatant measured by H33258. The results

obtained (Table 2) show that increasing the length of the incubation time had no significant effect on the amount of DNA solubilised from the samples.

3.5.2 Effect of increasing incubation temperature.

Since increasing the incubation time did not significantly increase the amount of DNA solubilised the effect of increasing the incubation temperature was investigated. Female WKY animals (n = 3) with a mean weight of 191g and a mean blood pressure of 113mmHg were culled at 12 weeks of age. The aortae were dissected, cleaned, homogenised in NTE and divided into aliquots. All aliquots were extracted with 10µl of extraction buffer and incubated for 30 minutes at 37^{o}C, 50^{o}C or 70^{o}C. Subsequent assay of these aliquots showed that increasing the temperature above 37^{o}C significantly increased the amount of DNA solubilised (Table 3). Unfortunately the samples incubated at 50^{o}C and 70^{o}C exhibited an instability of fluorescence not seen at 37^{o}C, suggesting that the double-stranded DNA helix was being denatured at these temperatures.

3.5.3 Effect of increasing the concentration of extraction buffer.

The possibility of significantly increasing DNA solubilisation without affecting the native DNA structure by increasing the concentration of extraction buffer was

TABLE 2.

Incubation Time (mins).	ng DNA	Comparison with; 10	30	60	90
10	1315.0 ± 118.8	_	NS	NS	NS
30	1554.3 ± 117.2	NS	_	NS	NS
60	1580.0 ± 125.0	NS	NS	_	NS
90	1737.1 ± 86.7	NS	NS	NS	_

Effect of increasing the incubation time on the amount of DNA solubilised from aortic homogenates, using the H33258 assay system.

NS = no significant difference.

TABLE 3.

Incubation Temp. (°C).	ng DNA	Comparison with; 37	50	70
37	1362.5 ± 11.6	_	P <0.001	P< 0.001
50	1775.0 ± 20.6	P <0.001	_	NS
70	1825.0 ± 29.5	P < 0.001	NS	_

Effect of different incubation temperatures on the amount of DNA solubilised from aortic homogenates using the H33258 assay system.

investigated. Twelve week old female WKY animals (n = 3) were weighed (mean = 175g) and their blood pressure measured (mean = 103mmHg) prior to culling. The aortae were excised, cleaned and homogenised in NTE, aliquots of the aortic homogenate were then divided into four groups A-D. Each group was extracted by the addition of extraction buffer to give a final concentration of;

A - 0.91N NH_4OH/0.18% Triton X-100

B - 1.67N NH_4OH/0.33% Triton X-100

C - 2.31N NH_4OH/0.46% Triton X-100

D - 2.86N NH_4OH/0.57% Triton X-100

and incubated at 37^{o}C for 30 minutes. Following incubation all of the samples were diluted to the same final concentration of 0.025N NH_4OH/0.005% Triton X-100, to avoid the problem of fluorescence quenching. Samples were then centrifuged and the supernatants assayed by H33258. The results obtained (Table 4) indicated that DNA solubilisation is increased by increasing the initial concentration of extraction buffer, although this does not achieve significance below 2.31N NH_4OH/0.46% Triton X-100 and decreases above this concentration. This pattern of increasing fluorescence correlates well with that described by Downs and Wilfinger (1983) who suggested that maximum fluorescence was achieved using a concentration of between 1N and 2N NH_4OH and that a concentration of 3N NH_4OH resulted in a decrease in flourescence.

TABLE 4.

Concn. NH_4OH	ng DNA	Comparison with; 0.91	1.67	2.31	2.86
0.91N	1420.0 ± 36.5	_	NS	P<0.01	NS
1.67N	1553.3 ± 108.5	NS	_	NS	NS
2.31N	1960.0 ± 119.7	P<0.01	NS	_	P<0.01
2.86N	1413.3 ± 93.9	NS	NS	P<0.01	_

Effect of increasing the concentration of extraction buffer on the amount of DNA solubilised from aortic homogenates using the H33258 assay system.

3.5.4 Validation of improved DNA solubilisation.

3.5.4.1 Effect of dilution.

The use of increased initial concentrations of NH_4OH/Triton X-100 necessitates diluting samples with large volumes of NTE to achieve the same final concentration. Accordingly it is important to ensure that the increase in fluorescence detected does not result from a dilution-related enhancement of assay sensitivity. Aliquots of calf thymus DNA (5µg) were made up to 2ml, 4ml, 6ml and 8ml in assay buffer and their DNA content measured fluorometrically by H33258. The results of this experiment (Table 5) show that dilution factor does not affect the sensitivity of the assay system, since all dilutions gave a total DNA value similar to that obtained spectrophotometrically.

3.5.4.2 Electrophoresis of DNA.

The fact that dilution does not affect H33258 sensitivity does not in itself provide evidence that the increase in fluorescence detected with increasing concentrations NH_4OH/Triton X-100 actually corresponds to an increase in solubilised DNA. For this to be the case an increase in the fluorescence of the supernatant should correlate closely with a decrease in the fluorescence, of the pellet. Accordingly pellets were dried carefully, digested with proteinase K and run on agarose gels containing ethidium bromide. During

TABLE 5.

Final vol(ml)	ng DNA	Comparison with; 2	4	6	8
2	4940 ± 85.0	NS	NS	NS	NS
4	5027 ± 57.2	NS	NS	NS	NS
6	5220 ± 138	NS	NS	NS	NS
8	5040 ± 115	NS	NS	NS	NS

Effect of dilution factors on the amount of calf thymus DNA measured by the H33258 assay system.

electrophoresis ethidium bromide intercalates with the DNA and the pattern of fluorescence can be visualised under ultra-violet light and recorded by polaroid photography (Freeman et al, 1986). Figure 12 shows that the amount of DNA retained in the pellet decreases with increasing concentration of extraction buffer up to a concentration of 2.31N NH_4OH/0.46% Triton X-100 after which no further decrease can be detected. This provides qualitative, but not quantitative (Ribeiro et al, 1989) visual evidence that the increase in fluorescence detected by H33258 is indeed related to an increase in DNA in the supernatant which results from the increased efficiency of DNA solubilisation.

3.6 Application to other vessels.

All of the validation studies were performed using aortic homogenates from twelve week old normotensive WKY animals. The results obtained from these vessels in terms of optimum extraction conditions, may not be representative of the results obtained from small resistance vessels or indeed from hypertensive animals.

In order to investigate the behaviour of this assay system in resistance vessels the small intestine was removed from two female WKY animals (mean weight 194g; mean blood pressure 120mmHg). Ten mesenteric arteries, second or third order branches, were dissected (total length 164.78mm). These vessels were homogenised in NTE and the DNA solubilised using increasing concentrations of extraction buffer. The pellets

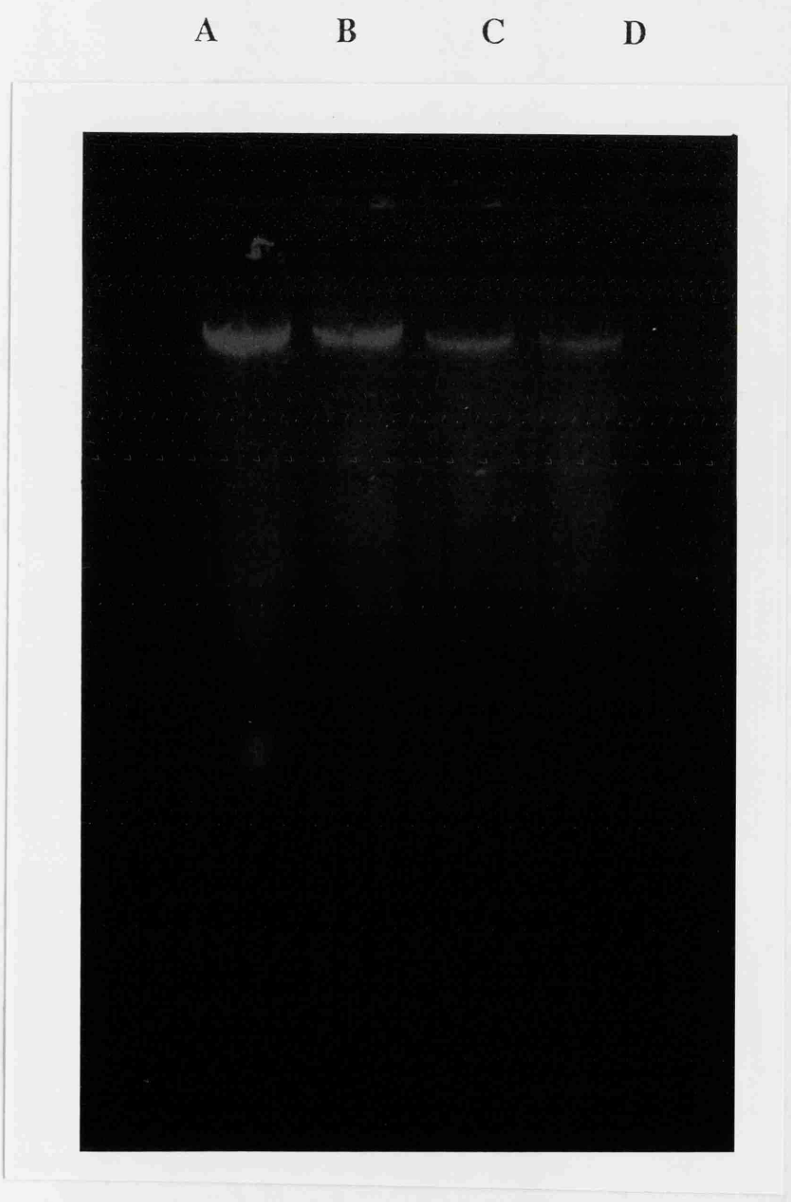
FIGURE 12

Detection of unextracted DNA from aortic homogenates.

DNA was extracted from aliquots of an aortic homogenate using increasing concentrations of extraction buffer.
The pellet was digested free of proteins, run on an agarose gel and the DNA remaining in this pellet was visualised with ethidium bromide.

A = 0.91N NH_4OH / 0.18% Triton X-100.
B = 1.67N NH_4OH / 0.33% Triton X-100.
C = 2.31N NH_4OH / 0.46% Triton X-100.
D = 2.86N NH_4OH / 0.57% Triton X-100.

FIGURE 12



were digested and electrophoresed in agarose in the same manner as the aortic homogenates. The resulting pattern of fluorescence, Figure 13, shows that increasing the concentration of extraction buffer has a similar effect on increasing solubilisation efficiency in mesenteric arteries as has already been noted in aortic homogenates.

The applicability of this solubilisation method for hypertensive animals was investigated in the following manner. Aortae were removed from both a young (5 week) and an old (30 week) SHR animal and extracted as previously described. The pattern of solubilisation found in normotensive animals was observed in young and old hypertensive animals (Figure 14).

3.7 Manipulation of Samples.

The total length of small resistance vessel to be dissected from the experimental animals could be as little as 50mm. This would be homogenised in 1ml of NTE buffer and a 100µl aliquot of the homogenate removed for analysis of DNA content. Using the optimum solubilisation conditions the final volume would be in the order of 6ml and thus the samples would be too dilute for direct assay. Sample concentration was therefore necessary and because of the large number of samples to be handled needed to involve both a concentration and a storage step.

FIGURE 13

Detection of unextracted DNA from mesenteric artery homogenates.

DNA was extracted from aliquots of a mesenteric artery homogenate using increasing concentrations of extraction buffer.
The pellet was digested free of proteins, run on an agarose gel and the DNA remaining in this pellet was visualised with ethidium bromide.

A = 0.91N NH_4OH / 0.18% Triton X-100.
B = 1.67N NH_4OH / 0.33% Triton X-100.
C = 2.31N NH_4OH / 0.46% Triton X-100.
D = 2.86N NH_4OH / 0.57% Triton X-100.

FIGURE 13

A B C D

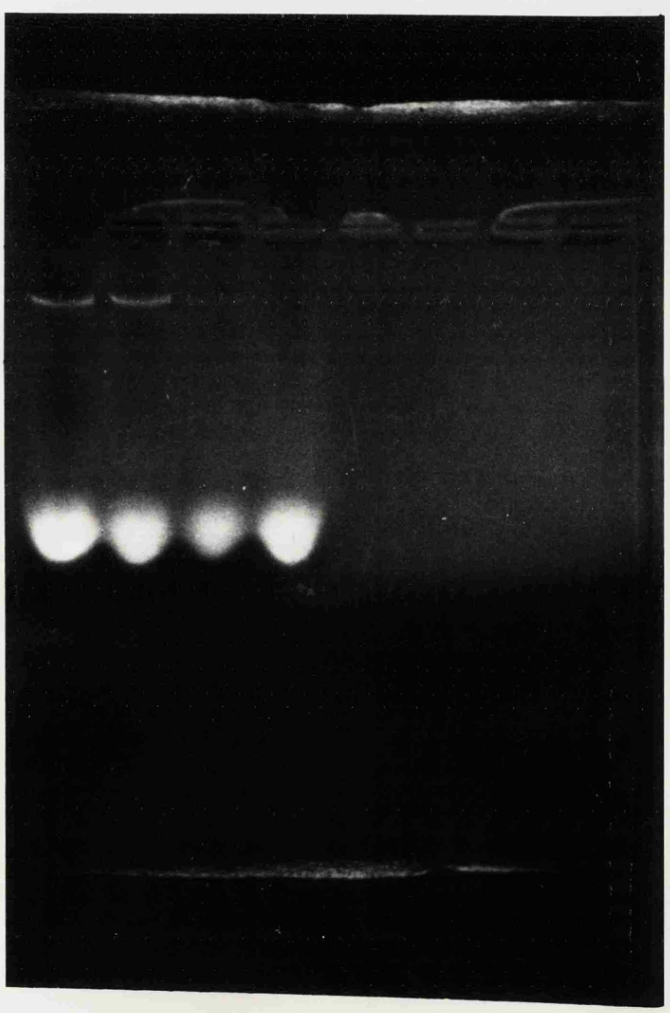


FIGURE 14.

Detection of unextracted DNA from hypertensive rats.

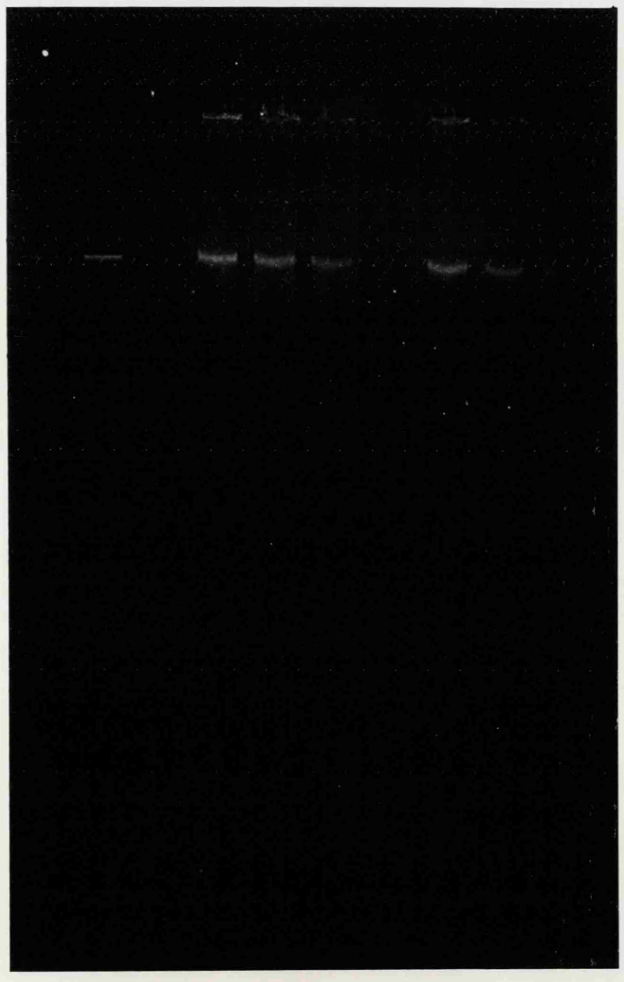
DNA was extracted from aortic homogenates of both 5 and 30 week old SHR's using increasing concentrations of extraction buffer.
The pellet was digested free of proteins, run on an agarose gel and the DNA remaining in the pellet visualised with ethidium bromide.

5 Week old SHR.
A = 0.91N NH_4OH / 0.18% Triton X-100
B = 1.67N NH_4OH / 0.33% Triton X-100
C = 2.31N NH_4OH / 0.46% Triton X-10

30 Week old SHR.
X = 0.91N NH_4OH / 0.18% Triton X-100
Y = 1.67N NH_4OH / 0.33% Triton X-100
Z = 2.31N NH_4OH / 0.46% Triton X-100

FIGURE 14.

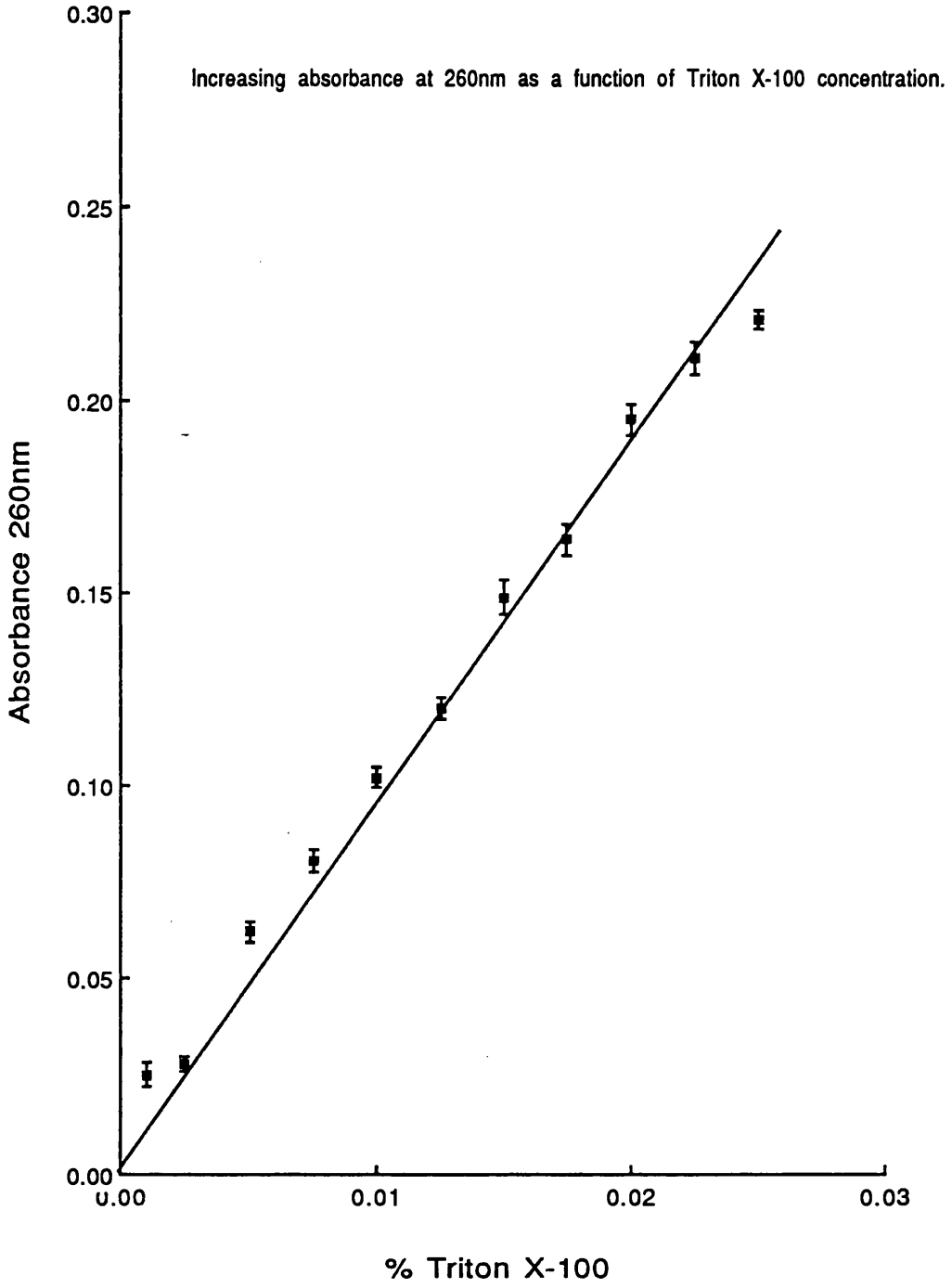
5ng DNA 50ng DNA A B C X Y Z



3.7.1 Concentration by Ultrafiltration.

The method chosen for the concentration of DNA samples was an Amicon stirred cell ultrafiltration system. A slight quenching effect of NH_4OH/Triton X-100 had already been noted, and it was important to determine that concentration of these buffer constituents did not occur using this system. The membrane chosen for the amicon system has a molecular weight cut-off point of 30,000 and allowed free passage of NH_4OH through the membrane to waste. However it was possible that Triton X-100 might be retained by the membrane and concentrated in the sample. Preliminary studies showed that Triton X-100 in NTE absorbs light at a wavelength of 260nm, and the optical density at this wavelength increases linearly with increasing concentration of Triton X-100 (Figure 15). This information was used to investigate Triton X-100 concentration by the amicon system. The absorbance of a 2ml solution of NH_4OH/Triton X-100 at 260nm was measured as 0.112. This solution was then added to the amicon system and concentrated to a volume of 500µl. The absorbance at 260nm of the eluted solution was 0.121 whilst that of the concentrate was 0.113. In order to check that the absorbance at 260nm of the NH_4OH/Triton X-100 solution was not influenced by the presence of NH_4OH the absorbance at 260 nm of this buffer constituent alone was recorded (Optical density of NH_4OH at 260nm = 0.008). Thus NH_4OH does not affect the optical absorbance at 260nm and there is no evidence to suggest that concentration of either of these buffer constituents occurs

FIGURE 15.



Optical density at 260nm of increasing concentrations of Triton X-100.

with the use of the amicon stirred cell system.

Studies of the effective recovery of DNA from the amicon system was performed in a series of eperiments using aortic homogenates from 12 week old female WKY animals. The DNA in the aliquots was solubilised and then measured by H33258. The remaining solution was then concentrated by the ultrafiltration method and the DNA content of the concentrate remeasured by H33258. It can be seen from Table 6 that the efficiency of DNA recovery from the amicon system is 92% (mean of 15 samples).

