THE PRESSOR EFFECTS OF ANGIOTENSIN II

A thesis presented in part fulfillment of the requirements for admittance to the degree of Doctor of Philosophy of the University of Glasgow

by

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SUMMARY

A method has been developed in which intravenous infusions can be given and arterial pressure and metabolic balance measured for up to 21 days in conscious unrestrained rats.

Using the method, a comparison was made of the direct vasoconstrictor and slow pressor effects of angiotensin II (AII). Both were related to concurrent plasma AII concentrations. Nine female Wistar rats were infused intravenously firstly with 5% dextrose for 3 days, then AII at 20ng/kg/min at 2.4mls/day for 7 days and finally with dextrose for 3 days. Nine control rats received dextrose throughout. AII raised mean arterial pressure (MAP) progressively reaching a peak on the 7th day, 49.7 mmHg higher than control ($p< 0.001$). MAP in control rats did not change significantly. The direct pressor response to AII (30,90 and 270ng/kg/min, each for 1 hour) did not change during prolonged AII infusion, although it was enhanced 4 hours after stopping prolonged infusion at a time when MAP was normal. Plasma angiotensin II levels during the progressive rise were 6 times those of control rats. In contrast, the pressor response to an hourly infusion of AII at 270ng/kg/min raising plasma levels to 32 times those of control, was on average 45.3 ± 3 mmHg. This was close to the maximum direct effect since infusion at 810ng/kg/min raised MAP by an additional 9.3 mmHg only. Estimates of plasma AII were confirmed by chromatography. Infusion of AII reduced rather than increased the proportion of angiotensin III. Because changes of plasma angiotensin II concentration during prolonged infusion were closer to the physiological range of AII, this suggests that the slow pressor effect of AII may be more important than the direct effect in longterm regulation of MAP.

Saralasin, an effective antagonist of AII, has a rapidly-acting agonist pressor effect. Prolonged infusion of saralasin has been used to assess the role of AII as a pressor hormone, but it is not known if saralasin has an additional

slow developing pressor action like angiotensin II. This was tested in 8 female Wistar rats. Dextrose was infused intravenously for 2 days, followed by saralasin at 10µg/kg/min for 4 days and then dextrose for 2 days. MAP was recorded continuously. Saralasin gradually raised MAP in all rats. After 4 days, MAP was 22 mmHg higher than in the control period. The diurnal variation of arterial pressure also increased during saralasin - MAP increased during the night between 9 p.m. and 9 a.m. and decreased during the day between 9 a.m. and 9 p.m. Variability of pressure also increased.

A second group of rats was infused with AII at 20ng/kg/min for 4 days with continuous recording of arterial pressure. AII, as well as raising MAP slowly, also increased variability and diurnal rhythm of pressure. Saralasin and AII had no effect on food and water intake or on sodium balance. These findings, together with the structural similarities between AII and saralasin, suggest that both peptides are acting by a similar mechanism to raise arterial pressure. These properties of saralasin may also compromise interpretation of experiments in which the inhibitor is given by prolonged infusion to test the role of AII in the maintenance of MAP.

CHAPTER 1

REVIEW OF THE LITERATURE

1 RENIN AND ANGIOTENSIN

1.1 DISCOVERY

The idea that the kidneys might liberate some substance which could raise arterial pressure was the logical extrapolation of observations made by two famous scientists, Richard Bright and Charles Brown-Sequard in the 19th century. It was Bright (1836) who first implicated the kidney in the aetiology of hypertension when he drew attention to the common association of renal disease with a 'hard full pulse' and cardiac hypertrophy. Then in 1856 Brown-Sequard proposed that organs, in particular the kidney, released substances into the blood to act at other sites within the body. Influenced by these ideas, two Swedish physiologists, Robert Tigerstedt and Per Bergman, began to investigate the possible endocrine function of the kidney. They prepared crude saline extracts of rabbit kidneys which they injected into other rabbits. The extract contained an agent which they called renin and which raised arterial pressure. Further experiments showed that section of the spinal cord did not abolish the pressor effect and that it was only from the cortex that the extract could be produced (1898). It is interesting, but less well known, that in the same year Livon (1898) working in Marseilles, showed that extracts of various organs, including the kidney, raised blood pressure after injection into curarized dogs.

These discoveries remained relatively unnoticed for 40 years until the classical experiments of Harry Goldblatt in 1934. He showed that experimental hypertension as sustained as human hypertension could be produced by placing adjustable clips on both renal arteries of dogs. Goldblatt suggested that a humoral mechanism could be raising pressure and this revived interest in a pressor substance of renal origin.

At the end of the 1930's, three different groups confirmed Tigerstedt

and Bergman's original work (Pickering and Prinzmetal 1938; Landis, Montgomery and Sparkman 1938; Hessel 1938) and the first assay for renin was established (Pickering and Prinzmetal 1938). This assay compared the pressor effects of kidney extracts in the unanaesthetized rabbit with the blood pressure increase produced by a standard extract prepared from normal rabbit kidneys. Pickering and Prinzmetal also made the very important observation that some anaesthetic agents, e.g. urethane, ether and nembutal, reduced the pressor effect of renin considerably.

It was then found independently by Braun-Menendez and Page that the renal pressor extract was itself inactive, but was an enzyme releasing the pressor substance from substrate. Working in Cleveland, Page (1939) added renin to blood perfusing isolated ears of rabbits. He noticed that if tachyphylaxis occurred in a first ear, and the same blood was used to perfuse a second ear, that renin had no pressor effect, but that the addition of fresh blood restored the response. He concluded that the presence of renin activator was necessary for a response. Then in 1940, Page and Helmer began looking for a renin activator. They concluded from their studies that the renin activator was the substrate on which renin acts and began a search for products resulting from the interaction of renin and renin-activator. This search culminated in the extraction of a pressor substance which they called 'angiotonin'. Slightly earlier in Argentina, Braun-Menendez and his colleagues were working towards the same end. In 1938, Fasciolo, Houssay and Taquini showed that when the ischaemic kidneys of dogs with chronic hypertension were grafted into the neck of normal and nephrectomised dogs, that a rise in blood pressure of between 30 and 70 mmHg occurred, while grafting the kidneys of normal animals had no effect. They subsequently showed that the venous blood from these acutely ischaemic kidneys had vasoconstrictor properties. Extracts of the blood contained a pressor substance, which they called 'hypertensin' which was formed in vitro when blood proteins were incubated with renin (Braun-Menendez,

Fasciolo, Leloir and Munoz 1939). Braun-Menendez drew the important conclusion that the production of hypertensin by renin was enzymatic, a process which Page had called activation. The dual nomenclature, angiotonin and hypertensin persisted for almost 20 years until in 1958, Braun-Menendez and Page issued a joint communication in which they compromised calling the substance angiotensin.

1.2 PURIFICATION OF ANGIOTENSIN

With the discovery of angiotensin and its possible relationship to arterial hypertension, scientists turned their efforts to the elucidation of its chemical composition. Until purification could be achieved, the compound could not be prepared and chemical analytical methods to determine its concentration in blood and other body fluids would be unreliable. The first partially successful attempt was that of Plentl and Page (1945) who attempted purification by formation of metallic salts. Their attempts at synthesis, however, were unsuccessful. The next preparation by Edman (1945) was said to have been the purest angiotensin for the next 10 years (Bumpus and Smeby 1968). This purification relied on the precipitation with ethanol of the proteins formed during incubation of renin with renin substrate. The pressor fraction was adsorbed on alumina, the eluate precipitated with nitranilic acid and separation achieved by electrodialysis. Ten years later, with better methods for inhibiting angiotensinase activity Skeggs, Marsh, Kahn and Shumway (1954b) and Peart (1955) obtained pure angiotensin polypeptide. Identification of the amino acid sequence of angiotensin soon followed. Skeggs, Marsh, Kahn and Shumway (1954a) reported the existence of two forms of hypertensin, which they designated 'hypertensin I', formed as a result of the action of renin on its substrate and 'hypertensin II' formed by the action of hypertensin converting enzyme on hypertensin I (Skeggs, Kahn and Shumway 1956). The amino acid sequence of this form of hypertensin I was found to be:-

asp-arg-val-tyr-ileu-his-pro-phe-his-leu

(Skeggs, Marsh, Kahn and Shumway 1955), the result of the reaction of pig renin and horse renin-substrate. Meanwhile Elliott and Peart (1956) had isolated and purified hypertensin, produced by the reaction of rabbit renin and ox serum. The amino acid sequence of this hypertensin differed only by the presence of valine in place of the fifth amino acid, isoleucine. Peart (1956) suggested that this represented a species difference since Skeggs et al (1955) had used pig renin and horse serum. Soon after, Skeggs, Lentz, Kahn, Shumway and Wood (1956) demonstrated that angiotensin II was an octapeptide formed by the removal of the histidyl-leucine fragment from the C-terminal end of the angiotensin I molecule.

The determination of the amino acid sequence of angiotensin II and modern methods of peptide synthesis have allowed the production of synthetic angiotensin II, which is now used for most investigations where angiotensin II is required.

1.3 DESTRUCTION OF ANGIOTENSIN

It has long been evident that there are substances in blood and tissues which are capable of destroying angiotensin (Braun-Menendez, Fasciolo, Leloir, Munoz 1940). Indeed in 1944, Plentl and Page showed that proteolytic enzymes such as carboxy peptidase A, chymotrypsin, trypsin, and pepsin all rapidly inactivate angiotensin. The term 'angiotensinase' has been used to describe these enzymes although their multiplicity suggests that they are non-specific. Three main groups of angiotensinases have been described:-

i) Aminopeptidases or Angiotensin 'A' - these enzymes which are calcium dependent hydrolyse the molecule at the N-terminal bond. Glenner, McMillan and Folk (1962) described such an enzyme in rat kidney microsomes which they named aminopeptidase A. A similar enzyme, found in human plasma and washed hemolyzed red cells, was named angiotensinase A (Khairallah, Bumpus, Page and Smeby 1963). The latter enzyme was subsequently shown to be two separate enzymes (Khairallah and Page 1967), angiotensinase A_1, the peptidase specific to

Asn^1 AII and angiotensinase A_2, the peptidase specific to asp^1 AII.

ii) Endopeptidases, or angiotensinase 'B'- these hydrolyse the middle of the peptide and were discovered by Regoli, Riniker and Brunner (1963). A similar plasma endopeptidase, inhibited by diisopropyl-fluorophosphate (DFP) was described by Khairallah and Page (1967).

iii) Carboxypeptidases or angiotensinase 'C' - these hydrolyse the C-terminal bond of angiotensin II. Lentz, Skeggs, Woods, Kahn, Shumway (1956) showed that pancreatic carboxypeptidase A released phenylalanine from AII. Evidence of carboxypeptidase activity has been found in aqueous extracts of rabbit liver (Johnson and Ryan 1968).

Although aminopeptidases and endopeptidases are present in plasma and serum, degradation of angiotensin II in blood is now known to be a slow process of minor significance compared with the rapid catabolism of the peptide in tissue vascular beds (Hodge, Ng and Vane 1967; Bakhle, Reynard, and Vane 1969, Ng and Vane 1968).

1.3:1 Fate of angiotensin in the body.

Angiotensin II has a very short biological half-life, according to early estimates, about 10 minutes (Corcoran, Kholstaedt, Page, 1941). However, as pure angiotensin and better means of detection became available, estimates of the half-life became very much shorter, more recent estimates by radioimmunoassay being of the order of one minute (Cain, Catt, Coghlan and Blair-West 1970). This accords with the brief pressor response following intravenous injection of angiotensin II (Peart 1955). In order to ascertain the various vascular beds responsible for the removal of angiotensin II, Hodge, Ng and Vane (1967) infused angiotensin II and showed that the liver, head, kidneys and hindquarters removed up to 75% of the activity in one circulation, while the lungs removed none. Use of isotopically labelled angiotensin (Bumpus, Smeby, Page and Khairallah 1964) to determine the regional distribution of angiotensin, showed that there was a high concentration in the uterus, adrenals and kidneys.

After 30 minutes, the uterine concentration had dropped, the brain concentration had risen and the kidneys and adrenals retained substantial activity. One possibility was that the more prolonged effects of angiotensin were only relevant to the kidneys, adrenals and the central nervous system. But difficulty interpreting these experiments is that the label may become separated from the original peptides or from the peptide fragments after enzymatic degradation.

1.4 ANGIOTENSIN II, A BLOOD BORNE OR LOCAL TISSUE HORMONE?

In vertebrate evolution, some of the earliest hormones were local tissue hormones. Later, in evolution, when the circulation developed, these hormones were spilled into the blood to produce effects at a distance. The renin-angiotensin II system may have developed in this way (Brown, Fraser, Lever and Robertson 1968). Indeed there is some evidence that angiotensin II may be generated by renin within the blood vessel walls and within the tissues of the brain and the kidney as well as in the circulation (Malik and Nasjletti 1976; Swales 1979).

Vessel wall preparations of the aorta and large arteries have been shown to contain renin-like activity which generates angiotensin I at a physiological pH. If such activity reflects equivalent activity at the resistance vessel receptor site and if substrate and converting enzyme were also present, it is possible that high concentrations of angiotensin II would be formed locally and could thus modulate both blood pressure and the pressor response to injected angiotensin II (Swales 1979).

Another site where local formation of angiotensin II could exert an influence on blood pressure is in the brain. Renin-like activity has been detected in every part of the brain examined (Ganten, Hutchinson, Schelling, Ganten and Fischer 1976), although it has been identified by one group as cathepsin D (Reid 1977). Thus there is considerable debate as to whether angiotensin I or II is generated in the brain in vivo (Reid 1977, Ganong 1977, Ganten 1978) and whether it plays a role in the pathogenesis of hypertension.

A considerable amount of evidence now suggests that angiotensin II is generated locally within the kidney and can influence renal function under conditions of sodium and water restriction (Levens, Peach and Carey 1981).

There exists the possibility then that renin and angiotensin act in the body as local tissue hormones. It is important, however, to note that although many of the tissues studied contain enzymes which produce angiotensin I when incubated with naturally-occurring or synthetic substrate, it does not follow that these enzymes have a physiological role in the formation of angiotensin and in the regulation of blood pressure. In the sections which follow I shall consider evidence that angiotensin II is also a blood borne hormone.

1.5 PHARMACOLOGICAL AND PHYSIOLOGICAL EFFECTS OF ANGIOTENSIN II.

Angiotensin II has many actions. Although their existence is not disputed, there is considerable controversy as to whether they are pharmacological curiosities or physiological phenomena. Demonstration of a pharmacological effect does not necessarily reveal a physiological mechanism. I have tried to distinguish the two forms of experiment. The discussion below concerns angiotensin II, its pressor actions, its nervous effects and its interrelationships with sodium, blood pressure and aldosterone.

1.5:1 Pressor action

The immediate pressor effect of renin, subsequently shown to be due to formation of angiotensin II was the earliest to be demonstrated (Tigerstedt and Bergman 1898) and remains the most studied of its actions (DeBono, Lee, Mottram, Pickering, Brown, Keen, Peart and Sanderson 1963). DeBono et al (1963) showed that angiotensin II was approximately 6-8 times more potent as a pressor agent than noradrenaline on a weight basis and 40-60 times stronger on a molar basis.

In intact animals, including man, the characteristic response to an intravenous injection of angiotensin II is a sharp rise in systemic arterial

pressure within 20 to 30 seconds, which reaches a peak in 1-2 minutes and returns to normal within 3 minutes. The height of the response is dose-dependent. Evidence that tachyphylaxis to angiotensin II could occur was provided by Bock and Gross (1961) who demonstrated that large doses of angiotensin in dogs diminished or abolished the pressor response to subsequent injections of angiotensin II. It is likely that tachyphylaxis is due to occupation of receptors by angiotensin. This has been supported by the observation (Khairallah, Page, Bumpus, and Turker 1966; Bohr and Uchida 1967; Walter and Bassenge 1969) that treatment of tissue showing tachyphylaxis to angiotensin II with a solution containing angiotensinase activity, or with Dowex resin, diminishes the tachyphylaxis.

As well as its direct vasoconstrictor pressor effect, angiotensin II raises arterial pressure gradually when given in low dose for a prolonged period. This was first shown by Dickinson and Lawrence in 1963. It is the main subject of my thesis and will be discussed in greater detail later.

Mechanism of the direct vasoconstrictor action of angiotensin II

The contractile apparatus in vascular smooth muscle is activated by a rise in the cytoplasmic contraction of ionized calcium. Thus, a vasoactive substance, like angiotensin II, initiates contraction by its ability to raise intracellular ionized calcium (Daniels and Kwan 1981). Calcium can enter the cell through two types of ion channels in the membrane, (1) potential-operated channels which admit calcium when they are caused to open by depolarization and (2) receptor-operated channels (ROC's) associated with the receptors for a stimulant substance. These receptor-operated channels appear to be a separate and distinct population from the ion channels although sometimes activation of a receptor may affect the opening of potential sensitive channels (Bolton 1979). Opening of either channels increases the permeability of the membrane to calcium which triggers contraction, possibly by releasing more calcium from bound sites on the side of the membrane and on the sarcoplasmic reticulum

(Bolton 1979). Thus, an agonist may affect calcium movements by depolarization and/or occupation of receptors both of which may affect calcium binding or transport by internal membranes (Daniels and Kwan 1981; Benham and Bolton 1981).

Angiotensin II has been shown to cause depolarization and contraction of spiral strips of carotid arteries of sheep (Keatinge 1966). The peptide also contracts the same arteries in the presence of depolarizing concentrations of potassium, but without any detectable electrical changes, suggesting that the action of angiotensin II is, at least in part, independent of changes in membrane potential (Keatinge, 1966).

A combination of angiotensin II with its receptor thus causes an increase in the permeability of the membrane to calcium. The movement of calcium and possibly sodium across the membrane into the cell is probably responsible for the depolarization sometimes seen in certain vascular smooth muscles exposed to angiotensin II. As well as movement of extracellular calcium into the cell, angiotensin II may also displace calcium by receptor activation from bound sites within the cell which explain its ability to produce contraction without depolarization (Bolton 1979). The site of this bound calcium may be the plasma membrane, mitochondrion or endoplasmic reticulum as well as calcium bound to the receptor. Angiotensin II has been shown to increase the efflux of ^{45}Ca from a preparation of vesicular cell fragments of rabbit aorta (Baudouin, Meyer, Fermandjian and Morgat 1972). The vesicular fragments were absent of mitochondrial contamination since no cytochrome c. oxidase activity was detected, suggesting the cell membrane and endoplasmic reticulum are the only important internal stores of calcium.

The mechanism whereby activation of receptors by angiotensin II causes release of internal bound calcium is not yet clear, but could involve either a calcium induced release of bound calcium or biochemical changes within the cell, e.g. changes in cyclic nucleotide levels. Volicer and Hynie (1971) showed

that vasoconstriction induced by angiotensin II in rat aorta is associated with decreased adenylate cyclase activity while some recent work (Vesely 1981) has shown that angiotensin II in physiological concentrations enhanced guanylate cyclase activity in rat aorta. These biochemical effects, however, do not occur with a time course comparable to other drug effects and it therefore seems unlikely that they are involved in the pathway between receptor activation and contraction of the smooth muscle cell (Bolton 1979).

1.5:2 Effects of angiotensin II mediated by the nervous system.

The idea that there might be a link between angiotensin II and the nervous system came from the early work of Tigerstedt and Bergman (1898) who concluded that the principal mechanism of renin's pressor action was neural, involving peripheral components of the autonomic nervous system.

The subject lay dormant for several years. Then considerable evidence appeared showing that angiotensin II acted on the central and peripheral nervous system at several levels.

1) Central nervous system.

a) Pressor effects. In the early 1960's cross-perfusion experiments in dogs indicated that injection of AII into the circulation of the donor or into the arterial supply to the head of the recipient, resulted in a systemic pressor response in the recipient, although the latter was connected to its head by the spinal cord only (Bickerton and Buckley 1961). This centrally mediated pressor response was inhibited by piperoxan, a sympatholytic agent. In these early experiments, the doses of angiotensin II utilized were large and unphysiological. Subsequent investigations, however, have shown that doses of peptide nearer the physiological range of AII induce a centrally mediated pressor response: administration of AII into the vertebral circulation of conscious rabbits, in doses which had no effect if administered intravenously, resulted in an increase in peripheral blood pressure (Yu and Dickinson 1965). An increase in blood pressure induced by angiotensin adminstration centrally has been confirmed in

the dog (Lowe and Scroop 1969), cat (Severs, Daniels, Smookler, Kinnard, Buckley 1966), and rat (Fink, Haywood, Bryan, Packwood and Brody 1980) and the effects observed are mediated mainly by increased efferent sympathetic activity and partly by withdrawal of parasympathetic discharge. The rise in pressure is due primarily to increases in peripheral resistance, although sometimes increased cardiac output contributes (depending on the species) (Severs et al 1966; Lowe and Scroop, 1969)

Various neural ablation and recording procedures were then used to elucidate the site at which angiotensin exerts its pressor effects. These revealed that the area postrema, a medullary circumventricular organ, is a likely site of action of AII in the dog (Gildenberg, Ferrario and McCubbin 1973; Joy and Lowe 1970), and rabbit (Yu and Dickinson 1971). The area postrema, a zone where the blood brain barrier is deficient (Wislocki and Putman 1924), has intimate connections with the nucleus of the solitary tract (Morest 1967) part of the vasomotor centre, the latter also receiving fibres from the glossopharyngeal nerve and carotid sinus nerve (Gabriel and Selter 1970). The interconnection has led to the suggestion that angiotensin may act centrally to modulate the baroreceptor reflex (Sweet and Brody 1970) and there is some supportive evidence for this (Fukiyama 1973; Goldstein, Heitz, Schaffer and Brody 1974, Marker, Miles and Scroop, 1980). Some recent work in the rat (Haywood, Fink, Buggy, Phillips and Brody 1980; Fink et al 1980) has implicated periventricular structures surrounding the 3rd ventricle rather than the area postrema as the central site for pressor action of blood-borne angiotensin in the rat.

b) Dipsogenic and feeding responses. The administration of angiotensin II into the central nervous system stimulates drinking in several species (Epstein, Fitzsimons and Rolls 1970; Fitzsimons, Kucharczyk and Richards 1978). The area of the brain most sensitive is the subfornical organ (Simpson and Routtenberg 1973), application of 0.1 ng angiotensin II to this area eliciting a drinking response. This amount of angiotensin II, however, is large and would

produce a high concentration diluted in a small volume. It is therefore unlikely to be physiological. However, as well as direct injection of angiotensin II into the brain, systemic infusion in physiological amounts does induce drinking in the water replete animal (Trippodo, McCaa and Guyton 1976; Fitzsimons et al 1978).

Angiotensin II, as well as stimulating drinking, also acts directly on the central nervous system to suppress feeding (Epstein et al 1970; McFarland and Rolls 1972). Later studies, however, (Rolls and McFarland 1973) showed that the suppression of feeding depends on the thirst inducing property of the substance, i.e. angiotensin does not act directly on feeding systems but rather acts on the thirst mechanisms which, in turn, suppress feeding.

c) Effects on the pituitary Increased circulating levels of angiotensin have been shown to increase the plasma concentration of arginine vasopressin (Bonjour and Malvin 1970; Keil, Summy-Long and Severs 1975; Mouw, Bonjour, Malvin and Vander 1971). Some investigators, however, have been unable to observe such an increase (Claybaugh, Share and Shimizu 1972; Shade and Share 1975) and so there exists controversy as to whether angiotensin II is of physiological importance in the control of vasopressin secretion. Padfield and Morton (1977) measured circulating levels of arginine vasopressin and corresponding angiotensin II levels in physiological states in man and during infusions of angiotensin II. Angiotensin II was found to stimulate secretion of AVP but only at supraphysiological concentrations. Cowley, Switzer and Skelton (1981) infused angiotensin II at physiological rates into dogs for 7 days and concluded that circulating angiotensin II was not involved in the longterm control of AVP secretion.

2) Action of angiotensin II on the sympathetic nervous system.

In the early 1960's, evidence accumulated suggesting that some of the effects of angiotensin II result from an action on the autonomic nervous system. Zimmerman (1962) showed that sympathectomy greatly reduced the response to

intra-arterial angiotensin injected into an isolated hind-limb preparation. Angiotensin II was also found to induce ganglionic stimulation (Kaneko, McCubbin and Page 1961) and facilitate responses to nerve stimulation and drugs or reflexes which evoked the release of noradrenaline (McCubbin and Page 1963; Kaneko, Takeda, Kouji and Ueda 1966). Pretreatment with reserpine, which depletes catecholamines, diminished the effect of angiotensin II on blood pressure (Baum 1963). This facilitation of angiotensin II was partly thought to be due to a direct action on the effector, leading to an increase of their sensitivity to noradrenaline and there exists some evidence for this (Zimmerman 1978). However, in addition to this postsynaptic component, angiotensin has presynaptic actions which increase the release of transmitter noradrenaline. Evidence for this was first presented by Zimmerman and Whitmore (1967) who showed that angiotensin augments the overflow of noradrenaline evoked by sympathetic nerve stimulation. There has been considerable controversy concerning the mechanism of this effect, the main postulates being that angiotensin II inhibits neuronal uptake of noradrenaline (Khairallah 1972) and that it enhances the average release of transmitter per impulse (Benelli, Bella and Gandini 1964). It is now accepted that the latter is of greater importance than the former (Starke 1977). The mechanism of this peripheral adrenergic facilitation is not known, although it is suggested that angiotensin can affect the neuronal membrane, possibly resulting in a change of calcium flux (Zimmerman 1981). Zimmerman (1981) in a recent review discusses the possible importance of central and peripheral adrenergic facilitation in certain pathophysiological conditions.

1.5:3 Aldosterone stimulation

The first evidence to suggest a relationship between the renin-angiotensin system and aldosterone was histological. Deane and Masson (1951) showed that injections of partially purified solutions of renin caused enlargement of the zona glomerulosa of the adrenal cortex in rats. Then in the late 1950's there was

the speculation of Gross (1958) followed by a series of elegant studies in several laboratories in the 1960's showing the existence of an aldosterone stimulating hormone of renal origin, probably renin (Davis, Carpenter, Ayers, Holman and Bahn 1961) and that injected angiotensin II increased aldosterone (Laragh, Angers, Kelly and Lieberman 1960; Biron, Koiw, Nowaczynski, Brouillet and Genest 1961). There is now little doubt that angiotensin II stimulates aldosterone synthesis by the adrenal cortex and is probably the major factor responsible for the control of aldosterone secretion in response to changes in sodium balance (Fraser, Brown, Lever, Mason and Robertson 1979; Brown, Casals-Stenzel, Cumming, Davies, Fraser, Lever, Morton, Semple, Tree and Robertson 1979). The heptapeptide, des-Asp^1-angiotensin II may be important in stimulating aldosterone in the rat (Semple and Morton 1976).

1.5:4 Renal effects.

Infusion of angiotensin at low doses produces sodium and water retention (Brown and Peart 1961, 1962; Malvin and Vander 1967; Waugh 1972). This may be due to stimulation of renal tubular sodium reabsorption as well as to the haemodynamic changes produced by angiotensin II (Johnson and Malvin 1977). Studies with the competitive antagonist Sar^1 Ile^5 angiotensin II and the converting enzyme inhibitor, SQ 14225 in sodium depleted dogs have suggested that the direct effect of angiotensin may be more important than the changes in aldosterone secretion in regulating renal haemodynamics and electrolyte excretion during chronic sodium deprivation (Hall, Guyton, Smith and Coleman 1979, Hall, Guyton, Smith and Coleman 1980).

1.6 INHIBITORS OF THE RENIN ANGIOTENSIN SYSTEM

Major advances in our understanding of the role of the renin-angiotensin system in blood pressure control and salt and water homeostasis have been made as a result of the identification and synthesis of specific inhibitors of renin, converting enzyme and angiotensin II.

1.6:1 Renin inhibitors

As early as 1953, Wakerlin, Bird, Brennan, Frank, Kremen, Kuperman and Skom (1953) showed that immunization of dogs with hog kidney extracts containing renin was successful in preventing the development of renal hypertension. Although objections were raised that the extracts were far from pure and could react with other renal components, this work initiated the search for substances which might inhibit the enzyme. Improved methods have enabled complete purification of hog (Corvol, Devaux, Ito, Sicard, Duclox and Menard 1977), human (Galen, Devaux, Guyenne, Menard and Corvol 1979) and dog (Dzau, Slater and Haber 1979) kidney renin, and the latter has been used to raise specific antibodies for use as physiological probes (Haber 1980). Some work in the dog with antibody Fab fragments, which avoid problems with immune complexes or complement activation, looks promising (Haber 1980).

Compared with other acid proteases, renin acts on a relatively narrow region of substrate. This property of renin has enabled the synthesis of octapeptides containing the leucyl-leucine bond. These act as competitive renin antagonists (Haber 1980). Although this work on renin inhibitors is in its preliminary stages, they should prove exciting tools for the future. I shall describe an experiment with a new renin inhibitor.

1.6:2 Inhibitory angiotensin II analogues

The recognition of the critical importance of position 8 in the biological activity of angiotensin II was a key step in the development of angiotensin II blocking agents (Khairallah, Toth and Bumpus 1970). Khairallah et al (1970) observed that Ala^8 -Ang II, given in concentrations >500 ng/ml specifically blocked the contractile response of angiotensin on the isolated guinea-pig ileum. Various amino acids have been substituted in this position - alanine, phenylalanine, isoleucine, threonine and O-methylthreonine. The introduction of sarcosine into position 1 created a much more effective inhibitory peptide (Pals, Masucci, Sipos, Denning 1971a) by preventing the degradation of the analogue by aminopeptidase A and resulting in a substantial increase in the half-life (Bumpus

1977). These analogues, however, all contain a certain degree of agonist activity, sarcosine^{1}-isoleucine^{8} AII having more agonist activity than sarcosine^{1}-alanine^{8} AII and sarcosine^{1}-threonine^{8} AII analogue having less (Bumpus 1977; Saltman, Fredlund and Catt 1976). This agonist activity was important in one of my experiments and I discuss it in more detail later.

Sar^{1}, Ala^{8} angiotensin II has been used extensively in man and animals. A hypotensive effect has been consistently observed in hypertensive patients with raised plasma levels of renin and angiotensin II (Brown, Brown, Fraser, Lever, Morton, Robertson, Rosei and Trust 1976), although patients with low plasma renin levels may exhibit a mild pressor response to the analogue, a result of its partial agonist properties (Case, Wallace, Keim, Sealey and Laragh 1976). The reduction of blood pressure with saralasin is related to the plasma concentration of angiotensin II before its infusion (Brown et al 1976). In addition, sarcosine^{1}, alanine^{8} angiotensin II produces an initial transient pressor response which is associated with a rise in the plasma concentration of noradrenaline (McGrath, Ledingham and Benedict 1977).

1.6:3 Converting enzyme inhibitors

Bakhle (1968) first observed that bradykinin-potentiating factor, a mixture of peptides from the venom of the South American pit viper, Bothrops jaracara, inhibited the conversion of angiotensin I by particles of canine lung. Several of these peptides were synthesised (Engel, Schaeffer, Gold, and Rubin 1972). However, many subsequent studies have been carried out with a proline derivative 'captopril' (SQ 14225). This compound is an effective antihypertensive agent in certain clinical and experimental situations. However, all converting enzyme inhibitors suffer from one grave disadvantage as scientific tools. This is their doubtful specificity; the contribution of bradykinin potentiation to the blood pressure fall produced by converting enzyme inhibition is uncertain. Thurston and Swales (1978) showed that converting enzyme inhibitor produced a further blood pressure fall in animals pretreated with

saralasin to block the renin-angiotensin system, thus suggesting an additional vasodepressor mechanism.