3.7.2 Effect of Storage.

In order to make the assay methods managable for large numbers of samples storage, either in the form of crude homogenate or in the form of solubilised DNA, must be employed. The effect of such storage on the measurement of DNA by H33258 was therefore investigated.

The aorta of a female WKY animal was dissected, cleaned and homogenised in NTE and aliquots of this homogenate were divided into five groups. Four of these groups were stored as the crude homogenate at -20^{o}C and their DNA content assayed after 24 hrs, 7 days, 14 days or 28 days. The remaining group was immediately extracted and its DNA content assayed fresh. The remainder of the DNA extract was divided into aliquots and stored at -20^{o}C for 24 hrs, 7 days, 14 days or 28 days prior to re-assay. Storage of aortic samples in the form of crude homogenate appears to result in an increased fluorescence compared to fresh samples, although this

TABLE 6.

Sample	Total DNA initially (ng)	Total DNA after concn. (ng)	% Recovery
A	2840	2535	89
B	3120	2986	96
C	3300	2962	90
D	2980	2981	100
E	2820	2795	99
F	3020	2866	95
G	3400	3208	94
H	2960	2937	99
I	3500	2698	77
J	3420	2949	86
K	3380	3470	100
L	3180	2437	77
M	3380	3204	95
N	2820	3036	100
O	3160	2582	82

Effect of amicon filtration on DNA recovery.

DNA content was measured using the H33258 assay system.

increased fluorescence is not affected by the period of storage (Table 7). It is possible that this increased fluorescence is actually related to an increase in the DNA content of the samples, the inclusion of a freeze-thaw step prior to solubilisation may indeed improve the efficiency of the extraction procedure. A similar trend is however seen in aortic samples stored in the form of solubilised DNA (Table 8) and may be due to some renaturation of the DNA following solubilisation with strong alkali solution. A similar experiment was performed using mesenteric arteries (n = 12) from the same female rat although in the case of these small resistance vessels no significant change in fluorescence could be detected following storage as either crude homogenate (Table 9) or as extracted solubilised DNA (Table 10). These results, contary to the studies by Mates et al (1986), indicate that storage of samples over long periods of time does not result in a diminished capacity to enhance the fluorescence of H33258 dye. However, to ensure that samples were always comparable with each other storage at -20^{o}C was routinely included as part of the assay procedure.

3.8 Measurement of DNA Synthesis.

The experiments in this section were performed in order to determine the optimum conditions for the incorporation of exogenous [^{3}H]-thymidine into DNA, which is regarded as a measure of DNA synthesis.

TABLE 7.

Storage Time (days)	ng DNA	Comparison with; 0	1	7	14	28
0	1044.0±123	_	P<0.01	P<0.01	NS	P<0.05
1	2195.0±263	P<0.01	_	NS	P<0.05	NS
7	1746.0±95.5	P<0.01	NS	_	NS	NS
14	2343.0±271	NS	P<0.05	NS	_	NS
28	2394.0±75.4	P<0.05	NS	NS	NS	_

Effect of storage at -20°C on the amount of DNA assayed in aortic homogenates using the H33258 assay system.

TABLE 8.

Storage Time (days)	ng DNA	Comparison with; 0	1	7	14	28
0	1044.0±123	_	P<0.05	NS	P<0.01	P<0.01
1	1637.0±89.3	P<0.05	_	NS	NS	NS
7	1544.0±138	NS	NS	_	NS	NS
14	1256.0±119	P<0.01	NS	NS	_	NS
28	1427.0±89.9	P<0.01	NS	NS	NS	_

Effect of storage at -20°C on the amount of DNA assayed in extracts of aortic homogenates using the H33258 assay system.

TABLE 10.

Storage Time (days)	ng DNA	Analysis of Variance
0	345.4 ± 26.7	NS
1	291.6 ± 31.8	NS
7	352.5 ± 14.2	NS
14	152.5 ± 18.8	NS
28	264.7 ± 48.0	NS

Effect of storage at -20°C on the amount of DNA measured in extracts of mesenteric artery homogenates using the H33258 assay system.

3.8.1 General assay conditions.

The ability of isolated, dissected blood vessels to continue to synthesise DNA *in vitro* depends on a number of physiological conditions being met. Vessels should be maintained in a physiological buffer and at a constant 37^{o}C in order to mimic a natural environment as closely as possible and thus ensure viability even after long incubation periods. The need to incubate large numbers of vessels simultaneously prompted the design of a sample chamber. This chamber holds up to 24 separate vessel samples, can be easily fitted into a waterbath and offers the advantage of maintaining the samples in an atmosphere of 95% oxygen, 5% carbon dioxide.

3.8.2 Investigation of optimum incubation time.

The method described by Loeb *et al* (1986) was designed for use with large vessels and demonstrated that [^{3}H]-thymidine incorporation was linear over the 2 hour time course chosen by Loeb and his co-workers. This incubation period was too short for use with resistance vessels because the low count values obtained (data not shown) made the method inaccurate. Consequently a time course experiment was designed to define the optimum incubation period for both large (aorta) and small resistance vessels (mesenteric arteries).

Female WKY animals at 12 weeks of age (n = 12) with a mean weight of 188g and a mean blood pressure of 102mmHg were

culled and the aortae dissected. Each aorta was divided into two longditudinal halves which were weighed and their length measured. All aorta halves were incubated with [^{3}H]-thymidine for 2, 4, 7, 10, 18 or 22 hours. Following incubation samples were extracted with hot PCA and the incorporation of [^{3}H]-thymidine determined by scintillation counting. The results are shown in Figure 16 and demonstrate linear incorporation throughout the 22 hour incubation period.

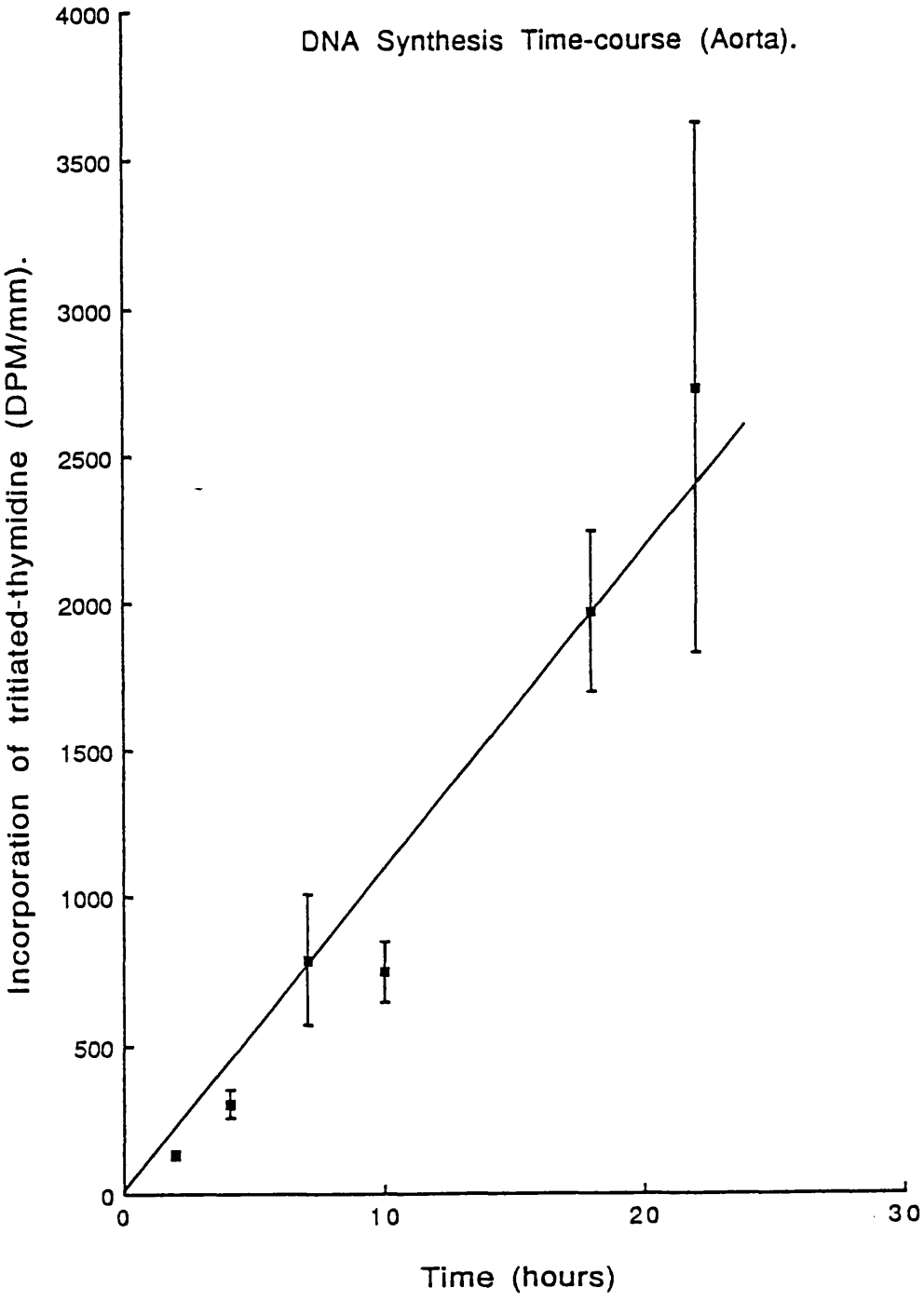
Ninety-six mesenteric arteries were dissected from 8 female WKY animals and incubated with [^{3}H]-thymidine in the same manner as the aortic samples. Figure 17 shows that incorporation of [^{3}H]-thymidine into these arteries is linear up to 18 hours but appears to be decreased at 22 hours.

Thus 18 hours was the optimum incubation time for all vessel types used in these studies.

3.8.3 Efficiency of hot acid extraction.

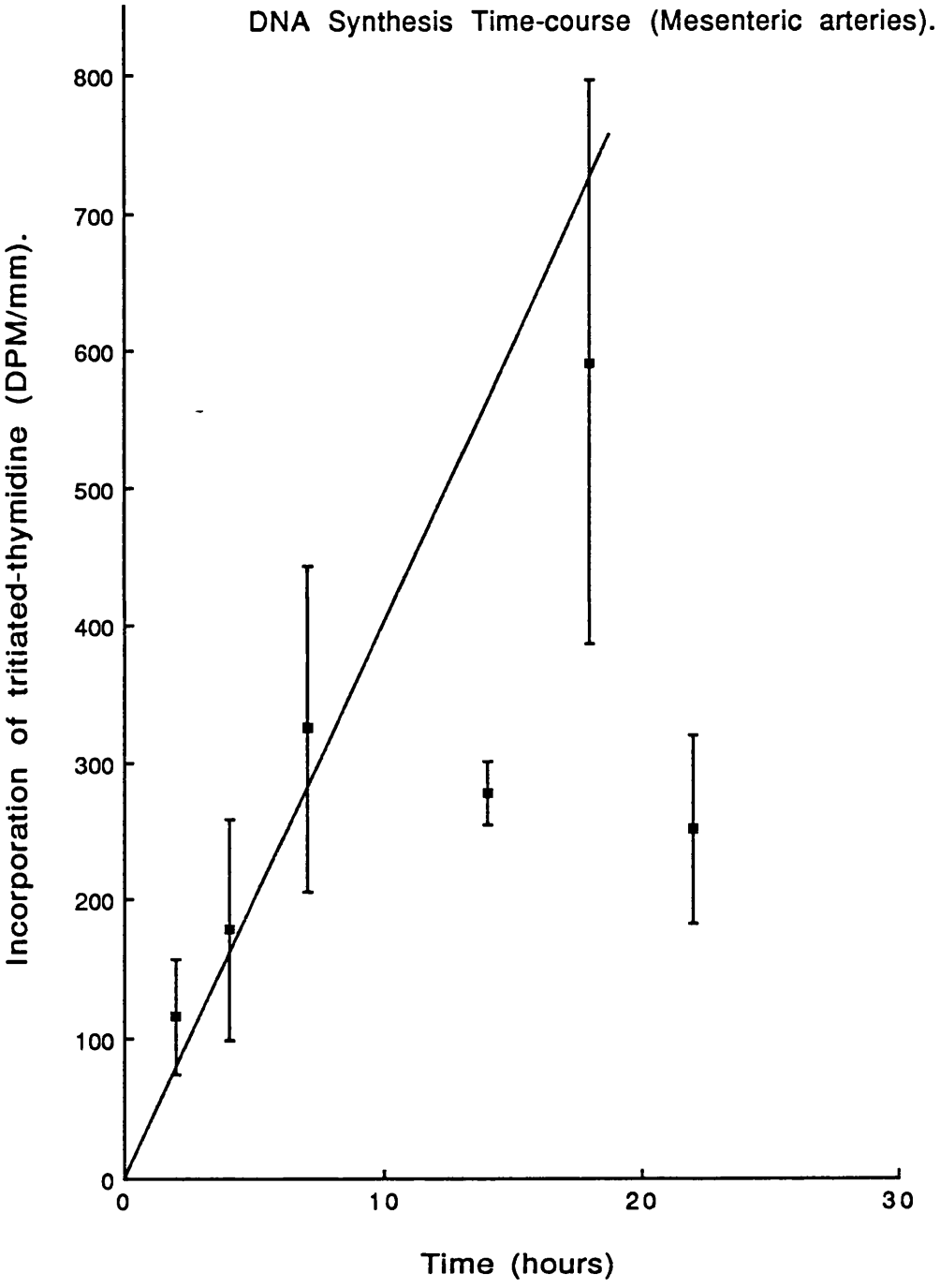
Total incorporation of [^{3}H]-thymidine into DNA (see Section 2.5) is determined by adding the counts obtained as described by Loeb et al (1986), (representing extracted DNA) to those which remain in the pellet after the hot acid extraction, (representing the non-extracted DNA). In order to validate the assertion that these counts are indeed incorporated into DNA the protein content of the pellets was digested and the DNA precipitated with ethanol. Both the ethanol supernatant and the precipitate were counted in the liquid scintillation counter and the distribution of [^{3}H]-

FIGURE 16.



Effect of increasing incubation time on the incorporation of [3H]-thymidine into samples of aortic tissue.

FIGURE 17.



Effect of increasing incubation times on the incorporation of [^{3}H]-thymidine into mesenteric arteries.

thymidine between the two phases determined. Results (n = 4) show that only 1% of the counts detected were present in the ethanol supernatant representing free [^{3}H]-thymidine. Thus it can be accepted that the counts detected in the final pellet do represent incorporation of [^{3}H]-thymidine into DNA which has not been extracted from the tissue sample.

The percentage of the total tissue DNA which was not effectively extracted by the hot acid procedure was similar in both the normotensive and the hypertensive animals. However the extraction was more efficient in the small resistance vessel compared to the aortic homogenates (Hypertensive animals: mesenteric arteries - 4.8% aorta - 15.5% n=14). The amount of DNA in aortic homogenates which was not extracted by the hot acid procedure ranged from less than 5% to greater than 30%. Therefore it is important that the final pellet counts were added to the counts obtained from the first supernatant to assess the total incorporation of [^{3}H]-thymidine into DNA and give a reliable index of DNA synthesis.

CHAPTER FOUR.

EXPERIMENTAL HYPERTENSION.

4.1 Choice of experimental model

The experimental model chosen was the two-kidney, one-clip (2K 1C) renovascular model of hypertension originally described by Goldblatt et al in 1934. This model has been used extensively for hypertensive studies within the department (Marks et al, 1979; Mistry et al, 1983; Edmunds et al 1987). Consequently there was not only considerable experience in handling this model, but also a wealth of knowledge regarding the course of development of hypertension (Thurston and Swales 1976). Other advantages of the 2K, 1C renovascular model are that the operative proceedure is quick, relatively simple to perform and that the onset of hypertension enjoys a high success rate.

4.2 Incidence of Hypertension.

Investigation of the biochemical events associated with structural vascular change inevitably requires the study of animals throughout the course of development of hypertension; from the early stages, at which animals would not necessarily exhibit a raised blood pressure, through to the established phase of renovascular hypertension.

It was important, therefore, to have accurate information about the incidence of hypertension. Consequently a parallel group of animals were set up and their blood pressure monitored routinely every seven days. Results obtained from this parallel group demonstrated that 80% of the experimental

animals had developed hypertension i.e. exhibited a blood pressure of $\geq$ 140mmHg by the fourth week following renal artery clipping. Of these hypertensive animals over 40% had developed hypertension by 7 days post-clipping. It was also noted that 25% of animals died prior to reaching 28 days post-renal artery stenosis. The death of these animals was presumably due to the development of malignant hypertension. These results agree closely with the incidence of hypertension reported in the literature (Russell 1982; Mistry 1984).

4.3 Development of Hypertension

The development of hypertension in experimental animals was assessed by both measurement of the blood pressure and the presence or absence of cardiac hypertrophy.

4.3.1 Blood Pressure measurements.

Three days post-renal artery constriction, there was no significant difference between the blood pressures of the control and experimental animals (control - 103.5 ± 4.5 mmHg; clipped - 115.5 ± 7.5 mmHg), although the blood pressure of the clipped animals was higher. This slight elevation was due entirely to one animal which exhibited a blood pressure of 200mmHg, which is more than three standard deviations greater than the mean value of the group. The blood pressures of all other clipped animals at three days

were normally distributed. Exclusion of this animal from the experimental group on a statistical basis gives a new mean blood pressure of 109.2 mmHg, which is within 6 mmHg of the control group.

By 7 days post-renal artery clipping however, the blood pressure in the experimental animal group was significantly increased ($p \leq 0.01$). Blood pressure continued to increase steadily up to 14 days after which no further increase was observed (Figure 18).

4.3.2 Measurement of Cardiac Hypertrophy.

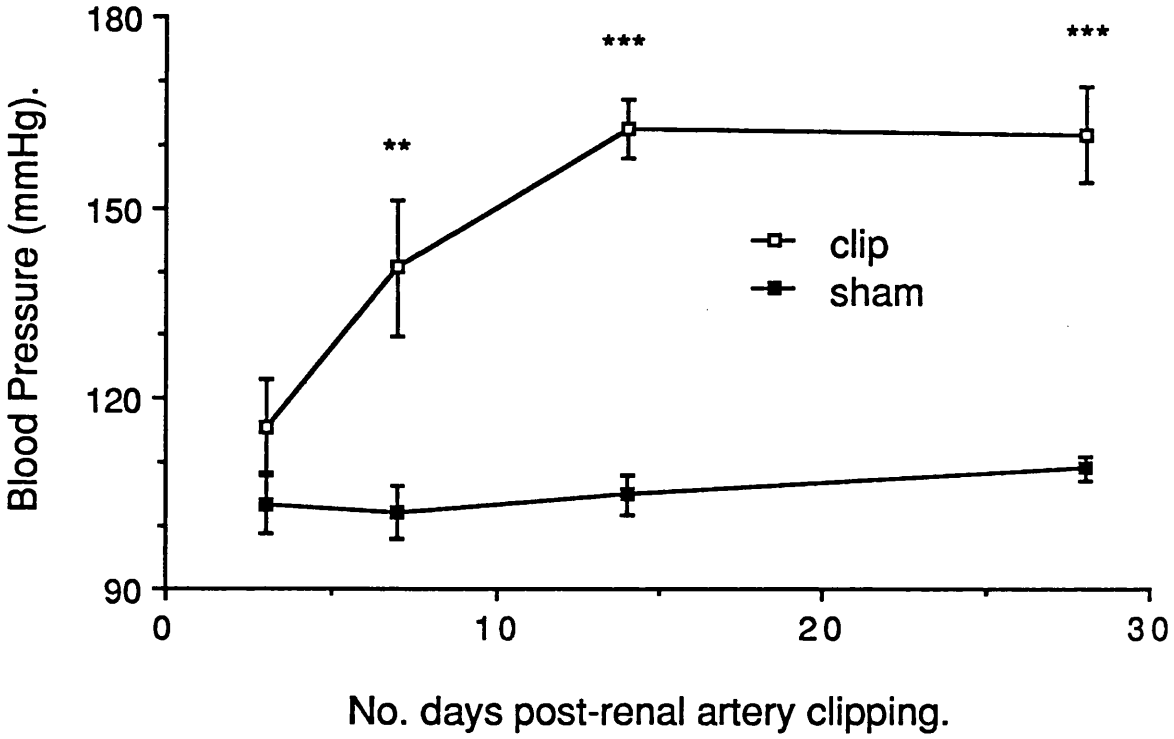
A further indication of the development of hypertension in the experimental rat is the development of cardiac hypertrophy. There was no significant difference in heart weights between clipped and control animals of animals at 3 days. However, the hearts of experimental animals were significantly heavier at 7 days ($P \leq 0.05$), and continued to increase in weight up to 14 days after which no further increase occured (Table 11). Heart weight alone is a poor index of cardiac hypertrophy because of differences in body weight and greater sensitivity can be achieved using the heart weight / body weight ratio.

4.3.2.1 Heart weight / Body weight ratio.

Heart weight was therefore, indexed against body weight to give a measure of cardiac hypertrophy (Figure 19a). The use

FIGURE 18.

Blood Pressure Measurements.



Effect of renal artery constriction on the blood pressure of control and experimental rats.

Comparisons were made using a T-test for unpaired samples.

** represents $P \leq 0.01$

*** represents $P \leq 0.001$

TABLE 11.

The body weight, heart weight and tibial lengths (mean ± SEM) in control (sham) and experimental (clip) rats following renal artery constriction.

A minimum of 10 animals were used in each group.

* represents $P \leq 0.05$ compared to control rats.

TABLE 11.

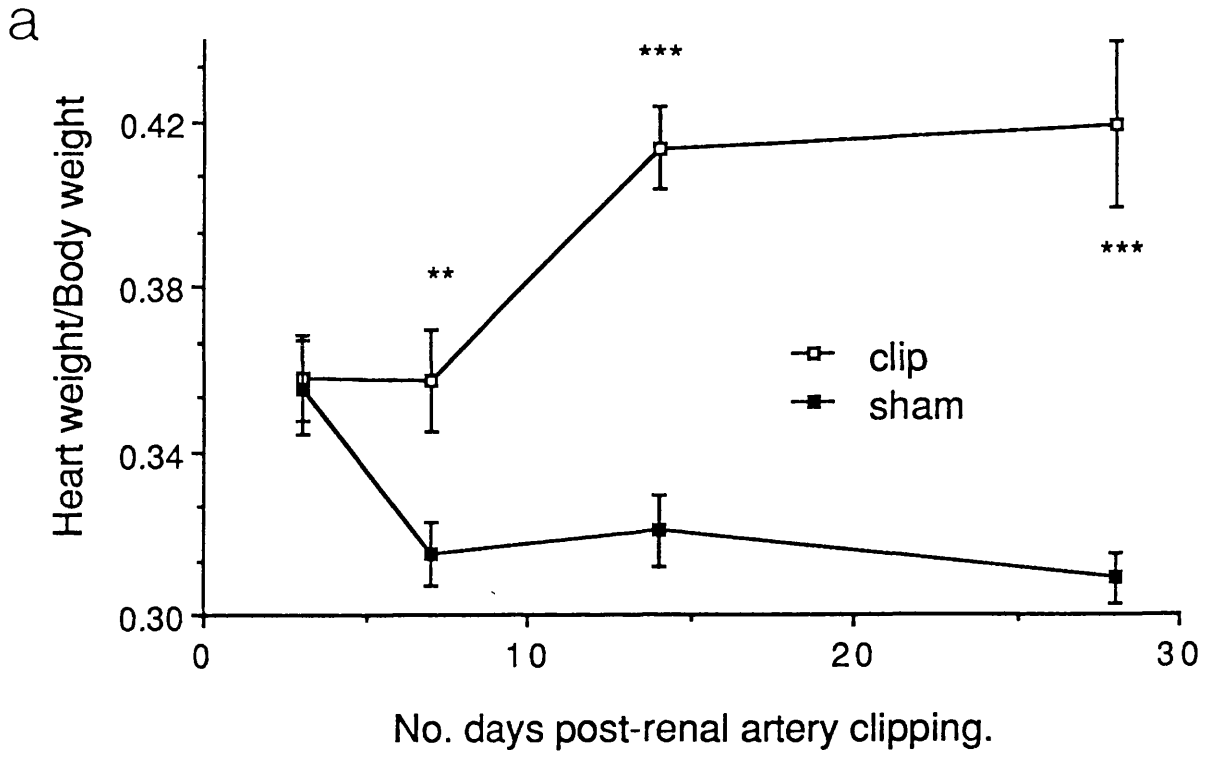
		Heart Weight. (g)	Body Weight. (g)	Tibial Length. (cm)
3 Days.	Sham	0.663 ± 0.031	186.3 ± 5.9	2.98 ± 0.04
	Clip	0.673 ± 0.026	187.8 ± 3.6	3.04 ± 0.04
7 Days.	Sham	0.638 ± 0.016 *	203.0 ± 3.8	3.12 ± 0.02
	Clip	0.684 ± 0.016	193.1 ± 7.9	3.06 ± 0.02
14 Days.	Sham	0.675 ± 0.020 *	211.6 ± 6.7	3.16 ± 0.04
	Clip	0.801 ± 0.027	194.6 ± 7.9	3.14 ± 0.02
28 Days.	Sham	0.785 ± 0.022 *	254.3 ± 5.0 *	3.34 ± 0.02
	Clip	0.917 ± 0.034	223.5 ± 11.1	3.28 ± 0.02

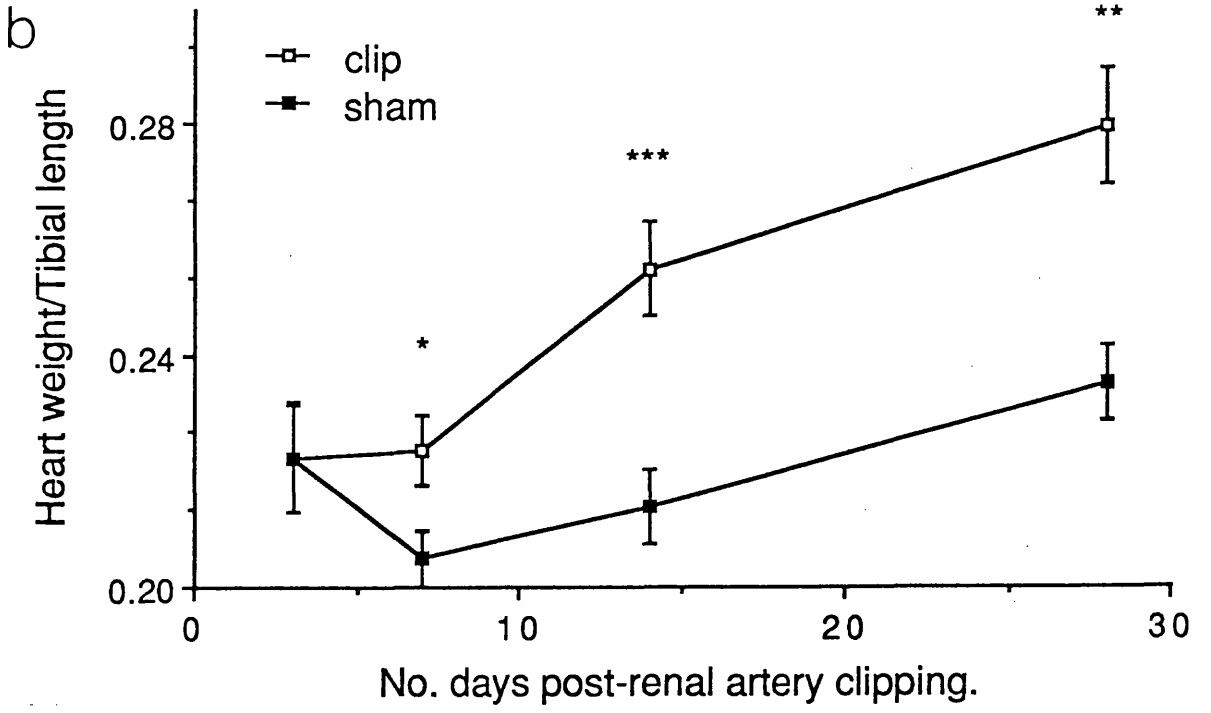
FIGURE 19

The heart weight / body weight ratio (a) and the heart weight / tibial length ratio (b) in control (sham) and experimental (clip) rats.

* = $P \leq 0.05$
** = $P \leq 0.01$
*** = $P \leq 0.001$

FIGURE 19.


a
Heart weight/Body weight
0.42
0.38
0.34
0.30
0
10
20
30
**
***
***
clip
sham
No. days post-renal artery clipping.


b
Heart weight/Tibial length
0.28
0.24
0.20
0
10
20
30
clip
sham
*
***
**
No. days post-renal artery clipping.

of this index depends on the assumption that both the experimental and control rats grow at the same rate. Unfortunately this is not always the case. Animals of the 2K, 1C renovascular model grow more slowly than their corresponding controls and may even lose weight (Bing et al 1984). Thus although the experimental and control rats were well matched for weight prior to the induction of renovascular hypertension (Table 11), the experimental animals had a significantly lower body weight at 28 days ($P \leq 0.05$, Table 11). As a consequence the use of heart weight/body weight tended to lead to an overestimation of cardiac hypertrophy.

4.3.2.2 Heart weight / Tibial length ratio.

There was no significant difference in tibial length between the experimental animals and the sham animals at any stage after renal artery constriction (Table 11). The tibial length of an animal is independent of factors such as general health and nutrition (Berg and Harmison, 1958) and the heart weight/tibial length ratio is considered to be a more reliable index for the assessment of cardiac hypertrophy. Figure 19b shows that unlike the heart weight/body weight index heart weight/tibial length demonstrates similar age-related changes in cardiac mass in both the control and the experimental rats and is not affected by the weight loss which occurs in renal hypertensive rats.

4.4 Changes in Plasma Renin Concentration.

Plasma renin concentration (PRC) was significantly higher in the experimental animals compared to the sham animals during the development of hypertension (Table 12). Since plasma renin concentration levels are not normally distributed, the values in the experimental hypertensive rats and the controls were compared using the Wilcoxon rank sum test for non-parametric data. With the exception of day 7, there was a significant elevation of PRC in the experimental animals compared to the controls. These results are similar to those obtained previously by other workers (Morton and Wallace, 1983).

4.5 Changes in DNA synthesis - Aorta.

Aortae dissected from animals at three and seven days post-renal artery clipping were of similar lengths (7 days; sham - 44.8 ± 2.8 mm; clipped - 43.7 ± 3.1 mm) and weights (7 days; sham - 31.9 ± 2.7; clipped - 32.7 ± 2.4 mg) and thus exhibited similar wet weight/unit length ratios. However, by 14 days the aortae of experimental animals were both heavier and longer than those of the control animals, more importantly the weight per unit length ratio was also increased ($P \leq 0.005$), indicating that growth had occurred. Similar results were obtained at 28 days post-renal artery clipping although the weight per unit length ratio did not achieve significance.

TABLE 12.

		Mean Plasma Renin Concn. (ng/ml/hr) ± SEM.	P
3 Days.	Sham	17.09 ± 2.05	< 0.05
	Clip	32.37 ± 5.74	
7 Days.	Sham	16.52 ± 2.78	NS
	Clip	41.78 ± 14.95	
14 Days.	Sham	9.41 ± 1.46	< 0.01
	Clip	92.43 ± 48.05	
28 Days	Sham	9.72 ± 2.69	< 0.05
	Clip	45.61 ± 18.86	

Plasma renin concentration of control (sham) and experimental (clip) rats following renal artery constriction.

4.5.1 Aortic Uptake of [^{3}H]-thymidine.

The uptake of [^{3}H]-thymidine represents the sum of thymidine and metabolites of thymidine taken into the vessel but not incorporated into DNA. Uptake can be determined from the amount of radioactivity present in the first supernatant (Section 2.5) and was expressed as dpm/mg wet weight tissue/hr and dpm/mm length tissue/hr. There was no significant difference in uptake of [^{3}H]-thymidine between the aortae of experimental animals compared to control animals at any stage during the course of development of hypertension (Table 13).

4.5.2 Aortic Incorporation of [^{3}H]-thymidine.

The incorporation of [^{3}H]Thymidine into DNA was determined from the sum of radioactivity present in the second supernatant and in the final pellet (Section 2.5). Incorporation of [^{3}H]-thymidine was expressed as either dpm/mm length tissue/hr or dpm/µg tissue DNA/hr. At three days post-renal artery constriction no significant difference in incorporation could be detected, although the results obtained from the experimental animals were consistently greater than those seen in the control animals. However by seven days there was a significant increase in thymidine incorporation into the aortae of experimental animals compared to the control animals (dpm/mm/hr - $P \leq 0.001$;

TABLE 13.

	Dpm/mgweight/hr ± SEM	Dpm/mmlength/hr ± SEM
Sham	84.3 ± 21.5	43.8 ± 4.42
3 Days.		
Clip	68.8 ± 9.91	46.7 ± 4.40
Sham	67.2 ± 6.82	44.5 ± 4.81
7 Days.		
Clip	63.9 ± 7.57	45.6 ± 5.51
Sham	88.4 ± 9.55	60.1 ± 6.52
14 Days.		
Clip	61.1 ± 11.4	47.2 ± 6.37
Sham	73.1 ± 10.1	52.8 ± 6.28
28 Days.		
Clip	57.4 ± 13.3	47.2 ± 10.1

Uptake of [^{3}H]-thymidine into aortae from both control and experimental rats following renal artery constriction.

dpm/μgDNA/hr - $P \leq 0.02$), and remained elevated at day 14. Incorporation of thymidine into the aortae of experimental animals had however, fallen back to control levels by 28 days after renal artery constriction (Figure 20).

4.5.3 Aortic Protein Content.

Aortic protein concentration increased steadily in all animals throughout the study. However the rate of increase in protein content was greater in the experimental animals and by day 28 these animals exhibited a significantly greater protein content than did their corresponding controls (protein/length; sham - 50.78 ± 3.02 μg/mm; clipped - 59.88 ± 1.50 μg/mm; $P \leq 0.02$ - Figure 21a).

4.5.4 Aortic DNA Content.

No change in DNA levels was detected in the aortae of animals during the first fourteen days following renal artery clipping. At 28 days the DNA content of aortae from experimental animals was slightly raised compared to control animals although this change failed to reach statistical significance (Figure 21b).

4.5.5 Aortic DNA / Protein Ratio.

The ratio of DNA to protein in the aortae of experimental and control animals was similar, (e.g 7 days; sham - 0.014 ±

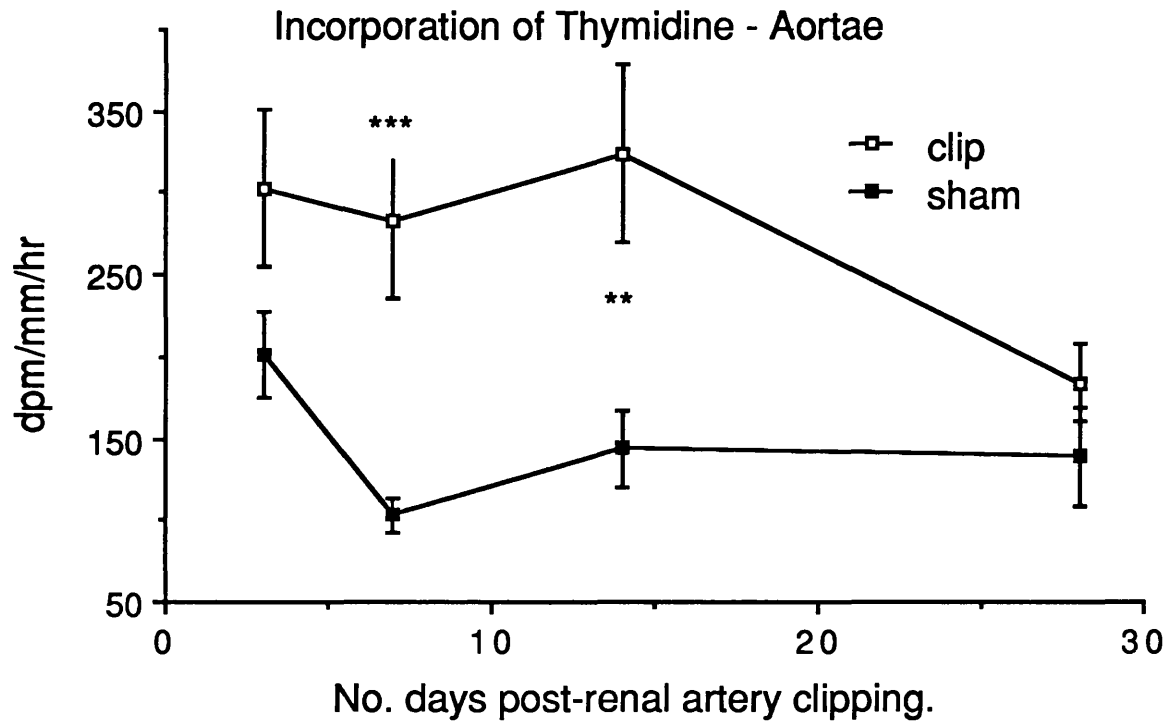
FIGURE 20

The rate of incorporation of [^{3}H]-thymidine into aortic DNA from control and experimental rats.

** = $P \leq 0.01$
*** = $P \leq 0.001$

FIGURE 20.

a


Incorporation of Thymidine - Aortae
350
250
150
50
dpm/mm/hr
***
**
clip
sham
0
10
20
30
No. days post-renal artery clipping.

b

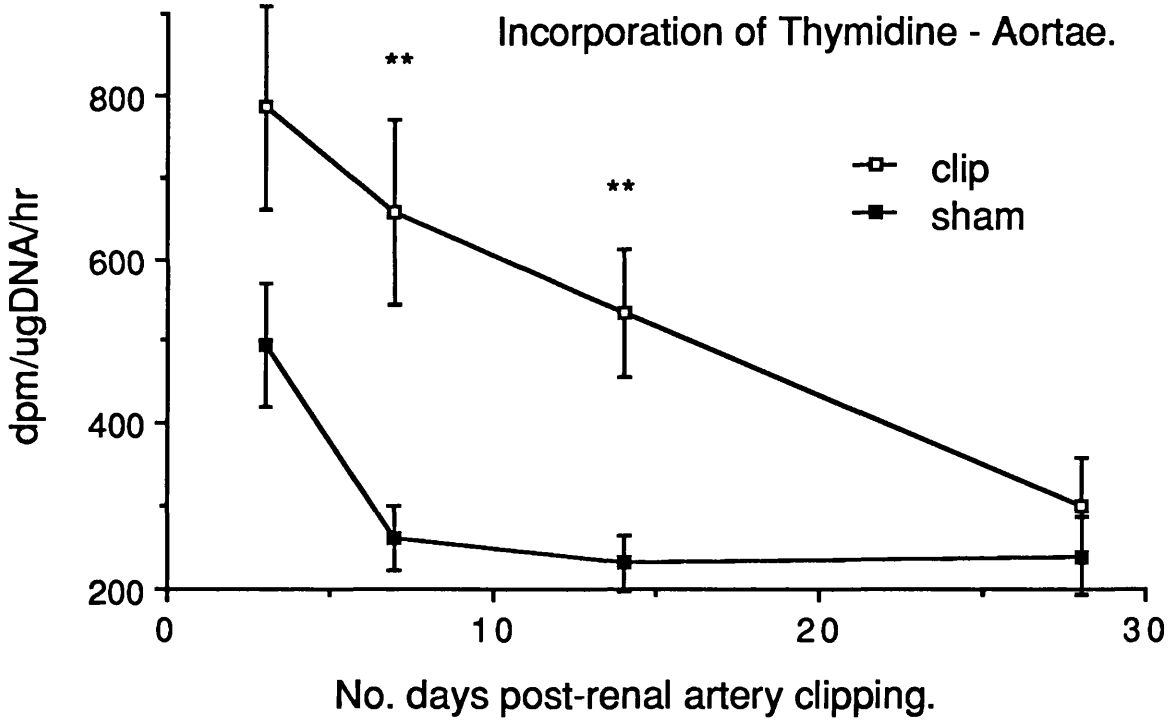

Incorporation of Thymidine - Aortae.
800
600
400
200
dpm/ugDNA/hr
**
**
clip
sham
0
10
20
30
No. days post-renal artery clipping.

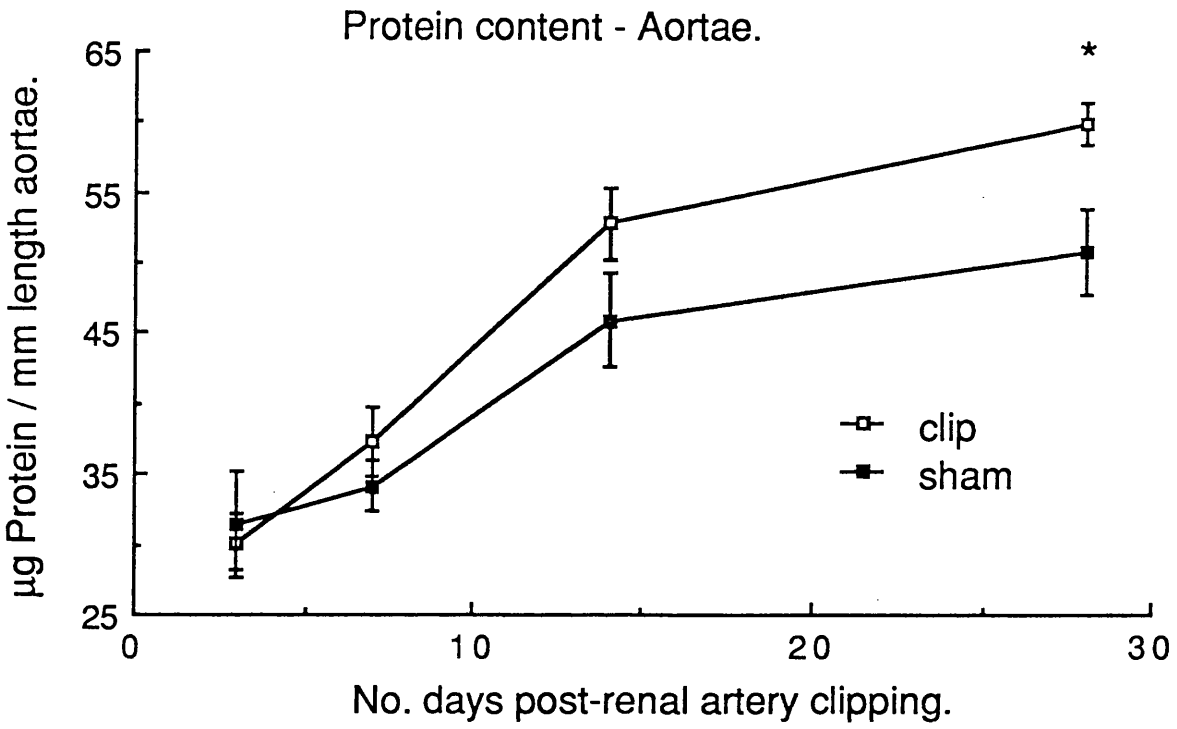
FIGURE 21

The protein content (a) and DNA content (b) of aortae from control and experimental rats.