1.7 THE SLOW PRESSOR EFFECT OF ANGIOTENSIN II

As mentioned previously, angiotensin II infused at subpressor doses into animals for periods of days, results in a slow rise of pressure. This action was first demonstrated by Dickinson and Lawrence (1963) in the rabbit. Infusion of angiotensin II at doses between 3 and 10 ng/kg/min for 4 days raised arterial pressure slowly by 5-30 mmHg. Dickinson and Yu later showed that adrenalectomy did not prevent the hypertension, but that a variety of sympathetic nervous system blocking agents did attenuate or abolish the response (Dickinson and Yu 1967a; Yu and Dickinson 1971), thus implicating the nervous system in the mechanism of the slow effect. Since then the slow effect has been studied in a variety of species.

Koletsky, Rivera-Velez and Pritchard (1965) produced sustained hypertension by infusing angiotensin II at 10 ng/kg/min intermittently (12 hours/day) into rats for 5 days. Three months later, the rats had developed hypertension. McCubbin, Demoura, Page and Olmsted (1965) were first to demonstrate the effect in the dog. They infused angiotensin II at 10µg/kg/day into dogs for 2 weeks and showed an increase of 45 mmHg in arterial pressure. Arterial pressure showed great lability during the infusion and the rate of fall of pressure on stopping the infusion was dependent on the duration of the infusion, being slower with longer infusions. Studies in dogs have indicated that the magnitude of the rise of pressure during a continuous infusion of angiotensin II at 5ng/kg/min is related to the sodium intake of the animals, a larger intake producing a greater rise of pressure (Cowley and McCaa 1976; DeClue, Cowley, Coleman, McCaa and Guyton 1976) and that this form of hypertension can be prevented by dietary salt restriction (Cowley and McCaa 1976). Plasma aldosterone levels, however, were not elevated unless the rate of AII infusion was increased to 15-23ng/kg/min. These results suggest that the

rise of arterial pressure occurs independently of changes in aldosterone secretion. Bean, Brown, Casals-Stenzel, Fraser, Lever, Millar, Morton, Petch, Riegger, Robertson and Tree (1979), infusing dogs with AII at 3ng/kg/min for 2 weeks, also concluded that a persistent increase in aldosterone was not important. Systemic arterial pressure in these animals was increased by 26 mmHg by the end of the infusion, which also caused a shift in the relationship of plasma angiotensin II and arterial pressure, so that a given plasma concentration of the peptide maintained a higher pressure.

The slow pressor effect has also been observed in man. Ames, Borkowski, Sicinski and Laragh (1965) maintained a small, but constant increase of blood pressure with a decreasing rate of angiotensin infusion. Infusion of AII constantly at 2ng/kg/min for 66 hours into man raised blood pressure by 15/4 mmHg (Oelkers, Schoneshofer, Schultze, Brown, Fraser, Morton, Lever and Robertson 1975).

The idea that the slow pressor effect might be important in the pathogenesis of hypertension was first envisaged by Dickinson and Lawrence (1963). They suggested that angiotensin might specifically constrict the cerebral vessels and increase the cerebrovascular resistance, thus altering the intrinsic activity of the medullary vasoconstrictor centre. I propose to discuss the possible role of the slow pressor effect in the pathogenesis of chronic renal hypertension later in this thesis.

1.8 PURPOSE OF THE PRESENT STUDY

My main purpose was to study the slow pressor effect of angiotensin II in more detail. The rat was chosen because it is small, cheap and robust and because it is easier to make hypertensive by renal artery clipping and injection of DOCA than are dogs and rabbits. It was therefore necessary to develop a technique whereby continuous intravenous infusion could be given and blood pressure could be recorded continuously for periods of up to 3 weeks in conscious unrestrained animals. Although Koletsky et al (1965) had produced

sustained hypertension with intermittent infusions of angiotensin II, blood pressure was not recorded in the early stages of infusion and the infusions were not continuous.

Because there were no techniques for continuous recording and infusion, it was my first task to develop them.

If the slow effect occured in the rat, I proposed to determine its magnitude, compare it with the direct vasoconstrictor effect in the rat and search for a mechanism. Finally, if possible, I hoped to determine the contribution of the slow effect to the pathogenesis of chronic renal hypertension. In the event, the slow pressor effect was more marked than I had expected and was associated with increased diurnal variation of arterial pressure. Also during an early stage of the experiment on renal hypertension I discovered an interesting and previously unrecognised action of saralasin. This drew my attention away from the final objective.

CHAPTER 2

METHODS.

My main technical problem was to develop a method capable of giving continuous intravenous infusions in the conscious unrestrained rat, and at the same time to measure arterial pressure continuously and estimate metabolic balance, all for periods of up to 21 days. I had most difficulty with the arterial and venous catheters but eventually succeeded with them positioned in the aorta and inferior vena cava. These and the earlier unsuccessful techniques are described.

2.1 RATS AND THEIR CAGES.

Throughout all of the studies to be described, female rats from an inbred Wistar strain were used. They weighed 180g to 220g and were supplied by Olac Ltd. (Sussex). Female rats in this weight range are approaching the plateau of their growth curve and rarely gain more than 5g in body weight in a week. This is important in longterm studies using catheters for blood pressure measurement, since the size and fit of catheters is critical to their function and patency.

At first rats were housed in groups of four in plastic cages with aluminium lids in an animal house of constant temperature (18-22^{o}C) and lighting (12 hour light/darkness cycle). Rats studied in the conscious state were transfered to the laboratory in which the measurements were to be made at least one week before the start of the experiment.

Following catheterisation they were housed individually in plastic cages with aluminium lids, designed to minimise damage to catheters. After a period of recovery, they were placed singly in metabolic cages for acclimatization. They remained in the metabolic cages for the duration of the experiment. The laboratory was kept at a temperature of 20-25^{o}C.

2.2 CATHETERISATION.

Arterial and venous catheters were needed for blood pressure recording and intravenous infusion.

2.2:1 Carotid artery and jugular vein catheters.

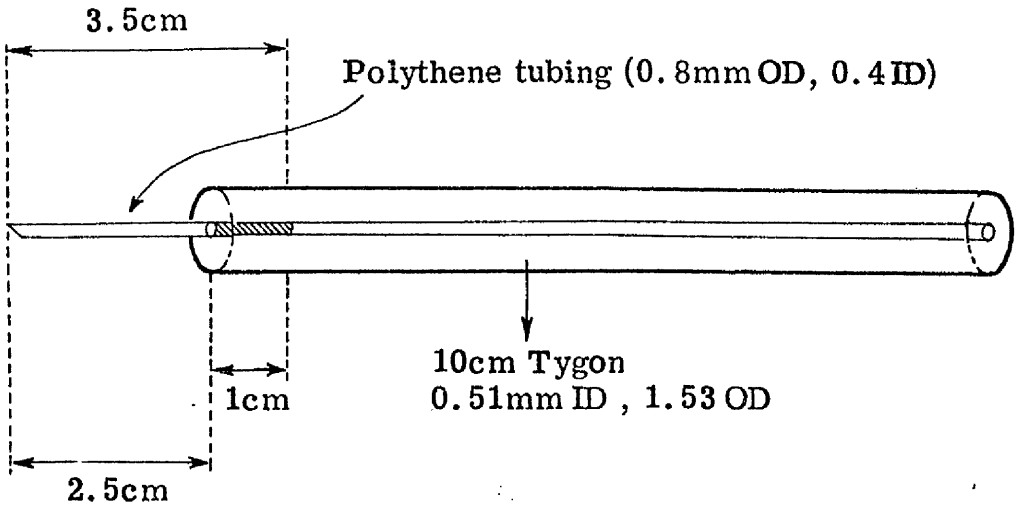
Preliminary studies were carried out using the carotid artery and jugular vein for catheterisation. Two types of arterial catheters were tested, one type of venous catheter.

i) Construction of carotid and jugular catheters. The form of the three catheters is illustrated in Figure 2.1. Their construction is detailed in Appendix 1.

ii) Implantation of carotid artery and jugular vein catheters. Catheters were filled with heparin (1000 units/ml) and the end plugged with 1 cm of stainless steel wire (0.7 mm diameter in type 1 carotid catheter, 0.9 mm in type 2 carotid catheters and 0.5mm in jugular catheters) which had been carefully rounded at one end to prevent damage to the catheters. Plugs and catheters were sterilised overnight in a 5% hibitane/alcohol solution. Instruments used for the operative technique were sterilised by boiling.

Rats were anaesthetised with ether. Before the operation both the front and back of the neck were shaved and cleaned with 5% hibitane/alcohol solution. A 1-2 cm longitudinal incision was made at the front of the neck. The left carotid artery was freed by blunt dissection. Three pieces of thread (4/0 silk) were passed beneath the artery. The most distal thread from the heart was tied in a tight knot, the other threads tied loosely. Gentle traction was exerted on the lower tie by artery forceps to stop the blood flow. The tip of the catheter was trimmed to form a blunt bevel 2.0 to 2.5 cm from the join with the tygon tubing. Using small scissors an incision was made in the artery between the two sutures. The catheter was then inserted, moved carefully up to the lower tie and tied into place with the loose thread. The lower tie was released and the catheter pushed gently into the artery until the whole of the

Carotid Catheter Type 1



Carotid Catheter Type 2

Identical to type 1 except materials are different. A 10cm length of transparent vinyl tubing was used instead of tygon and 3.5 cm length of polythene tubing (0.38mm ID, 1.09mm OD)

Jugular Catheter

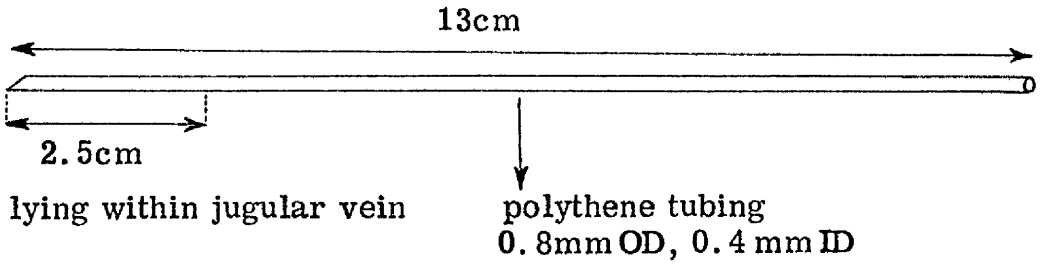


Figure 2.1 Carotid artery and jugular vein catheters

2.5 cm polythene tip lay within the vessel. All of the threads were tied firmly round the catheter to hold it in place. The ends of the sutures were sewn into the underlying muscles and tied tightly again.

The right jugular vein was exposed. Three lengths of thread were then passed under the vein and tied loosely. Gentle traction was applied to the upper and lower ties by means of artery forceps and an incision was made in the vein. The end of the catheter was trimmed to form a blunt bevel and then inserted 2.5 cm into the vein. It was tied firmly in place and then into the underlying muscle as for the carotid catheter. Both catheters were passed subcutaneously to the back of the neck by means of a hollow needle. The skin was sutured and rats received ampicillin (125 mg) subcutaneously.

iii) Performance of carotid and jugular catheters. Carotid and jugular catheters were not a success for longterm recording and infusion. There were three main problems.

a) Removal of venous catheters. Jugular catheters were frequently pulled out by tangling in bars of the cage beneath the food hopper. This was avoided by a metal barrier which prevented access to this part of the cage.

b) Occlusion of arterial catheters. In order to maintain patency both arterial and venous catheters were flushed with heparin. Blood was allowed to flow back and the deadspace of the arterial catheter was filled with fresh heparin (1000 units/ml) daily. The venous catheter was flushed with 0.04 ml of heparin (1000 units/ml) so that the rat received systemically 40 units of heparin/day. In spite of this, carotid type 1 catheters remained patent for only 5 days on average after implantation while type 2 catheters remained patent for only 3 days on average. Pulse pressure was reduced, sometimes gradually, during this time.

To prolong patency, different concentrations of heparin were tested and the number of daily flushings increased. In one experiment, arterial catheters were filled with 5000 units/ml of heparin instead of 1000 units/ml. In another,

catheters were flushed three times daily instead of once. The first increased the duration of patency to 7 days on average for both types of arterial catheters, but also increased the incidence of haematomas. Interestingly, with the second, the catheters became blocked earlier rather than later.

c) Macroscopic kidney damage. The third and perhaps most serious problem was the effect of carotid artery catheters on the kidney. Postmortem examination showed that in some rats there was macroscopic damage to the kidneys, in particular small areas of infarction. In 9 rats implanted with type 1 carotid catheters, 3 showed damage to the kidneys; 2 of 7 rats bearing type 2 carotid catheters had damaged kidneys, but 6 rats bearing jugular catheters only did not have damaged kidneys. Thus, the arterial catheter was sometimes damaging the kidneys. Daily flushing may have dislodged clots formed at the catheter tip.

My general conclusion from this experiment was that carotid and jugular catheters may be suitable for short-term experiments (a possibility I have not tested) but they were not suitable for the longterm infusion studies I had in mind.

2.2:2 Aortic catheterisation

Attention now turned to aortic catheters. The method was modified from the technique of Weeks and Jones (1960) (Browning, Ledingham and Pelling 1970).

i) Construction of catheters. The catheter consisted of 4 pieces of polythene, A,B,C and D welded together by heating and had 3 anchor points used to secure the catheter to the tissues by sutures. Figure 2.2 shows a catheter. The manufacture and materials used in these catheters are detailed in Appendix 2.

ii) Testing of the catheters. The constructed catheter was tested under pressure by sealing the end of part A (Figure 2.2) and then forcing saline into the catheter through a syringe with a filed down serum 5 needle inserted into

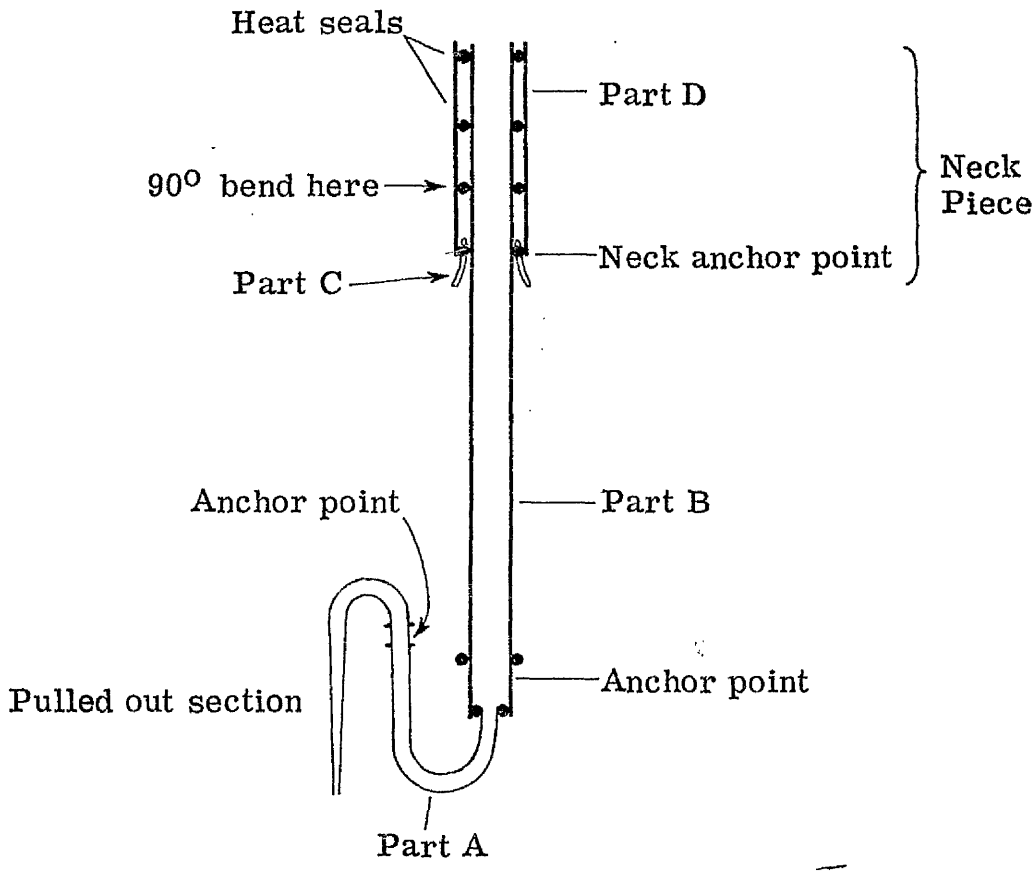


Figure 2.2 Aortic catheter. Materials used in the construction of the catheter are detailed in appendix 2.

the top of the catheter. If there was any leakage, the catheter was discarded.

The main points of difference from the earlier published methods (Browning et al 1970) are that this aortic catheter has an additional anchor point on part A to aid securing and it has been bent at the neck, so that the catheter emerges from the neck of the rat at right angles to the neck.

2.2:3 Vena caval catheterisation

The vena caval catheter described here is new but was developed in consultation with Ms. Susan Gofford from Professor Ledingham's Department, The London Hospital.

Construction of catheters. The vena caval catheter, like the aortic catheter, also consisted of 4 pieces of polythene, welded together by heating. Figure 2.3 shows a constructed catheter. The construction of the catheter and the materials used in its making are detailed in Appendix 3.

2.2:4 Implantation of aortic and IVC catheters (Figure 2.4)

Catheters were sterilised overnight in a 5% hibitane/alcohol solution. Before implantation, they were soaked in sterile saline to remove traces of antiseptic solution. Instruments used for the operative technique were sterilised by boiling.

Rats were anaesthetised with ether. Before the operation the abdomen and a small area at the back of the neck were shaved and cleaned with 5% hibitane/alcohol solution. An incision was made from pubis to xiphisterum along the midline through the skin and body wall to expose the gut. The gut was reflected to the right and covered with sterile saline soaked gauze. The aorta was carefully exposed with the fingers, removing any connective tissue and fat. The psoas muscle to the left of the aorta was cleared of fat and connective tissue.

The aortic catheter was positioned approximately as shown in Figure 2.4. A sharpened guide needle was passed through the psoas muscle to the subcutaneous tissue of the back. The needle was then turned and passed

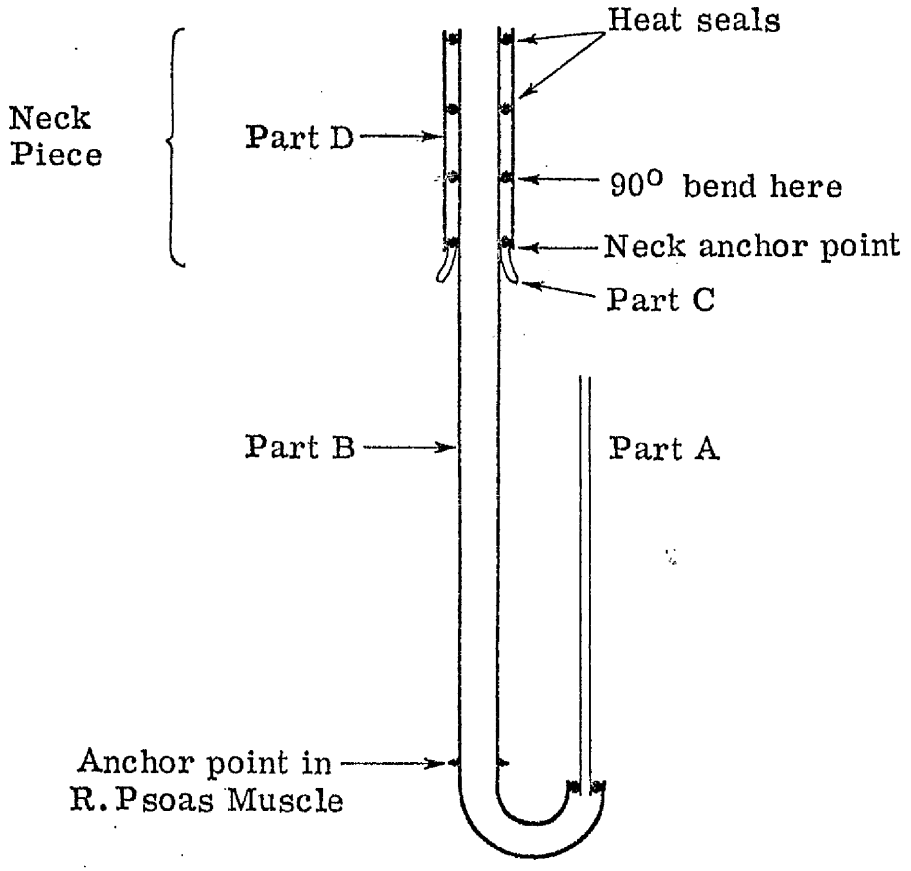


Figure 2.3 Vena caval catheter. Materials used in the construction of the catheter are detailed in appendix 3

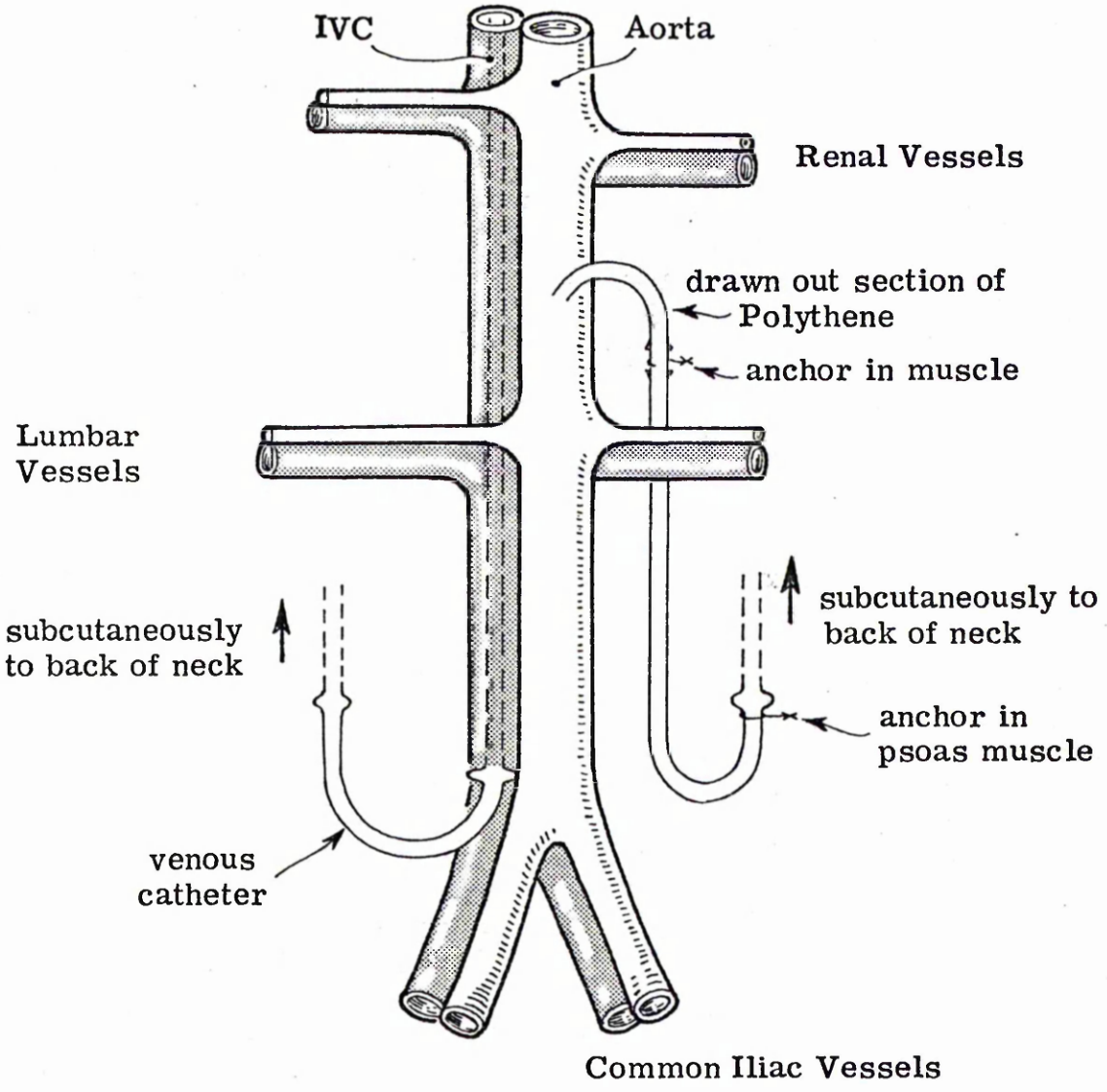


Figure 2.4 Position of aortic and vena caval catheters

subcutaneously up the back of the rat and brought to the surface at the back of the neck between the ears. The top of the catheter (neck piece) was pushed onto the end of the guide needle. A long thread was tied to the anchor point at the bottom seal of the aortic catheter. The guide needle and attached catheter were then pulled carefully through to the neck. Care was taken not to pull any intra-abdominal structures, e.g. ureter, into the muscles of the posterior abdominal wall. The needle was removed and the catheter positioned so that it lay flat on the posterior body wall. It was anchored into the psoas muscle using the attached thread. The left lumber vein was then freed from the body wall and the top of the S-bend was passed under it, so that the final straight portion of the catheter was lying on top of the aorta and parallel to it (Figure 2.4). A thread was attached to the anchor point on part A of the catheter and was then tied into the muscle. The free end of the catheter was trimmed to form a bevel of about 45 degrees at a point 5 mm above the aortic bifurcation. A 1 ml syringe attached to a 15 cm length of polythene tubing (I.D. 0.58mm; O.D. 0.96mm) was connected to the end of the catheter by a piece of filed needle tubing (serum V) and the tubing and catheter filled with sterile saline. The catheter was now ready to be inserted.

The aorta was occluded above the level of the renal arteries. Using a number 20 gauge sterile hypodermic needle, a hole was made in the aorta 3-5 mm below the level of the renal veins. Care was taken not to pierce the posterior wall of the aorta. This was best achieved by previously bending the number 20 needle in a right angle about 5 mm from the end of the needle with the bevelled tip of the needle on the side away from the hub of the needle, thus  . The needle was removed and the free end of the catheter inserted through the hole into the aorta and gently fed down the vessel. The aorta was released slowly and if any bleeding occurred through the site of insertion, pressure was gently maintained until a clot had formed. In practice there was little bleeding.

The bifurcation of the inferior vena cava into the iliac veins was then carefully exposed as well as the right psoas muscle. The guide needle was passed through the right psoas muscle and then subcutaneously to the back of the neck as in the aortic catheterisation procedure. A thread was tied to the anchor point of the IVC catheter. The catheter was then pushed onto the guide needle and pulled carefully through to the neck. A 1 ml syringe attached to a 15 cm length of polythene tubing (I.D. 0.58, O.D. 0.96 mm) was connected to the end of the catheter by a piece of filed needle tubing (serum V) and the tubing filled with sterile saline. The tip of the catheter was trimmed to 5 cm and a straight edge left at the tip. The catheter was then gently pulled back through the muscle until the tip was lying just above the bifurcation of the IVC. A number 20 gauge hypodermic needle was bent as previously described. A hole was made in the IVC at the bifurcation and the tip of the catheter was introduced into the hole. The catheter was carefully pulled back through the muscles at the back of the neck and in doing so the tip was carefully passed down the IVC until the A-B seal was lying at the hole in the bifurcation. (Postmortem studies showed that when 5 cm tubing was introduced into the IVC in this way, the tip lay between the renal veins and the right atrium). The gut was replaced and the abdominal musculature sutured. Antibiotic (polymyxin, bacitracin, neomycin dispray; Stuart Pharmaceuticals) was applied to the sutures and the skin was closed.

The rat was turned over and from the point of exteriorisation of the catheters, the skin was incised longitudinally until the neck anchor points on the catheters were exposed, usually 1.5 to 2 cm. A suture was tied round this anchor point and tied into the muscles of the neck, thus preventing the rat from pulling the catheters out of the aorta or the IVC. A second suture was tied round the 90^{0} bends and this too was tied into the muscle. Antibiotic was applied to the wound and the skin sutured.

The end of the catheters were plugged with 1 cm lengths of stainless

steel wire (0.7 mm diameter) which had been carefully rounded at one end to prevent damage to the catheters. Rats received ampicillin (125 mg) subcutaneously.

The day after implantation, the catheters were filled with 0.04 ml of 1000 units/ml heparin.

2.2:5 Performance of aortic and IVC catheters.

Aortic and IVC catheters proved to be highly successful. Patency was maintained by daily flushing of catheters. 0.04 ml of sterile saline was injected first, followed by 0.04 ml heparin (1000 units/ml) in the aortic catheter and 0.05 ml of heparin (1000 units/ml) in the venous catheter.

During my first year of work there was considerable variation in the duration of catheter patency. However, with time most catheters remained patent for at least 3 weeks. Of the last 30 rats catheterised with the intention of prolonged infusion, 20 were satisfactory for infusion and recording for 2-3 weeks after implantation of catheters; 7 were satisfactory for 1-2 weeks after implantation and 3 for 0-1 week after implantation. Postmortem studies in 28 rats showed no macroscopic damage to the kidneys in marked contrast to the results obtained earlier with carotid and jugular catheters.

Aortic and IVC catheters are thus clearly suitable for longterm infusion studies with measurement of arterial pressure.

2.3 MOBILITY OF CATHETERISED RATS IN THEIR CAGES

2.3:1 Small restraining cage.

Previous studies involving infusion of substances and measurement of arterial pressure in rats have used small restraining cages (Riegger, Lever, Millar, Morton and Slack 1977). Most commonly the cages are perspex cylinders which are adapted to prevent movement of the rat in any direction. This method, perhaps suitable for short-term studies, is clearly not suitable for prolonged infusion studies. I needed a technique where the rat would be less restrained.

2.3:2 Spring and balance system.

This part of the technique was developed by Dr Jorge Casals-Stenzel of Schering AG in Berlin. Before starting work in Glasgow, I spent one month with him in Berlin learning the technique.

After implantation of catheters, rats were allowed a period of acclimatization in metabolic cages (Techniplast (R), Forth-Tech Services Ltd. Midlothian, Scotland) (Figure 2.5). A 10.5 cm length of perspex cylinder was added to the top of the standard cage to raise its height to 56.5 cm. This allowed the rat to stand on its hind legs without reaching the upper rim. The cage was closed by a perspex lid with a 10 cm hole in its centre.