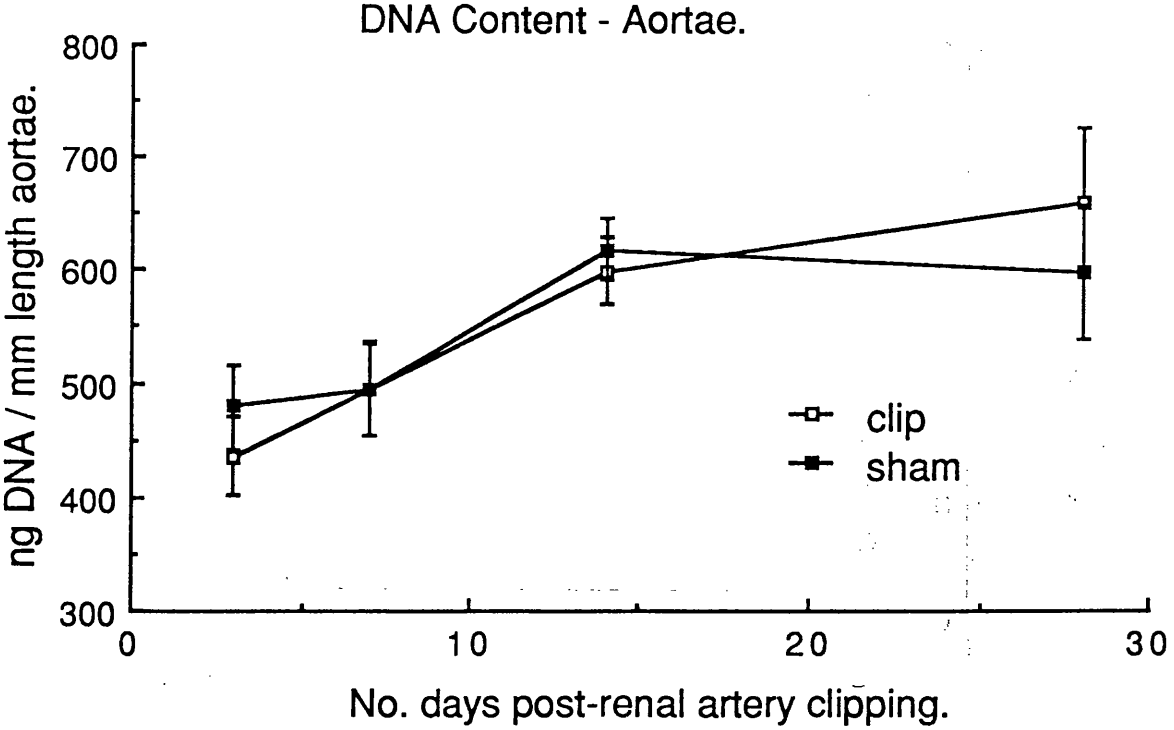
* = $P \leq 0.05$

FIGURE 21.

a


Protein content - Aortae.
*
65
55
45
35
25
µg Protein / mm length aortae.
0
10
20
30
clip
sham
No. days post-renal artery clipping.

b


DNA Content - Aortae.
800
700
600
500
400
300
ng DNA / mm length aortae.
0
10
20
30
clip
sham
No. days post-renal artery clipping.

0.002; clip - 0.012 ± 0.001), and remained unchanged throughout the duration of the experiment.

4.6 Changes in DNA synthesis - Mesenteric Artery.

Four mesenteric arteries (approximately 250 - 300 μm) were dissected from each animal (Section 2.4.2). The total length of these arteries from both the experimental and the control groups were similar at all stages during the period of the experiment.
e.g. 14 days; sham - 77.4 ± 2.1; clip - 78.9 ± 2.0 mm.

4.6.1 Mesenteric Artery Uptake of [^{3}H] Thymidine.

There were no significant differences in the uptake of [^{3}H]-thymidine into the mesenteric arteries of experimental compared to control animals, at any stage during the development of hypertension (Table 14).

4.6.2 Mesenteric Artery Incorporation of [^{3}H]-thymidine.

Incorporation of [^{3}H]-thymidine into mesenteric artery samples was expressed as dpm/mm length/hr and dpm/μg tissue DNA/hr. At 3 days post-renal artery clipping the experimental animals were already showing an increased incorporation of [^{3}H]-thymidine into mesenteric arteries although this was only statistically significant when expressed as dpm/mm/hr ($P \leq 0.05$). Arteries from experimental animals continued to

TABLE 14.

		Dpm/mmlength/hr	P
3 Days.	Sham	13.96 ± 2.07	NS
	Clip	16.35 ± 2.75	
7Days.	Sham	16.20 ± 2.84	NS
	Clip	17.23 ± 2.75	
14 Days.	Sham	08.99 ± 1.88	NS
	Clip	09.89 ± 1.38	
28 Days.	Sham	09.98 ± 1.68	NS
	Clip	09.18 ± 1.28	

Uptake of [^{3}H]-thymidine into mesenteric arteries from both control and experimental rats following renal artery constriction.

incorporate [^{3}H]-thymidine into DNA at a much greater rate than those from control animals up to 14 days post-renal artery clipping, after which the rate of incorporation returned to control levels (Figure 22).

4.6.3 Mesenteric Artery Protein Content.

The protein content of mesenteric arteries from control and experimental animals were similar in the early stages during the development of hypertension. However in the established phase of hypertension (28 days) the mesenteric arteries of experimental animals had a significantly higher protein content ($P \leq 0.05$) compared to the controls (Figure 23a).

4.6.4 Mesenteric Artery DNA Content.

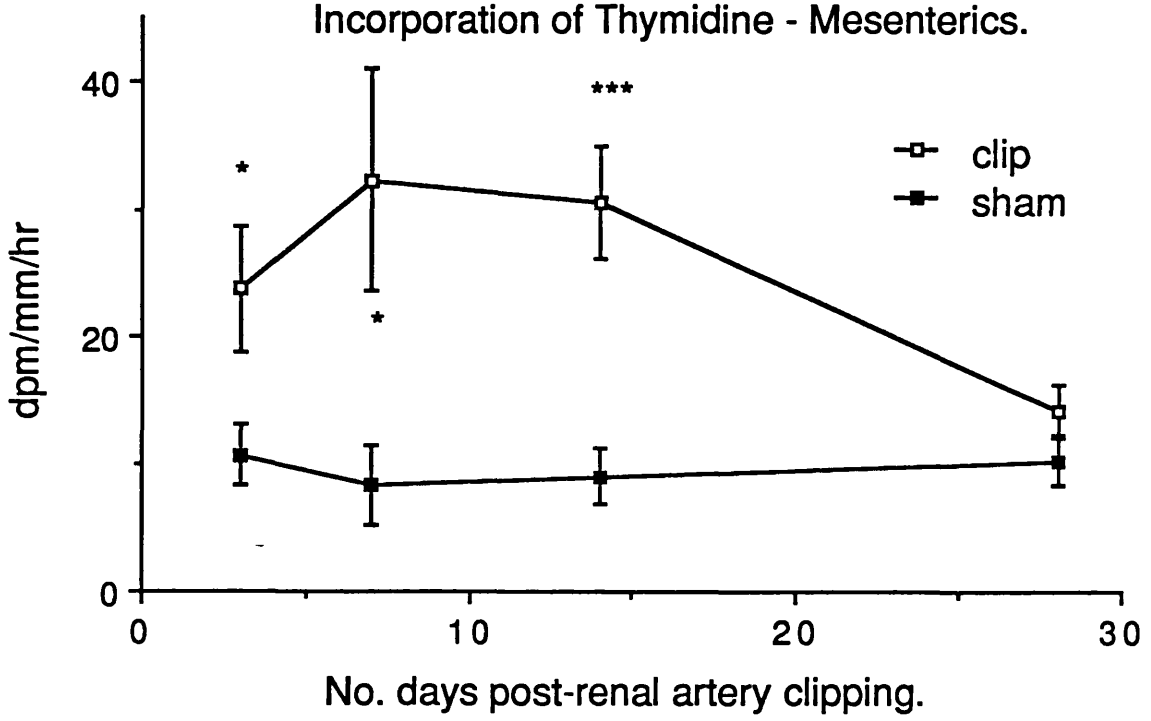
There was no significant difference in the DNA content of mesenteric arteries from the experimental animals compared to control animals throughout the first fourteen days following renal artery clipping. At 28 days however, when the blood pressure of the experimental animals had reached a plateau, the mesenteric artery DNA content of experimental animals was significantly raised compared to the controls ($P \leq 0.05$, Figure 23b).

FIGURE 22.

The rate of incorporation of [^{3}H]-thymidine into mesenteric artery DNA from control and experimental rats.

* = $P \leq 0.05$
*** = $P \leq 0.001$

FIGURE 22.


Incorporation of Thymidine - Mesenterics.
dpm/mm/hr
40
20
0
0
10
20
30
*
*
***
clip
sham
No. days post-renal artery clipping.

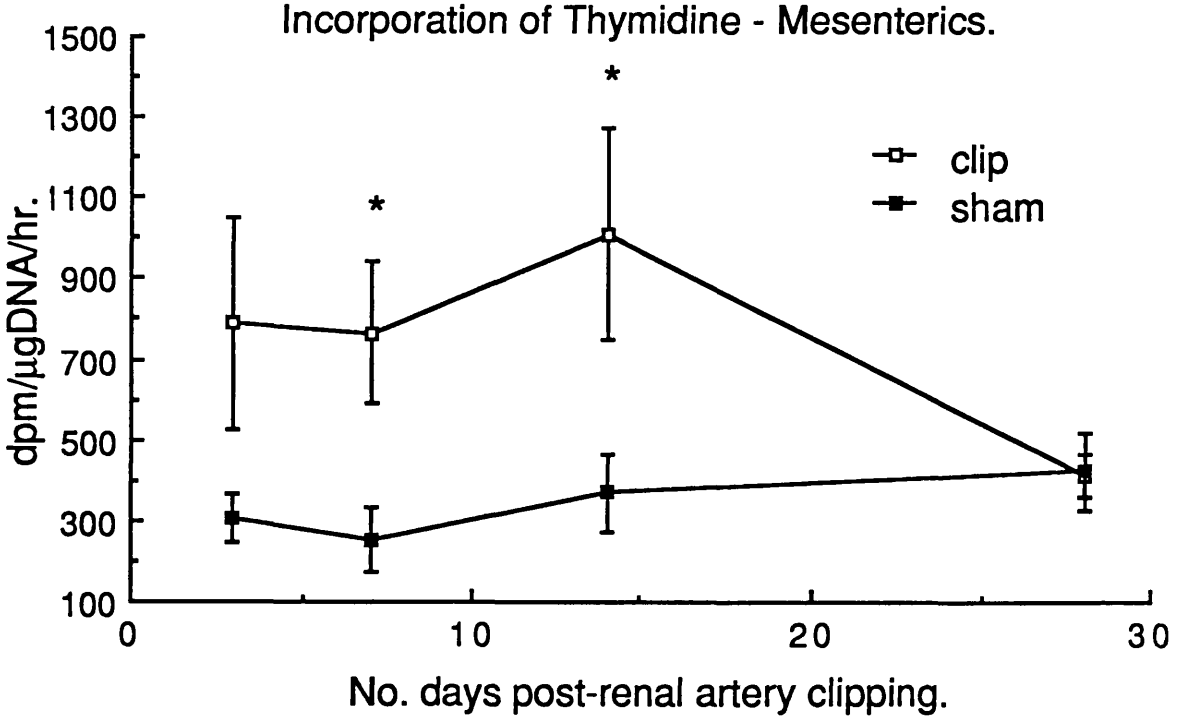

Incorporation of Thymidine - Mesenterics.
dpm/µgDNA/hr.
1500
1300
1100
900
700
500
300
100
0
10
20
30
*
*
clip
sham
No. days post-renal artery clipping.

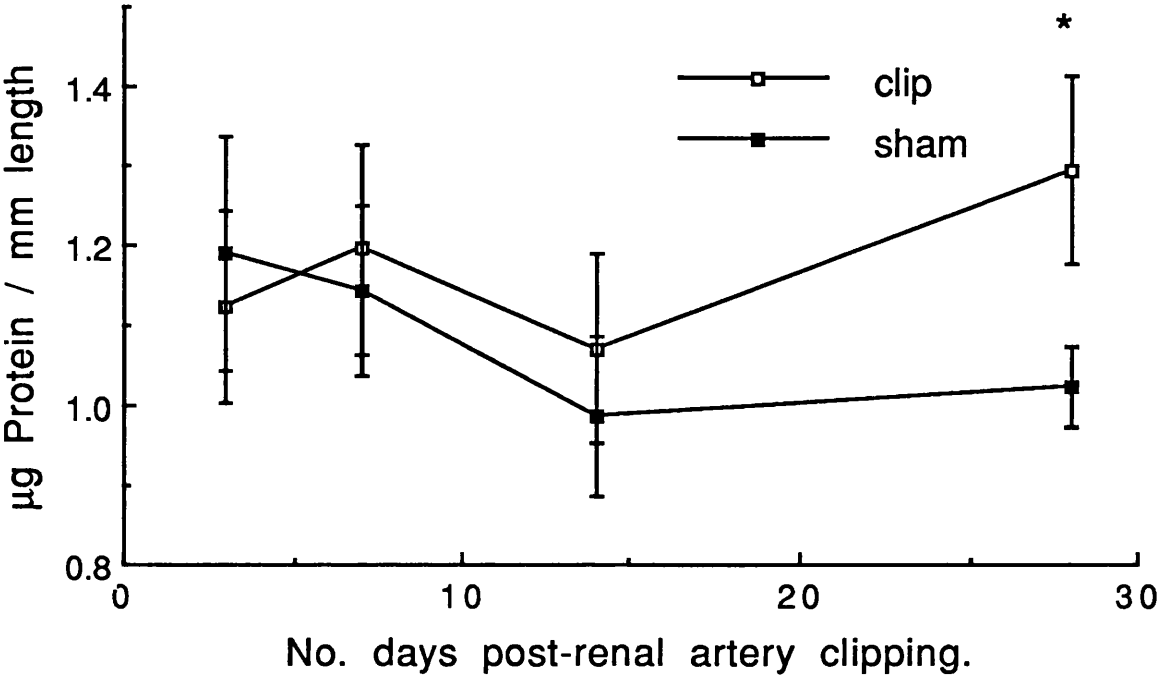
FIGURE 23.

The protein content (a) and DNA content (b) of mesenteric resistance vessels from control and experimental rats.

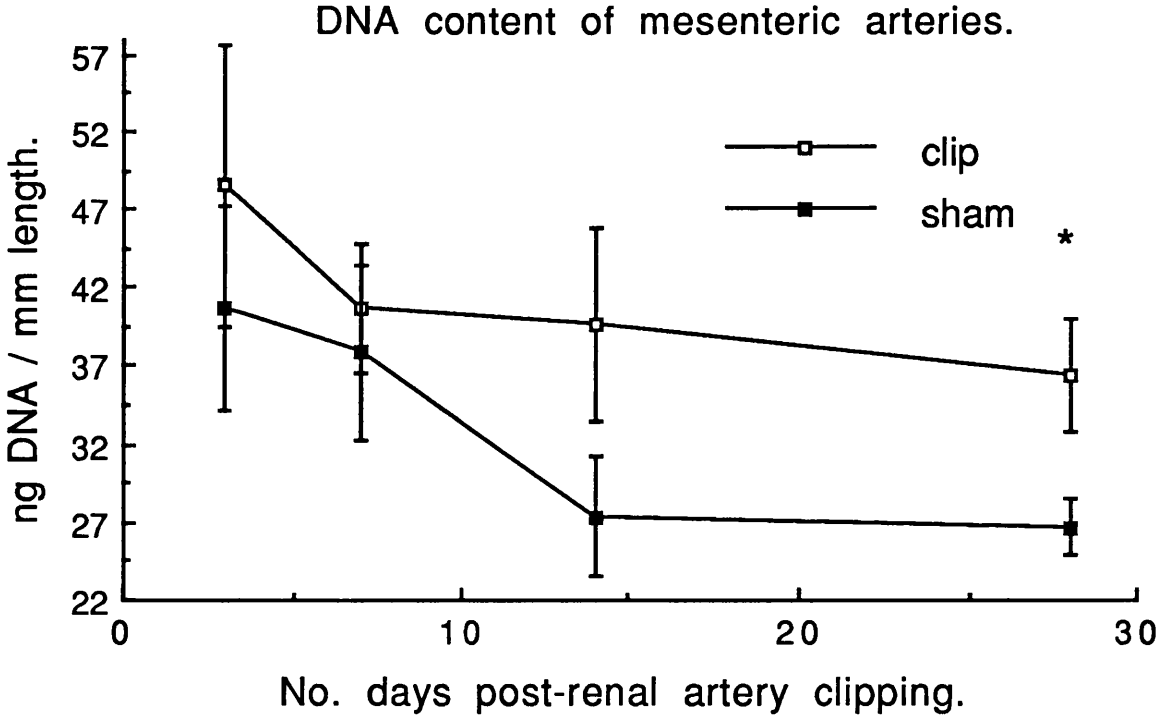
* = $P \leq 0.05$

FIGURE 23.

a


Protein Content of mesenteric arteries.
µg Protein / mm length
1.4
1.2
1.0
0.8
clip
sham
*
0
10
20
30
No. days post-renal artery clipping.

b


DNA content of mesenteric arteries.
ng DNA / mm length.
57
52
47
42
37
32
27
22
clip
sham
*
0
10
20
30
No. days post-renal artery clipping.

4.6.5 Mesenteric Artery DNA / Protein Ratio.

The ratio of DNA to protein was similar in both the control and the clipped animals and remained unchanged throughout the course of the experiment.

4.7 Changes in DNA synthesis - Subcutaneous Vessels.

The lengths of the subcutaneous vessels dissected were similar in both the control and the experimental animals at all stages.
e.g. 14 days; sham - 74.1 ± 2.5, clipped - 74.6 ± 2.9 mm.

4.7.1 Subcutaneous Vessel Uptake of [^{3}H]-thymidine.

There was no significant difference in the uptake of [^{3}H]-thymidine into the subcutaneous vessels of the control or the experimental animals during the study (Table 15). Interestingly values obtained in these vessels were similar to those obtained using the small resistance vessels of the mesenteric bed.

4.7.2 Subcutaneous Vessel Incorporation of [^{3}H]-thymidine.

In contrast to results obtained for aortae and mesenteric arteries there was no change in the incorporation of [^{3}H]-thymidine into the subcutaneous vessels. Vessels from the

TABLE 15.

		Dpm/mmlength/hr	P
3 Days.	Sham	13.40 ± 4.74	NS
	Clip	12.40 ± 4.35	
7Days.	Sham	8.25 ± 1.42	NS
	Clip	10.72 ± 2.34	
14 Days.	Sham	9.90 ± 1.25	NS
	Clip	8.64 ± 1.38	
28 Days.	Sham	9.42 ± 1.82	NS
	Clip	8.82 ± 1.55	

Uptake of [^{3}H]-thymidine into subcutaneous vessels from both control and experimental rats following renal artery constriction.

experimental animals consistently maintained a rate of incorporation of [^{3}H]-thymidine which was comparable to control values (Figure 24).

4.7.3 Subcutaneous Vessel Protein Content.

Protein content in the subcutaneous vessels of experimental and control animals were similar throughout the course of this work, (Table 16).

4.7.4 Subcutaneous Vessel DNA Content.

There was no significant difference in the DNA content of the subcutaneous vessels from the experimental and the control animals at any stage in the development of hypertension (Table 16).

4.7.5 Subcutaneous Vessel DNA / Protein Ratio.

In keeping with the mesenteric arteries there was no difference in the DNA/protein ratio of the subcutaneous vessels between experimental and control animals at any time following renal artery constriction (Table 16).

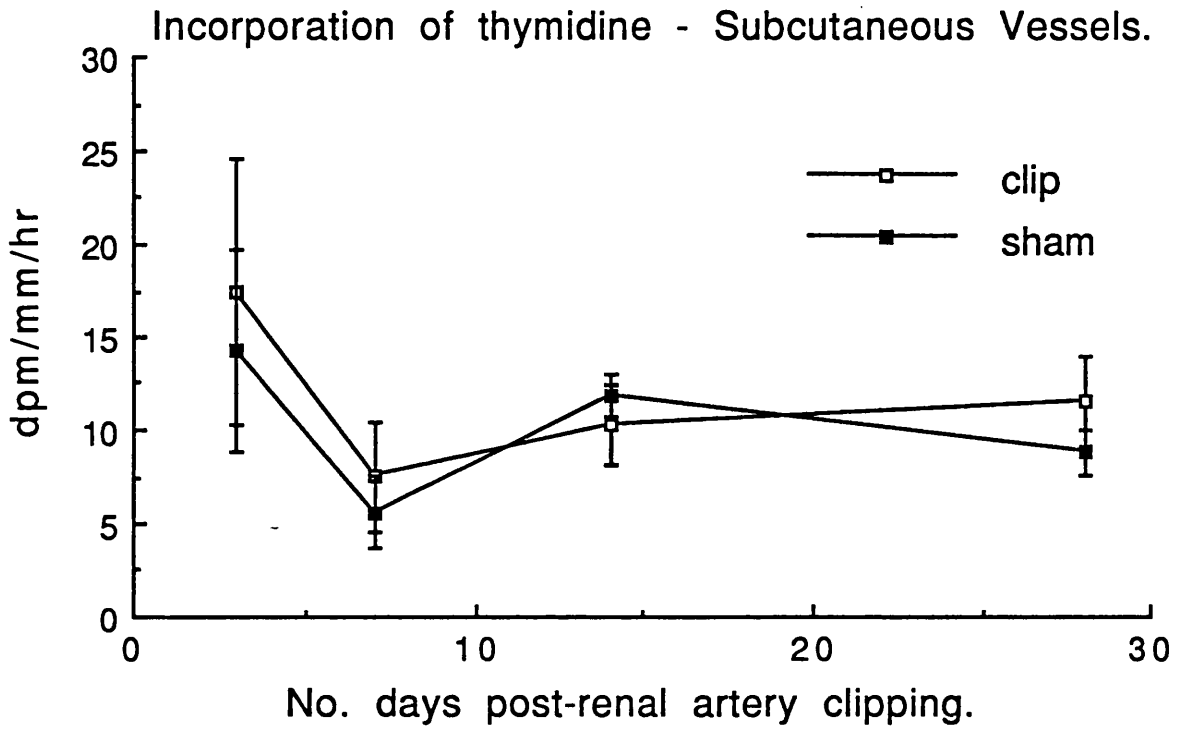
4.8 Summary of Results.

It is interesting to note that the induction of 2K, 1C renovascular hypertension does not affect all vascular beds

FIGURE 24.

The rate of incorporation of [^{3}H]-thymidine into subcutaneous artery DNA from control and experimental rats.

FIGURE 24.


Incorporation of thymidine - Subcutaneous Vessels.
dpm/mm/hr
30
25
20
15
10
5
0
0
10
20
30
clip
sham
No. days post-renal artery clipping.

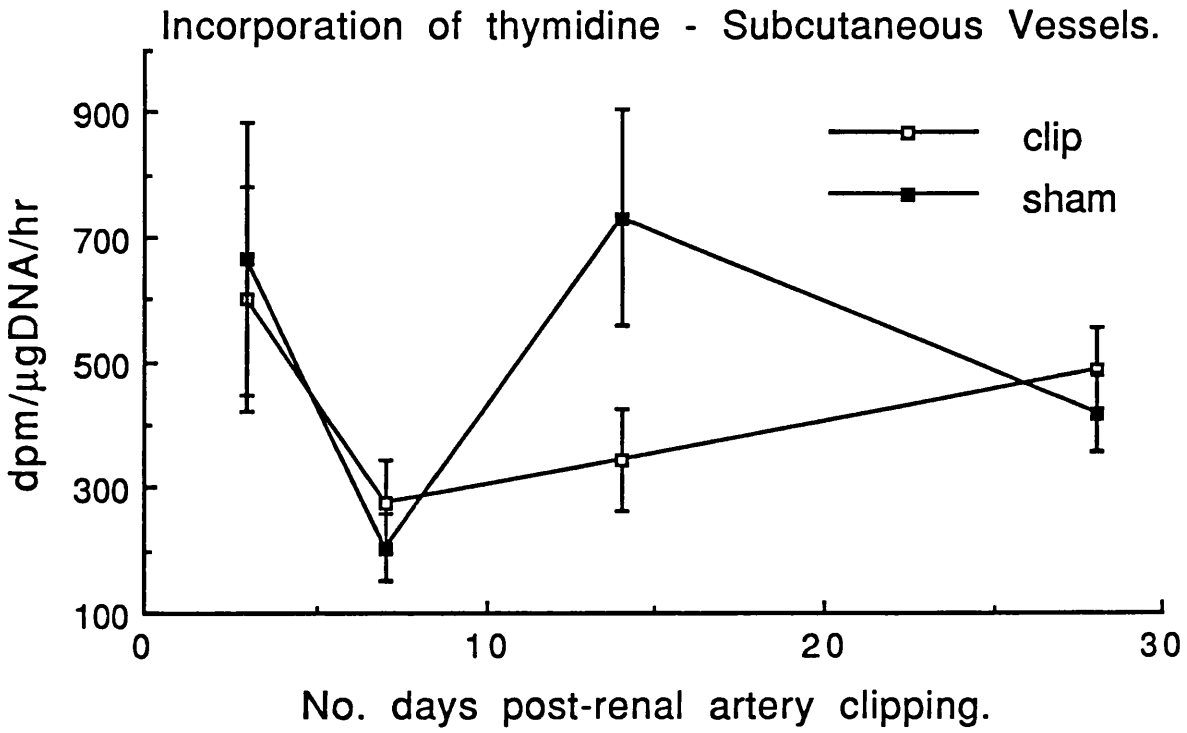

Incorporation of thymidine - Subcutaneous Vessels.
dpm/µgDNA/hr
900
700
500
300
100
0
10
20
30
clip
sham
No. days post-renal artery clipping.

TABLE 16.

The protein content , DNA content and DNA/protein ratio of subcutaneous resistance vessels from both experimental and control rats following renal artery constriction.

in a similar manner. Large conduit arteries like the aorta, and small mesenteric arteries showed an increase in the rate of [^{3}H]-thymidine incorporation prior to any significant rise in blood pressure. By contrast the [^{3}H]-thymidine incorporation in subcutaneous vessels remained at control levels throughout the development of hypertension.

The increased rate of DNA synthesis was associated with a significant increase in protein content in both the aorta and the mesenteric arteries but the DNA content was increased only in the mesenteric vessels. The DNA/protein ratio remained unchanged in all vessels studied.

CHAPTER FIVE.

GENETIC HYPERTENSION.

5.1 Choice of Genetic Model.

The Wistar derived Okamoto-Aoki strain of Spontaneously Hypertensive (SH) rats is the most widely studied animal model of essential hypertension. This genetic hypertensive strain together with the Wistar-Kyoto (WKY) normotensive control strain were both being bred in established on-site colonies at Leicester University. Thus animals were both readily available on a cost-effective basis and furthermore, information regarding the parentage of the animals used was easily obtainable. The animals from these colonies have been used previously for hypertension research within the department (Izzard and Heagerty 1989; Watt and Thurston 1989) and this considerable experience in handling and a knowledge about the course of development of hypertension made the SH rat the best choice of a genetic model for this study.

5.2 Development of Hypertension.

The course of development of hypertension in the SH rats was assessed by blood pressure measurement and the development of cardiac hypertrophy.

5.2.1 Blood Pressure Measurements.

In order to investigate the development of hypertension in these genetic strains of rat it was neccessary to study

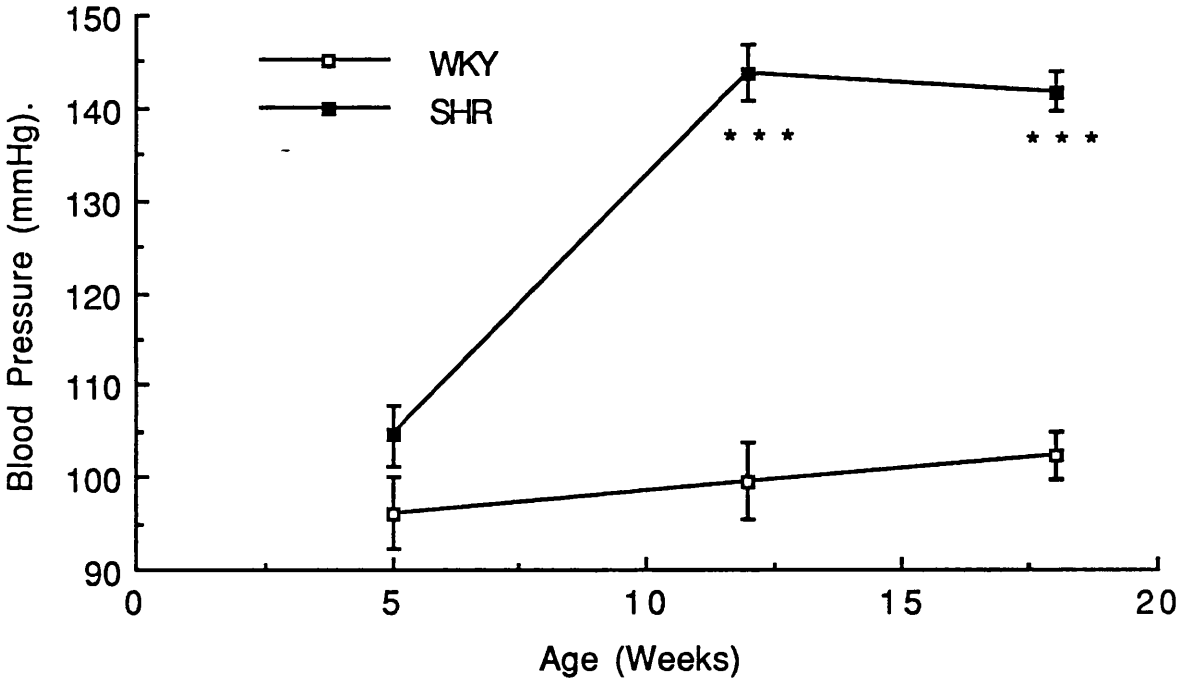
animals from an early age. Animals were studied at 5 weeks of age, just fourteen days after weaning, through to 18 weeks of age when hypertension is well established. At 5 weeks of age there was no significant difference in the blood pressures of the SH and WKY animals, although the blood pressure of the SH rats was already slightly elevated (WKY - 96.0 ± 4.0; SH - 104.5 ± 3.5 mmHg). By 12 weeks of age however the picture had changed with the SH animals exhibiting very significantly elevated blood pressures ($P \leq 0.001$). After this age blood pressure failed to rise any further giving results at 18 weeks of age very similar to those obtained at 12 weeks (Figure 25).

5.2.2 Development of Cardiac Hypertrophy.

The development of cardiac hypertrophy is a further indication of a raised blood pressure and therefore the heart weight was measured in each animal. The hearts of the SH animals were significantly heavier than those of the WKY rats at all the ages studied (Figure 26b). However although the SH and WKY animals were age-matched, SH rats are usually heavier than their WKY controls. Thus the increased heart weight of the SH rats could relate more to a difference in body weight rather than to the presence of cardiac hypertrophy. Thus in isolation heart weight may not be a reliable index of cardiac hypertrophy in SH rats.

FIGURE 25.

Blood Pressure Measurements.



Effect of increasing age on the blood pressure of SH and WKY rats.

Comparisons were made using a T-test for unpaired samples.

*** represents $P \leq 0.001$

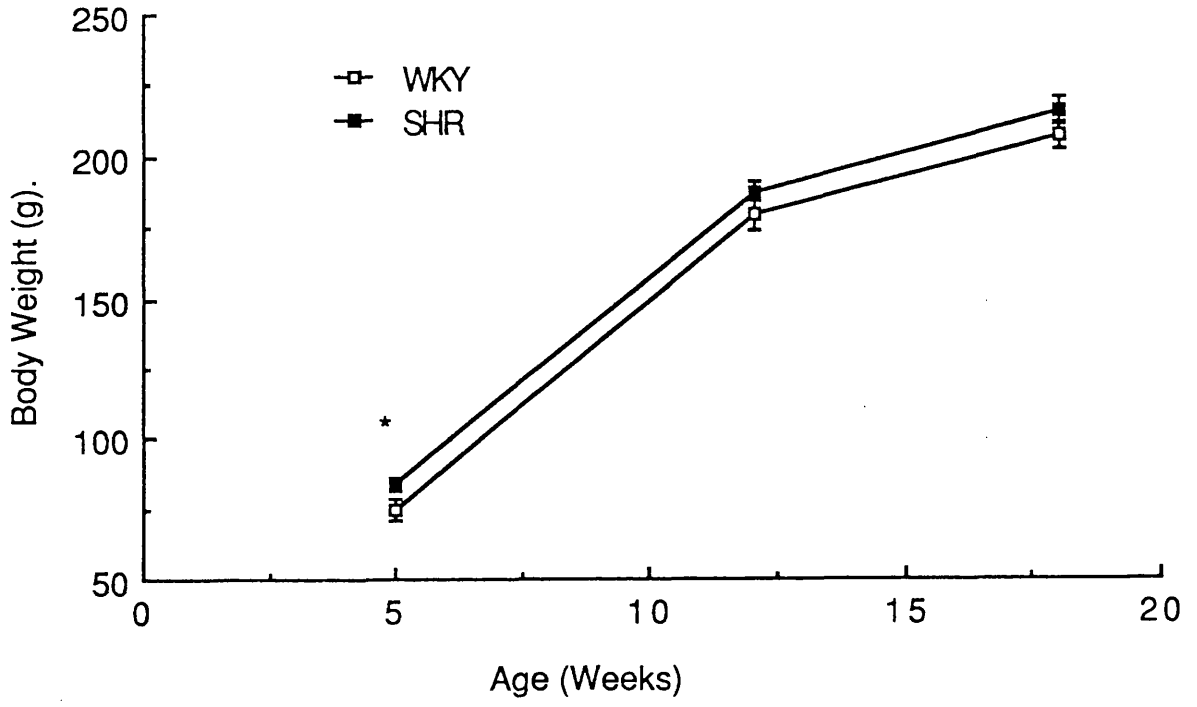
FIGURE 26.

Effect of increasing age on both body weight (a) and heart weight (b) in SH and WKY rats.

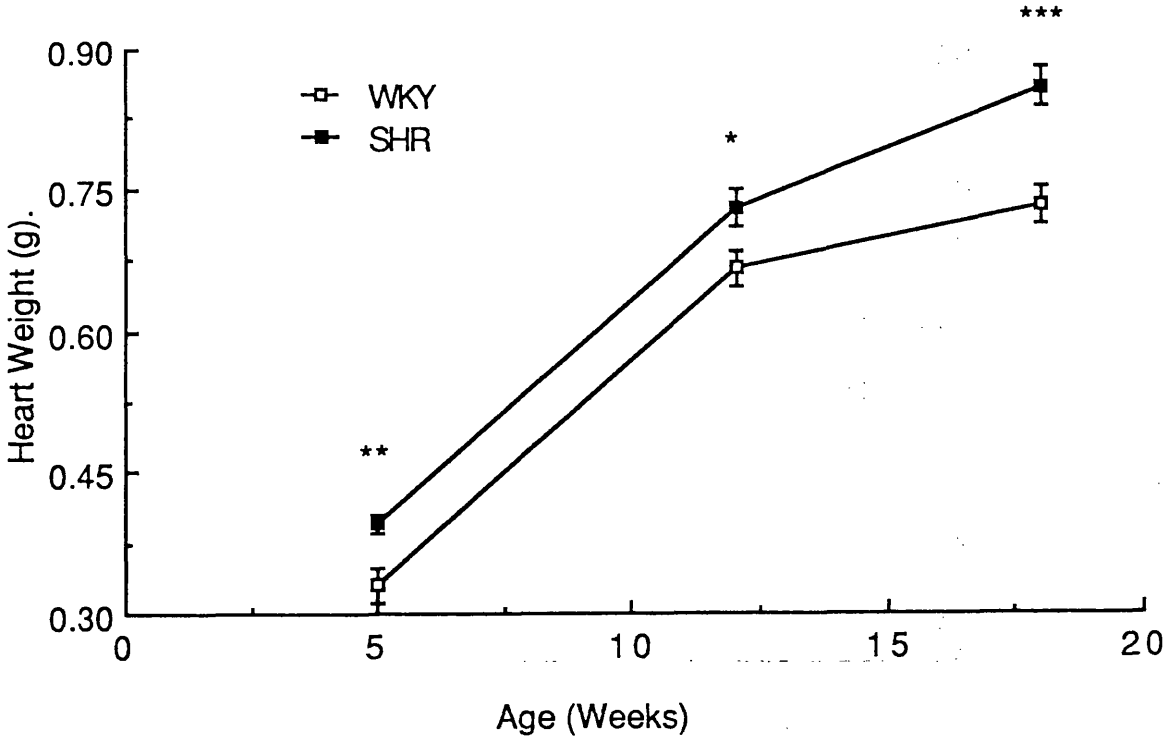
* = $P \leq 0.05$
** = $P \leq 0.01$
*** = $P \leq 0.001$

FIGURE 26.

a


250
200
150
100
50
Body Weight (g).
WKY
SHR
*
0
5
10
15
20
Age (Weeks)

b


0.90
0.75
0.60
0.45
0.30
Heart Weight (g).
WKY
SHR
**
*
***
0
5
10
15
20
Age (Weeks)

5.2.2.1 Heart Weight / Body Weight Ratio.

Figure 26a shows that the changes in body weight in the SH rats parralleled the changes in the body weight of WKY rats. However, the SH rats were consistently heavier than their age-matched WKY controls, although this difference only achieved significance at 5 weeks of age.

The use of heart weight/body weight as an index of cardiac hypertrophy thus would take account of the increased body weights of the SH rats. Heart weight/body weight ratio was only significantly different in the SH rats compared to the WKY rats at 18 weeks of age, when hypertension was well established. The heart weight/body weight ratio of the SH rats was however, consistently greater than that of the WKY rats (Figure 27a). However, when the heart weight/body weight index is used it is possible that the heavier weight of the SHR is actually masking the detection of cardiac hypertrophy in these animals.

5.2.2.2 Heart Weight / Tibial Length Ratio.

As expected the tibial length of the animals increased with age but there was no significant difference in tibial length between the SH and WKY rats at any age (Table 17). The heart weight/tibial length ratio was significantly increased in the SH compared to the WKY rats at all ages (Figure 27b). Thus the heart weight/tibial length ratio may be a more sensitive index of cardiac hypertrophy than the heart weight/body

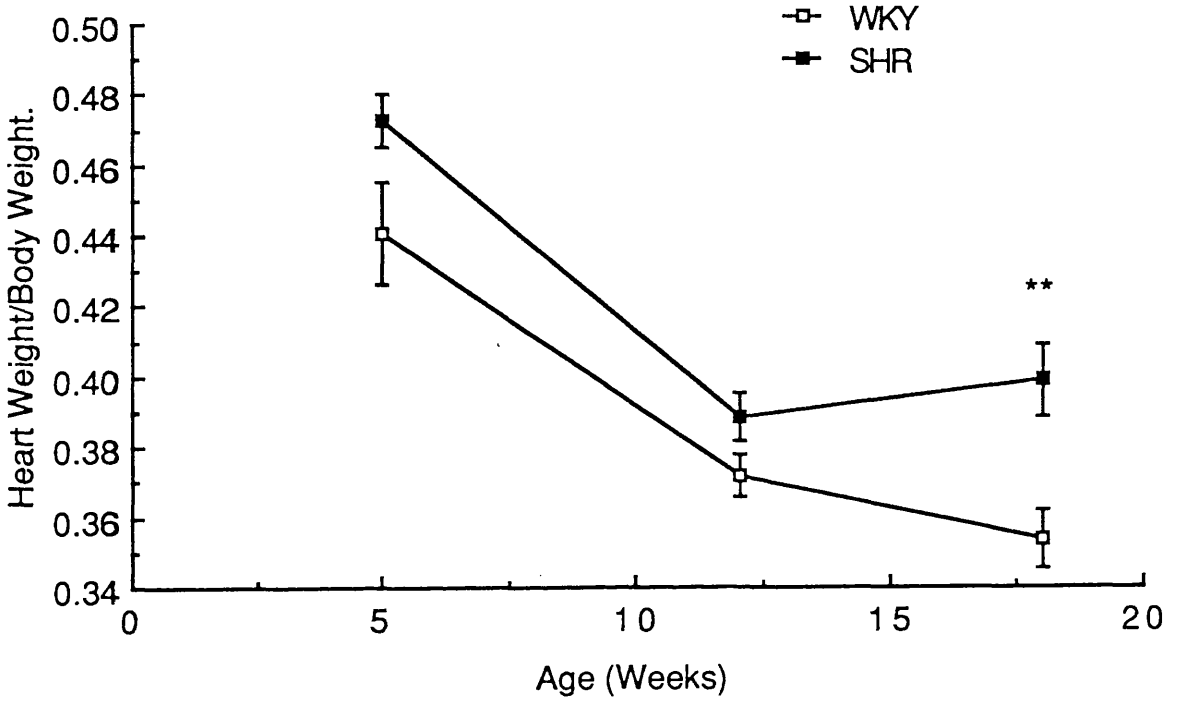
FIGURE 27.

The heart weight / body weight ratio (a) and heart weight / tibial length ratio (b) in SH and WKY rats at different ages.

* = $P \leq 0.05$
** = $P \leq 0.01$
*** = $P \leq 0.001$

FIGURE 27.

a


WKY
SHR
Heart Weight/Body Weight.
0.50
0.48
0.46
0.44
0.42
0.40
0.38
0.36
0.34
0
5
10
15
20
Age (Weeks)
**

b

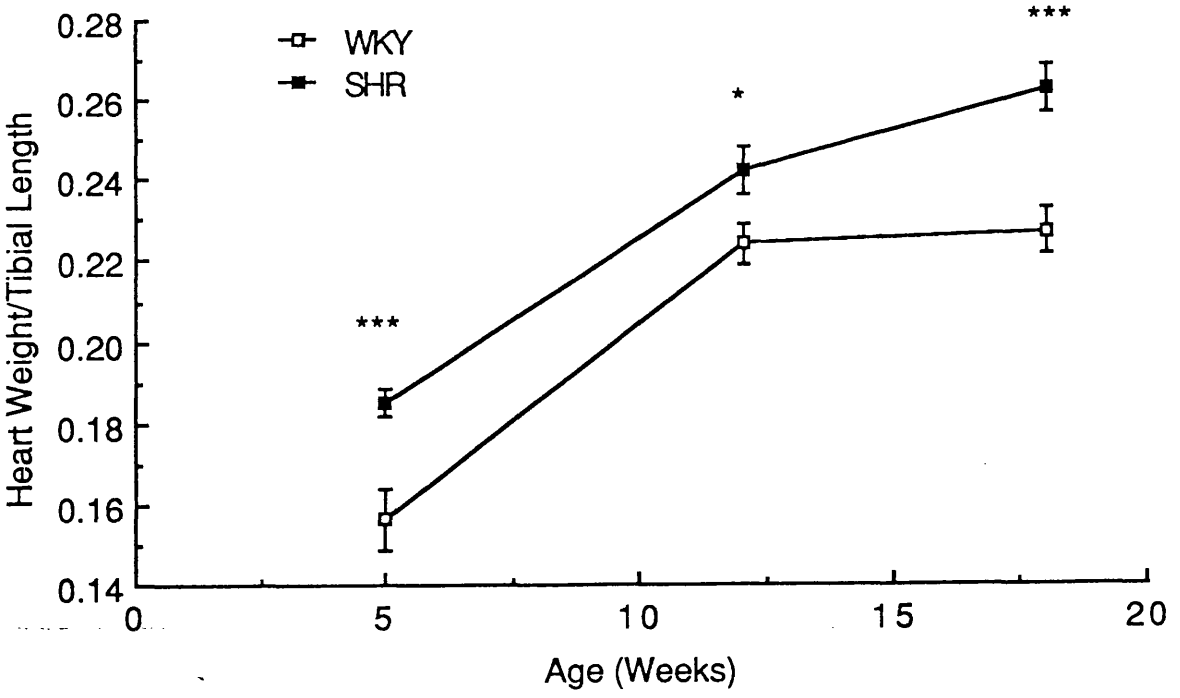

WKY
SHR
Heart Weight/Tibial Length
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0
5
10
15
20
Age (Weeks)
***
*
***

TABLE 17.

The body weight, heart weight and tibial lengths (mean ± SEM) in SH and WKY rats at different ages.

* represents $P \leq 0.05$ compared to WKY rats.

TABLE 17.

		Heart Weight. (g)	Body Weight. (g)	Tibial Length. (cm)
5 Weeks.	WKY	0.330 ± 0.020 *	74.7 ± 3.4 *	2.10 ± 0.03
	SHR	0.396 ± 0.010	84.1 ± 2.4	2.13 ± 0.02
12 Weeks.	WKY	0.666 ± 0.018 *	179.5 ± 5.0	2.00 ± 0.02
	SHR	0.729 ± 0.019	187.7 ± 3.6	3.01 ± 0.02
18 Weeks.	WKY	0.733 ± 0.020 *	207.5 ± 4.5	3.22 ± 0.02
	SHR	0.859 ± 0.021	216.0 ± 5.0	3.27 ± 0.01

weight ratio.

5.3 Changes in DNA Synthesis - Aorta.

The aortae removed from SH and WKY rats at 5 weeks of age were of similar weights (WKY - 11.8 ± 3.2; SH - 13.6 ± 2.7 mg) and lengths (WKY - 32.2 ± 1.3; SH - 35.5 ± 0.8 mm) and exhibited similar weight per unit length ratios. The weights and lengths of all aortae were greater in the SH rats at 12 weeks of age compared to the WKY rats but again the weight per unit length ratio was not significantly different. However, aortae removed from 18 week old SH animals were considerably heavier than those from the WKY animals (WKY - 24.2 ± 1.2; SH - 31.9 ± 1.6 mg; $P \leq 0.01$) with a significantly increased weight per unit length ratio (WKY - 0.584 ± 0.019; SH - 0.680 ± 0.016; $P \leq 0.001$) suggesting increased growth in the aortae of the SH animals.