After the period of acclimatization, rats were connected to the spring and balance system (Figure 2.5). This comprised a 25 cm length of stainless steel spring through which passed extensions of catheters connecting the rat with blood pressure recorder and infusion pump. The spring allowed freedom of movement, prevented tangling and protected the catheters. 60 cm lengths of polythene tubing were used for the arterial extension (I.D. 0.58mm; O.D. 0.96mm); 120 cm lengths of tygon tubing (I.D. 0.5mm; O.D. 1.53mm) for the venous extensions; 2.5 cm lengths of stainless steel needle tubing joined catheters as shown in Figure 2.6. A 3.0 cm length of silastic tubing fixed to the end of the spring and fitting snugly over the connection, lay flush with the neck and served to protect the catheters at a potentially vulnerable point. Arterial and venous extension catheters were filled with heparin (1000 units/ml) and 5% dextrose, respectively. The spring was attached to a metal rod, pivoted at its mid-point to a vertical stand 9 cm higher than the cage top. The weight of the spring and attached rat were counterbalanced by screwed weights (Figure 2.5). Thus, when the rat stood, the spring and catheters rose. When not in use for recording, but when connected to the rat, the arterial and venous catheters, emerging from the top of the spring were plugged, coiled and taped. Otherwise the arterial catheter was connected to a pressure transducer for blood pressure

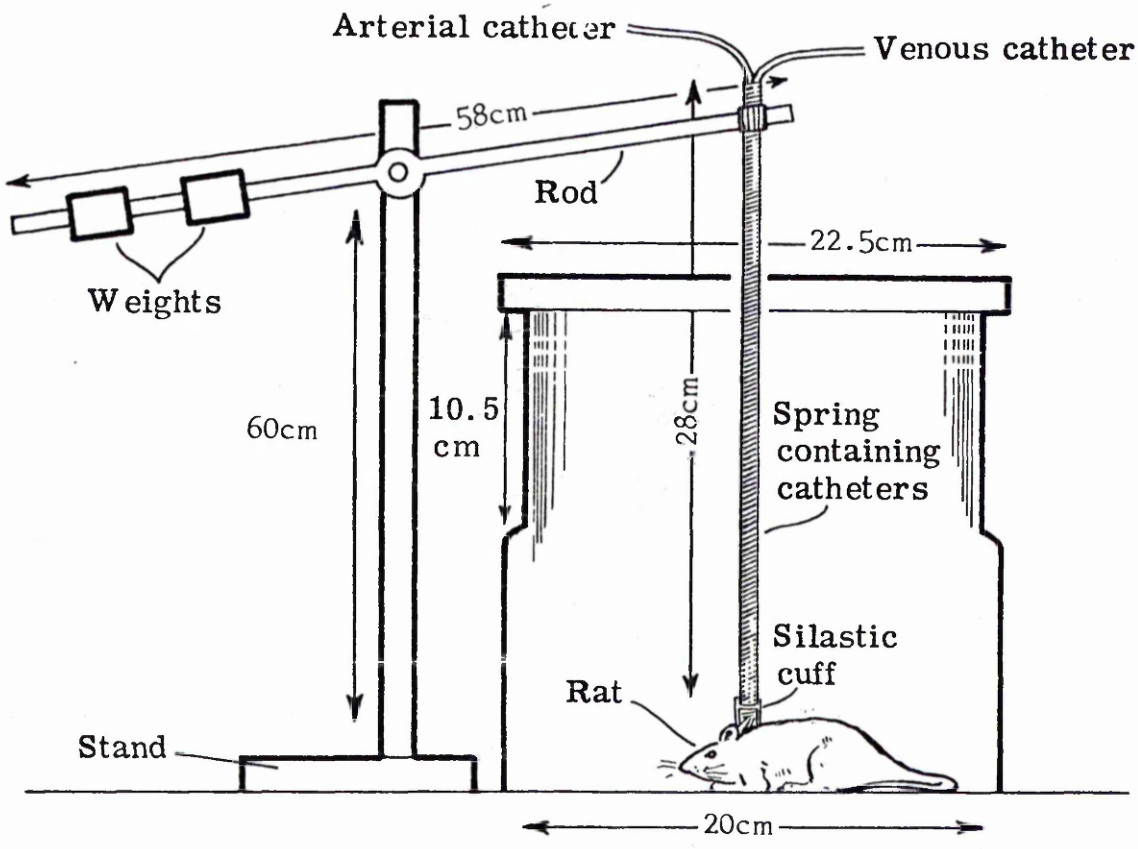


Figure 2.5 Upper part of metabolic cage with the spring and balance system. The metabolic details are given later in Figure 2.8 Brief details : rat's catheters are connected to elongated catheters, which are protected by the spring.

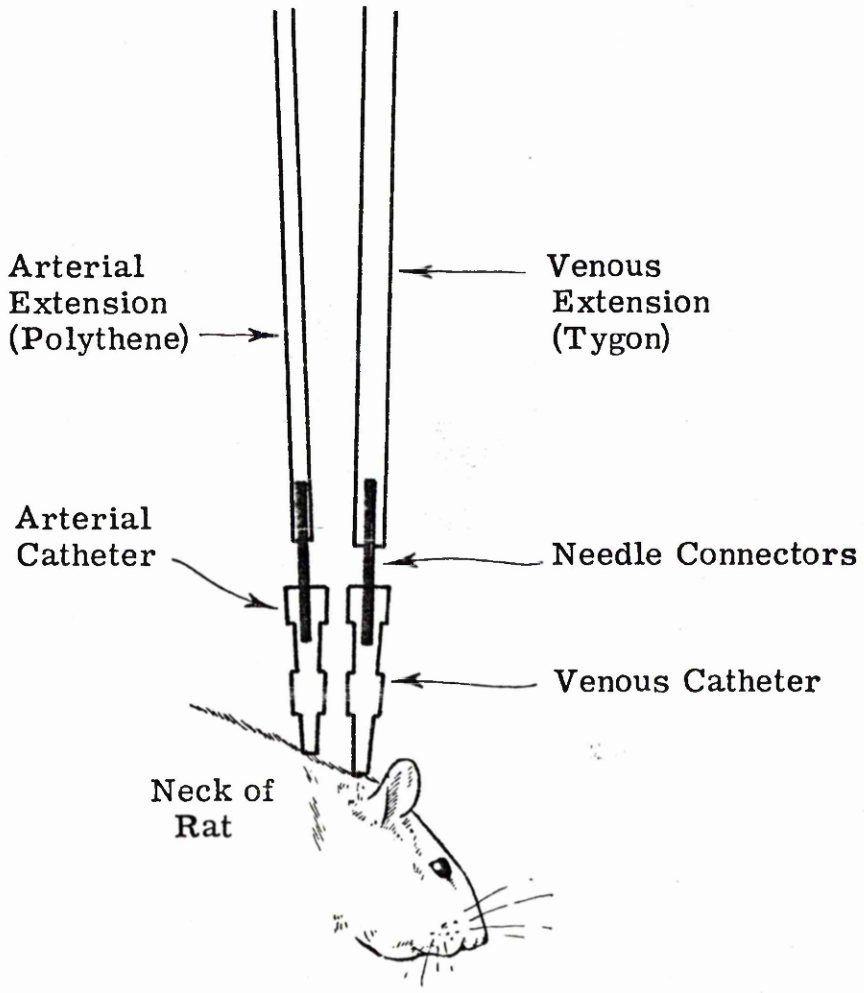


Figure 2.6 Details of connection of rat's catheters to elongated catheters.

recording and the venous catheter to a pump for continuous infusion. A plastic swivel at the top of the spring prevented tangling and twisting of the spring.

2.3:3 Connection of rat to spring and balance system.

In initial studies, rats were connected to the spring balance system under light ether anaesthesia. The catheters were flushed as previously described and the extensions, filled with heparin and 5% dextrose, were connected by needle tubing. The extensions were threaded through the spring until the silastic tubing lay flush with the neck of the rat. The rat was then placed in the metabolic cage and the spring attached to the pivoted rod. In later studies, rats were conscious during connection.

2.3:4 Comparison of small cages and large cages.

Small cylindrical cages, while suitable for short-term infusions are clearly unsuitable for infusions lasting longer than 12 hours. The act of restraining may impose considerable stress on the animal and there may be more difficulty in maintaining steady state conditions. I tested the effect of different cages on the level of arterial pressure. Six rats with aortic and IVC catheters were placed in small cylindrical cages 5 days postoperatively and arterial pressure measured for 30 mintues. A further 6 rats were placed in large cages 5 days postoperatively and mean arterial pressure measured for 30 minutes. Figure 2.7 shows the effect on pressure. The 6 rats in small cages had a mean pressure of 120 $\pm$ 2.8 mmHg while those in the large cages had a mean pressure of 106.6 $\pm$ 2.6 mmHg.

2.4 MEASUREMENT OF ARTERIAL PRESSURE AND HEART RATE.

Arterial catheters were connected to Elcomatic EM 750/751 transducers via a length of polythene tubing (800/100/200 Portex) and needle tubing (Star Everett serum V). The transducer was connected to EM 720 pen recorder (Elcomatic, Kirktonfield Road, Neilston, Glasgow G78 3PL) and calibrated against a standard mercury manometer. A damping mechanism was built into the recorder circuit to give a true mean arterial pressure. Heart rate was

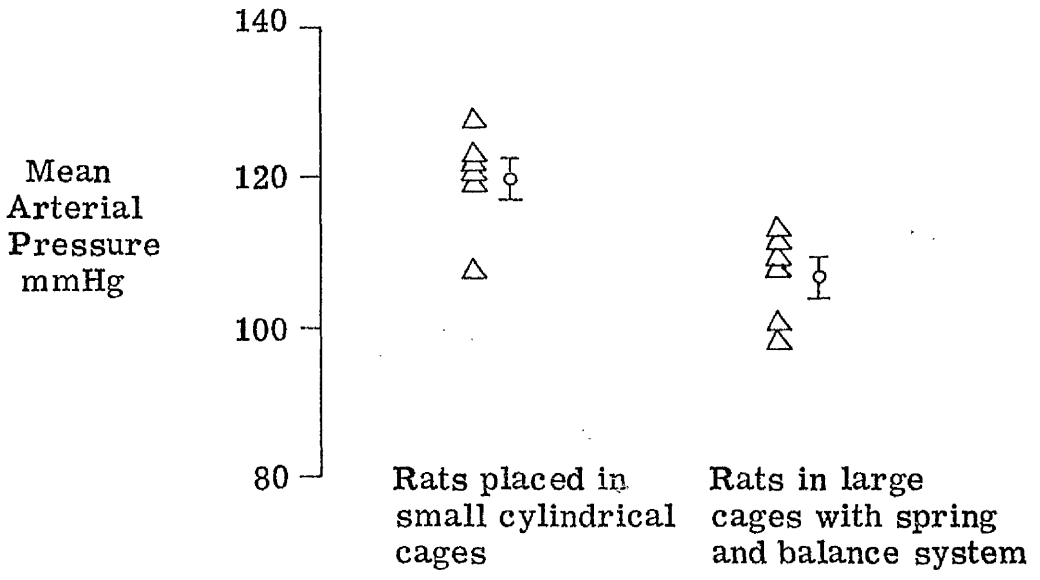


Figure 2.7 Comparison of large and small cages. Mean arterial pressure (MAP) in six rats placed in small cylindrical cages compared with MAP in six different rats placed in large metabolic cages with spring and balance system ($t = 3.5$ $p<0.01$)

measured during recording of arterial pressure by speeding up the trace until the individual pulse waves were visible and countable.

2.5 CONTINUOUS INTRAVENOUS INFUSION

Continuous intravenous infusion was given by a Dascon pump (Table 2.1) with a variable flow rate. Usually I chose a rate of 2.4 mls per 24 hours. Details of the reagents used for infusion are given in Table 2.1

2.5:1 Angiotensin II

Asp^1-Val^5-angiotensin II amide was dissolved in 5% dextrose, for stock solutions, at a concentration of 10µg/ml. It was frozen in aliquots and stored at -20°C until used. Longterm infusions were diluted from the stock to a concentration of 2.4µg/ml. Fresh infusate was made up daily.

The potency of stock solutions and the daily infusates were tested at the end of the experiment by bioassay in the anaesthetised rat using freshly prepared standard angiotensin II, as described by Peart (1955). With this method, no solution tested showed deterioration greater than 10%. I did these assays myself.

2.5:2 Saralasin.

Saralasin was supplied in two forms (1) in solution and (2) in powder form (Table 2.1).

Solutions were frozen at -20° and a new vial defrosted as needed. Saralasin in powder form was stored in an air-tight, moisture-free, container. The saralasin was then placed in another container filled with dry Rite as saralasin is hydroscopic. It was stored at room temperature (15-30°C) and protected from light. Once dissolved it was frozen at -20°C.

Saralasin was tested in conscious rats. Firstly control intravenous injections of angiotensin II (50 or 100 ng) were made and the pressor response noted. Saralasin was then infused at the rate used in experiments for 2 hours and the angiotensin II intravenous injections repeated.

2.5:3 Renin inhibitor (Table 2.1)

Table 2:1 Pumps used and solutions to be infused.

Dascon Pumps. Model 300, Uden, Holland.

Angiotensin II - Hypertensin^{R} - Ciba,
Ciba Laboratories, Horsham, West Sussex, England.

Saralasin, Norwich-Eaton Pharmaceuticals,
Norwich, New York, 13815

Renin Inhibitor, H77 - provided by Dr M. Szelke,
University of London.

Renin inhibitor H.77, a peptide analogue of renin substrate, was stored at -20°C. The inhibitor was allowed to defrost at room temperature for 30 minutes before being weighed out. Ability of the analogue to inhibit renin was tested in vitro by Dr Brenda Leckie using a system based on the antibody trapping method of Poulson, Burton and Haber, (1973) in which plasma renin reacts with angiotensinogen at pH 7.0 to produce AI. I did not do these tests myself.

2.6 METABOLIC BALANCE TECHNIQUE

Sodium balance was measured in some of the experiments and the following techniques were used.

2.6.1 Metabolic cages.

Rats were maintained in the modified metabolic cages described earlier - Figure 2.8. Rats sat on a supporting grid through which the urine and faeces fell. The latter were then separated automatically by a funnel into different containers. Drinking water was accessible from a bottle at the side of the cage, overflow being collected in a further container. Food was accessible from a detachable food hopper. The cage was designed so that the rat had to crawl into the food hopper to obtain a powdered diet. However, because of the spring and catheters, vertical bars were specially fitted between the cage wall and the food hopper to prevent rats entering the hopper and tangling their catheters.

2.6:2 Food and water.

Rats were fed on a pellet food diet (Oxoid Ltd. Modified Diet 41B) whose composition is given in Table 2.2. Diet 41B contained 1mEq sodium per 10 grammes food. On average a rat consumed 1.5-2.5mEq sodium per day. Rats drank tap water which, in Glasgow, contains less than 0.1 mEq sodium/l.

2.6:3 Daily balance studies.

Rats were allowed to become familiar with the balance procedure for 3 days before implantation of catheters and then for a further 3 days after

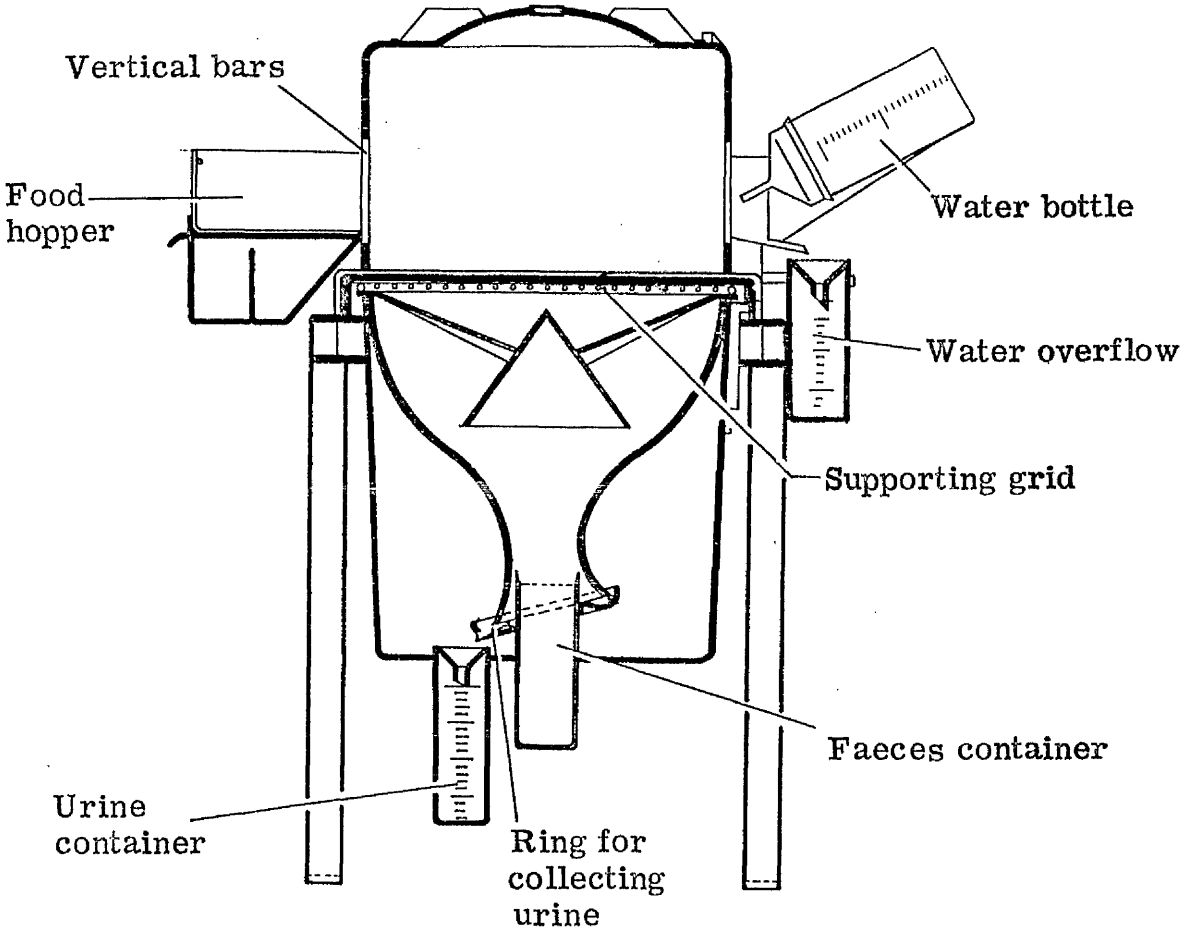


Figure 2.8 Details of metabolic cage. Rats occupy upper compartment with grid as floor. Faeces and urine pass through grid to separate containers. Rats have separate access to food and water with a trap for water overflow.

Table 2:2 Composition of pellet food diet, 41B.

Ingredients	% Composition	Vit.suppl	Per kilo diet.
Sussex ground oats.	40	Ca-D-pantothenate	0.5
Wholemeal flour	46	Vitamin B12	3.4
White fish meal.	8	Choline Chloride	25
Dried yeast	1	Vitamin E	1.2
Dried skimmed milk	3	(alpha Tocopherol Acetate)	
Mineral Supplement	1	Vitamin K	
Vitamin supplement	1	(Menadione)	0.5

Calculated nutrients.	%
Crude protein	15.91
Crude fibre	5.05
Oil (ether extract)	3.17
Calcium	0.85
Phosphorus	0.67
Chlorine	0.34
Sodium	0.23
Potassium	0.50

Mineral supplement (calculated elementary analysis)

	%
Calcium	21.55
Chloride	18.21
Sodium	11.79
Phosphorus	3.58
Magnesium	1.19
Urea	0.93
Manganese	0.026
Copper	0.023
Iodine	0.023
Cobalt	0.006
Potassium	0.006

Vitamin supplement.	per kilo diet
Vitamin A	3928 I.U.
Vitamin D3	982 I.U.
Thiamine	0.5 mgm
Riboflavin	1.5 mgm
Nicotinic acid	2.5 mgm

implantation. The balance technique was as follows: at 11.00 a.m. water bottles were weighed to determine water intake during the previous 24 hours. The water overflow was also weighed but in practice overflow was less than 1% of intake. The water bottle was emptied, refilled with fresh tap water and reweighed. A clean empty overflow container was weighed. The food hopper was removed and weighed and any spilled food swept up and weighed - this was never more than 0.2g from an average daily intake of 16 grammes. The bottom part of the cage was removed and replaced by a clean cage. The cage was rinsed with distilled water and the washings added to the urine specimen before photometry. Urine and faeces containers were removed and replaced with clean weighed containers. The faeces were separated from any food which had fallen into the faeces container. The spilled food was weighed. The faeces were then homogenized in 100 ml of distilled water. An aliquot of the homogenate was centrifuged (2000g for 20 minutes) and the supernatant stored.

2.6:4 Electrolyte determination.

The sodium concentration of the urine and faeces were determined with an IL Flame Photometer. I have done all the extractions and photometry myself and the coefficient of variation for 60 replicate measurements of the same sample of urine was 0.7%

2.6:5 Calculations.

The intake of sodium was calculated on the basis of daily food consumption. The sum of urinary and faecal sodium excretion were taken as the total excretions. Balance was calculated as (weight of food x sodium concentration) - sodium excretion.

2.7 MEASUREMENT OF ANGIOTENSIN II BY RADIOIMMUNOASSAY.

Angiotensin II plasma levels were determined using a modification (Morton, Casals-Stenzel, Lever, Millar, Riegger and Tree 1979) of a radioimmunoassay for angiotensin II in the rat (Powell-Jackson and MacGregor 1976). In some experiments the individual plasma concentrations of angiotensin

II and angiotensin III were determined separately following chromatography (Semple & Morton 1976).

2.7:1 Collection of samples.

Rat arterial blood (1 ml) was drawn rapidly into an ice cold syringe containing 0.1 ml of an aqueous solution of 0.1 M ethylenediaminetetra-acetic acid (disodium salt) and 0.05M o-phenanthroline and were immediately chilled in ice. This solution completely inhibits rat plasma converting enzyme and angiotensinases (Powell-Jackson and MacGregor 1976) and was used with arterial blood in a ratio of 1:10. Blood samples were centrifuged as soon as possible, in practice within 5 minutes, at $5^{o}C$ and 2000g for 20 minutes and the plasma stored at $-20^{o}C$ to await extraction.

I did not do many of the radioimmunoassays reported here, but I know the principle of the technique and have assayed a few samples.

2.8 PREPARATION OF RENAL HYPERTENSIVE ANIMALS

Rats were made hypertensive by constriction of the left renal artery with a 0.007" or 0.008" silver clip, the right kidney being left in situ (one-clip, two-kidney hypertension).

2.8:1 Construction of renal artery clips.

Clips were constructed from a strip of silver (0.38mm x 1.0mm). A 1 cm length of silver was cut from the strip and the edges filed until smooth. The piece was then bent at its mid-point around a feeler gauge using artery forceps to the required size, 0.007" or 0.008". One end was then bent upwards at an angle of 45^{o} to be used for holding during implantation and the clip's gap was checked once more. Clips were sterilized in hibitane/alcohol solution overnight and then soaked in sterile saline before implantation.

2.8:2 Clipping of renal artery

Rats were anaesthetised with ether, the abdomen shaved and cleaned with hibitane/alcohol solution. An incision was made from 2-3 cm above the pubis to the xiphisterum in the midline through the skin and abdominal wall to

expose the gut, which was reflected to the right and covered with saline soaked gauze. The left renal artery was exposed and cleared of connective tissue. The clip was placed on the artery. If the kidney blanched, the clip was removed and a smaller clip (0.007" or 0.006") applied. The gut was then replaced and the abdominal musculature sutured. Antibiotic was applied topically and the skin was closed. Rats received ampicillin (125 mg) subcutaneously.

2.8:3 Measurement of systolic blood pressure.

Systolic blood pressure was measured weekly, following application of the clip, by tail plethysmography using a W & W 8005 recorder (Wand W Electronic Instruments, CH-4002 Basel, Munchenstein, Switzerland). The rat was warmed in an incubator at 37°C for 20 mins. It was wrapped in a towel with its tail protruding. The pressure cuff was pushed over the tail up to the root, followed by the pulse detector. Figure 2.9 shows the arrangement of cuff and detector and two recordings of systolic pressure.

2.9 STATISTICAL METHODS

Student's t-test was employed for the direct comparison of means. The non-parametric Mann-Whitney U-test was also used to assess statistically significant differences between groups.

Dose-response curves were represented by the regression line of response on the logarithm of the dose. Differences between the slopes of dose-response curves were analysed by F-test.

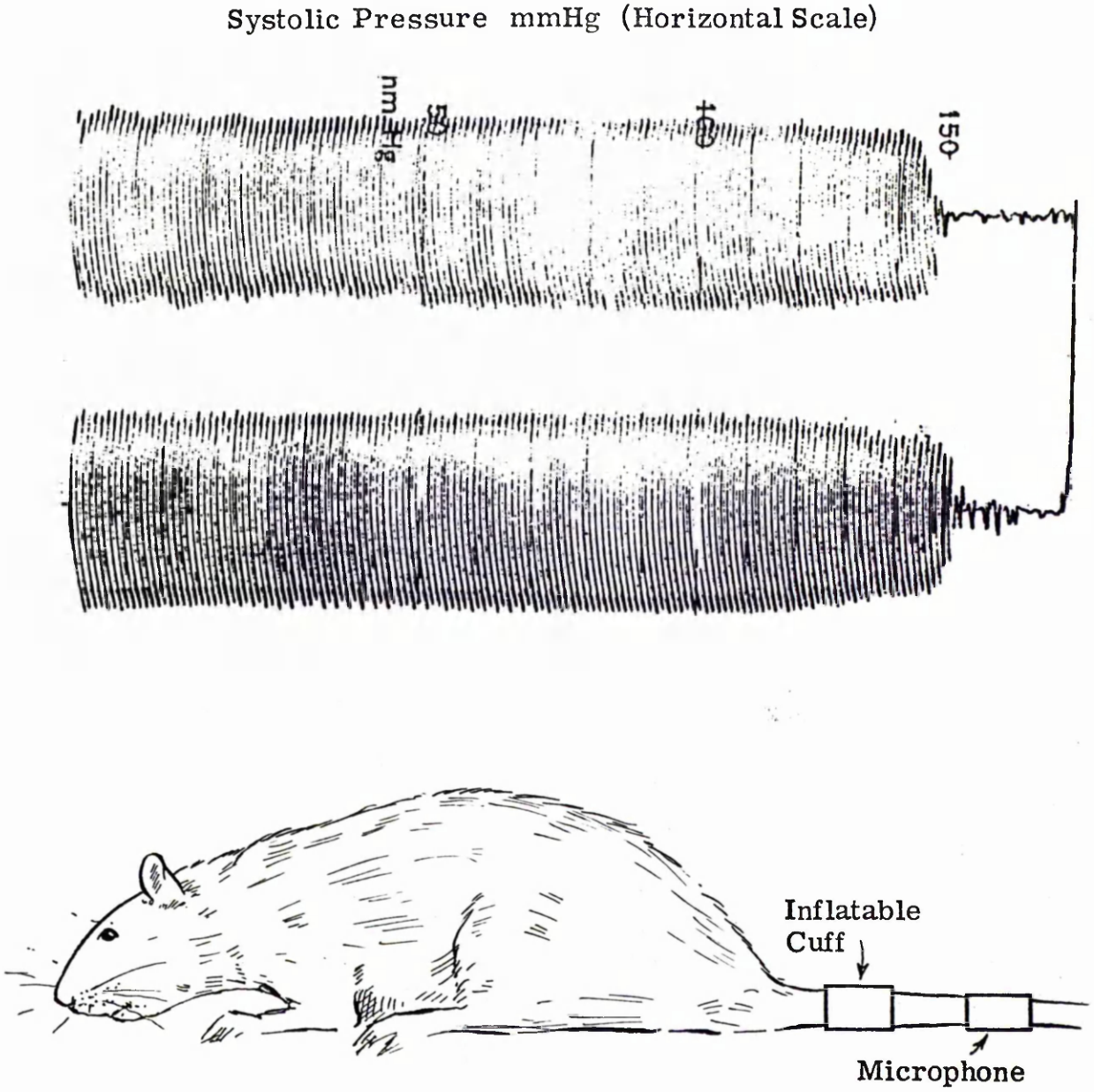


Figure 2.9 The position of the pen recorder on the horizontal scale is determined by cuff pressure. The pen moves across the calibrated paper during deflation of the cuff. Arterial pulsation in the vertical plane is recorded by the microphone. Systolic pressure is the point on the horizontal scale at which pulsation ceases.

CHAPTER 3

SLOW PRESSOR EFFECT OF ANGIOTENSIN II IN THE RAT.

3.1 INTRODUCTION

Angiotensin II raises blood pressure rapidly by direct vasoconstriction (DeBono et al 1963) and gradually when given by constant infusion at a dose below the threshold of the direct pressor effect. This slow pressor action has been shown in dogs (Bean et al 1979; Cowley and McCaa 1976; Cowley and DeClue 1976; McCubbin et al 1965), rabbits (Dickinson and Lawrence 1963; Dickinson and Yu, 1971) and man (Oelkers, Schoneshofer, Schultze and Bauer 1978; Ames et al 1965) and has been implicated in the pathogenesis of chronic renal hypertension (Dickinson and Lawrence 1963; Brown, Fraser, Lever, Morton, Robertson and Schalekamp 1977). Its mechanism is uncertain, but a nervous element is likely (Ferrario, Gildenberg, and McCubbin 1972), resetting of baroreceptor reflexes and increased cardiac output also possibly contributing (Cowley and McCaa 1976).

Our purpose here was to examine the slow pressor effect of angiotensin II in the conscious rat, to compare it with the maximum direct pressor response and to relate both to concurrent plasma concentrations of angiotensin II. Sodium balance was also measured since angiotensin II sometimes produces sodium retention (DeBono et al 1963) and the gradual rise of pressure is enhanced by increased dietary sodium (Cowley and McCaa 1976). Water intake was measured as well, as the dipsogenic effect of angiotensin occurs at low dose and may be important physiologically (Mann, Johnson and Ganten 1980).

3.2 EXPERIMENT 1

EFFECT OF PROLONGED ANGIOTENSIN II INFUSION ON ARTERIAL PRESSURE, SODIUM BALANCE, WATER INTAKE AND THE RESPONSE TO BRIEF INFUSION OF ANGIOTENSIN II.

Eight groups of rats were studied (Table 3.1).

3.2:1 Experimental design (Groups 1 and 2)

Before implantation of catheters, all rats were placed in metabolic cages for 3 days for acclimatization. Aortic and IVC catheters were implanted on day 4 using methods described earlier (Chapter 2.2:4). Rats were allowed to recover in ordinary rat cages on day 5 (Figure 3.1). On day 6 they were returned to their metabolic cages for acclimatization. The experiment began on day 9 when they were connected to the spring and balance under light ether anaesthesia. 5% dextrose in water was infused at 2.4 ml/24 hours in all rats from day 9 until day 11. Rats in group 1 then received angiotensin II at 20 ng/kg/min for 7 days until day 18, when 5% dextrose was again infused until day 21. Rats from group 2 received 5% dextrose throughout (Table 3.1 and Figure 3.1).

Sodium balance. Sodium balance was measured from the 6th to the 21st day as described in Chapter 2 (2:6).

Dose-response studies. Studies testing the direct pressor effect of angiotensin II were carried out in rats of group 1 and 2 on days 11,17,18 (Figure 3.1). In each study, angiotensin II in 5% dextrose was infused at successive rates of 30,90,270 ng/kg/min each for one hour. Mean arterial pressure was measured continuously for one hour before commencement of the infusion and during the infusion. Average basal pressure was determined by measuring the pressure from the trace at 5 minute intervals, the mean basal pressure was then calculated as the mean of 12 readings. During infusion, blood pressure was measured from the trace at 5 minute intervals and the mean of the resultant 12 measurements used to calculate the change of pressure at each rate of infusion.

Table 3.1 Groups of rats and experimental protocol.

Group number	n	Protocol
1	9	Rats received dextrose for 3 days, then AII at 20 ng/kg/min for 7 days; and finally dextrose for 2.5 days.
2	9	Rats received dextrose for 12.5 days.
3	7	Rats received brief dose-response infusions of AII up to 810 ng/kg/min.
4	6	Rats received 5% dextrose at 1 ml/hour for 1 hour and then sampling for estimation of plasma angiotensin II concentration.
5	10	Rats received AII in dextrose at 20 ng/kg/min for 1 hour - then sampling for estimation of plasma AII concentration.
6	6	Rats received AII in dextrose at 270 ng/kg/min for 1 hour - then sampling for estimation of plasma AII concentration.
7	8	Rats received AII in 5% dextrose at 20 ng/kg/min for 7 days - then sampling for estimation of plasma AII concentration.
8	6	Rats received 5% dextrose for 7 days and then sampling for estimation of plasma AII concentration.