5.3.1 Aortic Uptake of [^{3}H]-thymidine.

The uptake of [^{3}H]-thymidine into the aortae of both SH and WKY rats was determined from the amount of radioactivity in the first supernatant (Section 2.5) and was expressed as either dpm/mg aortic wet weight/hr or dpm/mm aortic length/hr. There was no significant difference in the uptake of [^{3}H]-thymidine between the SH and WKY animals at any age (Table 18).

TABLE 18.

		Dpm/mgweight/hr ± SEM	Dpm/mmlength/hr ± SEM
5 Weeks.	WKY	90.9 ± 11.4	32.6 ± 4.26
	SHR	92.3 ± 7.17	34.6 ± 2.50
12 Weeks	WKY	103.2 ± 13.5	53.9 ± 6.19
	SHR	116.2 ± 20.9	66.1 ± 11.9
18 Weeks	WKY	111.6 ± 12.5	64.0 ± 6.63
	SHR	120.4 ± 21.8	82.0 ± 15.0

Uptake of [^{3}H]-thymidine into aortae of both SH and WKY rats at different ages.

5.3.2 Aortic Incorporation of [^{3}H]-thymidine.

The incorporation of exogenous [^{3}H]-thymidine into the native DNA of aortae was determined by combining the radioactivity in the final pellet to that in the second supernatant (Section 2.5). The rate of DNA synthesis was expressed as [^{3}H]-thymidine dpm/mm aortic length/hr and dpm/µg tissue DNA/hr. In contrast to the finding in the experimental hypertensive rat there was no significant difference in the incorporation of [^{3}H]-thymidine between the SH and the WKY animals at any age (Figure 28).

5.3.3 Aortic Protein Content.

The initial aortic protein content of the SH and WKY animals was similar (5 Weeks: WKY - 10.89 ± 1.19; SH - 12.02 ± 0.66 µg/mm) although the level was nonsignificantly higher in the SH rats. The protein content of all animals rose with increasing age but the rate of increase was greater in the SH than in the WKY rats. Similarly at 12 weeks of age this difference was maintained (WKY - 34.05 ± 1.46; SH - 37.97 ± 1.79 µg/mm) but only achieved significance ($P \leq 0.05$) in 18 week old rats (Figure 29a).

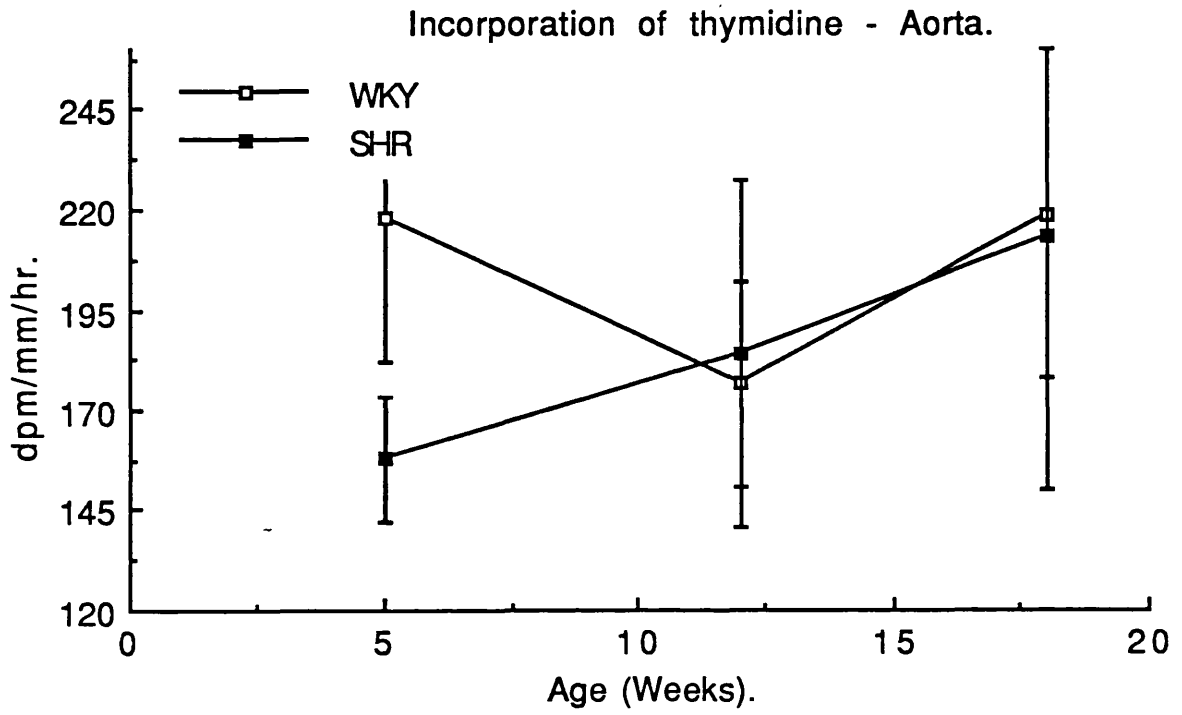
5.3.4 Aortic DNA Content.

There was no significant difference in the DNA content of aortae between SH and WKY rats at any of the ages studied

FIGURE 28..

The rate of incorporation of [^{3}H]-thymidine into aortic DNA from SH and WKY rats at different ages.

FIGURE 28.


Incorporation of thymidine - Aorta.
WKY
SHR
245
220
195
170
145
120
dpm/mm/hr.
0
5
10
15
20
Age (Weeks).

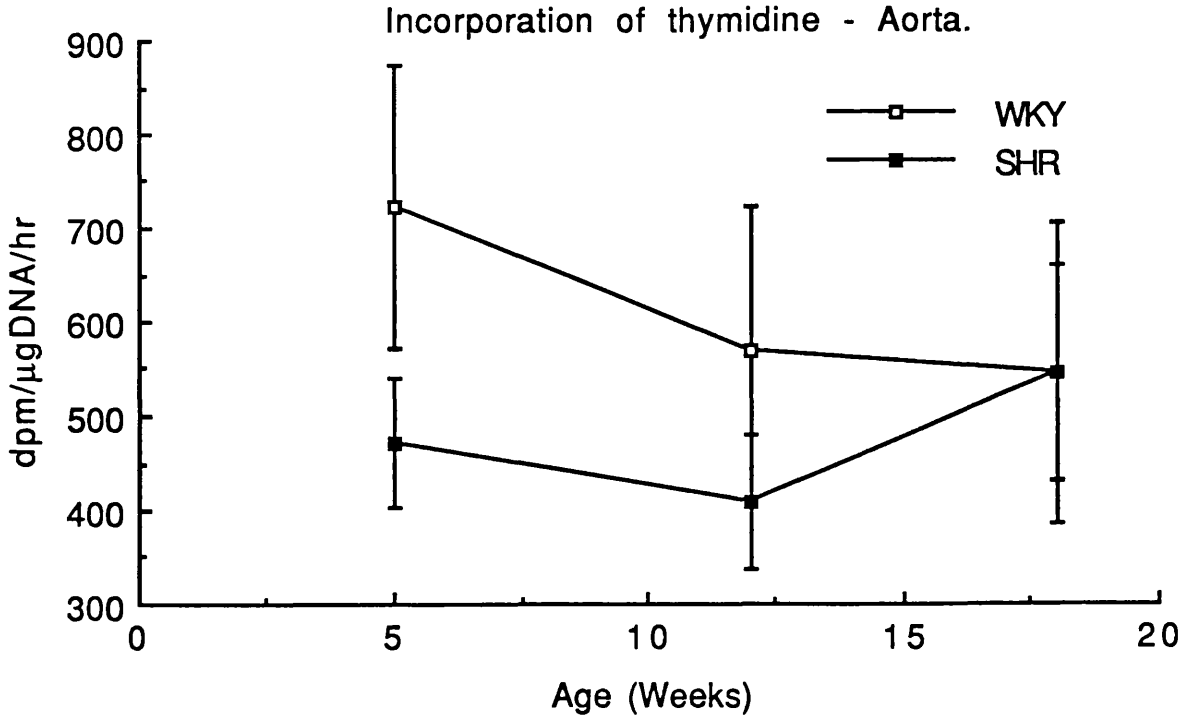

Incorporation of thymidine - Aorta.
WKY
SHR
900
800
700
600
500
400
300
dpm/µgDNA/hr
0
5
10
15
20
Age (Weeks)

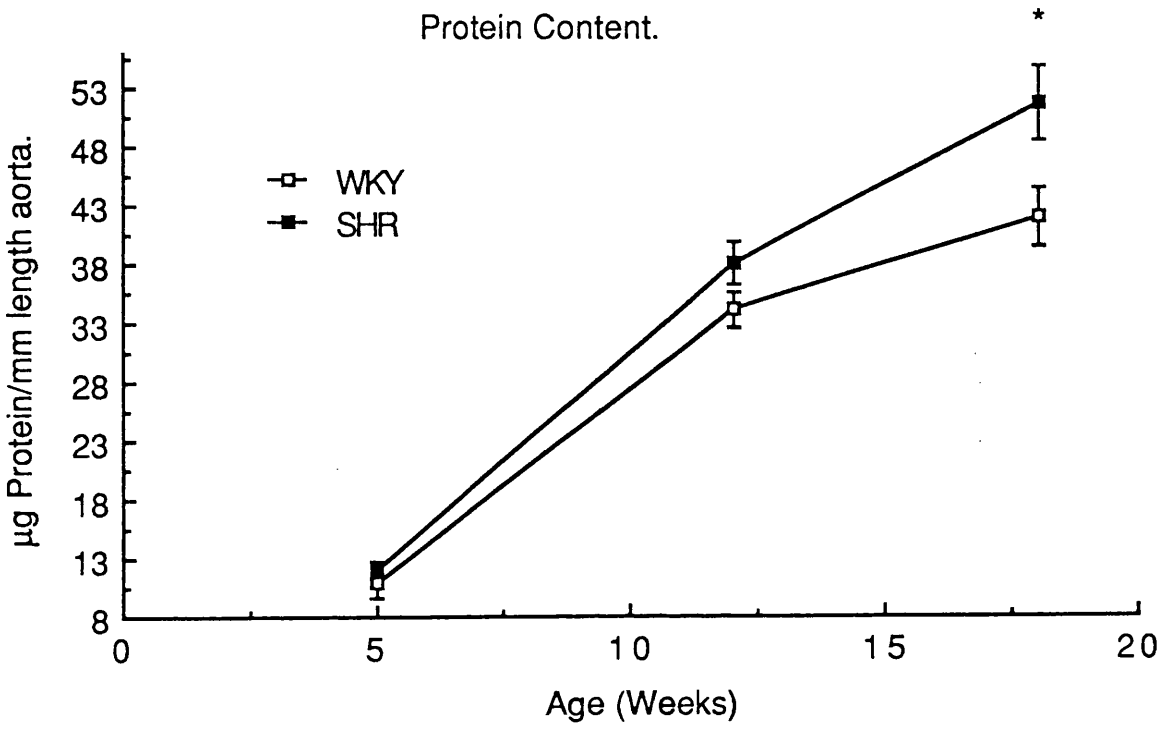
FIGURE 29.

The protein content (a) and DNA content (b) of aortae from SH and WKY rats at different ages.

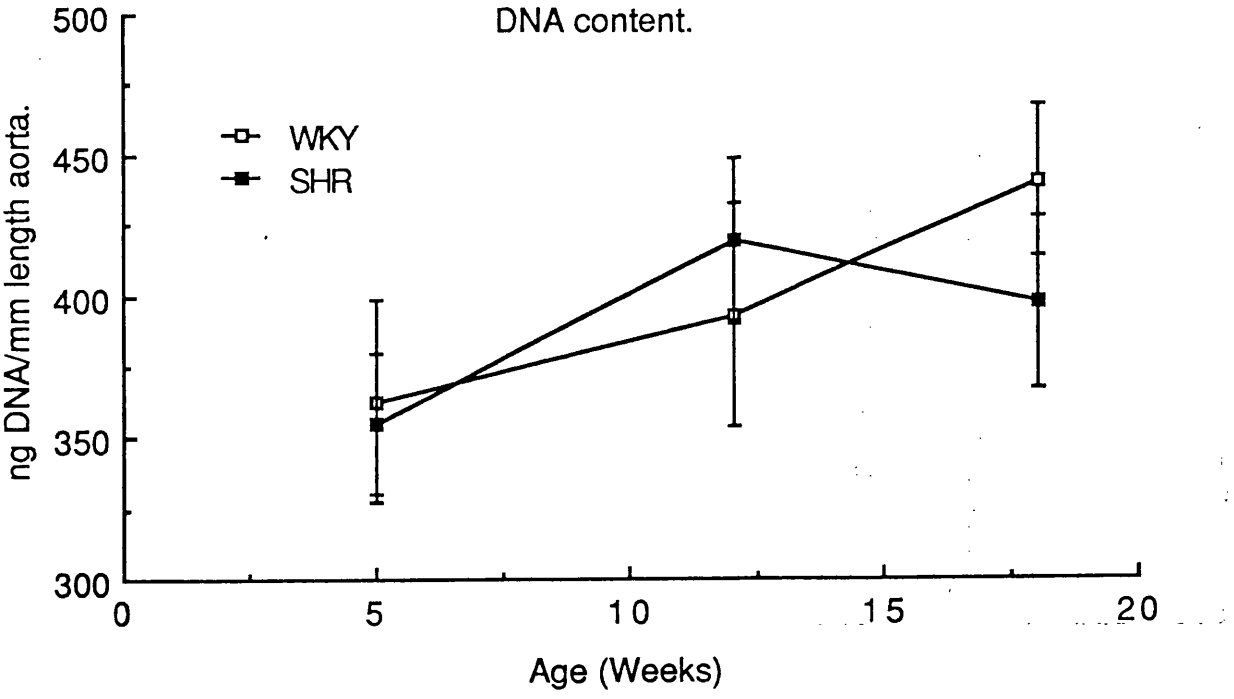
* = $P \leq 0.05$

FIGURE 29.

a


Protein Content.
*
µg Protein/mm length aorta.
53
48
43
38
33
28
23
18
13
8
WKY
SHR
0
5
10
15
20
Age (Weeks)

b


DNA content.
ng DNA/mm length aorta.
500
450
400
350
300
WKY
SHR
0
5
10
15
20
Age (Weeks)

(Figure 29b).

5.3.5 Aortic DNA / Protein Ratio.

The aortic DNA to protein ratio was similar in the 5 week old SH and WKY rats. The ratio fell with increasing age and this was more noticeable in the SH rats (Table 19) so that by 18 weeks of age there was a significant difference in the DNA/protein ratio between the two strains ($P \leq 0.01$).

5.4 Changes in DNA Synthesis - Mesenteric Artery.

Similar lengths of mesenteric artery ($\leq$ 250μm internal diammeter) were obtained from both SH and WKY rats (Section 2.4.2).

e.g. 5 weeks: WKY - 52.6 ± 1.0; SH - 54.6 ± 1.3 mm.

5.4.1 Mesenteric Artery Uptake of [^{3}H]-thymidine.

There was no significant difference in the uptake of [^{3}H]-thymidine between the SH and the WKY animals at either 5 or 12 weeks of age. However at 18 weeks the SH rats had a significantly increased uptake of [^{3}H]-thymidine (Table 20). The actual biological significance of this result is uncertain, it may represent an increase in the thymidine pool size in the SH animals or alternatively the difference may be the result of biological variability.

TABLE 19.

		DNA/ProteinRatio (μg/μg).	P
5 Weeks.	WKY	0.0378 ± 0.0047	NS
	SHR	0.0316 ± 0.0029	
12 Weeks.	WKY	0.0118 ± 0.0012	NS
	SHR	0.0111 ± 0.0006	
18 Weeks.	WKY	0.0107 ± 0.0007	< 0.01
	SHR	0.0077 ± 0.0006	

DNA/protein ratio of aortae from both SH and WKY rats at different ages.

TABLE 20.

		Dpm/mmlength/hr	P
5 Weeks.	WKY	16.00 ± 3.71	NS
	SHR	13.99 ± 1.61	
12 Weeks.	WKY	21.40 ± 3.16	NS
	SHR	21.90 ± 3.32	
18 Weeks.	WKY	21.68 ± 2.77	< 0.05
	SHR	33.20 ± 3.83	

Uptake of [^{3}H]-thymidine into mesenteric arteries from both SH and WKY rats at different ages.

5.4.2 Mesenteric Artery Incorporation of [^{3}H]-thymidine.

The rate of incorporation of [^{3}H]-thymidine into the DNA of mesenteric arteries was expressed as dpm/mm length/hr or dpm/μgDNA/hr. At 5 weeks of age there was no significant difference in incorporation of [^{3}H]-thymidine between the SH and the WKY animals. Suprisingly there was a significant increase in the rate of [^{3}H]-thymidine incorporation in the 12 week old WKY compared to the SH rats (dpm/mm/hr: WKY - 40.10 ± 10.2; SH - 18.15 ± 2.69; $P \leq 0.05$). However, this difference disappeared when the results were expressed as dpm/μgDNA/hr. There was no significant difference in [^{3}H]-thymidine incorporation between the two strains of rat at 18 weeks of age (Table 21).

5.4.3 Mesenteric Artery Protein Content.

Protein content of the mesenteric arteries increased slightly in both SH and WKY animals between 5 and 12 weeks of age and substantially between 12 and 18 weeks of age. However there was no significant difference in protein content between the two strains at any age (Figure 30a).

5.4.4 Mesenteric Artery DNA Content.

The DNA content of WKY animals was significantly increased compared to the SH animals at 5 weeks of age (WKY - 37.70 ± 3.49; SH - 27.30 ± 2.98 ng/mm; $P \leq 0.05$). At both 12 and 18

TABLE 21.

		Dpm/mm/hr		Dpm/μgDNA/hr	
	WKY	29.70 ± 5.91		1006.0 ± 283	
5 Weeks.			NS		NS
	SHR	33.70 ± 4.63		1470.0 ± 251	
	WKY	40.10 ± 10.2		1718.0 ± 573	
12 Weeks.			<0.05		NS
	SHR	18.15 ± 2.69		712.0 ± 127	
	WKY	25.60 ± 5.53		889.0 ± 197	
18 Weeks.			NS		NS
	SHR	30.20 ± 6.96		1176.0 ± 462	

Incorporation of [^{3}H]-thymidine into mesenteric artery DNA from SH and WKY rats at different ages.

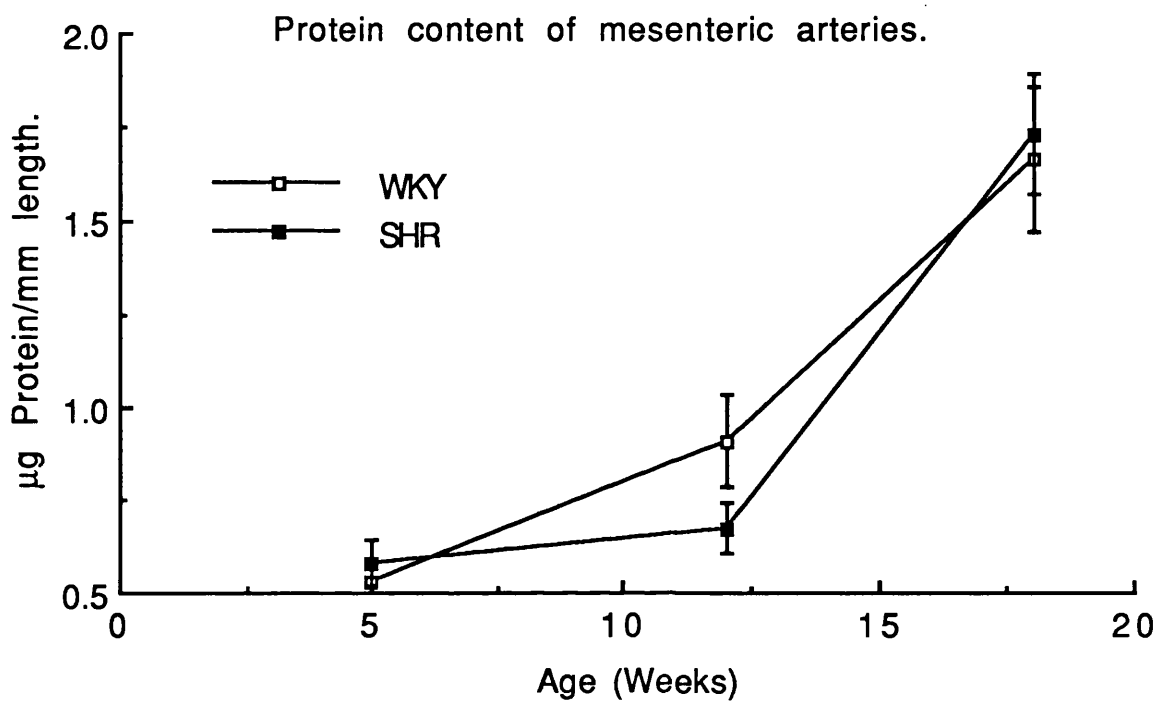
FIGURE 30.

The protein content (a) and DNA content (b) of mesenteric resistance arteries from SH and WKY rats at different ages.