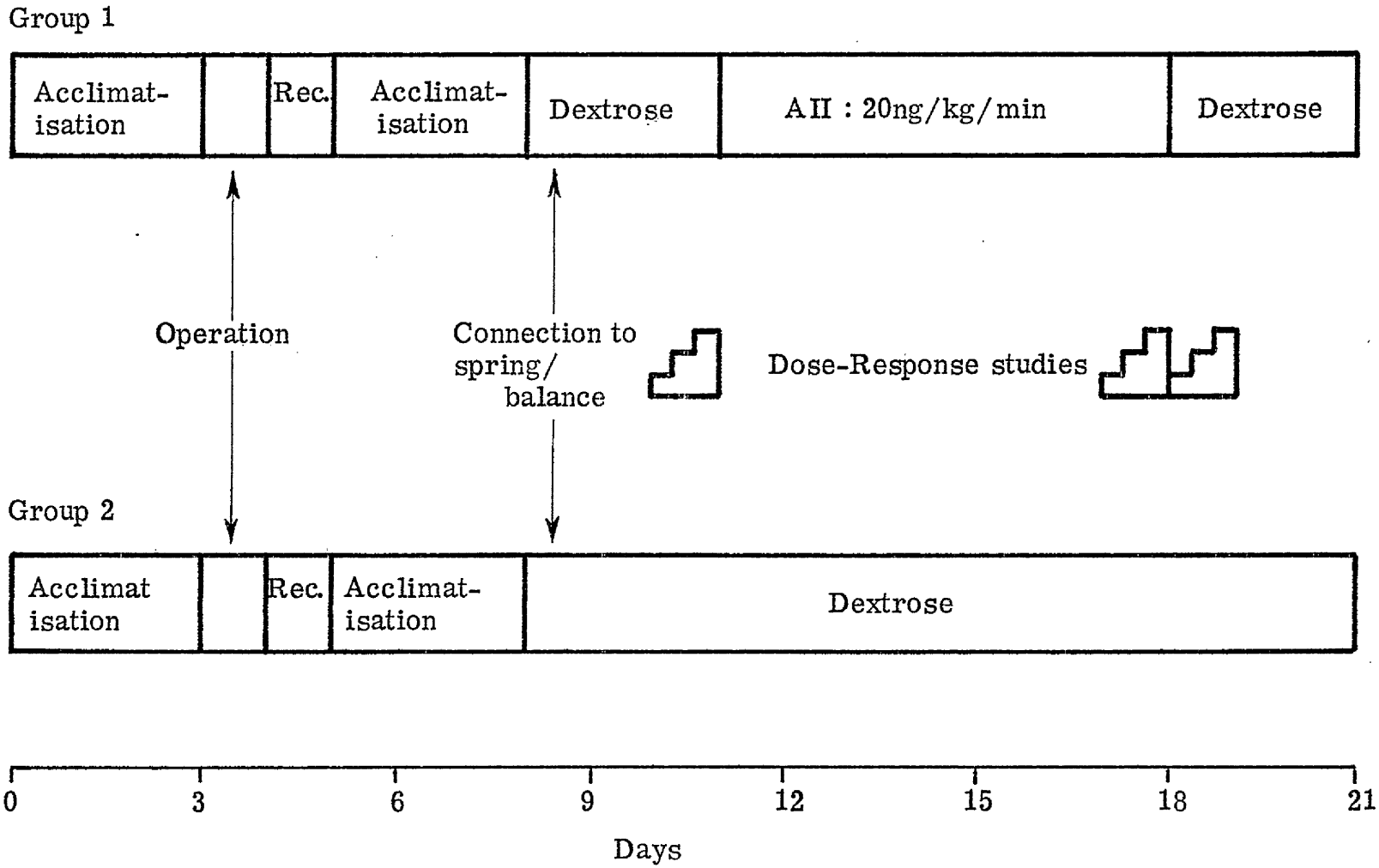


Figure 3.1 Experimental protocol. Rats in group 1 received angiotensin II in low doses Rats in group 2 received dextrose throughout as control.

A further 7 rats of group 3 not given prolonged infusion were used in a separate experiment to determine the maximum pressor response to brief infusion of angiotensin II. Catheters were implanted and rats were acclimatized as for groups 1 and 2. Angiotensin II was infused as in the dose-response studies except that an additional infusion was given at 810 ng/kg/min for one hour. Arterial pressure was recorded and expressed as before.

3.2:2 Analysis of results.

i) Mean arterial pressure (MAP) MAP was measured from day 9 until day 21. MAP was recorded for 40 minutes twice daily at 10.00 a.m. and at 2 p.m. Daily MAP was taken as the average of 80 measurements made at one minute intervals. The values quoted are the average of the daily means for all rats on a particular day.

ii) Heart rate. Heart rate was measured from day 9 until day 21, at the beginning and end of the two daily periods of mean arterial pressure recording. The daily heart rate was the mean of these measurements.

iii) Variability of blood pressure. Variability of pressure was assessed as the standard deviation for the 80 measurements made in individual rats during recording of daily mean arterial pressure and as the coefficient of variation for these measurements. The values quoted (Table 3.2) are the average of measurements in individual rats on a particular day.

3.2:3 Results.

i) Effects of prolonged AII infusion on mean arterial pressure. The effects of angiotensin II at 20 ng/kg/min on daily mean arterial pressure are shown in Figure 3.2 and Table 3.2. Mean arterial pressure did not increase during the first hour of angiotensin II at 20 ng/kg/min in rats of group 1, but on the morning of the following day it was significantly increased ($p < 0.01$, paired t-test; $p < 0.05$, Mann-Whitney U-test). Thereafter it rose progressively in each of 9 rats of group 1 reaching a peak towards the 7th day.

In a later experiment (3.3) 6 rats of group 7 were infused in the same

Table 3.2 Mean arterial pressure, standard deviation and coefficient of variation during prolonged AII and dextrose infusion in two groups of rats

		Group 1.				Group 2.			
Protocol	Day	MAP ± SEM mmHg	S.D.	C.V.%	n	MAP ± SEM mmHg	S.D.	C.V%	n
	9	108.9 ± 3.3	3.50	3.22	8	112.9 ± 2.4	4.69	4.04	7
Dextrose in both groups	10	104.4 ± 2.0	3.63	3.47	9	111.3 ± 2.4	5.42	4.93	9
	11	103.2 ± 2.4	3.19	3.09	9	106.2 ± 3.2	4.01	3.72	9
	12	118.1 ± 4.2*	6.22*	5.26	9	106.4 ± 2.2	3.69	3.48	9
Group 1:	13	129.7 ± 5.7**	6.83	5.19	9	108.2 ± 4.0	5.43	5.10	9
AII at 20 ng/kg/min	14	133.5 ± 4.4***	8.02	5.97	9	103.9 ± 1.4	4.81	4.61	9
	15	143.9 ± 2.9***	7.09***	4.93	9	107.7 ± 1.7	4.46	4.15	9
Group 2:	16	147.1 ± 4.5***	8.34*	5.70	8	109.9 ± 2.2	5.09	4.70	9
Dextrose	17	151.3 ± 5.0***	6.0*	3.95	8	111.9 ± 2.2	3.73	3.30	7
	18	152.9 ± 5.7***	5.81	3.88	8	115.4 ± 2.8	4.37	3.76	6
	19	104.8 ± 1.7	4.40	4.20	8	120.2 ± 6.2	5.38	4.51	4
Dextrose in both groups	20	106.2 ± 2.5	5.68	5.43	6	116.9 ± 9.1	4.15	3.64	2
	21	100.6 ± 2.4	4.10	4.13	4	111.9 ± 2.6	3.45	3.09	2

*p <0.05; **p <0.01; ***p <0.001; C.V.=coefficient of variation; S.D.=standard deviation.

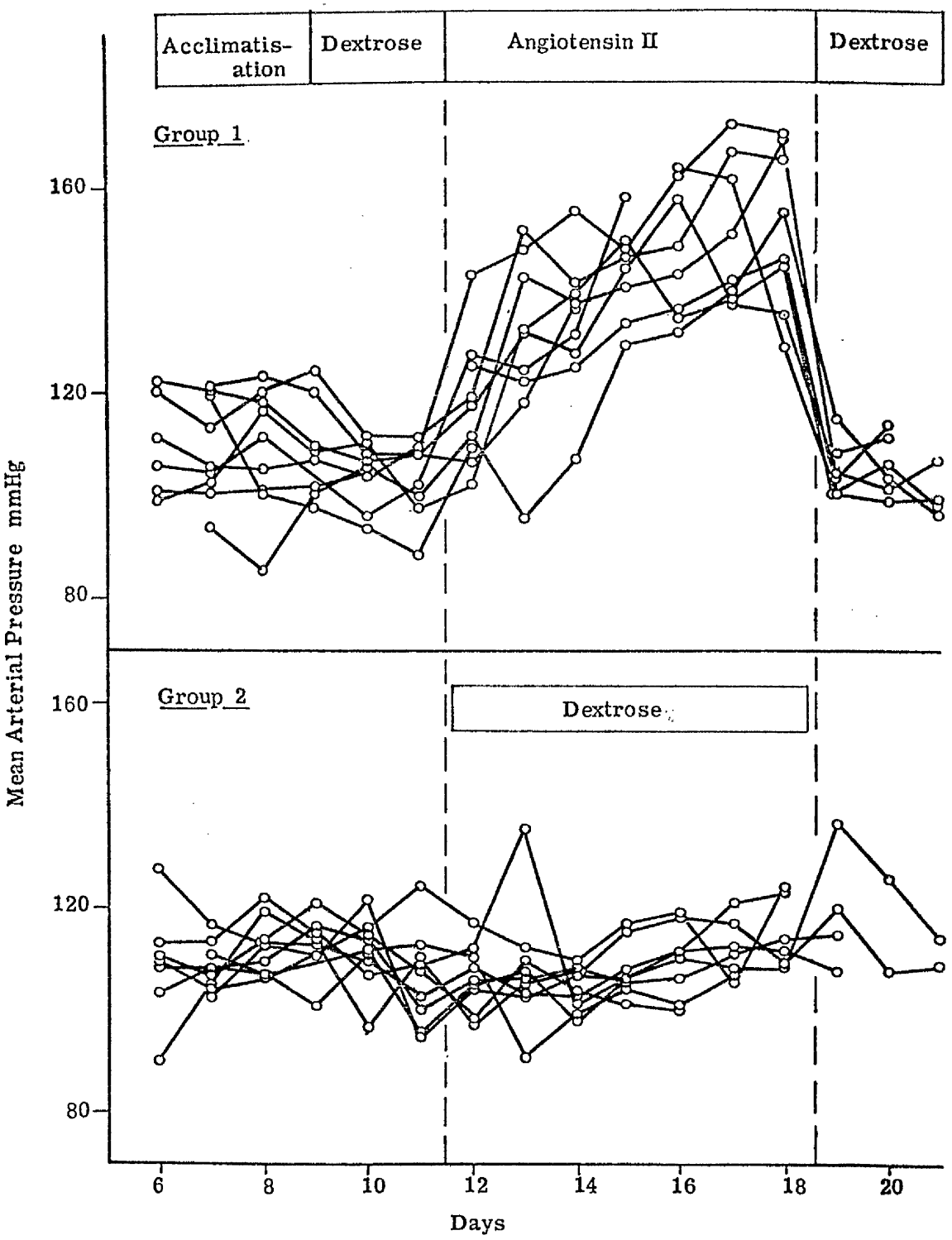


Figure 3.2 Upper Panel: Mean arterial pressure during a 7 day infusion of angiotensin II 20ng/kg/min. Lines join measurements from the same rat. Lower Panel: Measurements and infusions in 9 control rats receiving 5% dextrose instead of angiotensin II.

way in preparation for angiotensin II estimation. Blood pressure rose slowly and progressively in these also. Mean arterial pressure the day before angiotensin II was 107.5±1.1 mmHg ± SEM, in the 7 subsequent days 126.7±7.0, 141.2±6.4, 150.4±3.8, 145.8±7.4, 150.9±3.8, 156.3±3.7, 157.8±5.5 mmHg.

On returning to dextrose, arterial pressure fell in each rat of group 1, reaching control values within 4 hours. The rate of fall was faster than the previous rate of rise, but slower than the rate of fall on stopping a brief 1 hour infusion of angiotensin II at 270 ng/kg/min (Figure 3.3)

Mean arterial pressure in control rats (group 2) showed little change throughout the 14 day infusion (Figure 3.2). Similarly in a later experiment for estimation of plasma angiotensin II concentration (3.3), 6 rats of group 8 showed little change of arterial pressure during 7 days infusion with dextrose. Blood pressure on the first day was 107.3±0.7 mmHg and on the final day 100.4±1.1 mmHg.

ii) Variability of pressure. During the rise of mean arterial pressure, variability assessed as standard deviation, increased significantly ($p < 0.05$, t-test) on the 1st, 4th, 5th and 6th days of angiotensin infusion (days 12, 15,16 and 17 of the experiment) compared with dextrose infused controls. It decreased on return to dextrose infusion. However, the increase of variability assessed by coefficient of variation did not reach significance though on every occasion the coefficient of variation was higher in experimental animals than in controls (Table 3.2).

iii) Dose-response studies. Dose-response studies were done on the day before prolonged angiotensin II infusion began, on the 6th day of prolonged AII infusion and 4 hours after stopping prolonged angiotensin II infusion. Arterial pressure rose acutely during each dose-response study in each rat and the rise of pressure was related to the rate of infusion (Figure 3.4). Prolonged infusion shifted the curve upward. Thus a given rate of angiotensin infusion during prolonged infusion maintained a higher pressure than during the control period.

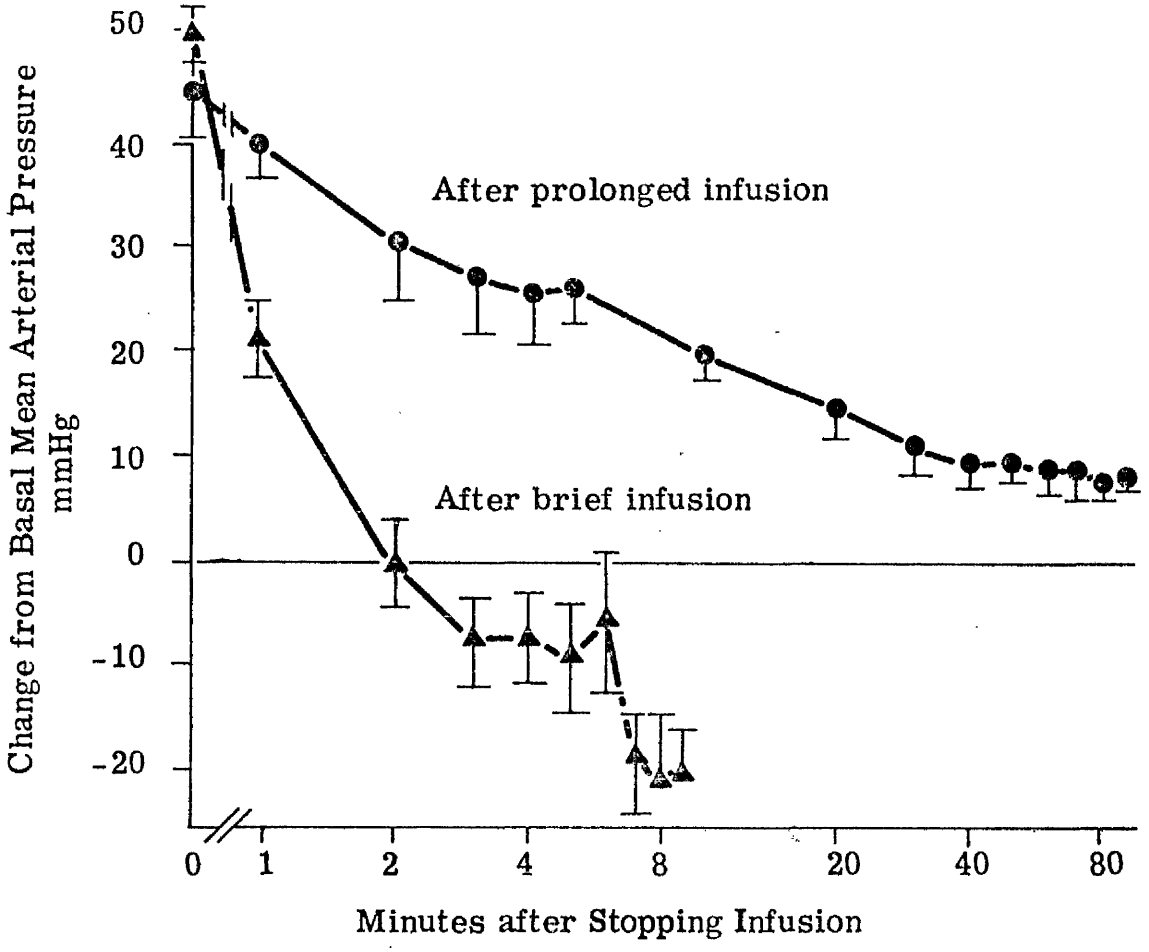


Figure 3.3 Fall in mean arterial pressure on stopping infusion of angiotensin II for 7 days at 20ng/kg/min. Comparison with the fall of pressure on stopping infusion at 270ng/kg/min for 1 hour. Results are expressed as change from initial blood pressure before angiotensin infusion began. Bars indicate 1SEM

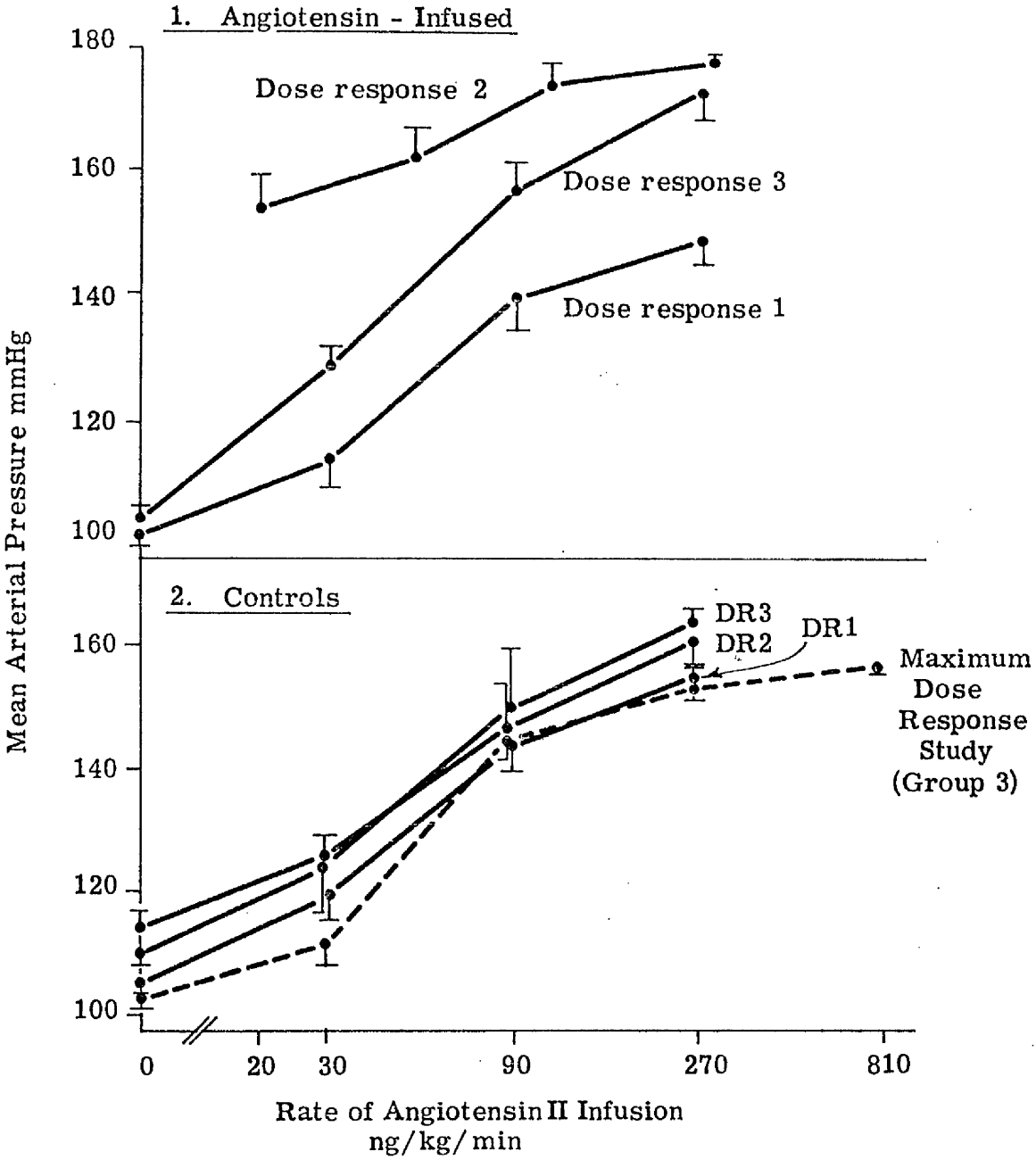


Figure 3.4 Upper Panel: 3 Dose-Response studies in rats of group 1 done before, during and after prolonged AII infusion (Days 11, 17 and 18 respectively) Lower Panel: 3 Dose-Response studies in rats of group 2 as above. Rats of group 3 received an additional infusion at 810ng/kg/min.

This occurred because arterial pressure at the beginning of the dose-response study was higher, not because the response (change of blood pressure for a given rate of infusion) was enhanced. Indeed the response was slightly diminished. The slope of the curve was not significantly different compared with dose-response 1 (F-test, F=0.294). On reverting to dextrose on day 18 following 7 days of low dose angiotensin II, blood pressure fell and for the third study, 4 hours after reverting to dextrose infusion the starting level of blood pressure was normal. On this occasion, at each rate of angiotensin infusion, there was now a significantly enhanced response ($F=5.958$, $p< 0.05$).

Dose-response studies in control rats showed little change during the experiment.

A separate experiment was done in a third group of rats (group 3) to establish the maximum direct pressor response to angiotensin. Angiotensin II was infused in 7 rats, as before, at the same three rates (30, 90, and 270 ng/kg/min) and at an additional rate of 810 ng/kg/min. The additional increase of pressure at the higher dose was only 3.7 mmHg (Figure 3.4). Thus, the rise of pressure at 270 and 810 ng/kg/min is probably close to the maximum acute pressor effect of angiotensin II. Interestingly, the rise of blood pressure produced by the 7 day infusion of angiotensin II at 20 ng/kg/min was 6.6 mmHg greater than that previously produced by one hour infusion at 270 ng/kg/min in the same rats, although the difference was not statistically significant.

iv) Heart rate Heart rate tended to decrease during the experiment in both control (group 2) and experimental (group 1) animals (Figure 3.5 and Table 3.3). The decrease was greater in the experimental group 1, but on no day during the experiment was heart rate significantly lower in experimental animals than in controls.

v) Food and water intake and sodium balance. Food and water intake were not significantly affected by prolonged infusion of angiotensin II (Figure 3.6). Sodium balance data were assessed in three ways. First, comparison

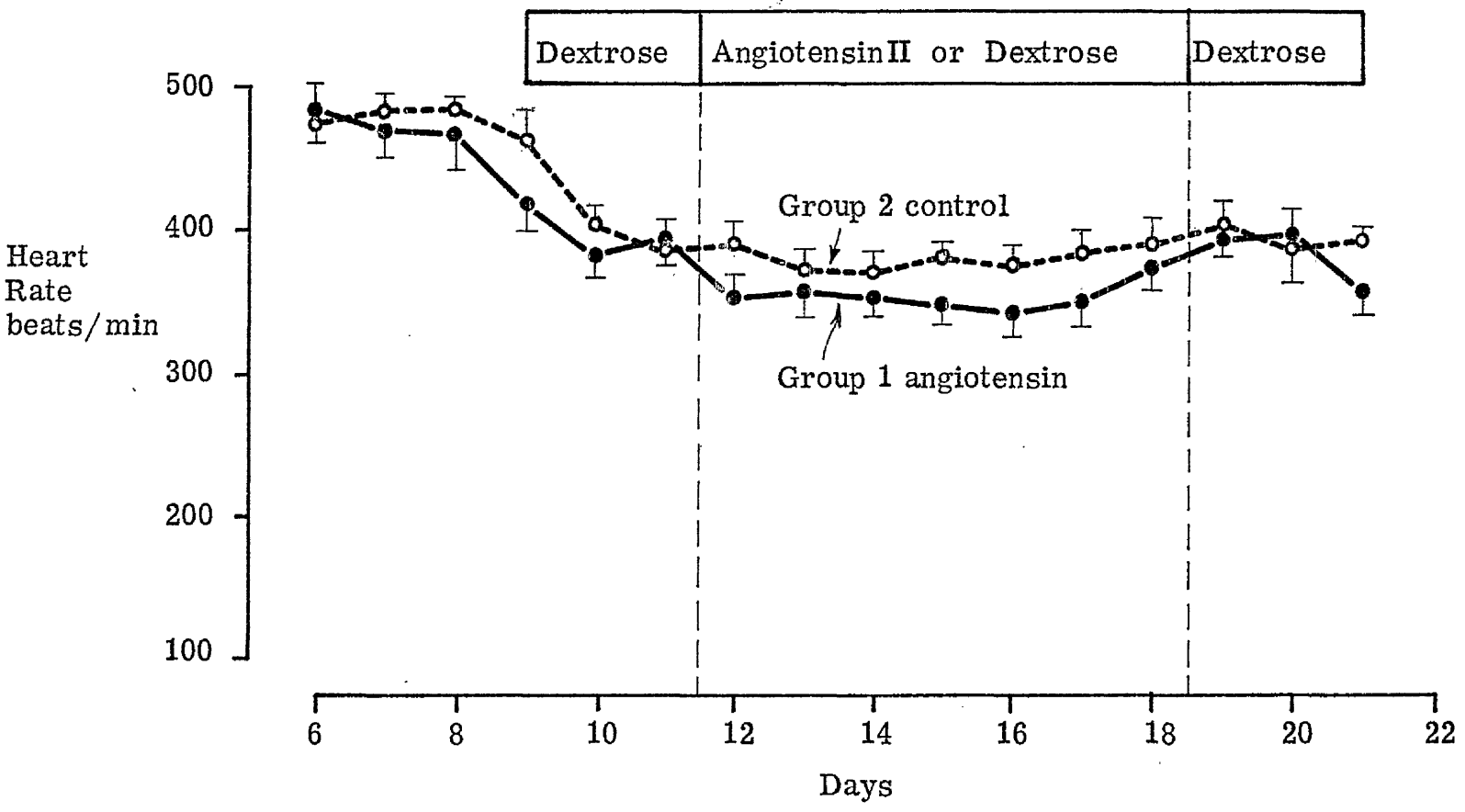


Figure 3.5 Heart rate during prolonged dextrose and angiotensin II infusion
Mean ± SEM

Table 3.3 Heart rate during prolonged AII infusion.

Day	HR - Group 1 Mean ± SEM	HR - Group 2 Mean ± SEM
9	419.0 ± 21.4	461.9 ± 21.8
10	383.8 ± 13.5	405.0 ± 12.1
11	394.6 ± 13.6	391.6 ± 14.5
12	353.1 ± 16.1	393.6 ± 13.8
13	359.1 ± 16.5	374.3 ± 13.5
14	353.8 ± 11.9	373.0 ± 13.2
15	347.9 ± 13.2	381.9 ± 8.3
16	342.9 ± 17.5	377.2 ± 9.7
17	331.7 ± 17.8	382.8 ± 17.9
18	372.5 ± 15.8	390.0 ± 18.4
19	393.7 ± 11.8	402.3 ± 17.9
20	393.8 ± 20.4	390.0 ± 29.8
21	358.0 ± 16.5	393.0 ± 6.5

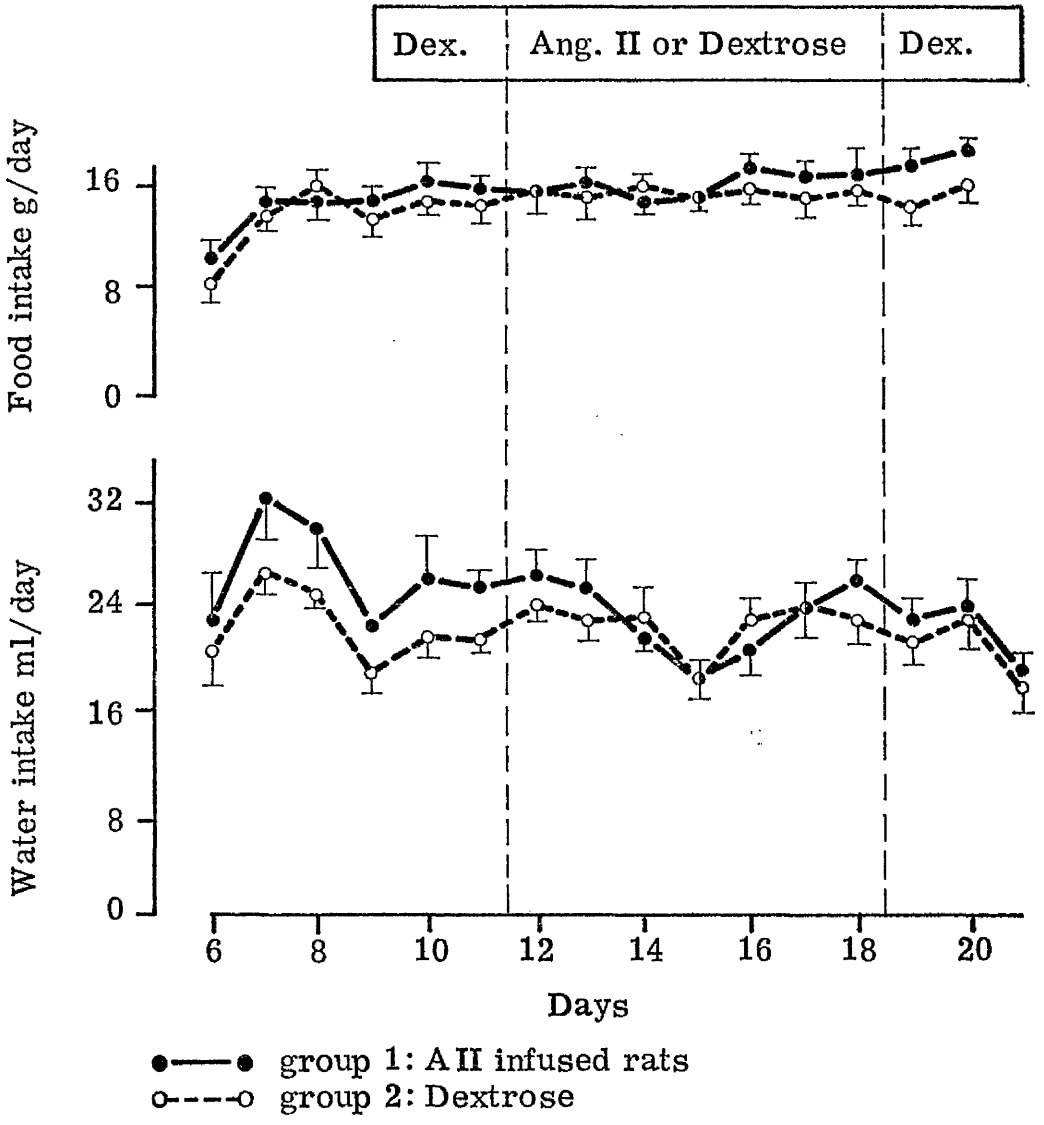


Figure 3.6 Daily food and water intake Mean ± SEM

was made of experimental and control groups. The only significant difference to emerge was during the last day of control of infusion (11th day of experiment) when both groups were receiving dextrose (Figure 3.7). On this day, control rats had a significantly negative balance compared with the experimental group (t-test, $p < 0.01$). The difference was mainly attributable to one control rat, which remained in negative balance consistently, on day 11 contributing a larger than usual deficit. A second comparison was of cumulative sodium balance during the 7 day infusion in the two groups. Again no significant difference was apparent (t-test $p > 0.4$). Finally, cumulative balance during the 6 days before infusion was subtracted from cumulative balance during the 7 day infusion and this difference was compared in control and experimental groups. Again the difference was insignificant (t-test, $p > 0.8$) (Table 3.4)

On stopping angiotensin infusion sodium balance became significantly positive in the experimental group (paired t-tests, $p < 0.01$ for comparison with the final day of angiotensin infusion). It was during this time that blood pressure fell.

3.3 EXPERIMENT 2.

PLASMA ANGIOTENSIN II LEVELS DURING ANGIOTENSIN INFUSION.

My object here was to demonstrate whether the plasma concentration of angiotensin II during the slow response was within or well above the physiological range.

3.3.1 Experimental design (Table 3.1)

Plasma angiotensin II concentration was measured in 5 groups of rats: group 4 received 5% dextrose at 1 ml/hour for 1 hour; group 5 received angiotensin II in 5% dextrose at 20 ng/kg/min for 1 hour; group 6 received angiotensin II at 270 ng/kg/min for 1 hour; group 7 received angiotensin II in 5% dextrose at 20 ng/kg/min for 7 days and group 8 received only 5% dextrose for 7 days.

Rats from groups 4, 5 and 6 were implanted with carotid type 1

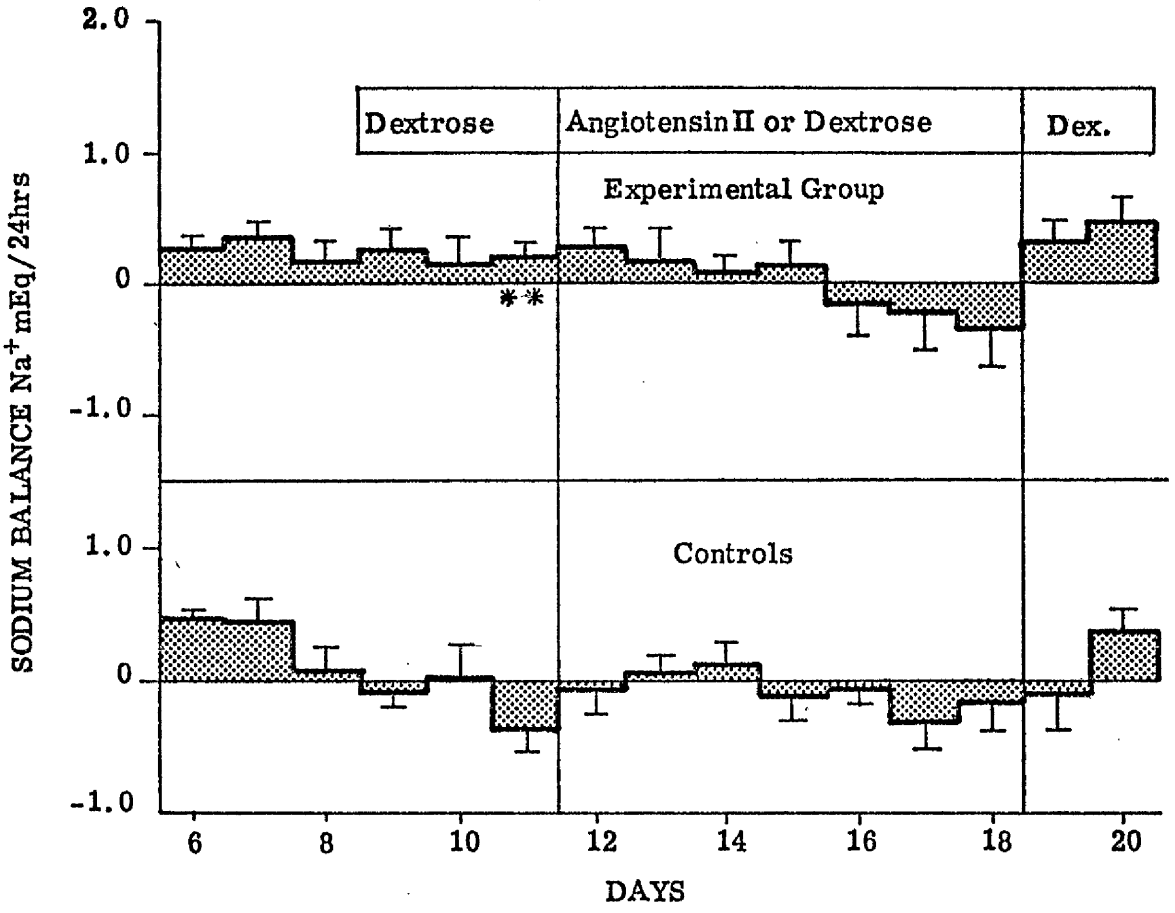


Figure 3.7 Sodium balance during prolonged dextrose and angiotensin infusions.

Table 3.4 Sodium balance.

	Group 1		Group 2.		
Day	n	mean ± S.E.	n	mean ± S.E.	
6	9	0.26 ±0.09	8	0.45 ± 0.08	
7	9	0.38 ± 0.09	8	0.45 ± 0.17	acclimatization.
8	9	0.18 ± 0.13	8	0.09 ± 0.16	
9	9	0.27 ± 0.15	8	-0.10 ± 0.12	
10	9	0.15 ± 0.24	8	0.01 ± 0.26	dextrose infusion.
11	9	0.21 ± 0.10	8	-0.38 ± 0.16	
12	9	0.30 ± 0.13	8	-0.07 ± 0.16	
13	9	0.18 ± 0.25	8	+0.05 ± 0.14	
14	9	0.08 ± 0.13	8	+0.10 ± 0.19	
15	9	0.16 ± 0.17	8	-0.13 ± 0.16	All 20 ng/kg/min
16	9	-0.12 ± 0.23	8	-0.06 ± 0.11	or dextrose.
17	8	-0.18 ± 0.26	8	-0.33 ± 0.19	
18	8	-0.30 ± 0.22	8	-0.18 ± 0.21	
19	8	+0.35 ± 0.16	7	-0.12 ± 0.26	
20	7	+0.50 ± 0.13	5	+0.37 ± 0.17	dextrose infusion
21	4	+0.30 ± 0.19	2	-0.19 ± 0.16	

Days 12-18: Mean cumulative balance 0.36 ± 0.76 Group 1

-0.62 ± 0.62 Group 2.

catheters and jugular vein catheters (2.2:1). The following day they were placed in small restraining cages (2.3:1) for infusion. Aortic and IVC catheters were implanted in rats from groups 7 and 8. Six days after the operation, they were connected to the spring and balance system and the infusion was begun. Mean arterial pressure was measured daily for 20 minutes in rats from groups 7 and 8.

At the end of each infusion, 1.0 ml of arterial blood was withdrawn for angiotensin II determination (2.7). Eleven of these samples were subjected to chromatography, separating angiotensin II and angiotensin III which were measured by radioimmunoassay.

3.3:2 Results

i) Effects of prolonged AII, prolonged dextrose and brief AII infusion on mean arterial pressure. Mean arterial presure rose slowly and progressively in 6 rats of group 7, (Table 3.5) as has been described, in the same way as rats of group 1 in the first experiment. Six rats of group 8 showed little change of arterial pressure during 7 days infusion with dextrose (Table 3.5). Mean arterial pressure rose rapidly in 6 rats from group 6.

ii) Plasma angiotensin II levels. Figure 3.8 shows the plasma angiotensin II levels for the different groups. Animals receiving control dextrose infusions for 7 days (Group 8) had lower plasma angiotensin II concentration than animals receiving control infusions for 1 hour (t-test, $p < 0.01$; $p < 0.01$, Mann Whitney) (group 4). This may be due to the fact that the rats in group 4 were placed in small restraining cages which cause considerable stress. The rats were also used the day after implantation of catheters and only had a short 30 minute period of acclimatization. All these factors would tend to raise the plasma angiotensin II levels and therefore may explain the difference between the two groups. In rats infused for 1 hour, angiotensin II at 20 ng/kg/min (group 5) produced a 2-fold increase of plasma angiotensin II concentration compared with controls infused with dextrose for 1 hour (t-test,

Table 3.5 **Mean arterial pressure during prolonged angiotensin II infusion and prolonged dextrose infusion (Groups 7 and 8) and brief angiotensin II infusion (Group 6)**

	MAP ± SEM mmHg.	
Control	107.5 ± 1.1	
Day 1	126.7 ± 7.0	
2	141.2 ± 6.4	Group 7 n = 6. Prolonged AII
3	150.4 ± 3.8	infusion at 20 ng/kg min for
4	145.8 ± 7.4	7 days.
5	150.9 ± 3.8	
6	156.3 ± 3.7	
7	157.8 ± 5.5	
Control	107.3 ± 0.7	
Day 1	104.9 ± 3.4	
2	101.6 ± 1.3	
3	101.6 ± 2.7	Group 8 n = 6. Prolonged dextrose
4	104.9 ± 1.9	infusion for 7 days.
5	108.6 ± 2.4	
6	103.6 ± 2.0	
7	100.4 ± 1.1	
Control	101.0 ± 3.5	Group 6 n = 6 AII infused at
1 hour later	146.3 ± 3.0	270 ng/kg/min for 1 hour.

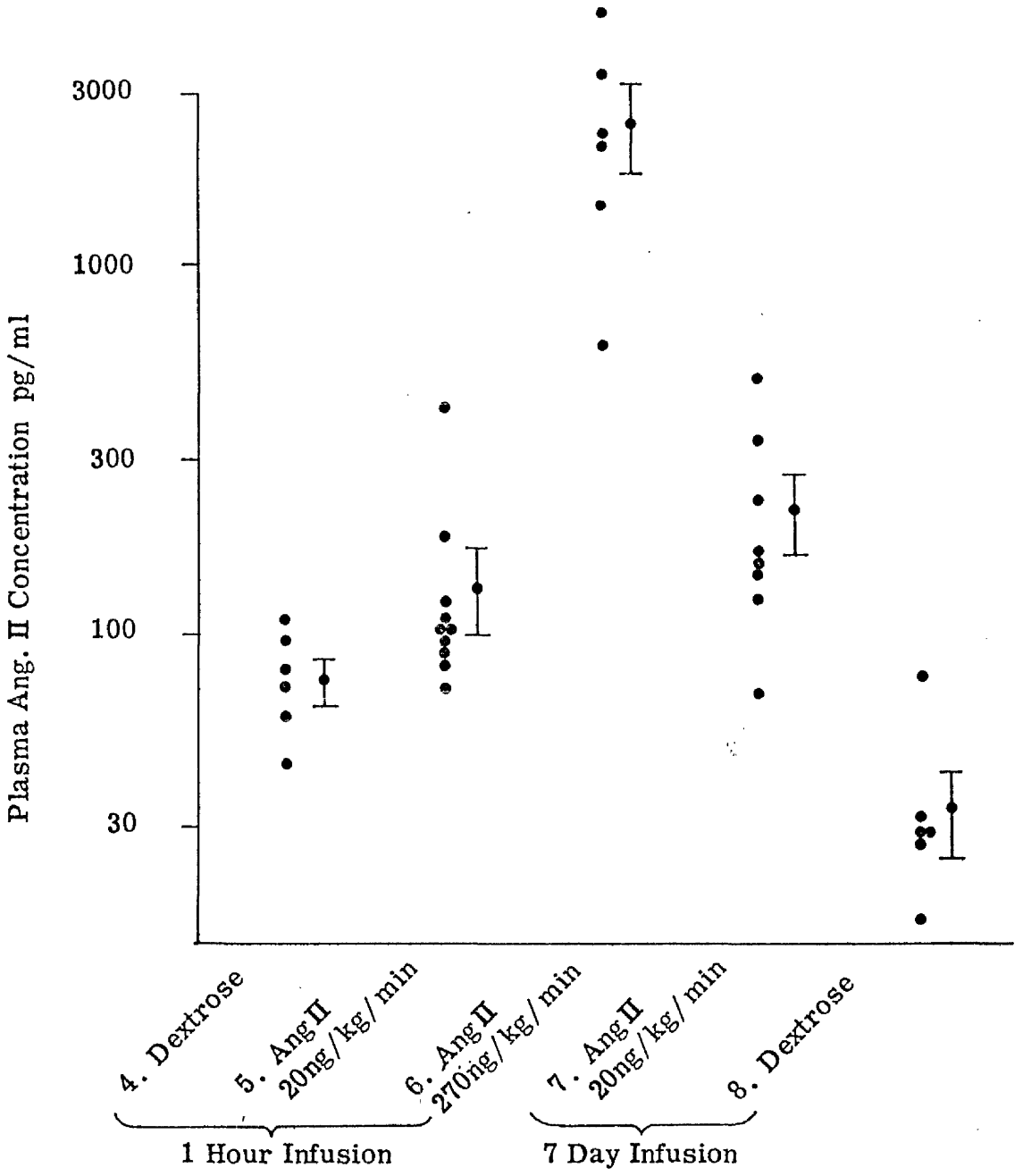


Figure 3.8 Plasma angiotensin II levels in 5 groups of rats.

$p > 0.1$; $p < 0.05$, Mann Whitney U test). Infusion at 270 ng/kg/min for 1 hour produced a 32-fold increase (t-test, $p < 0.01$; $p < 0.001$ Mann Whitney U Test). In rats infused for 7 days, angiotensin II at 20 ng/kg/min produced a higher concentration of peptide than did control infusions for 7 days (t-test, $p < 0.01$; $p < 0.001$, Mann Whitney U test). Animals infused with angiotensin II at 20 ng/kg/min for 7 days had a higher concentration of peptide than animals infused with AII for 1 hour at 20 ng/kg/min, although the difference was not significant.

Thus, infusion of angiotensin II at 20 ng/kg/min, a dose which over 7 days produced a large rise of pressure, produced only a small rise of plasma angiotensin II levels. Indeed levels in some rats were within the range found in dextrose-infused controls. As expected, brief infusion of AII at 270 ng/kg/min produced a much greater plasma concentration of peptide, but a similar rise of pressure.

Table 3.6 shows the plasma angiotensin II and angiotensin III levels from the II samples subjected to chromatography to separate angiotensin II and angiotensin III. The results confirm earlier reports (Semple and Morton 1976) that measurement of angiotensin II in rat plasma without chromatography overestimates the concentration of peptide measured as angiotensin II. Some of the excess can be accounted for by angiotensin III which is present in rat blood in relatively high concentrations and which reacts with the antiserum used in this assay.

It was interesting that infusion of angiotensin II decreased rather than increased the proportion of angiotensin III. During infusion of angiotensin II a higher proportion of the immunoreactive material chromatographed with angiotensin II, a lower proportion with angiotensin III. Thus, levels of circulating peptides measured as angiotensin II were not falsely elevated by an increased proportion of angiotensin III.

3.4 COMMENT.

In a general discussion at the end of this thesis I shall consider the

Table 3.6 Plasma angiotensin II and III levels

		Without Chromatography	With Chromatography	
		Total Angiotensin II pg/ml	Angiotensin II pg/ml	Angiotensin III pg/ml
Dextrose infused rats	1	32	15	0
	2	29	15	11
	3	17	14	8
	4	29	18	17
	5	77	55	9
	6	26	24	8
20 ng/kg/min Ang II for 1 hour.	7	87	68	9
	8	72	60	0
	9	107	87	0
20 ng/kg/min Ang II for 7 days.	10	68	55	0
	11	125	147	0

mechanism of the slow-pressor effect and its possible relevance in renal hypertension. This interim discussion is concerned here with points raised by experiments 1 and 2.

The slow response has been noted previously in rabbits (Dickinson and Lawrence 1963); dogs (McCubbin et al 1965, Cowley and DeClue 1976, Cowley and McCaa 1976, Bean et al 1979) and man (Oelkers et al 1978) but in none of these did it develop as markedly or as rapidly as in the rat. Its magnitude in the rat after 7 days was similar to the maximum direct response. Also the slow response developed at a plasma concentration of angiotensin II within or close to the physiological range. This implies that a small but sustained increase in the plasma concentration of endogenous angiotensin II could have a marked effect on blood pressure.

The rate of change of pressure on stopping infusion of angiotensin II was different for fast and slow responses. Arterial pressure fell to control values within 2 minutes of stopping brief pressor infusions of angiotensin II, but took 4 hours to reach control on stopping 7 day infusions at lower dose. The duration of angiotensin infusion is known to determine the subsequent rate of fall of pressure in rabbits (Dickinson and Yu, 1967b).

Dose-response studies testing the direct pressor response of angiotensin II during the slow rise of pressure showed an upward shift in the curve relating arterial pressure and rate of angiotensin infusion, a given rate maintaining a higher pressure. Bean et al (1979) showed this in the dog, demonstrating an upward shift in the curve relating arterial pressure and plasma angiotensin II concentration.

The third dose response study was begun 4 hours after stopping infusion of angiotensin II, at a time when arterial pressure was normal. The pressor response to angiotensin II during this study was enhanced. Skulan, Brousseau and Leonard (1974) made a similar observation in renal hypertensive rats. Two days after unclipping, when blood pressure had fallen to normal, the

pressor response to angiotensin II was increased. Ten Berg and de Jong (1980) suggested that the enhanced response was a result of altered reactivity of the cardiovascular system. One explanation for the altered response and for the rise of pressure is that structural changes occur in resistance vessels during (and possibly because of) the rise of arterial pressure and that these persist for a time when blood pressure falls. Folkow (1971) has discussed the importance of this mechanism and the way in which it can alter the response to vasoconstrictor agents.

Another explanation for the enhanced response derives from the theory of prior occupancy by endogenously generated angiotensin II (Marks, Thurston, Bing, and Swales 1979). During infusion of angiotensin II, endogenous renin and angiotensin II would be suppressed. On stopping the infusion there will be a period when endogenous angiotensin II is low because renin release is still suppressed and exogenously infused angiotensin II is no longer present in blood. Under these circumstances a high proportion of receptors will be unoccupied and the response to infused angiotensin II correspondingly larger.

Angiotensin II alters the urinary excretion of sodium and water (Brown and Peart 1962); stimulates drinking (Epstein et al 1970) and as a result of this latter action suppresses feeding (McFarland and Rolls 1972), but it is not certain whether these are physiological effects of the blood-borne peptide. Water and food intake and urinary sodium excretion did not change in our experiment during an infusion of angiotensin II which did raise blood pressure. Thus, thresholds of the urinary, dipsogenic and feeding effects of angiotensin II are likely to be higher than the threshold of the slow pressor effect. Mann et al (1980) consider the threshold for the dipsogenic effect in rats to be at or above 200 pg of angiotensin II/ml of plasma. In our experiment infusion of angiotensin II at 20 ng/kg/min for 7 days raised plasma angiotensin II concentration to 214 pg/ml, at which we failed to demonstrate an effect on drinking. However, the two studies are not necessarily incompatible as clear-cut effects on drinking

only occurred at higher plasma concentrations of angiotensin in the experiment of Mann et al (1980).

Separate measurements were made of plasma angiotensin II and angiotensin III as it was possible that angiotensin III might be formed from angiotensin II during infusion falsely elevating the estimate of angiotensin II-like material. In fact the opposite occurred, the plasma concentration of angiotensin II increased while that of angiotensin III decreased (Table 3.6). A possible explanation for this suggested by the recent demonstration (Garcia del Rio, Smellie and Morton 1981) that the nonapeptide, des-asp^{1} Ile^{5} angiotensin I, is present in rat blood and is converted directly in vivo to angiotensin III and does not form angiotensin II. If this nonapeptide is itself produced directly or indirectly by renin and if, as is likely, infusion of angiotensin II inhibits renin release, the nonapeptide and thence angiotensin III will decrease during infusion of angiotensin II.

Our experiment tests a role for angiotensin II as a blood-borne hormone. It has not tested roles which are thought to exist for angiotensin II generated locally in brain or blood vessels. Such actions cannot be quantitatively assessed by intravenous infusion of peptide.

3.4:1. Experiments 1 and 2: conclusions and future experiments.

Our main conclusions are that the slow pressor effect of angiotensin II develops at a plasma concentration of the peptide which is close to, or within, the physiological range and which has little or no effect on food or water intake or on urinary excretion of sodium and that the magnitude of the slow effect at 7 days is similar to the maximum direct pressor response produced by larger doses of angiotensin II. Experiments 1 and 2 have been written up and published in the American Journal of Physiology (Brown, Casals-Stenzel, Gofford, Lever and Morton, 1981). It has suggested a number of further experiments.

First, I would like to study the mechanism of the slow effect. There are several possibilities here (Brown, Fraser, Lever at al 1977, Dickinson and Yu

1971). An action of angiotensin II on the nervous system seems likely (Ferrario et al 1972), but there are many levels of the peripheral and central nervous system at which angiotensin II could act. I shall pursue this idea further in the rat. I plan to measure plasma noradrenaline and adrenaline during the slow rise of pressure to indicate whether there is overactivity of the sympathetic nervous system and then, by use of pharmacological agents such as bethanidine, an adrenergic neurone blocking agent or reserpine which depletes adrenergic neurones, to block the action of the sympathetic nervous system, before and during a prolonged infusion of low dose angiotensin II.

My second plan is to determine whether infusion of angiotensin II at low dose, for longer than one week produces a further increase in arterial pressure, possibly to the point where complications or malignant phase hypertension develop. With Ms. Sheila Clark I propose to infuse angiotensin at 20 ng/kg/min for 14 days. Experiments have begun, but so far only one rat has been completed. Figure 3.9 illustrates the rise of pressure over the 14 day period and compares it with the mean rise of pressure in the 9 rats of group 1 which received angiotensin II for one week only. Mean arterial pressure continued to rise after 7 days of angiotensin II infusion reaching a peak level of 176.2 mmHg at the end of the 14 day infusion. The fact that pressure continued to rise during this constant infusion suggests that the slow pressor effect of angiotensin II may have an important role to play in arterial pressure regulation and in the maintenance of some hypertensive states, although my experiments do not establish such a role.

There is also a need to assess the contribution of the slow effect to the maintenance of renal hypertension and the next chapter deals with some aspects of this interesting but unanswered question.

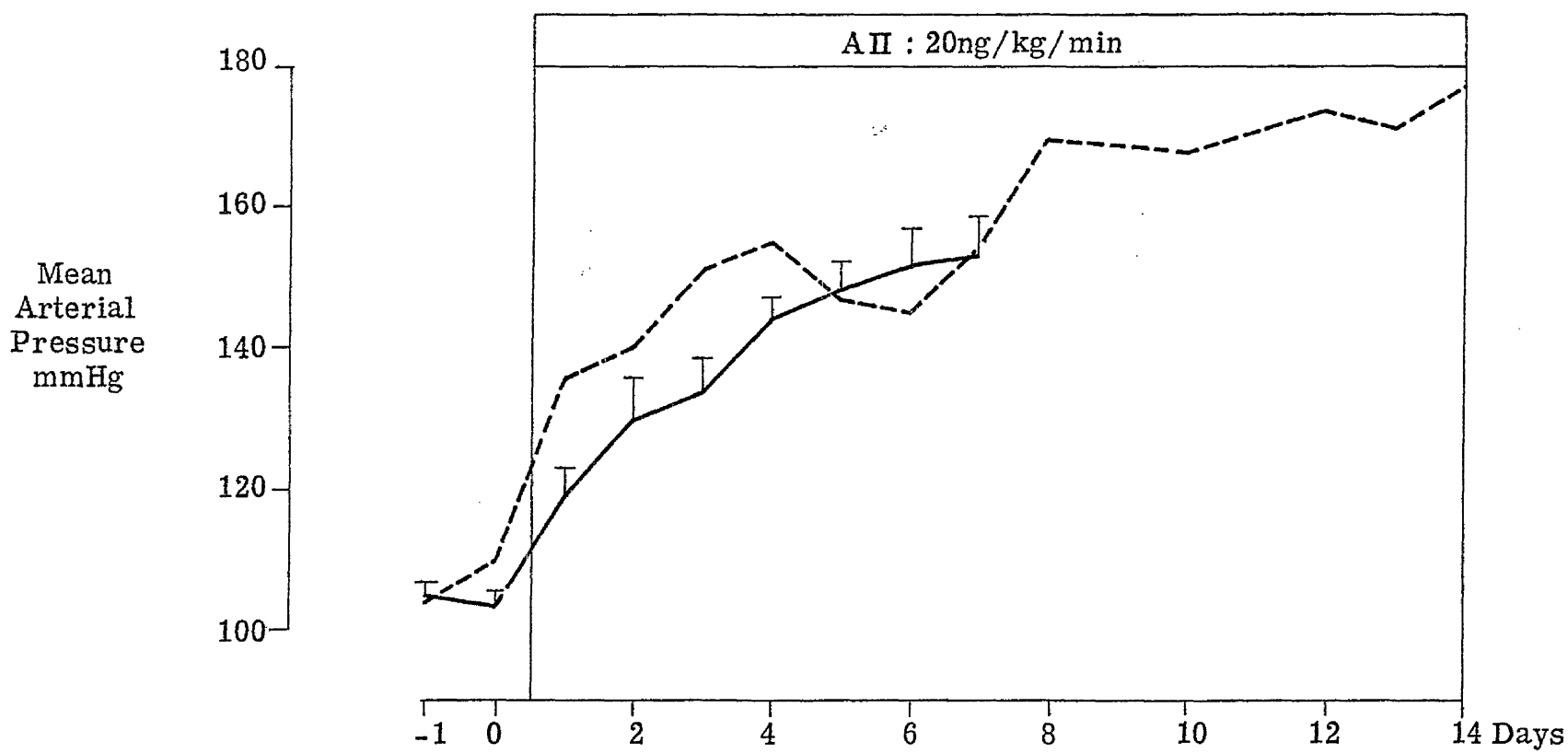


Figure 3.9 Mean arterial pressure (±SEM) during prolonged infusion of angiotensin II for 7 days (Group 1 —— n = 9) and for 14 days in one rat (- - - -)

CHAPTER 4

PROLONGED INFUSION OF SARALASIN IN NORMAL AND HYPERTENSIVE RATS.

4.1 INTRODUCTION.

With the synthesis of angiotensin II analogues, such as saralasin, tools were provided with which to study the role of angiotensin II in control of arterial pressure in normotensive and hypertensive states. Use of these and other antagonists of the renin-angiotensin system in the acute phase of renal hypertension have established fairly well that the rise of pressure at this stage is partly or wholly a result of the direct vasoconstrictor effect of angiotensin II (Pals, Masucci, Denning, Sipos and Fessler 1971b; Coleman and Guyton 1975; Thurston and Swales 1974; Freeman, Davis, Watkins and Lohmeier 1977; Masaki, Ferrario, Bumpus, Bravo and Khosla 1977). Dose-response studies also support the idea (Caravaggi, Bianchi, Brown, Lever, Morton, Powell-Jackson, Robertson and Semple 1976).

Chronic renal hypertension is less easy to explain. Blood pressure may be higher but plasma levels of renin and angiotensin II are not now high enough to raise blood pressure by acute vasoconstriction alone (Bianchi, Baldoli, Lucca and Barbin 1972; Brown, Cuesta, Davies, Lever, Morton, Padfield, Robertson, Trust, Bianchi and Schalekamp 1976). Attention therefore turned to additional mechanisms. One possibility was the slow pressor effect of angiotensin II. Prolonged infusion of saralasin for 12 hours into renal hypertensive rats was used to test this second hypothesis (Riegger et al 1977; Bing, Russell, Swales and Thurston, 1981). One group (Riegger et al 1977) found a gradual decrease of pressure to normotensive levels with saralasin; the other (Bing et al 1981) found no decrease. My third experiment had its origin in this disagreement. I felt it was important to resolve the matter before the role of a slow pressor effect of angiotensin II in renal hypertension could be properly assessed.

There were several differences between the experiments. At the time, none seemed particulary important, but the difference on which I decided to concentrate first was that one group (Riegger et al 1977) infused saralasin by day, the other group (Bing et al 1981) infused it by night. A possibility I considered was that saralasin produced a diurnal effect on arterial pressure, raising it by night, reducing it by day. If this diurnal effect was greater in hypertensive than in control animals, the difference would be explained.

My object, therefore, was to infuse saralasin for long enough to test this idea in normal and renal hypertensive rats. So far only 3 mildly hypertensive and one severely hypertensive rats have been infused and so I have not yet properly assessed the role of angiotensin in renal hypertension, but the results in normal rats were interesting and unexpected. The study of normal rats has been completed and a preliminary communication has been given (Brown and Lever, 1981). The findings are described below in experiment 3.

4.2 EXPERIMENT 3

EFFECT OF PROLONGED SARALASIN INFUSION ON ARTERIAL PRESSURE AND SODIUM BALANCE IN NORMAL RATS.

4.2:1 Experimental design.

Eight rats were studied. Figure 4.1 shows the protocol. Aortic and IVC catheters were implanted as previously described (2.2:4). After the usual period of recovery and acclimatization, rats were connected on day 6 to the spring and balance system. The experiment began on day 7 when 5% dextrose was infused for 2 days as control, then saralasin was infused at 10 µg/kg/min for 4 days, and finally dextrose for the last 2 days (Figure 4.1). Saralasin infusions were begun at 9.00 a.m. in 6 rats and at 9.00 p.m. in 2 rats.

Sodium balance was measured from the 7th day, as described in Chapter 2 (2:6). Mean arterial pressure (MAP) was recorded continuously from day 7 until the end of the experiment 8 days later. Traces were analysed as follows:

2-hourly mean values. A single value of MAP was obtained from the

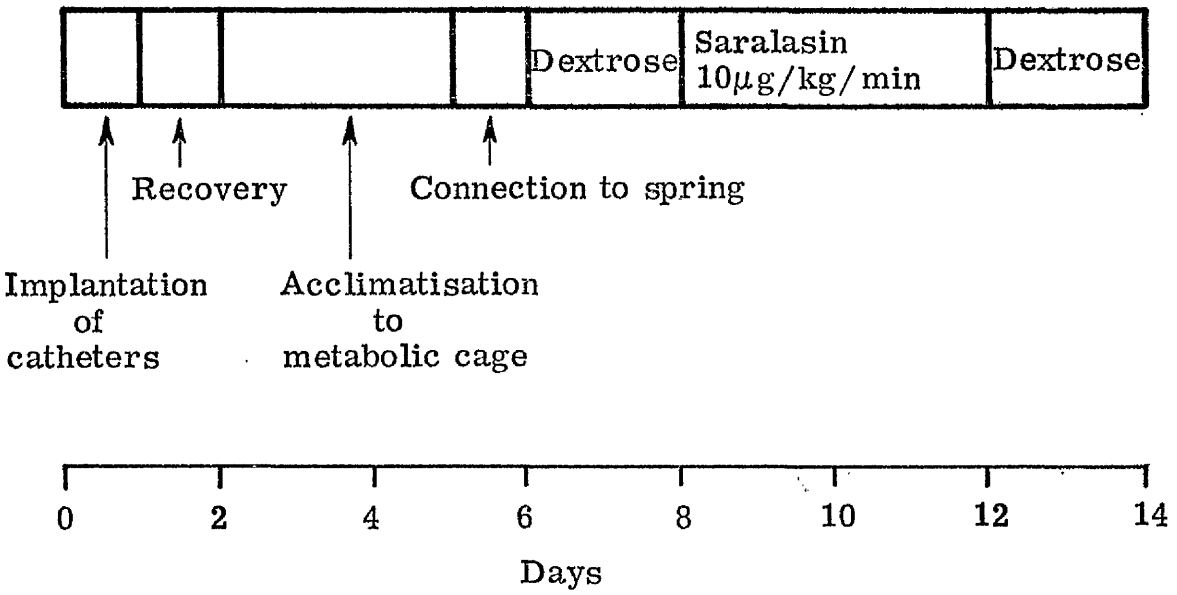


Figure 4.1 Experimental protocol

trace every 30 minutes. Each 24 hour period was then divided into twelve 2 hourly intervals i.e. 0.30-2.00; 2.30-4.00 a.m. etc. and the average of four 30 minute measurements during each 2 hour interval was calculated to give a 2 hour value for blood pressure for individual rats.