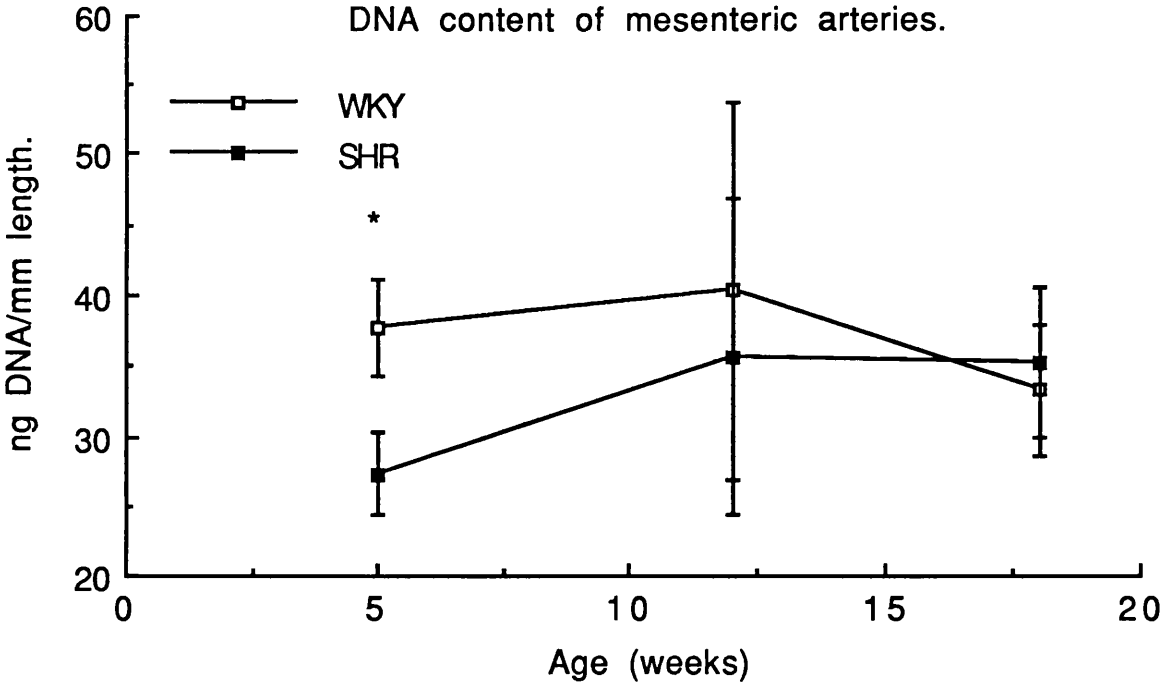
* = $P \leq 0.05$

FIGURE 30.

a


Protein content of mesenteric arteries.
2.0
1.5
1.0
0.5
µg Protein/mm length.
WKY
SHR
0
5
10
15
20
Age (Weeks)

b


DNA content of mesenteric arteries.
60
50
40
30
20
ng DNA/mm length.
WKY
SHR
*
0
5
10
15
20
Age (weeks)

weeks however, there is no significant difference in the DNA content of the two strains. This coupled with the lack of a significant increase in [^{3}H]-thymidine incorporation in the WKY animals, would indicate that this result has little biological importance. In summary then, the DNA content of both the WKY and SH animals was similar and changed little over the course of this study (Figure 30b).

5.4.5 Mesenteric Artery DNA / Protein Ratio.

The ratio of DNA to protein in the WKY animals is significantly increased compared to the SH animals at 5 weeks of age. This result is not suprising bearing in mind that there was a significant increase in DNA content. However there was no significant difference in the DNA/protein ratio between the two strains at 12 or 18 weeks of age (Table 22).

5.5 Changes in DNA synthesis - Subcutaneous Vessels.

The same length of subcutaneous vessel was obtained from SH and WKY animals.
e.g. 5 Weeks: WKY - 50.9 ± 0.8; SH - 52.2 ± 1.3 mm.

5.5.1 Subcutaneous Vessel Uptake of [^{3}H]-thymidine.

There was no significant difference in the uptake of [^{3}H]-thymidine into the subcutaneous vessels between the SH or the WKY rats at any age (Table 23).

TABLE 22.

		DNA/ProteinRatio (μg/μg).	P
5 Weeks.	WKY	0.0847 ± 0.0112	< 0.05
	SHR	0.0499 ± 0.0061	
12 Weeks.	WKY	0.0573 ± 0.0203	NS
	SHR	0.0715 ± 0.0258	
18 Weeks.	WKY	0.0230 ± 0.0054	NS
	SHR	0.0209 ± 0.0026	

Changing DNA/protein ratio of mesenteric arteries from both SH and WKY rats with increasing age.

TABLE 23.

		Dpm/mmlength/hr	P
5 Weeks.	WKY	10.04 ± 2.00	NS
	SHR	09.63 ± 1.00	
12 Weeks.	WKY	13.00 ± 3.64	NS
	SHR	18.10 ± 3.45	
18 Weeks.	WKY	13.23 ± 1.86	NS
	SHR	17.08 ± 1.54	

Uptake of [^{3}H]-thymidine into subcutaneous arteries from both SH and WKY rats at different ages.

5.5.2 Subcutaneous Vessel Incorporation of [^{3}H]-thymidine.

Incorporation of [^{3}H]-thymidine into the subcutaneous vessels was similar in SH and WKY rats at all ages (Figure 31).

5.5.3 Subcutaneous Vessel Protein Content.

The protein content of both the SH and WKY animals increased gradually with age but was not different between the two strains at any age (Table 24).

5.5.4 Subcutaneous Vessel DNA Content.

There was no significant change in the DNA content in either the SH or the WKY animals or between the strains at all ages studied (Table 24).

5.5.5 Subcutaneous Vessel DNA / Protein Ratio.

The ratio of DNA to protein gradually decreased in both strains as the rats aged but there was no difference between the strains at any age (Table 24).

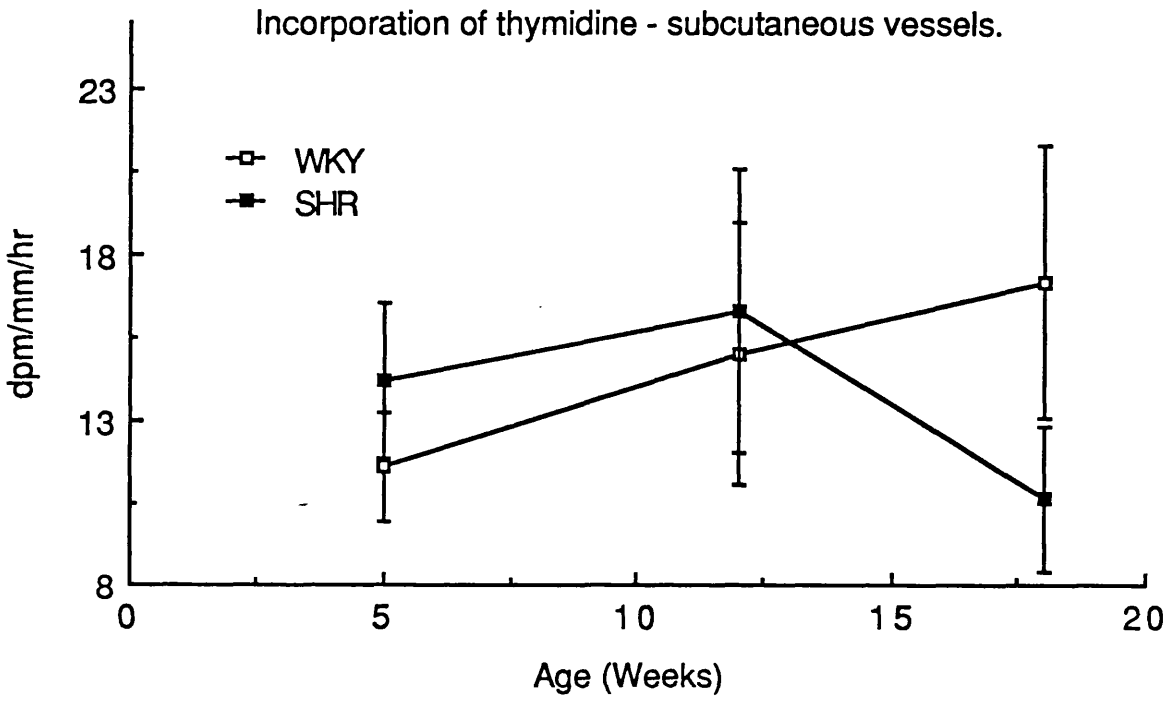
5.6 Summary of Results.

In summary these studies show that the rate of DNA synthesis

FIGURE 31.

The rate of incorporation of [^{3}H]-thymidine into subcutaneous resistance artery DNA from SH and WKY rats at different ages.

FIGURE 31.


Incorporation of thymidine - subcutaneous vessels.
23
18
13
8
dpm/mm/hr
WKY
SHR
0
5
10
15
20
Age (Weeks)

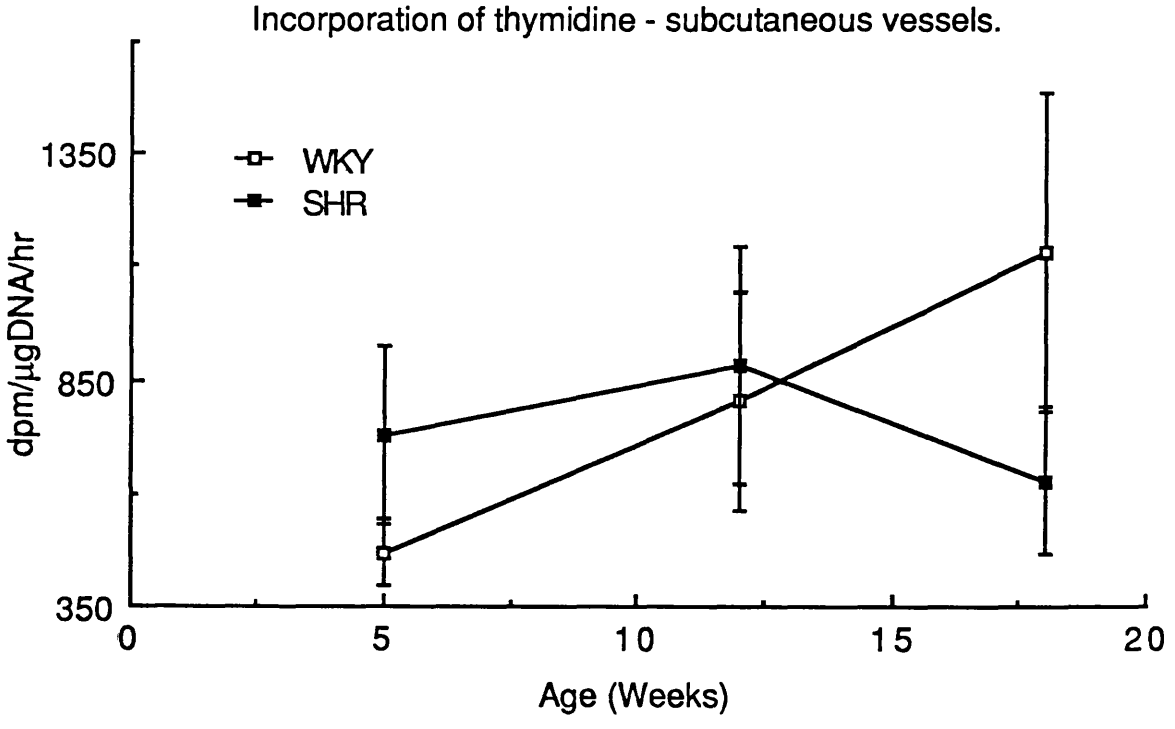

Incorporation of thymidine - subcutaneous vessels.
1350
850
350
dpm/µgDNA/hr
WKY
SHR
0
5
10
15
20
Age (Weeks)

TABLE 24.

The protein content, DNA content and DNA/protein ratio of subcutaneous reisitance vessels from both SH and WKY rats at different ages.

TABLE 24.

	5 Weeks.		12 Weeks.		18 Weeks.	
	WKY	SHR	WKY	SHR	WKY	SHR
Protein Content (μg/mm)	0.41 ±0.05	0.53 ±0.06	0.89 ±0.10	1.04 ±0.10	1.23 ±0.18	1.31 ±0.15
DNA Content (ng/mm)	25.07 ±1.45	27.50 ±3.40	27.20 ±8.29	24.80 ±6.38	17.35 ±1.72	20.34 ±2.32
DNA/Protein (μg/μg)	0.079 ±0.012	0.067 ±0.012	0.035 ±0.012	0.024 ±0.005	0.017 ±0.003	0.017 ±0.002

in all three vessels of the SH rat was not significantly different from the WKY at any age studied.

Protein content was significantly raised in the aorta at 18 weeks of age and this was accompanied by a concomitant fall in the DNA/protein ratio. Protein content was unchanged in all the small resistance vessels studied as was DNA content and DNA/protein ratio.

CHAPTER SIX.

DISCUSSION.

6.1 Introduction.

The relationship between vascular structural change and the rise in blood pressure has received much attention over the years, with studies generally falling into one of two categories - morphological studies of small vessels and biochemical studies of large arteries.

Morphological studies of resistance vessels have demonstrated an alteration in vascular structure in hypertension with an increased media to lumen ratio compared to normotensive vessels (Mulvany and Halpern, 1977; Warshaw et al, 1979; Mulvany et al, 1980; Smeda et al, 1988; Lee et al, 1989; Vial and Boyd, 1989). Although these studies have shown that structural hypertrophy occurs early in hypertension (Warshaw et al, 1979) this approach is limited because it can only detect established vascular change.

Since biochemical changes are a necessary step for growth the temporal relationship between structural change and the rise in blood pressure is best investigated by biochemical means. Measurements of protein and DNA content as well as the rate of DNA synthesis have been used to detect developing vascular structural change in both experimental (Rorive et al, 1980; Carlier et al, 1983; Loeb et al, 1986), and genetic (Loeb and Bean, 1986) hypertension. However, the use of large arteries limits the value of these studies because these vessels do not actively contribute to the peripheral vascular resistance.

The present study was therefore designed to investigate the

development of structural change in resistance arteries using biochemical markers of the growth process.

6.2 Methodology.

The initial thrust of this thesis was to establish reliable techniques which would permit biochemical measurements in resistance arteries. To this end the methodology described by Loeb et al (1986) for use in large arteries was investigated, however, this presented a number of problems when adapted for use in resistance arteries.

In their studies (Loeb et al, 1986) both aortic DNA and protein content measurements were performed on the final hydrolysis product. The DNA content of the perchloric acid (PCA) fraction was determined using the traditional Burton assay which lacks the requisite sensitivity for measurements in small vessels. Moreover, it became clear that PCA treatment not only produces single stranded DNA but reduces it to the form of apurinic acid which is unsuitable for the more sensitive fluorometric assay procedures. In addition measurements using the final hydrolysis product also presented problems because of incomplete DNA and protein recovery during the procedures and possibly incomplete hydrolysis. These problems were circumvented by the use of aliquots taken at the crude homogenate stage allowing the application of more sensitive methods for the analysis of DNA content.

Rates of DNA synthesis were determined by the measurement of

[^{3}H]-thymidine incorporated into the the native vessel DNA. Obviously small resistance arteries possess considerably smaller numbers of smooth muscle cells compared to large vessels and there will be a corresponding decrease in [^{3}H]-thymidine incorporation. Therefore it was necessary to extend the incubation time to increase the [^{3}H]-thymidine incorporation. A [^{3}H]-thymidine incorporation versus incubation time course was performed to ensure that incorporation remained linear over the 18 hour period chosen for these studies. In addition, to give a more complete picture [^{3}H]-thymidine incorporation into non-hydrolysed DNA was also measured.

Having established suitable and reliable methods, these were used to investigate the structural development in both experimental and genetic models of hypertension.

6.3 Experimental Hypertension.

The results of these studies confirm that the development of aortic medial hypertrophy in renovascular hypertensive rats is associated with an increase in the rate of DNA synthesis (Rorive et al, 1980; Carlier et al, 1983; Loeb et al, 1986; Loeb and Bean, 1986). In addition this work demonstrated an elevated rate of DNA synthesis at 3 days post-renal artery constriction, when blood pressure was not significantly different from that in the controls. This finding is in keeping with the studies of Loeb et al (1986) who also reported an increased rate of DNA synthesis prior to a change

in blood pressure at 5 days post-renal artery constriction. Thus it would appear that the stimulation of DNA synthesis may be independent of the rise in blood pressure.

In the aorta the cellular basis of growth appears to be smooth muscle cell hypertrophy. An increase in protein content of the aortae of hypertensive animals was detected at 21 days post-renal artery constriction by Loeb and co-workers (1986), and at 28 days in the present study. However in neither study was a significant increase in aortic DNA content observed, despite a significant elevation in the rate of DNA synthesis which ought to have lead to an increase in total DNA content. One explanation for this discrepancy could lie in the development of polyploidy. Thus, at 21 days post-renal artery constriction Loeb et al (1986) demonstrated a significant decrease in DNA/protein ratio which corresponds to the increase in protein content without a major change in DNA content. However at 28 days I could not demonstrate a change in the DNA/protein ratio despite an elevated protein content. In my study the mean DNA content of hypertensive aortae was marginally raised above that of control vessels but there was considerable individual variation. This may indicate that either an increase in DNA content had occured but was not detected or that the change in DNA content was just begining to become apparent. Indeed Owens and Schwartz (1983) demonstrated a significant change in aortic smooth muscle cell polyploidy from 2K, 1C hypertensive rats one month following renal artery constriction with a 200% increase after five months. Owens and Schwartz used flow

cytometry of isolated smooth muscle cells for their studies and this method has a high sensitivity for the detection of changes in cell ploidy whereas the measurement of total DNA is a relatively crude assay system. Altogether the evidence suggests that aortic medial hypertrophy in 2K, 1C hypertensive rats is characterised by smooth muscle cell hypertrophy coupled with a small increase in polyploidy.

Large vessel studies may not reflect what is happening in the small arteries which directly contribute to the total peripheral resistance and thus may have little relevance to the development of hypertension. Accordingly in this study the rates of DNA synthesis were measured in both the aorta and in resistance vessels. Interestingly these results show that the changes in DNA synthesis in the mesenteric arteries of hypertensive rats paralleled those found in the aorta. Thus, DNA synthesis was significantly increased 3 days after renal artery constriction before there was a significant rise in blood pressure. DNA synthesis rates remained elevated during the development of hypertension but fell back to control levels after blood pressure had plateaued in the established phase.

By contrast to the aorta, there was a significant increase in both protein and DNA content of mesenteric arteries at 28 days post-renal artery constiction. However the DNA/protein ratio remained unchanged. This would suggest that the increase in media volume detected in these vessels (Appendix D) consisted either of true hyperplasia or cellular hypertrophy coupled with a dramatic increase in cell ploidy.

Unfortunately the inherent limitations of our methods make it impossible to determine which of the two processes had occured in the mesenteric vessels although a uniform increase in polyploidy would seem unlikely.

The protein and DNA content of isolated smooth muscle cells measured by densitometry can be used to determine cell ploidy in small samples. This approach has been successfully applied using the aorta of 2K, 1C rats (Owens and Schwartz, 1983) and more recently mesenteric arteries from old spontaneously hypertensive rats (Lombardi et al, 1989). It may soon be possible to adapt this method for use with resistance arteries from younger animals. Other studies have utilised autoradiography of vessel sections in the early stages of development of hypertension where labelled nuclei in pairs or in small groups may be an indication of mitosis (Carlier et al, 1983). Alternative approaches have involved histological techniques such as the dissector method (Mulvany et al, 1985). Using the dissector method in resistance arteries from 1K, 1C renovascular rats Korsgaard and Mulvany (1988) found a significant increase in smooth muscle cell volume, although they also detected a small increase in the number of cells per unit wall length. In this model medial hypertrophy of mesenteric arteries depends on a combination of hyperplasia and cellular hypertrophy.

The picture becomes more complicated when other vascular beds are studied. By contrast to the mesenteric vessels there were no changes in the rate of DNA synthesis in the subcutaneous resistance vessels at any stage during the development of

hypertension. Similarly neither the protein or DNA content changed in these vessels. However morphological measurements of these vessels mounted in a myograph (Appendix D) demonstrated an increase in both the media/lumen ratio and media thickness in the hypertensive animals. On the other hand media volume was not significantly different from controls suggesting that the increase in media/lumen ratio may have been brought about by remodelling of the existing vessel structure (Heagerty et al, 1988) rather than by vessel growth.

This phenomenon is not unique to this vascular bed since Ono et al (1989) reported a significant increase in the wall to lumen ratio, without a corresponding increase in cross-sectional wall area in first and second order cremaster arterioles in 2K, 1C hypertension. Moreover these authors reported an absence of structural change in third or fourth order vessels but they did detect a mild functional vascular rarefaction at two weeks which had become structural rarefaction at eight weeks. Perhaps the failure to find true structural change can be explained by the fact that the increased total peripheral resistance in hypertension does not constitute a more-or-less equally raised arteriolar resistance in all vascular beds (Brod et al, 1962). A relatively minimal increase in resistance in some vascular beds may not stimulate growth processes of the resistance arteries.

The most interesting observation of this study is that medial hypertrophy occurred independently of the rise in

blood pressure. This is not a new finding since vascular mechanisms which operate independently of elevated pressure have been widely studied using the aortic coarctation model of hypertension (Nolla-Panades, 1962; Bell and Overbeck, 1979; Plunkett and Overbeck, 1985; Liu et al, 1988). These authors have variously reported structural changes in the abdominal aorta (Liu et al, 1988), cremaster resistance arteries (Plunkett and Overbeck, 1985) and renal arterioles (Liu et al, 1988) of the normotensive hindquarters of animals with coarctation hypertension.

The similarities between the 2K, 1C renovascular and aortic coarctation models do not stop here since both models have a similar course of development of hypertension (Nolla-Panades, 1962) and plasma renin concentration has also been reported to be elevated in the aortic coarctation model (Nolla-Panades, 1962; Overbeck et al, 1982). Thus the present study demonstrated a similar rise in plasma renin concentration of hypertensive animals throughout the development of hypertension. Overbeck and co-workers also demonstrated that sympathetic ablation does not prevent structural change of normotensive hindquarters vessels (Overbeck, 1980; Plunkett and Overbeck, 1988), indicating that a humoral factor or factors may be involved. These studies suggest that the increase in levels of plasma renin and hence angiotensin II, may play a different role in structural development than has been previously appreciated.

It is possible that previous comparisons with results obtained using the 1K, 1C model of renovascular hypertension

(Rorive et al, 1980; Carlier et al, 1983; Korsgaard and Mulvany, 1988) may not be entirely relevant since this is thought to be a low renin, volume dependant model of hypertension whose induction occurs independantly of the renin-angiotensin system (Loeb and Bean, 1986). The stimulus for growth in 1K, 1C hypertension may be related primarily to the increasing pressure whereas 2K, 1C hypertension appears to also involve the renin-angiotensin system.