Minute to minute variability of pressure. More frequent readings were used to assess variability of arterial pressure. Thirty measurements of arterial pressure made at 2 minute intervals between 9.00 a.m. and 10.00 a.m. each day were used in every rat to calculate variability which was expressed as before (Chapter 3.2:2) as the standard deviation and coefficient of variation for the 30 measurements.

4.2:2 Results.

Prolonged infusion of saralasin produced four main changes: a rapid pressor response, a gradual rise of pressure as with angiotensin II in low dose (Chapter 3), increased diurnal variation of pressure and increased variability of pressure assessed minute to minute.

1) Rapid pressor response. Infusion of saralasin at 10 µg/kg/min raised arterial pressure acutely in all rats from a mean level of 105.8 $\pm$ 3.0 mmHg before infusion to 126.8 $\pm$ 6.3 mmHg within 5 minutes ($p < 0.01$, paired t-test). Pressure then fell within 15 minutes to pre-infusion levels despite continuous infusion of the saralasin.

2) Gradual increase of blood pressure After this transitional increase in pressure and in the 6 rats which started saralasin infusion at 9.00 a.m., mean arterial pressure remained at control levels for 12 hours until approximately 10.00 p.m. when pressure began to rise. Thereafter it rose progressively, but with increased diurnal variation (described later) to reach a peak 21 mmHg higher than control on the 4th day (Figure 4.2). Mean arterial pressure for the group was 109 $\pm$ 0.4 mmHg the day before saralasin, 131 $\pm$ 0.6 mmHg on the 4th day of infusion ($n = 6$, paired t-test, $p < 0.001$). In the 2 rats starting saralasin infusion at 9.00 p.m. MAP also rose progressively. MAP for

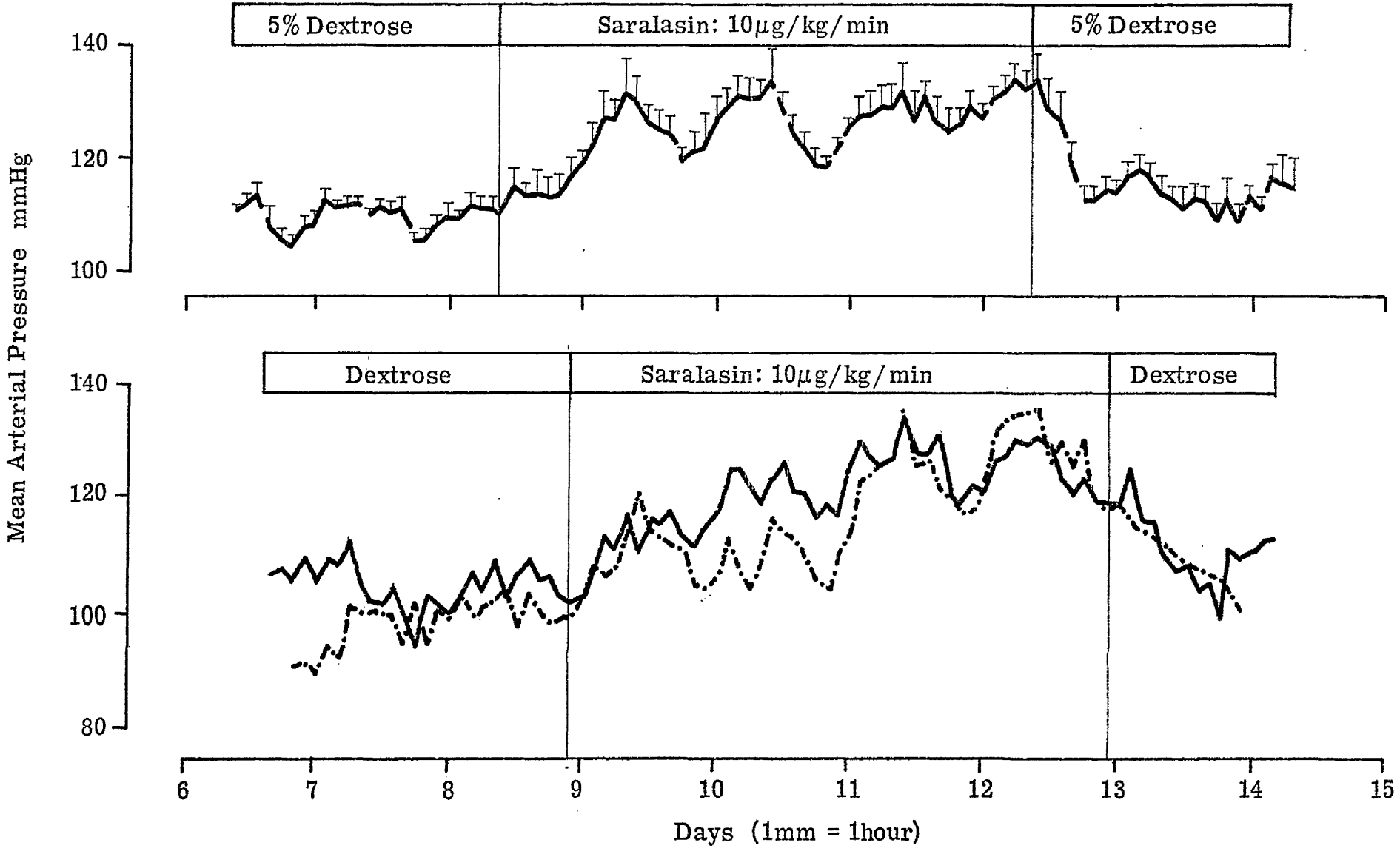


Figure 4.2 Mean arterial pressure at two hourly intervals (± SEM) in 6 rats starting saralasin by day (9am in upper panel. Lower panel shows, separately, data in 2 rats starting saralasin at night (9pm)

these was 100.3 ± 0.5 mmHg the day before saralasin infusion, 124.9 ± 0.9 mmHg on the 4th day of infusion.

On returning to dextrose, mean arterial pressure fell in each rat reaching control values within 6 hours. In the rats stopping saralasin at night, mean arterial pressure fell in these rats also reaching control values within 4 hours.

3) Diurnal changes of arterial pressure Mean arterial pressure varied during both the control and experimental periods, tending to increase during the night between 9.00 p.m. and 9.00 a.m. and to decrease during the day between 9.00 a.m. and 9.00 p.m. (Table 4.1). This normal diurnal variation increased during saralasin. The rise in mean arterial pressure during the night in the experimental period was significantly greater than the rise occurring in the same rats in the control period ($p < 0.05$, paired t-test), but on the 1st and 2nd days of saralasin only. The decrease in pressure during the day, however, was not significantly greater in the experimental period compared to that during the control period. The rises and falls of pressure were in themselves significant both during the control and experimental period ($p < 0.05$, paired t-test) (Table 4.1). Interestingly, in the rats starting saralasin at 9.00 a.m., mean arterial pressure remained at control levels until approximately 10.00 p.m. when it began to rise. Mean arterial pressure in these animals at 10.00 a.m. was not significantly different from mean arterial pressure at 10.00 p.m. ($p < 0.4$, paired t-test). Saralasin had thus prevented the usual decrease in pressure during the day.

The increase in diurnal rhythm of pressure was also apparent in the 2 rats which started saralasin infusion at night (Figure 4.2).

4) Minute to minute variability of pressure. Table 4.2 shows variability of mean arterial pressure assessed by standard deviation and coefficient of variation, before, during and after saralasin. Saralasin increased variability assessed by standard deviation on the 1st, 3rd and 4th days of its

Table 4.1 Diurnal changes of mean arterial pressure during saralasin.

Day		MAP mmHg.	MAP
7	9 p.m.	103.7	
			+8.7
8	9 a.m.	112.4	
			-7.7
	9 p.m.	104.7	
			+6.5
9	9 a.m.	111.2	
		**********	
	9 p.m.	112.1	
			+18.6*
10	9 a.m.	130.7	
			-12.4
	9 p.m.	118.3	
			+15.2*
11	9 a.m.	133.5	
			-16.4
	9 p.m.	117.1	
			+13.5
12	9 a.m.	130.6	
			- 6.7
	9 p.m.	123.9	
			+ 8.3
13	9 a.m.	132.2	
		**********	
	9 p.m.	111.1	
			5.4
14	9 a.m.	116.5	
			9.4
	9 p.m.	107.1	
			8.1
15	9 a.m.	115.2	

* $p < 0.05$
n = 6

Table 4.2 Standard deviation and coefficient of variation during prolonged saralasin infusion. (n = 8).

	Day	n	Standard deviation	Coefficient of variation
	8	8	4.24 ± 0.40	3.93 ± 0.34
Dextrose				
	9	8	4.84 ± 0.58	4.48 ± 0.5
	10	8	6.53 ± 0.84*	5.15 ± 0.61
	11	8	5.80 ± 1.06	4.39 ± 0.77
Saralasin				
	12	8	6.18 ± 0.60*	4.75 ± 0.53
	13	8	6.63 ± 0.68**	4.92 ± 0.47*
	14	7	5.64 ± 1.02	5.07 ± 0.89
Dextrose	15	3	6.33 ± 0.86	5.69 ± 1.07

*$p < 0.05$ significance when compared with day 8.

**$p < 0.01$ Less frequent but still significant changes were found when compared with day 9.

infusion and by coefficient of variation on the 4th day only. Neither change was marked or highly significant.

5) Food and water intake and sodium balance (Figure 4.3) Food and water intake were not significantly affected by prolonged infusion of saralasin. Urine volume, however, was significantly greater on the 2nd day of saralasin infusion compared to the 1st and 2nd days of dextrose ($p < 0.05$, paired t-test). Sodium balance was not significantly affected.

4.3 COMMENT

Saralasin infused into normal rats had initially a brief pressor response observed in the first few minutes of infusion. This response is well known and is probably partly mediated by angiotensin-like agonist action on vascular receptors (Pals et al 1971a) and also by an action on the central or peripheral autonomic nervous system (McGrath et al 1977).

After this acute response, saralasin had a second slow developing pressor action, similar in many ways to that seen previously with angiotensin II (Figure 3). The inhibitor raised mean arterial pressure gradually by 22 mmHg in 4 days. Arterial pressure in similar rats receiving angiotensin II at 20 ng/kg/min increased by 40.7 mmHg in 4 days (Chapter 3).

Saralasin also increased the diurnal variation of pressure. I did not know at the time if increased diurnal changes of pressure also occurred in the rats receiving angiotensin II because arterial pressure had not been recorded continuously in my earlier experiment (Chapter 3). I decided to infuse angiotensin II in a further group of rats with continuous measurement of arterial pressure.

4.4 EXPERIMENT 4

EFFECT OF PROLONGED ANGIOTENSIN II INFUSION ON DIURNAL CHANGES OF ARTERIAL PRESSURE.

4.4:1 Experimental design.

Six rats were studied. Figure 4.4 shows the protocol. Rats were

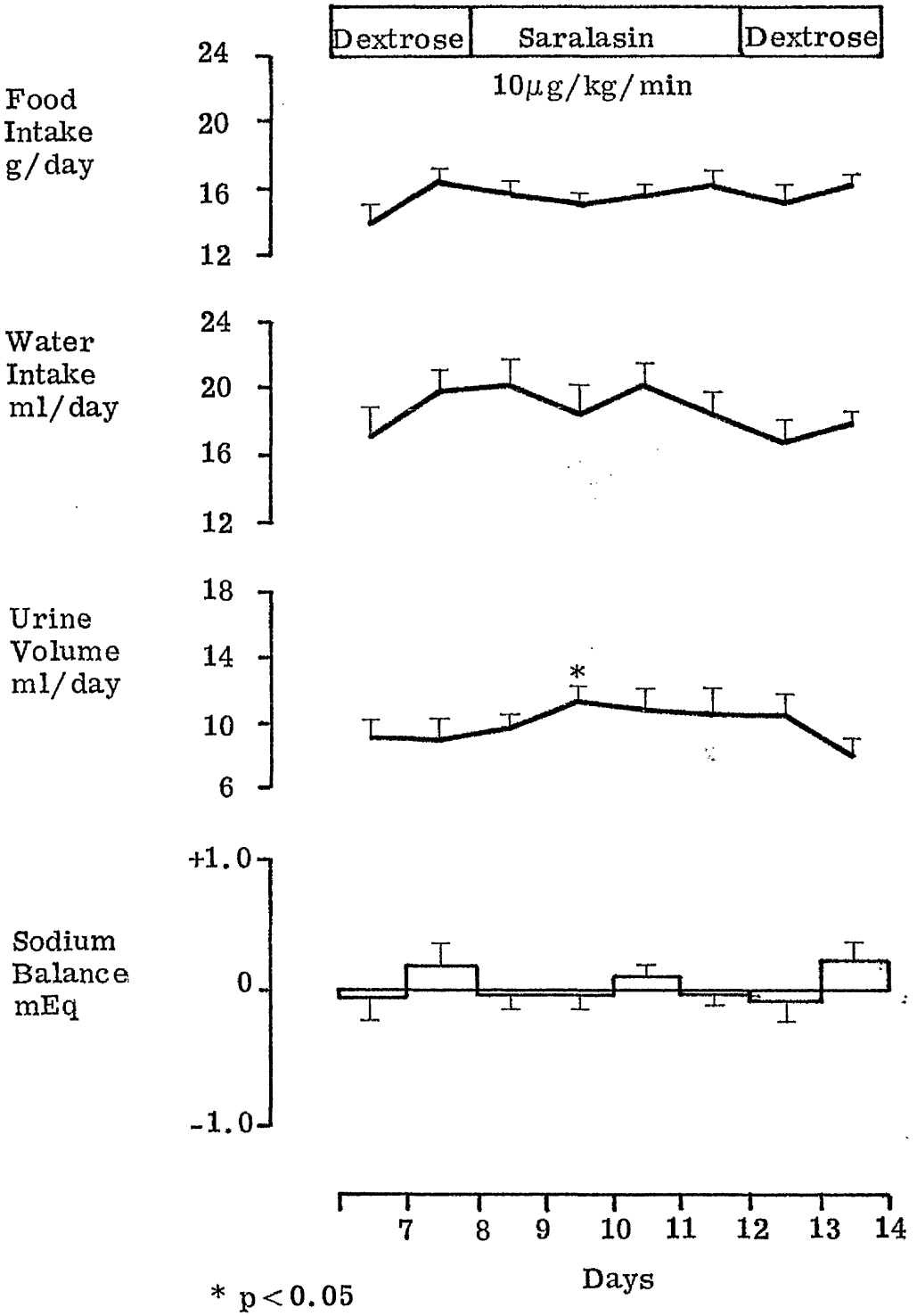


Figure 4.3 Food and water intake;sodium balance and urine volume during prolonged saralasin infusion

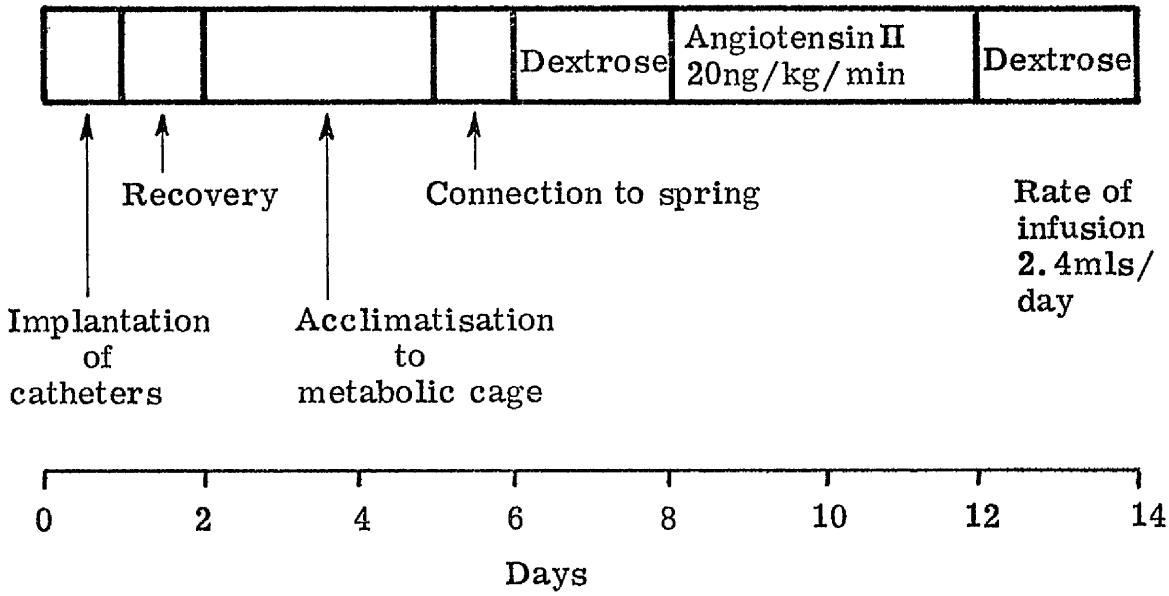


Figure 4.4 Experimental protocol

prepared as for experiment 4, in which saralasin was infused, and followed an identical protocol except that angiotensin II was infused at 20 ng/kg/min for 4 days instead of saralasin (4.2:1). Infusions were begun at 9.00 a.m. in all animals. Sodium balance was not measured as this had been done previously (Chapter 3) with negative results (Figure 3.7). MAP was recorded continuously from day 7 until the end of the experiment 8 days later. Traces were analysed as before (4.2:1)

4.4:2 Results

Prolonged infusion of angiotensin II produced similar changes to saralasin: a gradual rise of pressure and increased diurnal variation of pressure.

Infusion of angiotensin II at 20 ng/kg/min raised arterial pressure acutely in all rats, blood pressure increasing from a mean level of 109.1 $\pm$ 2.0 mmHg before infusion to 122.2 $\pm$ 3.3 mmHg within 10 minutes of starting the infusion ($p < 0.01$, paired t-test). Thereafter pressure rose progressively to reach a peak 41.7 mmHg higher on the 4th day of infusion (Figure 4.5) ($n = 6$, paired t-test, $p < 0.001$). On returning to dextrose, mean arterial pressure fell in each rat reaching control levels within 4 hours. Angiotensin II also increased the diurnal changes of pressure (Table 4.3). The rise in pressure during nights of angiotensin infusion were significantly greater than the change occurring in the same rats during the night in the control period ($p < 0.05$, paired t-test). The fall of pressure during the day was also greater than that during the day in the control period, but was significantly greater only on the 3rd day of angiotensin ($p < 0.05$, paired t-test).

4.5 COMMENT.

Angiotensin II infused into normal rats had an acute pressor effect observed in the first few minutes of infusion. Infused at this rate in my earlier experiment (Chapter 3) it had no initial pressor effect. We cannot explain this difference, but we do know from earlier experiments that this particular rate of infusion is near the threshold for the direct vasoconstrictor effect of angiotensin

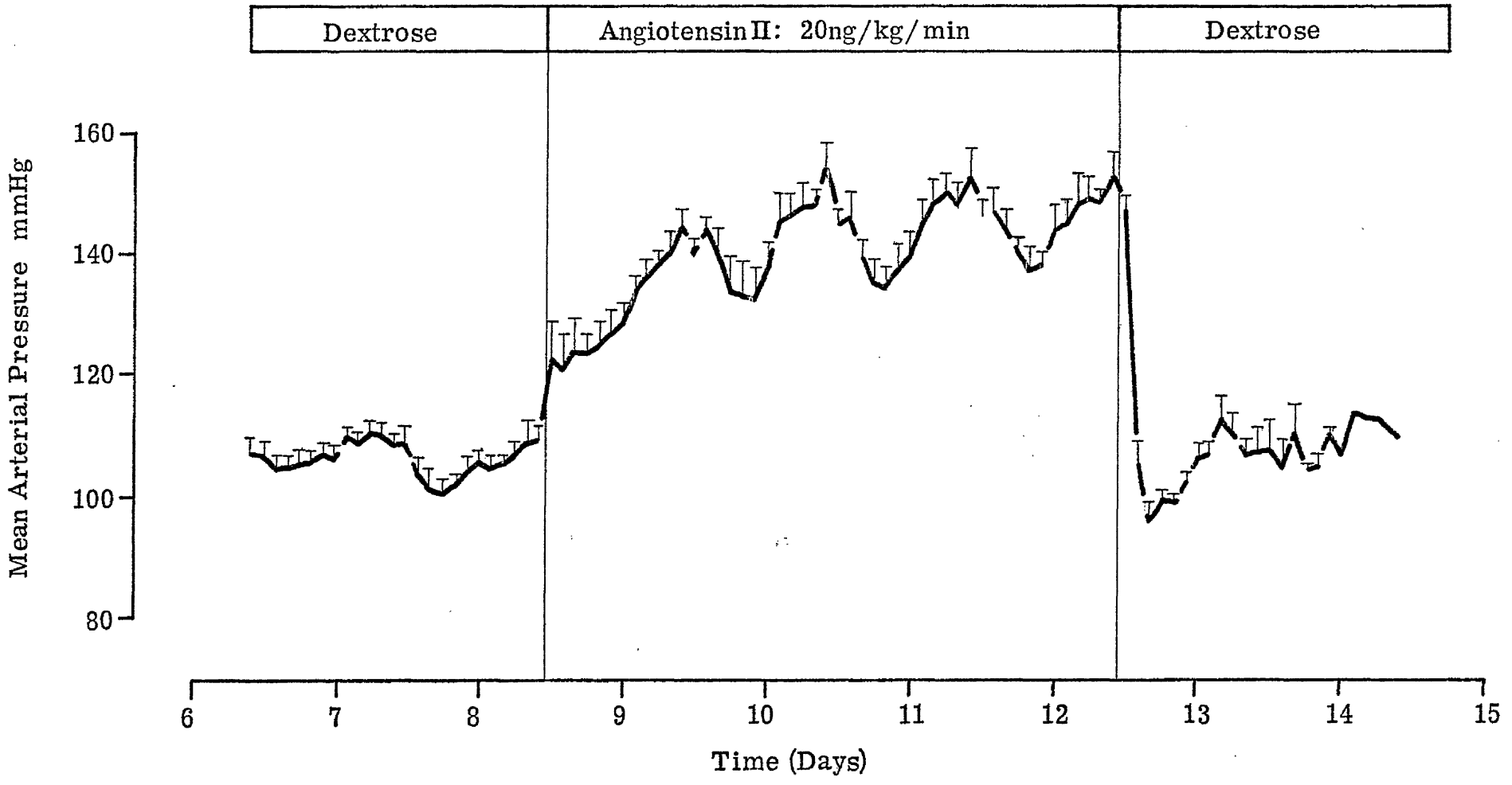


Figure 4.5 Mean arterial pressure at two hourly intervals during angiotensin infusion

Table 4.3 Diurnal rhythm of mean arterial pressure during angiotensin II

Day		MAP mmHg	Change in MAP
7	9 p.m.	104.6	
			+ 6.0
8	9 a.m.	110.6	
			-10.4
	9 p.m.	100.2	
			+ 8.9
9	9 a.m.	109.1	
	**********		
	9 p.m.	126.1	
			+18.2**
10	9 a.m.	144.3	
			-12.0
	9 p.m.	132.3	
			+20.6*
11	9 a.m.	152.9	
			-19.1*
	9 p.m.	133.8	
			+18.7*
12	9 a.m.	152.5	
			-15.8
	9 p.m.	136.7	
			+15.8**
13	9 a.m.	152.2	
	**********		
	9 p.m.	98.9	
			+13.9
14	9 a.m.	112.8	
			- 8.0
	9 p.m.	104.8	
			+ 8.7
15	9 a.m.	113.5	

*p< 0.05
**p< 0.01

II.

After this initial pressor effect, angiotensin II had a more marked second slow developing pressor action. Together the two effects raised arterial pressure gradually by 41 mmHg in 4 days. A similar increase occurred in 4 days in my two earlier experiments (Chapter 3). During the slow rise in this experiment, the diurnal variation of pressure was also increased. It was shown previously that variability of arterial pressure increased while sodium balance remained unchanged.

Thus angiotensin II and saralasin share several properties: both increase arterial pressure slowly and while this is occurring both increase the variability and diurnal variation of arterial pressure. Do the three effects of angiotensin II have a common mechanism and is that mechanism shared by saralasin?

I suspect that the three effects of angiotensin II do have a common nervous mechanism. Theoretically an effect of saralasin could be related to its action as an antagonist of angiotensin II or to an action as a partial agonist mimicing angiotensin II. Angiotensin II, on the other hand, acts only as an agonist. The similarity of the effects of the two agents suggests that those produced by saralasin are agonistic since there is the shared property. It is hardly likely that angiotensin II raises blood pressure at night in the rat as an agonist while saralasin produces the same effect by antagonising angiotensin II.

Normal rats infused with dextrose show a small diurnal variation of arterial pressure, being lowest in the early evening and highest in the early morning. This has been previously noted in rats (Manthorpe 1973). Man also has diurnal changes of arterial pressure, blood pressure falling markedly during sleep (Littler, Honour, Carter and Sleight 1975; Millar-Craig, Bishop and Raftery 1978). Since rats sleep for most of the day and become active at night, the fall in pressure during the day could be a result of sleep. I have noted on several occasions that blood pressure falls in the rat during the day when it sleeps rising when it wakes. Several reasons have been suggested for

the fall in arterial pressure during sleep in man (Littler et al 1975). The most likely, in my view, is that a reduction in sympathetic outflow leads to a reduction in peripheral resistance (Khatri and Freis 1969; Bristow, Honour, Pickering and Sleight 1969), increased baroreflex sensitivity also contributing (Smyth, Sleight and Pickering 1969). Thus, in the rat the fall in arterial pressure during the day may be the result of a reduction in sympathetic outflow and the rise at night a result of increased sympathetic outflow. If this is true, the extent to which saralasin and angiotensin II raise blood pressure is greatest when sympathetic activity is highest, during the night. They may achieve this by enhancing transmission in the sympathetic nervous system, (but the assumption must then be that the more activity there is, the greater will be the enhancement). It is known that angiotensin II enhances sympathetic activity by a presynaptic stimulant effect (Westfall 1977) and recent work by Wilcox, Lewis, Sever and Peart (1981) suggests that one part of the renal vasoconstriction which occurs with saralasin is mediated by renal nerves.

Our experiment also has implications for studies in which saralasin is used to test mechanisms of action of angiotensin II. As noted, the acute agonist effect of saralasin somewhat compromises interpretation of experiments testing the role of angiotensin II by short-term infusions of saralasin (Bumpus and Khosla 1977; Fagard, Amery and Lijner 1981). My present experiment suggests that the slow rise of pressure and increased diurnal variation may also compromise experiments in which saralasin is given longterm. The timing of experiments is clearly important, since in the normal rat, saralasin infused at night produces a different effect from saralasin infused during the day. I shall consider the implications of this later.

The original purpose of this part of my study was to resolve the different responses in two experiments in which saralasin was infused for 12 hours into renal hypertensive rats (Riegger et al 1977; Bing et al, 1981). The findings in normal rats were unexpected and interesting in themselves. I have

described them in a brief report (Brown and Lever 1981) and will shortly submit a full paper. Meanwhile I have not completed the study of renal hypertension: some preliminary results are given below.

4.6 EXPERIMENT 5

EFFECT OF PROLONGED SARALASIN INFUSION ON ARTERIAL PRESSURE IN FOUR RENAL HYPERTENSIVE RATS.

4.6:1 Methods

Rats were made hypertensive by the application, under ether anaesthesia, of a silver clip (2.8) to the left renal artery, the right kidney being left in situ. 35-60 days after application of the clip, catheters were implanted into the abdominal aorta and IVC and rats were allowed to recover in ordinary rat cages (Figures 4.6)

They were placed in metabolic cages on the second postoperative day (day 3) for 3 days for acclimatization and on day 6 were connected to the spring and balance, remaining conscious during the connection. They were infused with saralasin as described previously for normal rats (4.2:1). Infusions of saralasin were begun at 10.00 a.m. in 3 rats (mildly hypertensive) and at 9.00 a.m. on one occasion and at 9.00 p.m. on another in one severely hypertensive rat. MAP was recorded continuously from day 7 until the end of the experiment in these four animals. Traces were analysed as before.

4.6:2 Results

1) Mildly hypertensive rats As in normal rats, infusion of saralasin in three mildly hypertensive rats raised arterial pressure acutely from a mean level of 132.3 $\pm$ 2.1 mmHg to 158.3 $\pm$ 4.4 mmHg within 3 minutes of starting infusion. Pressure then fell to pre-infusion levels within 5 minutes. After this transitional increase, MAP remained at pre-infusion levels until about 10.00 p.m. when it began to rise progressively as in the control rats from a level of 135 $\pm$ 0.8 mmHg, the day before saralasin, to 150.4 $\pm$ 1.2 mmHg on the 4th day (Figure 4.7). There was a slight increase in diurnal variation of

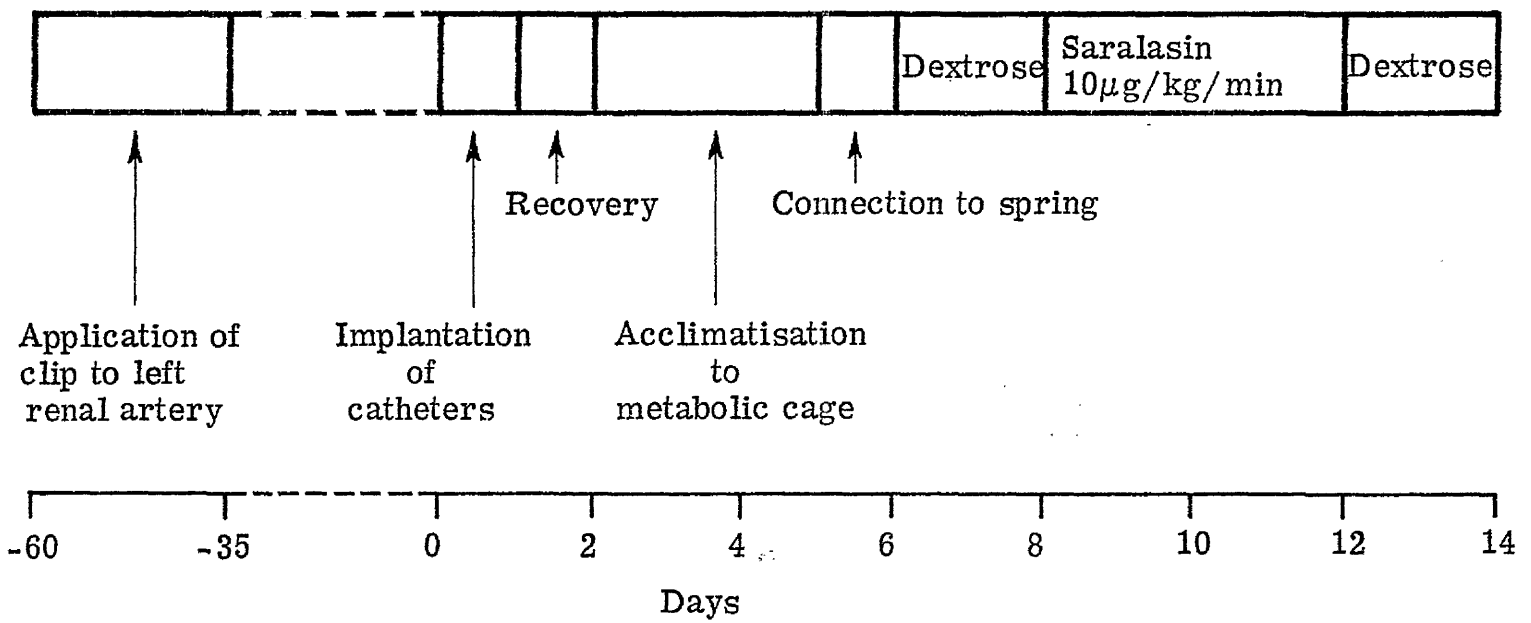


Figure 4.6 Experimental protocol

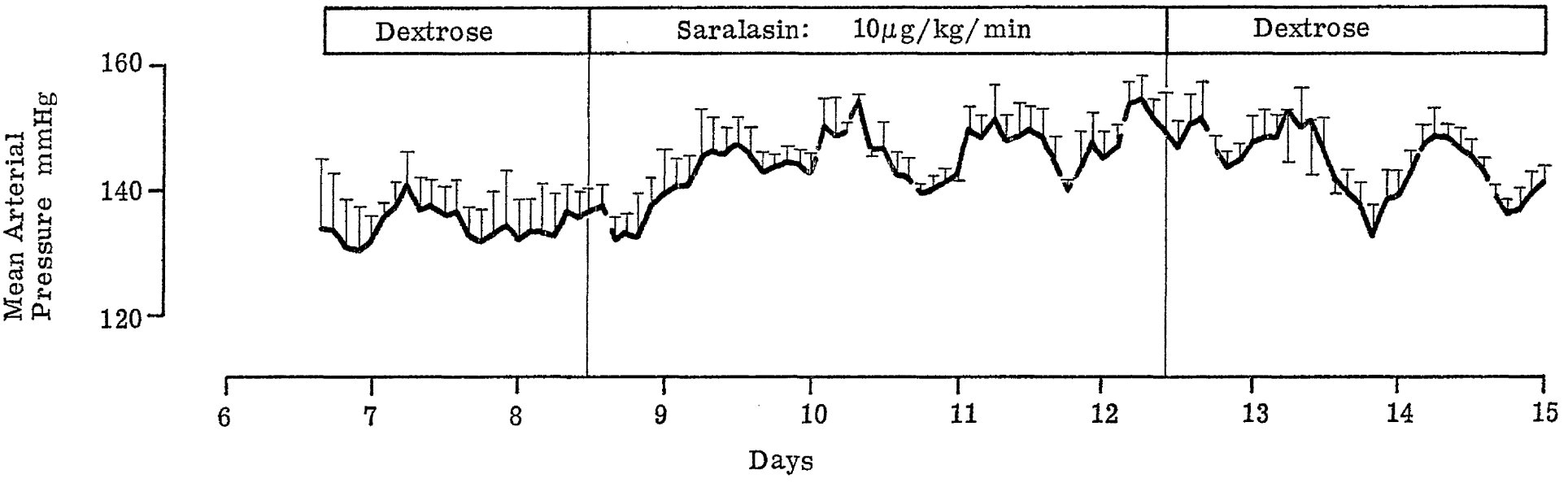


Figure 4.7 Mean arterial pressure at two hourly intervals during saralasin infusion

pressure during saralasin, but as far as could be judged in 3 animals this was not as marked as in normal rats receiving saralasin. On returning to dextrose, MAP did not fall to pre-infusion levels until at least one day later. Diurnal variation during this second control dextrose period was markedly increased.

2) Severely hypertensive rat. One severely hypertensive rat was infused, as described above, except that saralasin was infused for two 4-day periods, one starting in the morning, as in the experiment of Riegger et al (1977), the other starting at night, as in the experiment of Bing et al (1981). On stopping the first saralasin infusion, dextrose was infused for 5 days, instead of 2, to permit recovery. A second infusion of saralasin was begun on day 18 and was carried out as before.

Saralasin begun at night caused a rapid fall of arterial pressure (Figure 4.8). Mean arterial pressure before saralasin was 177 mmHg. Thirty minutes later it was 149 mmHg and 90 minutes later it was 127 mmHg. Thereafter it rose progressively over the 4-day period of saralasin infusion, but as in the control experiment, there was a diurnal variation of pressure. This diurnal variation was also apparent in the control period.

A different pattern emerged when saralasin was begun in the morning. Mean arterial pressure fell gradually over a 10 hour period (Figure 4.9). Before saralasin it was 185 mmHg; 10.5 hours later it was 127 mmHg. Thereafter it rose progressively as before (Figure 4.8). Figure 4.9 compares the time course of the decrease in pressure during the first 10 hours of saralasin infusion starting at night or in the morning.

4.7 COMMENT

No firm conclusions can be drawn from this small study. I include it here partly to show that it is possible, technically, to infuse renal hypertensive rats with saralasin for 4 days, measuring arterial pressure continuously. Also the findings in the severely hypertensive rat would be interesting and important if confirmed in a group of 6 more rats.

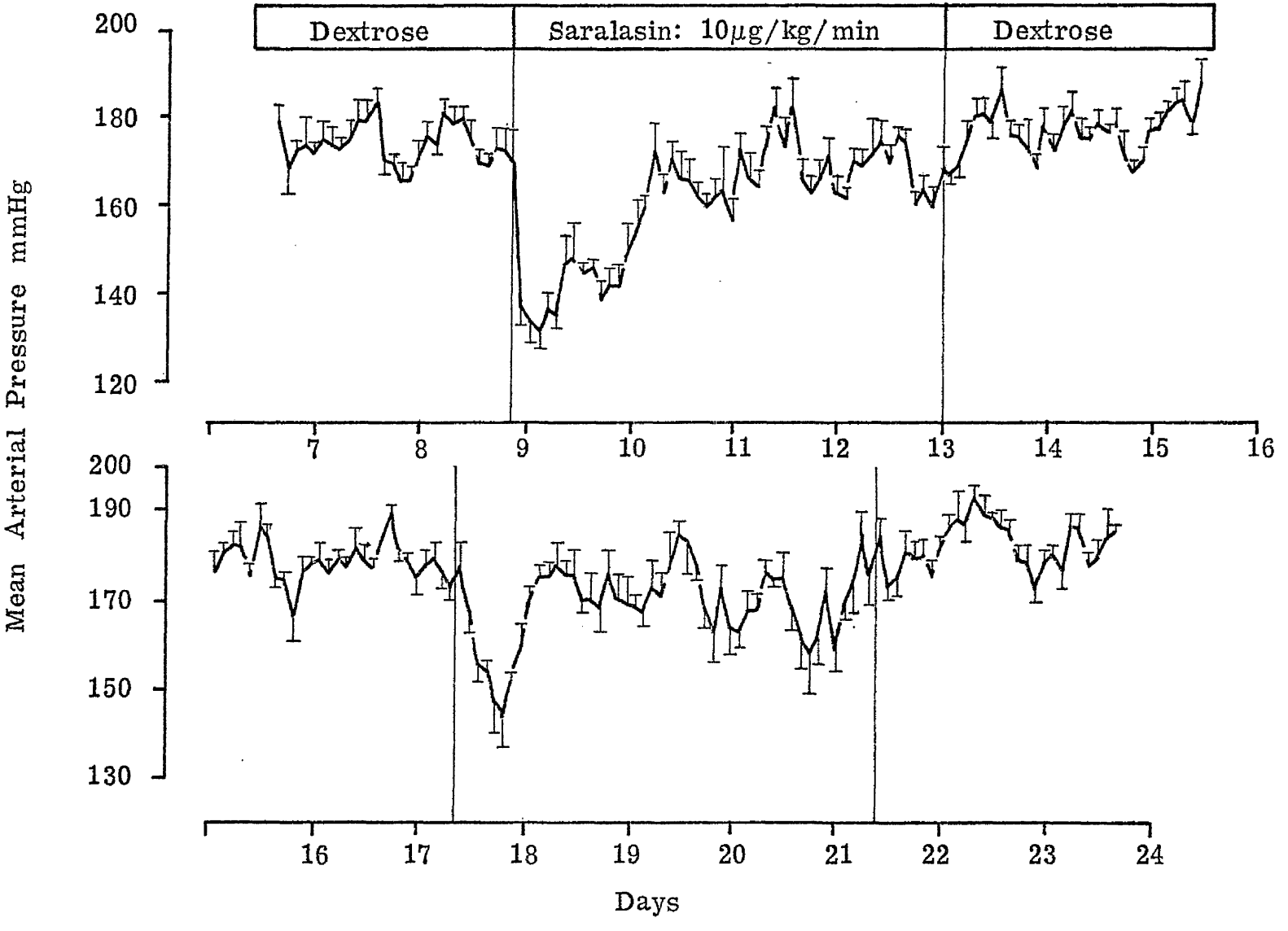


Figure 4.8 Mean arterial pressure during saralasin infusion begun at night (upper panel) and in the morning (lower panel)

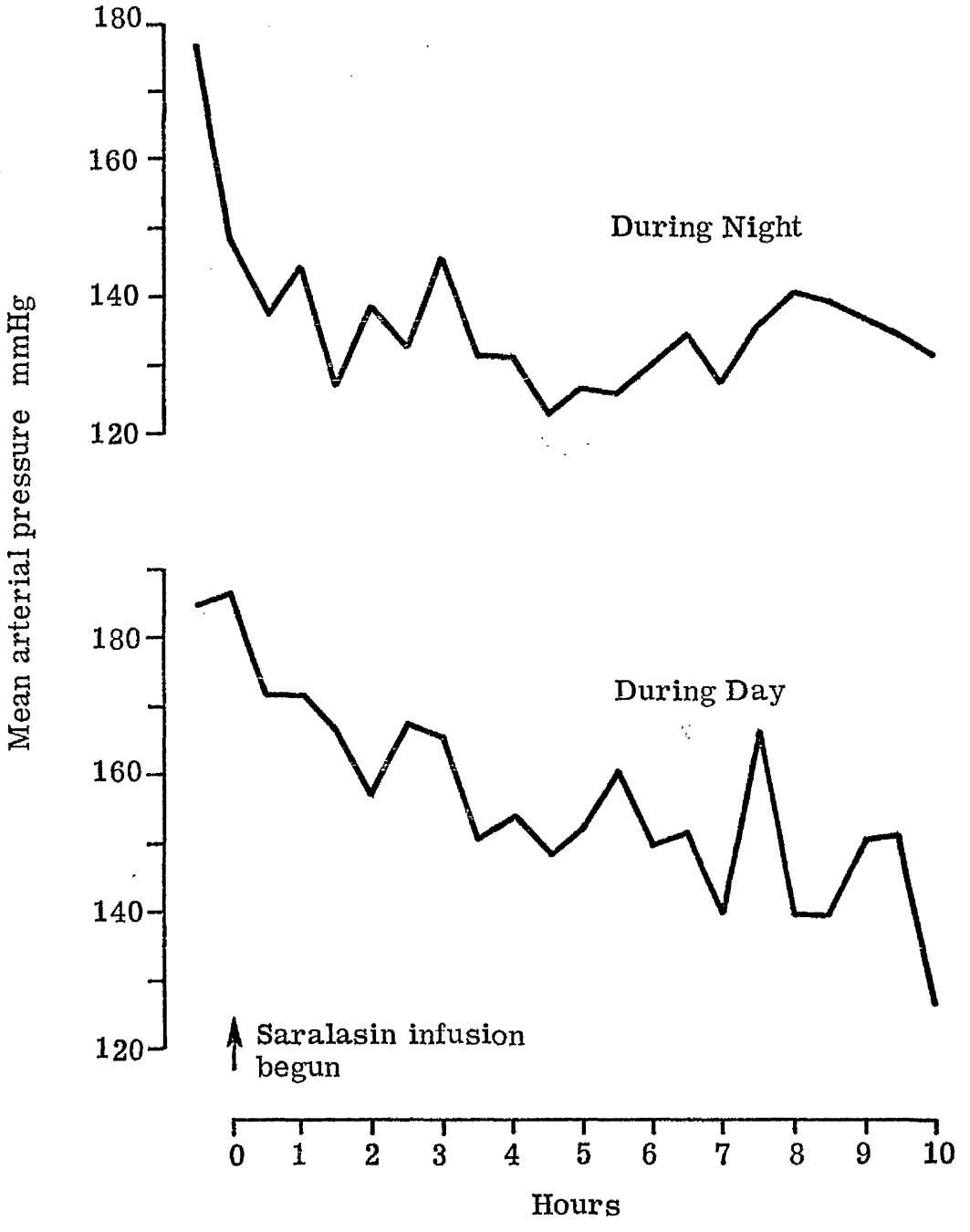


Figure 4.9 Time course of the decrease in pressure during the first 10 hours of saralasin infusion begun at night or in the morning.

No conclusions can be drawn from the study of 3 mildly hypertensive rats. Their hypertension was too mild for comparison with earlier studies. These rats did show a slow pressor response with increased diurnal rhythm during saralasin infusion, although neither effects were as marked as in the control rats. One severely hypertensive rat showed a different type of response depending on whether the infusion was begun at night or in the morning (though I have not excluded a difference resulting from the sequence in which infusions were given). After the abrupt fall in blood pressure with the first infusion beginning at night, there was little change of blood pressure for the following 12 hours as in the experiment of Bing et al (1981) where infusions were given at night. However, when the infusion was given during the day (Figure 4.9) blood pressure fell gradually as in the study of Riegger et al (1977) where infusions were also given during the day. Until more experiments are done, I cannot attribute the difference between experiments to the fact that saralasin was given by day in one and by night in the other. It remains a possibility. Meanwhile, my finding of a slow pressor response with saralasin, probably agonist in nature, raises another problem of interpretation: blood pressure may fall gradually in hypertensive rats, but not in normal rats infused with saralasin either because the antagonist action is greater or the agonist action is less in the hypertensive animals. That there was an agonist effect in the control group of normal rats given saralasin by Riegger et al (1977) is suggested here by the fall in pressure during the day in normal rats compared with the failure of blood pressure to fall in normal rats infused with saralasin by day (Figure 4.2).

For all these reasons, I am reluctant to place too much reliance on interpretation of the role of renin and angiotensin assessed by prolonged infusion of saralasin. I shall complete experiment 5 and repeat the experiment using a new renin inhibitor as described below.

CHAPTER 5

A NEW INHIBITOR OF RENIN: PRELIMINARY RESULTS IN THE RAT.

5.1 INTRODUCTION

Problems with saralasin as an agent for testing physiological and pathological roles of angiotensin II has led to a hunt for better agents blocking the renin angiotensin system. Angiotensin I converting enzyme inhibitors have been studied (Tree and Morton 1980) but their potential interaction with the kallikrein system (Thurston and Swales 1978) also clouds interpretation of their effects. Attention has therefore turned to renin inhibitors which have none of the agonist actions of saralasin or the kinin potentiation of converting enzyme inhibitors. The idea that competitive inhibitors of renin, made by synthesising substrate analogues, could inhibit the system is not new (Skeggs, Lentz, Kahn and Hochstrasser 1968), but they have not, as yet, been of sufficient potency and solubility for in vivo studies (Haber and Burton, 1979). The synthesis of a new peptide renin inhibitor, H77, (Szelke, Leckie, Tree, Brown, Grant, Hallett, Hughes, Jones and Lever, 1981) with a greater affinity for renin and increased solubility was opportune.

The inhibitor was tested in vitro by Dr Brenda Leckie. Figure 5.1 shows inhibition curves for one experiment in which dilutions of H77 were tested against human, dog and rat plasma in the same batch of assays. The efficiency of H77 against the renin in dog plasma was greater than against human renin, the inhibitory constant being about 0.3μM for dog renin as against 1μM in human. The inhibitory constant for the reaction of rat renin with its substrate was around 0.6μM. Thus, in vitro, H77 was most effective in dog plasma. Dr Malcolm Tree infused the inhibitor into sodium depleted dogs. It markedly reduced arterial pressure and plasma concentrations of angiotensin I and II when given at rates of 0.1 mg/kg/hour and higher (Szelke et al 1981).

My object in this preliminary study was to determine whether the

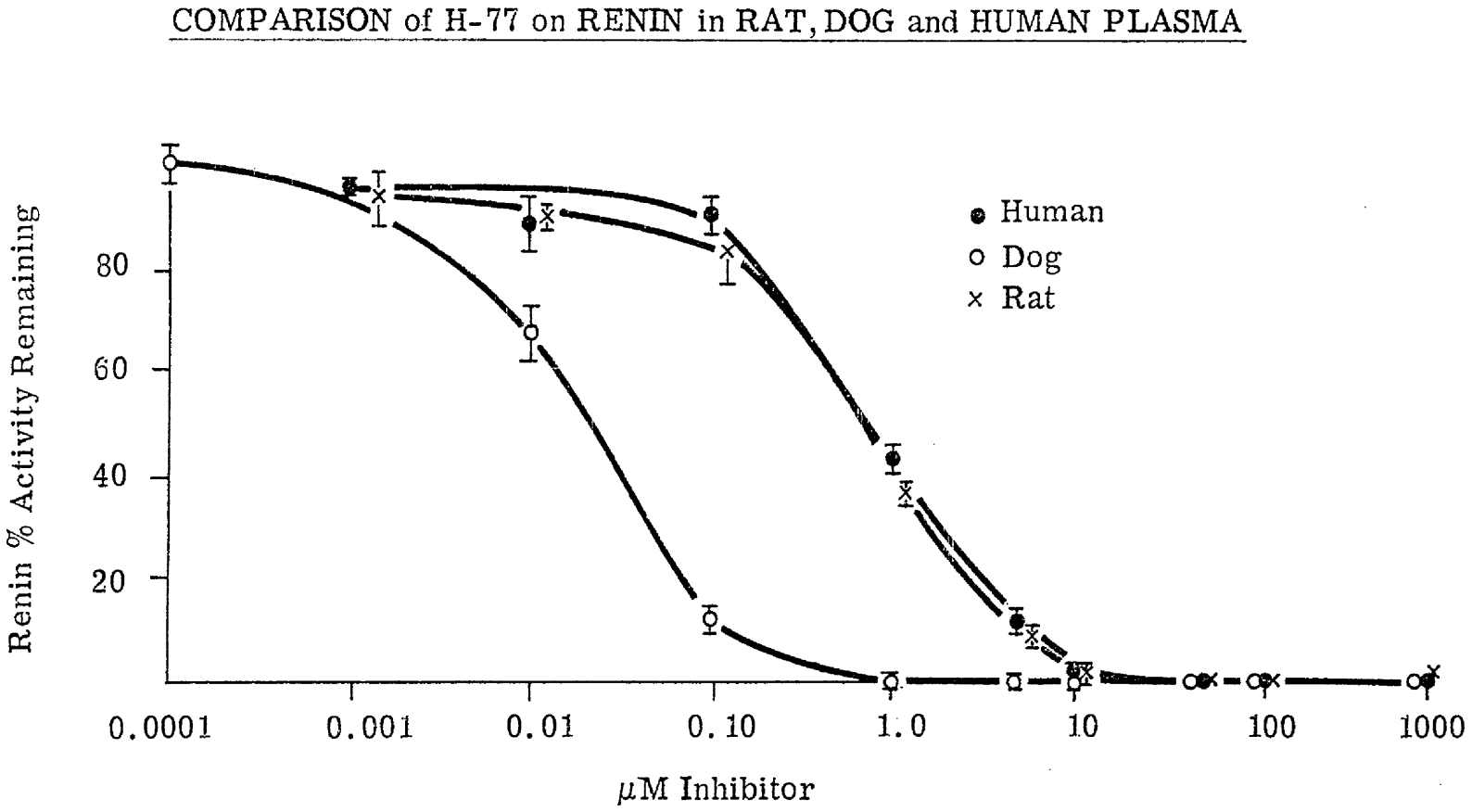


Figure 5.1 Comparison of H77 on renin in rat, dog and human plasma. Serial dilutions of H77 were tested for their inhibitory effect against standard preparations of human, rat and dog renin

inhibitor was also effective in the rat in vivo. Higher doses were tested as the in vitro data suggested that these might be needed.

5.2 EXPERIMENT 6

EFFECT OF H77 IN VIVO IN THE RAT

5.2:1 H.77 infusions in normal rats.

Four rats were studied. Aortic and IVC catheters were implanted and after recovery and acclimatization, rats were connected to the spring and balance system on day 6. Each rat then had three different intravenous infusions. These were given in random order on days 7,11 and 15. For one of these infusions (part 1 of the experiment, Figure 5.2), 5% dextrose was given at 1 ml/hour for 2 hours, mean arterial pressure being recorded continuously. After 1 and 2 hours, 0.5 ml arterial blood samples were taken for estimation of plasma angiotensin II concentration. Parts 2 and 3 of the experiment were carried out in the same way except that H77 at 1 mg/kg/h and at 10 mg/kg/h respectively were infused during the second half of the experiment (Figure 5.2). Samples for plasma angiotensin II measurement were taken as before. The order of the experiment was as follows: Rat 1 - part 1, part 2 then part 3; Rat 2 - part 2, part 3 then part 1; Rats 3 and 4 - part 3, part 1, part 2.

Mean arterial pressure was recorded continuously. The analysis was based on values at 5 minute intervals during the 2 hour infusion period.

Results.

H77 infused at 1 and 10 mg/kg/h had no significant effect on arterial pressure (Figure 5.3). The control infusion of dextrose was also without effect.

Plasma angiotensin concentration did not increase during the infusion of dextrose. Mean values for the 4 rats during the control period (Sample I) on the 7th, 11th and 15th days were 45.6 $\pm$ 5.3, 37.3 $\pm$ 1.9, 39.3 $\pm$ 1.4 pg/ml. H77 infused at 1 mg/kg/h and at 10 mg/kg/h had no effect on plasma angiotensin II concentration except possibly in one rat in which angiotensin II levels decreased from 42.3 pg/ml to 26.9 pg/ml (Figure 5.4).

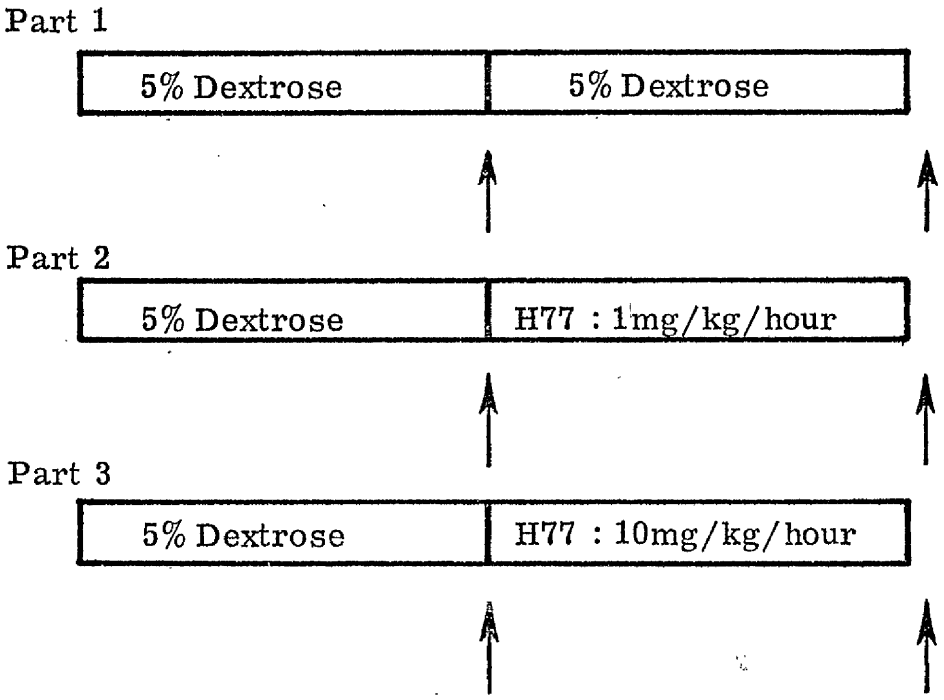


Figure 5.2 Experimental protocol

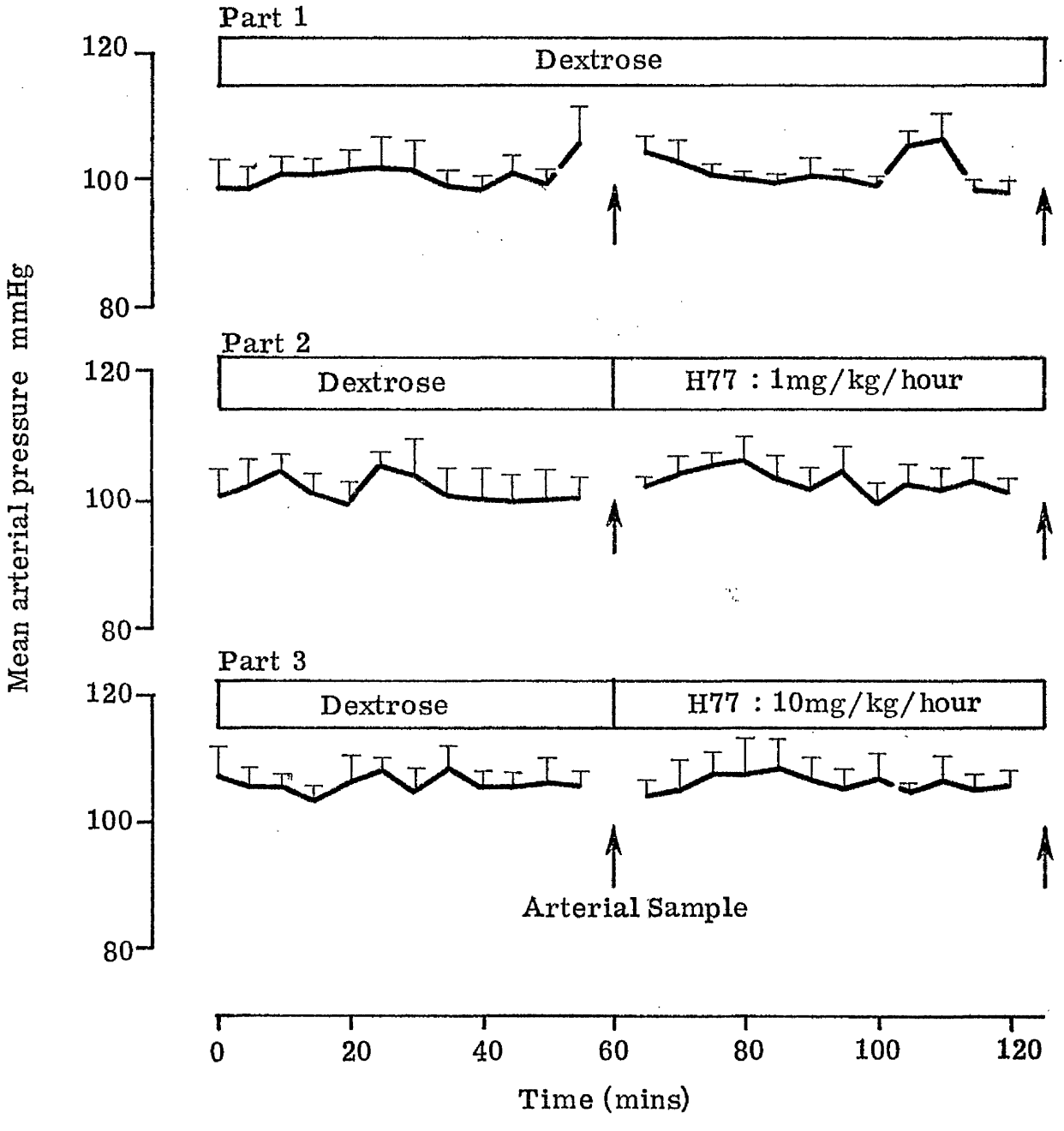


Figure 5.3 Mean arterial pressure during infusion of H77

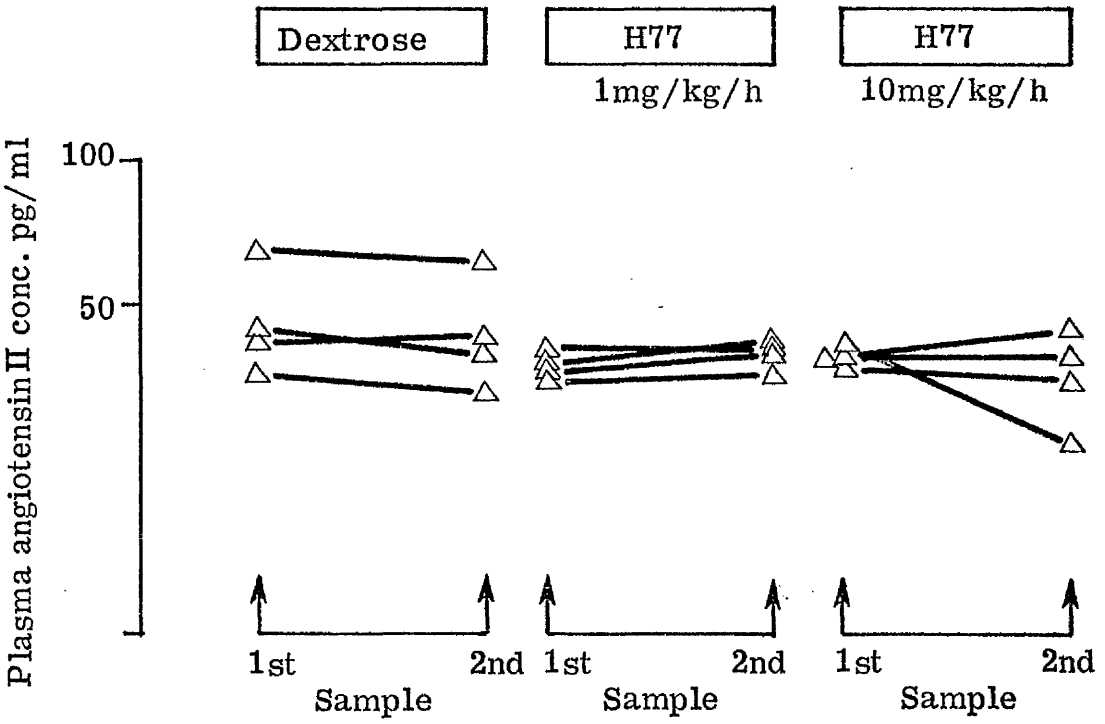


Figure 5.4 Plasma angiotensinII levels before and after infusion of H77

5.2:2 H77 infusions in renal hypertensive rats.