Recently angiotensin II has been shown to initiate a sequence of events associated with the signal transduction process which eventually leads to DNA synthesis. Studies of cultured vascular smooth muscle cells, usually isolated from rat thoracic aorta, have allowed us to gain a greater insight into the mechanisms by which angiotensin II mediates its effects in vascular smooth muscle. Angiotensin II has been shown to induce the rapid breakdown of phosphatidylinositol-4,5-bisphosphate to generate IP_3 in smooth muscle cells (Alexander et al, 1985), and in addition causes a rapid concentration-dependant increase in intracellular calcium concentration (Alexander et al, 1985; Berk et al, 1987). Griendling et al (1986) extended these studies and showed that angiotensin II stimulates sustained 1,2-diacylglycerol formation whilst other authors have demonstrated that angiotensin II stimulates the amiloride sensitive Na^+/H^+ exchange resulting in cellular alkalinisation (Morton et al, 1988; Lyall et al, 1988). Interestingly, Naftilan et al (1988) have shown that angiotensin II stimulates not only expression of the proto-oncogenes c-myc and c-fos (Taubman et

al, 1989) but also that several hours after exposure to angiotensin II an increase in platelet derived growth factor (PDGF) A-chain mRNA expression was observed. The latter observation may prove to be of great importance since PDGF is an established mitogenic agent (Frick et al, 1988; Geisterfer et al, 1988; Taubman et al, 1989).

More specifically Geisterfer et al (1988) have investigated the effect of angiotensin II on smooth muscle cells in terms of the induction of cellular hypertrophy or hyperplasia. The results obtained by these authors indicated that angiotensin II alone was neither mitogenic for smooth muscle cells nor did it potentiate the proliferative response of the cells to PDGF. However angiotensin II was shown to be a highly potent stimulator of protein synthesis affecting cellular protein content in a dose dependant manner causing cellular hypertrophy with an increase of between 20 and 38% in volume. Cellular hypertrophy is generally accompanied by nuclear polyploidy although the two phenomena are by no means invariably linked. Although there was no significant increase in either cell numbers or labelling index, Geisterfer et al (1988) found that angiotensin II induced a 50% increase in the fraction of cells in G_2 (i.e cells containing four times the haploid content of DNA but only 2N chromosomes) but there was no detectable increase in the fraction of cells in S phase (i.e. cells entering DNA synthesis).

Similarly Berk et al (1989) in their studies of the effects of angiotensin II on the capacity of smooth muscle cells to synthesise RNA, protein and DNA showed a significant dose

dependent increase in protein synthesis with a peak about 18 hours after exposure. Using [^{3}H]-uridine Berk et al also demonstrated that angiotensin II stimulates RNA synthesis which is a necessary precondition for protein synthesis to occur. On the other hand these workers were unable to demonstrate a significant increase in either [^{3}H]-thymidine incorporation or in the number of cells, and suggested that angiotensin II causes vascular smooth muscle cell hypertrophy rather than hyperplasia.

By contrast to these two extensive studies, Lyall et al (1988) reported that, in serum containing medium, angiotensin II produced a dose dependent increase in the smooth muscle cell number which could be blocked by the addition of the angiotensin II antagonist saralasin.

The discrepancy between this and other reports may have several explanations. Angiotensin II is readily degraded in cell media (Lyall et al, 1988) resulting in cells being exposed to a final concentration of angiotensin II which was considerably lower than that initially added to the medium. This may account for the inability of angiotensin II to potentiate the response of cells to PDGF. In addition serum is a relatively undefined component of cell culture media and differences in the serum used may lead to considerable variations in cellular response. Furthermore it is interesting to note that the studies which demonstrated cellular hypertrophy were performed using aortic vascular smooth muscle cells whereas those showing cellular hyperplasia used vascular smooth muscle cells isolated from

mesenteric arteries.

Other support for an important role for angiotensin II in the development of the medial hypertrophy comes from studies of the affects of treatment with Captopril, an angiotensin converting enzyme inhibitor which prevents the rise in DNA synthesis seen early in the development of 2K, 1C hypertension (Loeb and Bean, 1986). Moreover, converting enzyme inhibitors have been shown to reverse the morphological changes seen in the aortae of hypertensive animals (Levy et al, 1988). However whether similar results could be acheived in small resistance vessels subject to developing hyperplasia has yet to be determined.

These studies indicate that further investigation is required to determine the nature of the pressure independant stimulus inducing the rise in the rate of DNA synthesis which occurs in some vessels in 2K, 1C hypertension. In addition it is essential to study resistance vessels from a variety of vascular beds because the results obtained in this study indicate that not all peripheral vascular beds respond to the development of hypertension in the same way. Likewise different order arterioles from the same vascular bed have been shown to differ in their morphological response to hypertension (Owens et al, 1988; Lee et al, 1989; Ono et al, 1989).

6.4 Genetic Hypertension.

It is well established that the the rate of development of

genetic hypertension is considerably slower than that seen in experimental hypertension, in the order of weeks and months rather than days. Consequently this animal model was studied over a considerably prolonged period of time.

At five weeks of age there was a non-significant elevation of blood pressure of the SH rats compared to the WKY rats. By contrast, the elevated heart weight/tibial length index indicated that significant cardiac hypertrophy had already occured in the SH rats.

Investigation of the response of the aorta of SH rats to the developing hypertension demonstrated that growth does occur, by 18 weeks of age the aortae of SH rats exhibited an increased weight per unit length ratio compared to the WKY controls. Despite this change we were unable to demonstrate any increase in the rate of DNA synthesis in the SH rat compared to the WKY at any age. Similarly Loeb and Bean (1986) were unable to demonstrate an increase in the rate of incorporation of [^{3}H]-thymidine ino the aortae of SH rats at either 4 or 21 weeks of age. However they did find significant increases in aortic DNA synthesis in SH rats at 17 weeks of age. These authors suggested that the rate of DNA synthesis follows the rate of rise of blood pressure such that between 4 and 17 weeks when blood pressure is increasing the rate of DNA synthesis will also increase but by 21 weeks of age when blood pressure has stabilized aortic DNA synthesis would return to control levels.

If indeed this was the case, aortic DNA synthesis should be elevated in SH rats at 12 weeks of age, but we were unable to

demonstrate any such change. The considerable variation in the rate of DNA synthesis seen by us in both SH and WKY rats at all ages points to the possibility that the aortic DNA synthesis reported by Loeb and Bean at 17 weeks was the result of biological variability and their small group sizes. The failure to demonstrate an increase in DNA synthesis indicates only that medial growth of the aorta of SH rats is unlikely to be due to hyperplasia, but does not exclude the development of cellular hypertrophy.

Thus we were able to demonstrate both a significant increase in aortic protein content with a corresponding significant reduction in DNA/protein ratio in 18 weeks old SH rats. Similarly Owens and his co-workers have consistently demonstrated that aortic smooth muscle cells from SH rats with established hypertension contain at least 150% of the mean protein content (pg/cell) of their WKY controls (Owens et al, 1981; Owens and Schwartz, 1982). Although we were unable to detect any increase in either DNA synthesis or total DNA content, aortic smooth muscle cell polyploidy in SH rats has been consistently demonstrated (Owens et al, 1981; Owens and Schwartz, 1982; Owens, 1985 and 1987; Rosen et al, 1986). Furthermore Owens and Schwartz (1982) reported that the incidence of polyploidy in the aorta of 3 month old SH rats was twice that of age matched WKY rats and the incidence of polyploidy continued to increase up to seven months of age (Owens, 1985). Thus it is possible that changes in ploidy were occuring in our animals but were masked by the large individual variations in DNA levels measured by the

relatively insensitive DNA assay system.

Contrastingly the results obtained from the aorta were not mirrored by changes in the small resistance vessels of either the mesenteric or subcutaneous vascular beds. There was no change in the rate of DNA synthesis, protein or DNA content. However morphological studies (Appendix E) have demonstrated changes in media thickness and media volume in the mesenteric arteries of SH rats. In both 3 and 18 week old SH rats the media volume was significantly increased compared to WKY vessels although despite the increase in the media/lumen ratio and media thickness no change in media volume could be detected in either 6 or 12 week old SH rats.

Mulvany et al (1985) used the 'disector' technique to assess the development of structural changes in mesenteric arteries of 20 week old animals and demonstrated a 26% increase in the cross-sectional area of the vessel media in SH compared to WKY rats. They went on to suggest that the increase in medial area constituted cellular hyperplasia because there was no evidence of an increase in smooth muscle cell volume. In addition the numerical cell density, represented by the number of nuclei within the dissector (working on the assumption that all cells were mononuclear) divided by the dissector volume, was increased. They concluded therefore that the SH vessels contained more cells per unit vessel length than did the WKY vessels. However, they also reported that smooth muscle cells from SH rats had a smaller cell volume than those of WKY rats.

Owens et al (1988) approached the same problem by studying

enzyme dispersed smooth muscle cells from mesenteric arterioles. They determined that the individual protein content was reduced in SH compared to WKY rats suggesting a smaller cell volume, although this finding could not be repeated in the larger mesenteric arteries (First and second order branches). No change in the frequency of polyploidy has been demonstrated in the mesenteric arteries of SH and WKY rats (Owens et al, 1988; Black et al, 1988) although polyploidy does increase as a function of age in both strains (Lombardi et al, 1989).

Contary to first impressions these reports do not conflict with the results of the present study. None of the above studies were designed to investigate the relationship between the development of medial hypertrophy and the rise in blood pressure, but documented the cellular make-up of that medial hypertrophy in rats with established hypertension. The view that the medial hypertrophy of mesenteric arteries consists of cellular hyperplasia in the SH rat, albeit with these cells having a smaller cell volume than the corresponding WKY cells, is not disputed by the present study. Thus the larger number of smaller cells in SH rats contributed about the same total protein content as a smaller number of larger cells in WKY rats. Similarly the large variations in individual DNA content detected in both strains of rat may well have masked a slight change in total DNA content present in young animals with the increase in the frequency of polyploidy with age further masking this difference.

Unfortunately it is difficult to draw many conclusions about

the development of genetic hypertension as conflicting data regarding the SH rat abound. Perhaps the reason for this confusion lies not with the SH rat itself but with the WKY which is commonly used as a normotensive control. It has recently become known that unlike the SH rat the WKY breeding stocks were distributed before they were fully inbred and that basic parameters such as growth rate and blood pressure vary depending on the commercial supplier (Kurtz and Morris, 1987). Furthermore DNA fingerprinting has revealed not only genetic variability between WKY rats from different sources but also among WKY rats within a single breeding facility (Kurtz et al, 1989).

However, the results of the present study strongly suggest that there is no evidence of a growth stimulus in the small resistance vessels of SH rats from 5 weeks of age onwards in their postnatal life. It is possible however that a growth stimulus occurs prior to this period, either in very early postnatal life or perhaps in prenatal life. This view is supported by observations that despite the evidence that blood pressure was not significantly elevated, cardiac hypertrophy had already developed in 5 week old SH rats, and there is morphological evidence of a significant increase in the media volume of mesenteric arteries from 3 week old SH rats (Appendix E).

The first 40 - 50 days of life of SH rats were originally referred to as the 'prehypertensive' period by Okamoto and Aoki (1963) because the blood pressure had not reached an arbitrary 150 mmHg. This has subsequently been interpreted to

mean that there are no differences between SH and WKY rats during this period. However, it is becoming increasingly clear that this is not the case and several investigators have recorded differences showing the development of structural and functional changes previously associated only with the adult hypertensive state. Both Gray (1984) and Bruno et al (1985) have demonstrated significant differences in blood pressure between SH and WKY rats as early as the first postnatal day. Interestingly Sarah Gray has gone on to demonstrate structural changes in the aorta of both neonatal and foetal SH rats (Eccleston-Joyner and Gray, 1988). Moreover Morton et al (1990) found evidence of increased vascular responsiveness and increased membrane protein content in mesenteric arteries of 3 week old SH rats, which they felt indicated the presence of vascular hypertrophy.

Thus it would appear that the stimulus for vascular growth occurs very early in the life of the SH rat, prior to the onset of overt hypertension. In fact Yang et al (1989) were able to demonstrate an increase in the smooth muscle cell labelling index from mesenteric arteries of 1 week old SH compared to WKY rats, although this difference was no longer apparant at 2 weeks. Further evidence to support the idea of prehypertensive structural change can be drawn from the work of Smeda and co-workers who demonstrated increased cross-sectional quantities of arterial media in renal vessels from young SH rats (Smeda et al, 1988A) which could not be prevented by anti-hypertensive treatment in utero and postnatally for up to 21 weeks of age (Smeda et al, 1988B).

Similarly Aalkjaer et al (1989) demonstrated that prolonged anti-hypertensive treatment failed to normalise the morphological changes found in subcutaneous resistance vessels from essential hypertensives.

However it is possible that following the intial stimulus for growth further structural vascular modification does occur. Lundin and Hallback-Nordlander (1984) reported an increase in the resistance of perfused hindquarters of SH rats between 14 and 24 weeks of age which could be reduced by antihypertensive treatment. Similarly Christensen et al (1989) demonstrated that treatment of SH rats between 4 and 24 weeks of age tended to result in a reduction in the media/lumen ratio of mesenteric arteries. Media area remained unchanged however leading these authors to speculate that the change in media/lumen ratio, primarily due to increases in lumen diameter, represented rearrangement of the smooth muscle rather than a reduction in its mass.

It appears that medial hypertrophy of the aorta of SH rats which occurs postnatally and can be largely prevented by anti-hypertensive treatment (Owens, 1985 and 1987; Loeb and Bean, 1986) represents secondary growth occuring in response to increased blood pressure or wall stress. Whereas postnatal structural changes occuring in the small resistance vessels may represent rearrangement of vascular tissue rather than true growth.

CHAPTER SEVEN.

CONCLUSIONS.

7.1 Experimental Hypertension.

1. An increase in the rate of DNA synthesis occurs in both the aorta and mesenteric arteries of 2K, 1C renovascular hypertensive rats prior to a significant rise in blood pressure.

2. Plasma renin levels are consistently elevated throughout the development of 2K, 1C renovascular hypertension.

3. Not all peripheral vascular beds respond to developing renovascular hypertension in a similar manner.

4. Medial hypertrophy of the aorta in renovascular hypertension is primarily due to cellular hypertrophy coupled with an increase in polyploidy.

5. Medial hypertrophy of mesenteric arteries in renovascular hypertension is primarily due to hyperplasia.

6. Medial hypertrophy of subcutaneous vessels is primarily due to a reorganisation of vascular tissue rather than to growth.

7.2 Genetic Hypertension.

1. The initial stimulus for vascular DNA synthesis occurs prior to 5 weeks of postnatal life.

2. Medial hypertrophy of the aorta occurring postnatally in genetic hypertension primarily consists of cellular hypertrophy coupled with an increase in polyploidy.

3. Medial hypertrophy of resistance vessels occuring postnatally in genetic hypertension primarily involves structural reorganisation of vascular tissue as opposed to true growth.

APPENDICES.

APPENDIX A.

This appendix contains all the chemical reagents used in this thesis and their suppliers.

Amersham International plc, Buckinghamshire, England.
[methyl-^{3}H]Thymidine, [^{14}C]Deoxyribonucleic acid.
Amicon Corporation, Upper Mill, Gloucestershire.
Diaflo ultrafiltration membranes (YM30, 25mm).
BDH Chemicals Ltd, Poole, Dorset.
Ammonia solution (26%), Bromophenol Blue (water soluble), Perchloric Acid (70%).
BOC Ltd, Brentford, Middlesex.
5% Carbon Dioxide / 95% Oxygen, Nitrogen (Oxygen-free).
Fisons Scientific Apparatus, Loughborough.
Acetaldehyde, Diphenylamine, Folin and Ciocalteu's phenol reagent, Sodium lauryl sulphate (SDS).
Gibco BRL, Paisley, Scotland.
Antibiotic/antimycotic solution, Chicken serum, Dulbecco's modified Eagles medium 10X (DMEM), L-Glutamine, Hanks Balanced Salt solution, Newborn Calf serum, Sodium bicarbonate solution (7.5%), Trypsin solution (2.5%), Tryptose Phosphate Broth, Versene.
Pharmacia LKB Biotechnology, Milton Keynes.
Optiphase 'X' scintillation fluid.
Pierce and Warriner Chemicals, Chester.
Bicinchoninic Acid sodium salt.

Serono Diagnostics Ltd, Woking Surrey.

[^{125}I]Renin MAIA radioimmunoassay kit.

Sherman Chemicals, Sandy, Berkshire.

Bisbenzimide (Hoechst No. 33258).

Sigma Chemical Company Ltd, Poole, Dorset.

Agarose, Bovine serum albumin fraction V, Deoxyribonuclease I (DNase I from Bovine Pancreas, Type IV), Deoxyribonucleic acid (from Calf Thymus, Type I, sodium salt, highly polymerised), Ethidium Bromide, Protease (Proteinase K, from Tritachium album Type XI), Triton X-100 (Octyl Phenoxy Polyethoxyethanol).

APPENDIX B.

This appendix contains details of the equipment used in this thesis.

Analysis of Radioactivity.

All Beta-emitters ([^{3}H] and [^{14}C]) were counted using a Packard Tri-Carb Liquid Scintillation Analyzer, model number 2200CA.

All Gamma-emitters ([^{125}I]) were counted using a Packard Auto-Gamma, model number 5650.

Cell Culture.

All sterile procedures were carried out in a Gelaire BSB4 Class II cabinet. All cell cultures were maintained in a Flow Laboratories water-jacketed CO_2 incubator.

Centrifuges.

The following centrifuges were used throughout this work;
MSE: Chilspin I, Centaur 2, Microcentaur.

Fluorescence Measurement.

All determinations of the fluorescence of solutions were performed using a Perkin-Elmer LS-5B Luminescence Spectrometer, linked to a Pye-Unicam cell temperature controller.

Spectrophotometer.

All determinations of the optical absorbance of solutions were performed using a Pye-Unicam SP6-550 UV/VIS spectrophotometer.

APPENDIX C.

This appendix contains the chemical constitutions of the biological buffers and solutions described in the text.

1. Bicinchoninic Acid (BCA) Reagents.

Microreagent A.

$Na_2CO_3.H_2O$	8.00%	(w/v)
NaOH	1.60%	(w/v)
Na_2Tartrate	0.16%	(w/v)

Adjust to pH 11.25 with $NaHCO_3$

Microreagent B.

BCA-Na_2	4.00%	(w/v)

Microreagent C.

4% (w/v) $CuSO_4.5H_2O$	4	Volumes
Microreagent B	100	Volumes

2. Chick Embryo Fibroblast Media.

Stock solution.

(for 500ml)

DMEM (10X)	50ml
L-Glutamine (200mM)	10ml

$NaHCO_3$ (7.5%)	25ml
Antibiotic/Antimycotic solution	5ml
Sterile H_2O	410ml

Complete Media.

(for 100ml)

Tryptose Phosphate Broth	10ml
Newborn Calf serum	5ml
Chicken serum	1ml
Stock solution	84ml

3. Diphenylamine Reagent.

Diphenylamine	7.5g in 500ml Glacial Acetic acid
Concentrated H_2SO_4	7.5ml

(Can be stored foil-wrapped at room temperature).

Add $^1/_{200}$ of 2% (v/v) acetaldehyde immediately prior to use

4. NTE

NaCl	100	mM
EDTA	10	mM
Tris	10	mM

5. Physiological Salt Solution (PSS).

NaCl	119 mM
KCl	4.7 mM
$CaCl_2.2H_2O$	2.5 mM
$MgSO_4.7H_2O$	1.17 mM
$NaHCO_3$	25 mM
KH_2PO_4	1.18 mM
EDTA	0.026 mM
Glucose	5.5 mM

$CaCl_2$ (dissolved in H_2O) is added to the solution of all other constituents after gassing with 95%O_2/5%CO_2 for 10 minutes.

APPENDIX D

This appendix contains morphological data from our laboratory of resistance vessels from both control (sham) and experimental (2K, 1C) animals at 28 days post-renal artery constriction.

Mesenteric Arteries.

Parameter	Sham	2K, 1C	P
Vessel size ($L_{0.9}$)μm	221 ± 8	218 ± 9	NS
Media/Lumen Ratio (%)	4.81 ± 0.33	8.09 ± 0.70	0.001
Media Thickness (μm^2)	10.24 ± 0.47	15.98 ± 0.98	0.001
Media Volume (μ^3/mm)	7381 ± 358	15807 ± 4463	0.001

Subcutaneous Arteries.

Parameter	Sham	2K, 1C	P
Vessel size ($L_{0.9}$)μm	150 ± 5	113 ± 10	0.01
Media/Lumen Ratio (%)	9.76 ± 1.28	18.74 ± 2.49	0.01
Media Thickness (μm^2)	14.07 ± 1.71	22.01 ± 2.91	0.05
Media Volume (μ^3/mm)	7168 ± 946	9872 ± 1902	NS

APPENDIX E.

This appendix contains morphological data from our laboratory of mesenteric resistance arteries from both SH and WKY rats at different ages.

3 Week Old.

Parameter	WKY	SHR	P
Vessel size ($L_{0.9}$)μm	121.5 ± 6	135 ± 9	NS
Media/Lumen Ratio (%)	9.84 ± 0.77	10.17 ± 0.88	NS
Media Thickness (μm^2)	11.71 ± 0.61	13.33 ± 0.92	NS
Media Volume (μ^3/mm)	4982 ± 312	6357 ± 560	0.05

6 Week Old.

Parameter	WKY	SHR	P
Vessel size ($L_{0.9}$)μm	183.6 ± 10	169.2 ± 8	NS
Media/Lumen Ratio (%)	5.60 ± 0.35	7.92 ± 0.58	0.01
Media Thickness (μm^2)	10.12 ± 0.39	12.76 ± 0.71	0.01
Media Volume (μ^3/mm)	6272 ± 325	7189 ± 471	NS

12 Week Old.

Parameter	WKY	SHR	P
Vessel size ($L_{0.9}$)μm	198.9 ± 11	203.4 ± 10	NS
Media/Lumen Ratio (%)	5.34 ± 0.37	6.53 ± 0.48	NS
Media Thickness (μm^2)	10.78 ± 0.58	13.07 ± 0.85	0.05
Media Volume (μ^3/mm)	7342 ± 481	8993 ± 764	NS

18 Week Old.

Parameter	WKY	SHR	P
Vessel size ($L_{0.9}$)μm	252.9 ± 16	223.2 ± 14	NS
Media/Lumen Ratio (%)	3.35 ± 0.39	5.97 ± 0.35	0.001
Media Thickness (μm^2)	8.45 ± 0.70	12.97 ± 0.46	0.001
Media Volume (μ^3/mm)	7093 ± 544	9663 ± 686	0.05

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