Two rats were studied, one received H77 at 1 mg/kg/h, the top dose infused in the dog. The other received dextrose as a control. Forty to 50 days prior to the start of the experiment, a 0.008" silver clip was applied to the left renal artery, the right kidney was untouched (2.8). Aortic and IVC catheters were implanted 41 days after the application of the clip and rats were allowed the usual period of recovery and acclimatization before being connected to the spring balance system (3.2:1). The experiment began on day 7, 5% detrose was infused at 2.4 mls/24 hours for 2 days, then H77 at 1 mg/kg/h for 2 days and finally dextrose for a further 2 days. The control rat received dextrose throughout. Arterial samples for estimation of plasma angiotensin II concentration were taken on day 7 and day 10 (Figure 5.5). Mean arterial pressure was recorded continuously and analysed as before (4.2:1).

Results Figure 5.6 shows mean arterial pressure during prolonged dextrose and prolonged H77 infusion in renal hypertensive rats. MAP showed little change during H77 infusion compared with the control dextrose infused period. The control rat infused with dextrose, however, showed an increase in pressure over the 6 day period. MAP on day 7 was 131.8 $\pm$ 1.2 mmHg and on day 12 was 151.5 $\pm$ 0.8 mmHg.

Plasma angiotensin II levels fell from 71.1 pg/ml to 49.9 pg/ml during infusion of H77. The angiotensin II levels in the dextrose infused rats, however, increased slightly from 44.2 pg/ml to 51.9 pg/ml.

It was decided to test higher rates of infusion. One renal hypertensive rat was prepared as before. On day 7, dextrose was infused for 1 ml/h for one hour and then H77 at 100 mg/kg/h for one hour. Mean arterial pressure was recorded continuously. H77 raised arterial pressure by 41 mmHg, but the effect lasted for 6 minutes only. Blood pressure then fell. Although the infusion was stopped after 10 minutes, pressure continued to fall and the rat died 3 minutes later.

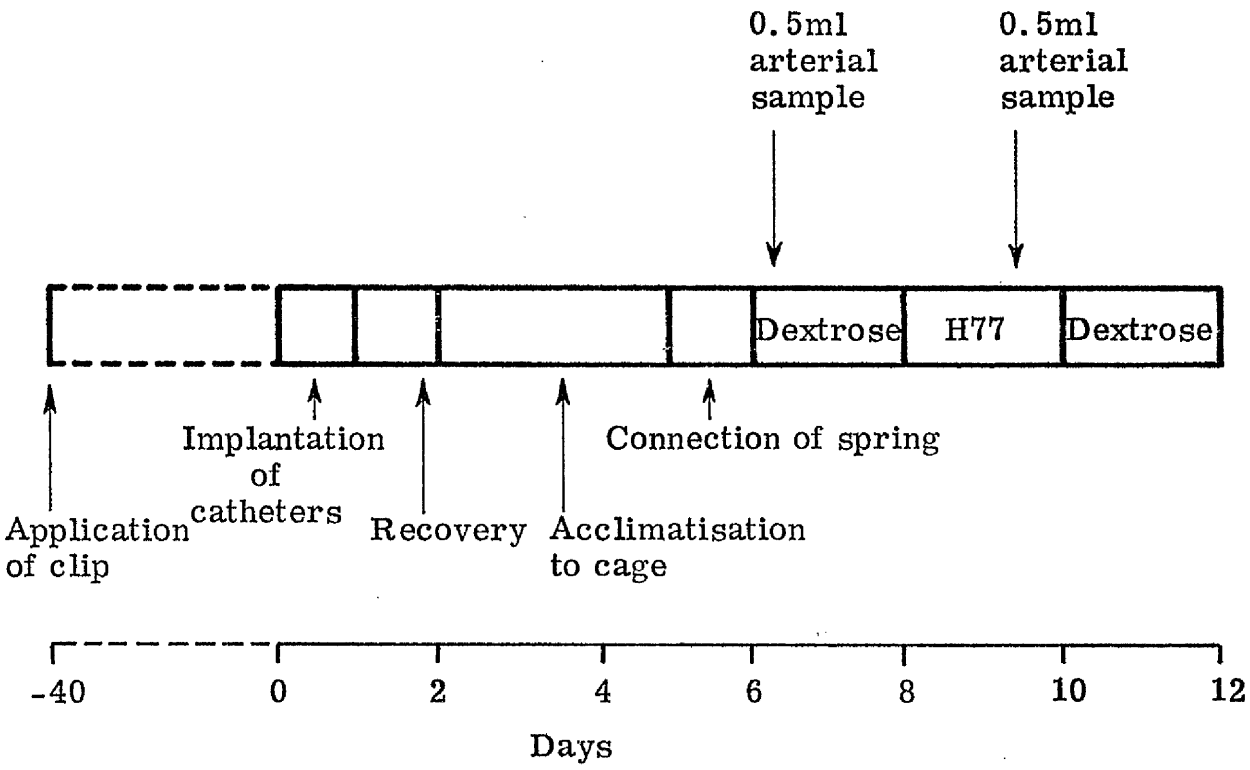


Figure 5.5 Experimental protocol

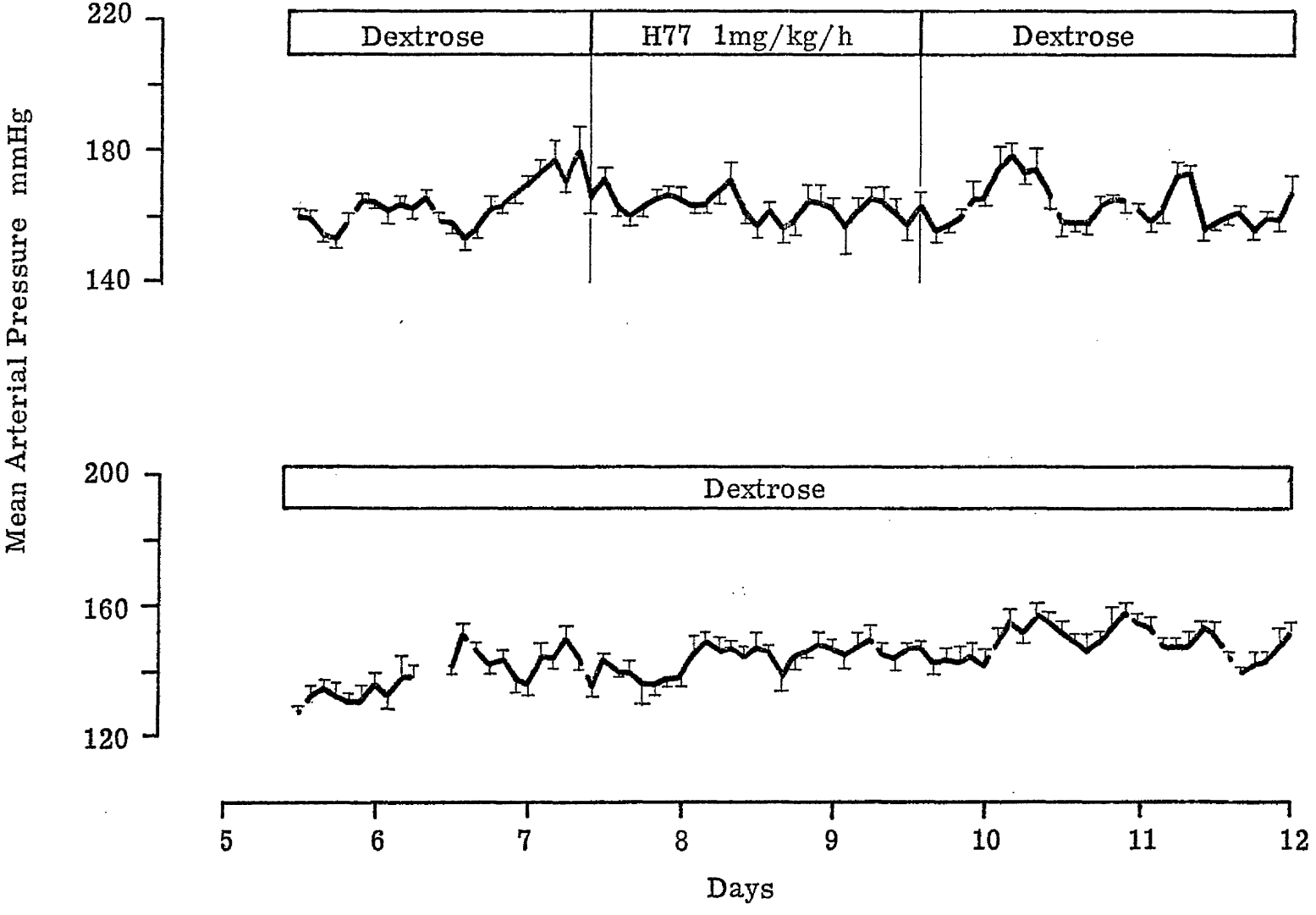


Figure 5.6 Mean arterial pressure during dextrose and H77 infusion in renal hypertensive rats

5.3 COMMENT.

In vitro testing of H77 showed that it is a more effective antagonist of dog renin than of human or rat renin. Dr Tree's experiment in the conscious dog and my own experiment in the conscious rat confirm this. The failure of H77 to reduce angiotensin II and blood pressure in the rat makes it less likely that the positive result in the dog is a non-specific effect of H.77 unrelated to its action as an inhibitor of renin.

My studies also suggest that the inhibitor is ineffective in the rat at a dose which is not toxic, but possibly toxic at doses which are more likely to be effective. Ferring Ltd. have done toxicity tests with H77 in the rat. The LD50 for intravenously injected inhibitor is 3 mg/rat. One rat died in my experiment. By the time of death it had received the largest dose, 3.8 mg.

The synthesis of new renin inhibitors is underway and in vitro tests already show one of these to be a more effective inhibitor of rat renin than is H77. Thus, there are prospects for testing effective inhibitors of renin in conscious rats. It is important that this is done, in my view, as the agonist action of saralasin and the kinin potentiation of converting enzyme inhibitor complicates analysis of the highly complex mechanism at work in renal hypertension (Swales 1979). The new renin inhibitor should be without these second effects.

CHAPTER 6

GENERAL DISCUSSION.

In earlier parts of this thesis I have discussed the differences between the fast and slow pressor effects of angiotensin II and have compared plasma angiotensin II levels during the two effects. I have discussed the diurnal effects of saralasin and angiotensin II and the in vitro and in vivo testing of a new renin inhibitor. I shall consider here in a more general discussion the mechanism of the slow pressor effect of angiotensin II and its possible roles in the pathogenesis of renal hypertension and in the regulation of arterial pressure.

6.1 SUMMARY OF MAIN FINDINGS.

The studies carried out here have shown that angiotensin II when given in low dose to conscious rats, raises arterial pressure gradually over a 7 day period. When the duration of the infusion was increased to 14 days, arterial pressure rose further. This slow response has been shown in a variety of species (see pages 29-31 in the Introduction), but in none did it develop as markedly or as rapidly as in the rat. Its magnitude in the rat after 7 days was similar to that of the maximum direct response, and after 14 days it was higher still. The slow pressor effect developed at a plasma concentration of angiotensin II within, or close to, the physiological range and at much lower levels than those required to produce a direct response of similar magnitude. The diurnal variation of arterial pressure also became more marked during prolonged angiotensin infusion and the variability of pressure increased.

Saralasin also produced a slow rise of pressure with increased diurnal variation and variability of pressure. Food and water intake and sodium balance remained unchanged by prolonged saralasin and angiotensin infusions. These findings, together with the structural similarities between angiotensin II and saralasin, suggest that both peptides are acting by a similar mechanism to raise arterial pressure.

6.2 POSSIBLE MECHANISMS OF THE SLOW PRESSOR EFFECT OF ANGIOTENSIN II.

A number of suggestions have been made as to the mechanism of the slow pressor effect of angiotensin II (Brown et al 1977). Some are discussed below.

6.2:1 Action of angiotensin II on the nervous system.

It is well know that angiotensin II has an action on both the central and peripheral nervous system (Yu and Dickinson, 1965; Lowe and Scroop 1969; Ferrario et al 1972; Westfall 1977) and there has been much to suggest that the nervous system participates in the slow effect. Drugs which interfere with nervous transmission prevent or attenuate the rise of pressure (McCubbin et al 1965; Yu and Dickinson 1971). Resetting of baroreceptors is partly responsible for the early rise of pressure produced by low dose angiotensin II in the dog (Cowley and DeClue 1976) and there is evidence that the baroreceptor reflex is modulated by central administration of angiotensin II (Fukiyama 1973, Goldstein et al 1974; Marker et al 1980). Further evidence for participation of the nervous system comes in this study from the increase in variability of pressure and increased activity of the rats in this study. Dogs in the study of McCubbin et al (1965) also showed increased variability of pressure. Increased diurnal changes of pressure during angiotensin II infusion may also be indicative of an action on the nervous system as described earlier (Chapter 4). Angiotensin II could thus be acting either centrally or peripherally or at both sites to raise arterial pressure. In the rat periventricular structures surrounding the 3rd ventricle have been shown to mediate the central component of the pressor action of blood borne angiotensin II (Fink et al 1980). This area is also a crucial site for the integration of circulatory, hormonal and behavioural responses involved in maintaining salt and water homeostasis in the rat (Buggy and Johnson 1977; Johnson and Buggy 1978; Brody, Fink, Buggy, Haywood, Gordon and Johnson 1978). The anteroventral 3rd ventricle (AV3V) region also

encompasses a variety of periventricular structures in the anterior hypothalamic pre-optic region of the forebrain, including the organum vasculosum of the lamina terminalis (OVLT), a highly vascularised area in which the blood brain barrier is relatively deficient. This site would be an ideal location of neural elements sensitive to concentrations of angiotensin in the blood perfusing the brain. It is, therefore, possible that angiotensin II is acting on such a site to produce its slow pressor effect. I discussed earlier the evidence that the area postrema mediates the central pressor effect of angiotensin II in other mammals.

6.2:2 An effect on salt and water excretion.

Angiotensin II alters urinary excretion of sodium and water (Brown and Peart 1962) and thus by acting directly or through aldosterone, could cause sodium and hence water retention, thereby raising blood pressure (Brown et al 1977). I did not, however, find sodium retention in rats during the slow rise, but the case for sodium retention should probably not be dismissed on this evidence as there are major difficulties in calculating cumulative balance properly in the rat (Mohring and Mohring 1972). Although the differences were not significant, rats receiving angiotensin II did have a greater cumulative balance than the control rats (Chapter 3). Work in other species has been conflicting. In man (Ames et al 1965; Oelkers et al 1978) and in the dog (Urquhart, Davis and Higgins 1963) prolonged angiotensin II infusion did produce sodium retention. In rabbit (Dickinson and Yu 1967), however, there was no evidence for a causative role for sodium. Sodium status is obviously important, however, since the pressor response to angiotensin II is increased during sodium retention (Slack and Ledingham 1976) and the slow rise in pressure in dogs is prevented by dietary salt restriction (Cowley and McCaa 1976). Also the rate and magnitude of the rise of pressure during prolonged angiotensin II infusion is greatly increased by increasing dietary sodium (Cowley and McCaa 1976, DeClue, Guyton, Cowley, Coleman, Norman and McCaa 1978).

Whether angiotensin II acts by stimulating aldosterone is also unclear. The balance of evidence suggests that during chronic angiotensin II infusion, the increase of aldosterone is not persistent or marked, and that a sodium retaining effect of angiotensin II is more likely to be renal than adrenal (DeClue et al 1976, Hall et al 1979, Bean et al 1979). At low doses angiotensin II promotes renal retention of salt and water, and this effect is compensated for by the accompanying rise of arterial pressure which promotes diuresis and natriuresis. In this study, although there was no early sodium retention, there was sodium loss (insignificant statistically) towards the end of the 7 day infusion which was probably a result of the high level of mean arterial pressure. I shall pursue the possibility that longer infusions result in a higher arterial pressure with greater sodium depletion and perhaps malignant phase hypertension.

It has been proposed that sodium and water retention results in expansion of plasma and extracellular fluid volume which raises cardiac output and, because of overperfusion of tissues, produces an autoregulatory vasoconstriction with decrease of cardiac output, hypertension then being maintained by vasoconstriction (Guyton 1969; Ledingham 1971). Evidence for an initial increase in cardiac output, however, is not available. In fact in the dog (Cowley and DeClue 1976), cardiac output was decreased initially and then increased during the 4th-5th day of prolonged low-dose angiotensin II infusion. There could be a contribution from autoregulation therefore, as the authors say, but it is not the whole explanation.

Thus, sodium status may modify the rate of rise of pressure, but this is a different issue from sodium retention being the primary cause of the rise of pressure. On balance, I favour a primary role for the nervous system. It is interesting then that the AV3V region, the area involved in the central pressor action of angiotensin II, is also involved in water and salt homeostasis (Johnson and Buggy 1978).

6.2:3 Structural changes during the slow effect.

The rise of pressure during prolonged infusion of angiotensin II could produce structural changes in the small resistance vessels which would help to maintain the hypertension. Folkow (1971) has suggested that the small resistance vessels, respond to raised pressure by increasing wall thickness and thus wall/lumen ratio, thus raising resistance to flow at maximum dilatation and increasing the response to vasoconstrictor agents. Koletsky, Rivera-Velez and Pritchard (1966) infused angiotensin into rats for 12 hours per 24 hours for 5 days and showed thickened arterioles with narrowed lumens. The rate of infusion in that study, however, was 100 ng/kg/min, five times the dose used in my experiment.

Changes in the vessels may explain the enhanced response to a pressor infusion of angiotensin II on stopping prolonged angiotensin II infusion. Tobian, Janecek, Tomboulian and Ferreira (1961) suggested that increased sodium in vascular tissue could heighten the response to vasoconstrictor agents. It is of interest, therefore, that prolonged angiotensin II infusion increased vascular sodium (Villamil, Nachev and Kleeman 1970).

6.2:4 A combination of mechanisms.

It is certainly not clear whether a combination of mechanisms or a single mechanism is responsible for the slowly developing pressor action of angiotensin II. The mechanisms outlined above are not mutually exclusive: angiotensin II acting through the nervous system could affect salt and water balance and changes of sodium and water could modify the rise of pressure. Also changes in sodium status could affect wall/lumen ratio and hence vascular tone.

It is clear that further work must be done to elucidate the mechanism of the slow pressor effect of angiotensin II. I hope to test the possibility that the nervous system is involved by using sympathetic nervous blocking agents. It would also be of interesting to try to produce the slow rise of pressure following ablation of the AV3V region. I shall, with Dr Stephen Ball, measure

plasma noradrenaline during the slow rise of pressure, seeking evidence of increased transmitter release. If noradrenaline rises, I would be particularly interested to see if propranolol prevents the slow rise of arterial pressure.

6.3 ROLE OF THE SLOW PRESSOR EFFECT OF ANGIOTENSIN II IN THE PATHOGENESIS OF RENAL HYPERTENSION.

There is considerable controversy about the mechanism by which sustained blood pressure elevation follows renal artery constriction. In the early phase of Goldblatt two-kidney one clip hypertension, plasma levels of renin and angiotensin II are raised sufficiently to account for the hypertension by direct vasoconstriction (Caravaggi et al 1976) and renin-angiotensin blockade lowers blood pressure to normal at this time (Pals et al 1971b; Miller, Samuels, Haber and Barger 1975; Coleman and Guyton 1975; Masaki et al 1977; Freeman et al 1977). In the later stages, however, plasma levels of renin and angiotensin II decline and are insufficient to raise blood pressure by direct vasoconstriction alone (Bianchi et al 1972; Brown et al 1977b). The mechanism responsible for maintaining the hypertension at this stage is unclear. Dickinson and Lawrence (1963) were the first to suggest that the slow pressor effect might be important in the pathogenesis of chronic renal hypertension.

In the experiments described here, dose-response studies testing the direct pressor response of angiotensin II during the slow rise of pressure showed an upward shift in the curve relating arterial pressure and rate of angiotensin infusion, a given rate maintaining a higher pressure. Bean et al (1979) demonstrated an upward shift in the curve relating arterial pressure and plasma angiotensin II concentration during the slow pressor effect in the dog. Thus, a constant increase in plasma concentration of angiotensin II maintained a progressively higher level of pressure. Data in a patient with renin-secreting tumour also suggests this (Brown, Fraser, Lever, Morton, Robertson, Tree, Bell, Davidson and Ruthven, 1973). Arterial pressure and plasma concentrations of angiotensin II are both increased in the disease, but arterial pressure is higher

than can be explained by the direct pressor action of angiotensin II. This state exists in chronic renal hypertension where blood pressure is higher for a given level of angiotensin II than in normal subjects in whom pressure is raised acutely by infusion of angiotensin II (Brown et al 1976b, 1977). It does not, however, follow that angiotensin II has produced the state in renal hypertension, although it is very likely to have done so in renin-secreting tumour. The slow pressor effect of angiotensin II and a hypertensive disease, renin-secreting tumour, resulting from excess of renin and angiotensin thus both produce changes similar to those observed in chronic renal hypertension. Although these findings are compatible with a role for the slow pressor effect in chronic renal hypertension, they do not establish it.

Work with inhibitors of the renin-angiotensin system to assess the role of angiotensin II in the pathogenesis of chronic renal hypertension has been conflicting. Many workers have shown that renin-angiotensin blockade has little or no effect on arterial pressure in these later stages of the hypertension (Thurston and Swales 1974; Gavras, Brunner, Thurston, Laragh 1975, Sen, Smeby, Bumpus, Tucotte 1979; Bing et al 1981) while others have produced a progressive fall in blood pressure with prolonged infusion of inhibitors (Riegger et al 1977; Bengis and Coleman 1979). The inhibitors used, however, do possess additional properties and therefore make interpretation of results difficult. To my mind, use of inhibitors in these experiments has neither proved nor disproved a role for angiotensin II in the pathogenesis of chronic renal hypertension. However, Thurston, Bing and Swales (1980) have recently reported an experiment in which a renal artery clip was removed from a hypertensive rat during blockade of the renin-angiotensin system. Blood pressure fell as in unblocked animals which strongly suggests that changes in the renin mechanism are not responsible for the fall of pressure when renal hypertension is reversed. It is my intention to repeat the experiment of Riegger et al (1977) using the new renin inhibitor which is being developed.

If the mechanism of the slow pressor effect of angiotensin II is a nervous one and the effect does play a role in renovascular hypertension, one would expect some evidence for the participation of the nervous system in renal hypertension. Indeed there is considerable evidence for its involvement. The development of a number of forms of experimental hypertension including renal hypertension produced by perinephritis can be prevented by the destruction of central catecholamine neurones (Chalmers, Dollery, Lewis and Reid 1974).

In the rat, the centrally mediated pressor activity of angiotensin II is dependent upon activation of angiotensin receptors located in the ventricular system in the pre-optic hypothalamic region. The integrity of this region, the AV3V region, is essential for the development and maintenance of hypertension in a number of models (Brody et al 1978). If the AV3V region is important in the mechanism of the slow pressor effect of angiotensin II, as discussed earlier, the findings discussed here suggest, if very indirectly, that the slow response may be involved in the pathogenesis of renal hypertension.

There was some evidence, however, suggesting that the mechanism of the slow pressor effect involved salt and water retention since sodium restriction prevented the rise of pressure in the dog (Cowley and McCaa 1976). The fact that dietary salt restriction has little effect on arterial pressure in experimental renovascular hypertension, on the other hand, tends to discount a role for the slow pressor effect of angiotensin II (Swales 1979).

In conclusion, the importance of the slow pressor effect of angiotensin II in the pathogenesis of chronic renal hypertension is very unclear. Some observations favour a role, but equally others are against it. Work with new renin inhibitors may be enlightening .

6.4 A PHYSIOLOGICAL ROLE FOR THE SLOW PRESSOR EFFECT OF ANGIOTENSIN II

As noted earlier, there are many ways in which angiotensin II might act physiologically: as a local tissue hormone in the extravascular spaces of the

kidney, brain and blood vessel wall or as a blood borne hormone. It is generally agreed that blood borne angiotensin II may influence arterial pressure primarily by its direct vasoconstrictor action, that it may alter urinary sodium excretion by a direct renal action and that it stimulates aldosterone. What has not been considered so often is the possibility that by its slow pressor action, angiotensin II is also an important longterm regulator of arterial pressure.

The slow effect develops with a 4-6 fold increase in plasma concentration. A comparable direct vasoconstrictor effect requires a 32-fold increase. Higher levels of angiotensin II were obviously required for an effect on sodium excretion and drinking than those found during the slow response in the rat. In the dog, a 2-fold increase in plasma angiotensin II produced a 22 mmHg rise of pressure over a 2 week period with only an initial transient effect on aldosterone. These results suggest that thresholds for the dipsogenic, electrolyte, steroidogenic and direct pressor effects of angiotensin II are higher than that for the slow pressor effect. It follows that the most likely consequence of a small rise of plasma angiotensin II within the physiological range is a slow rise of arterial pressure. It remains to be seen whether small increases of angiotensin II can be sufficiently sustained.

My thesis began with a description of the discovery of renin by Tigerstedt and Bergman in 1898. They also proposed that renin was a blood borne agent released from the kidney, producing an effect at a distance. This was one of the earliest descriptions of a hormone. Less well known and more relevant to the slow pressor effect was their idea on the mechanism by which renin raises blood pressure.

> "The experiments reported here indicated that the kidney (also) secretes a substance which affects vascular tone, probably via the peripheral vasomotor centres ..... It is possible, for example, that in renal disease, this substance is produced in larger quantities or is metabolized more slowly and leads to a permanent increase in vascular resistance."
>
> Tigerstedt and Bergman (1898)
> (translated by Axel Phillipi).

APPENDIX 1

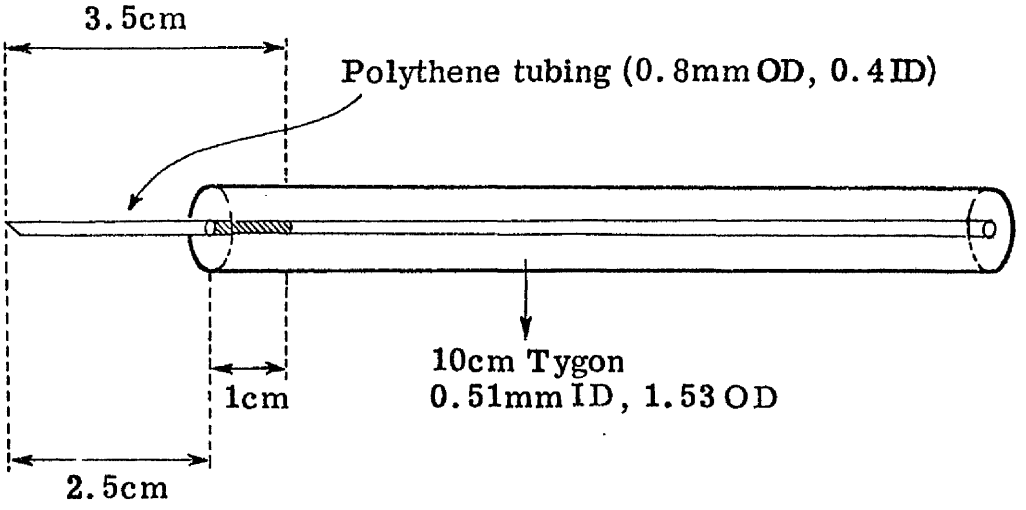
CONSTRUCTION OF CAROTID AND JUGULAR CATHETERS.

The form of the three catheters - carotid type 1 and type 2 and jugular catheter - is illustrated in Figure 1. Carotid catheter type 1 consisted of a 10 cm length of tygon (Table 1) and 3.5 cm length of polythene (Table 1). One end of the tygon tubing was enlarged by stretching over a number 21 gauge hypodermic needle which had been filed smooth. The polythene was then inserted 1 cm into the tygon tubing (Figure 1).

Carotid catheter type 2, consisting of 10 cm of NT/2 transparent vinyl tubing (Table 1) and 3.5 cm of polythene was constructed in an identical way to carotid type 1 catheter, the polythene tubing being inserted 1 cm into the NT/2 tubing.

The jugular vein catheter consisted of a 13 cm length of polythene tubing (Table 1 and Figure 1).

Carotid Catheter Type 1



lying within the carotid artery

Carotid Catheter Type 2

Identical to type 1 except materials are different. A 10cm length of transparent vinyl tubing was used instead of tygon and 3.5 cm length of polythene tubing (0.38mm ID, 1.09mm OD)

Jugular Catheter

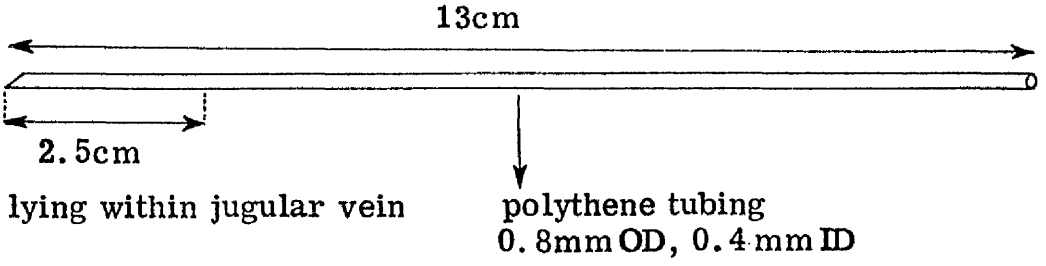


Figure 1 Carotid artery and jugular vein catheters

Table 1 Materials used in construction of carotid and jugular catheters.

CAROTID TYPE 1 CATHETER

- 10 cm length of tygon polythene: internal diameter 0.5 mm; outside diameter 1.53 mm. Norton Plastics, Akron, Ohio.

- 3.5 cm. length of polythene tubing: internal diameter 0.4 mm: outside diameter 0.8 mm - 800/100/140. Portex Ltd., Hythe, Kent, England.

CAROTID TYPE 2 CATHETER

- 10 cm length of NT/2 transparent vinyl tubing: internal diameter 0.91 mm, outside diameter 1.52 mm. Portex Ltd.

- 3.5 cm length of polythene tubing: internal diameter 0.38 mm; outside diameter 1.09 mm - 800/100/120. Portex Ltd.

JUGULAR VEIN CATHETER.

- 13 cm length of polythene tubing: internal diameter 0.4 mm; outside diameter 0.8 mm - 800/100/140. Portex Ltd.

APPENDIX 2 CONSTRUCTION OF AORTIC CATHETERS.

The catheter consisted of 4 pieces of polythene A,B,C and D; welded together by heating. In addition it had 3 anchor points used to secure the catheter to the tissues by sutures. Figure 2 shows a catheter and its main parts and Table 2 lists the materials used in making the catheter. Each part was prepared separately before assembly.

Preparation of catheter parts before assembly.

Part A. The polythene was threaded over the 35 gauge copper wire, previously siliconed to make the threading easier. The polythene was heated 4 cm from one end over a heating coil. When molten it was removed from the coil and pulled out firmly to give a thin walled section of tube about 3 cm long, as shown in Figure 2. The drawn out tubing was trimmed to give a 2.5 cm length of normal walled tubing before the thin walled portion. This was then threaded over the 35 gauge wire again and heated 2 cm from the end over a small soldering iron until pliable. The tubing was then compressed along the wire to form a small ridge or flange. The tubing was removed and allowed to harden in a ridge. A second ridge was made 0.5 cm from the first, as shown in Figure 2.

Part C This polythene was heated at one end until it was flared (Figure 2.). It was then widened over a length of 18 gauge tinned copper wire, stretching it sufficiently to push it over part B.

Part D. One end of the polythene was widened over a length of 16 gauge copper wire. As shown in Figure 2., the end was now sufficiently expanded to be pushed over parts B and C.

Assembly of the catheter.

Sealing part A to part B. A short length of 35 gauge copper wire was inserted inside the 26 gauge needle tubing. The needle tubing was then constricted over the wire so that the latter was held firmly and about 1 cm

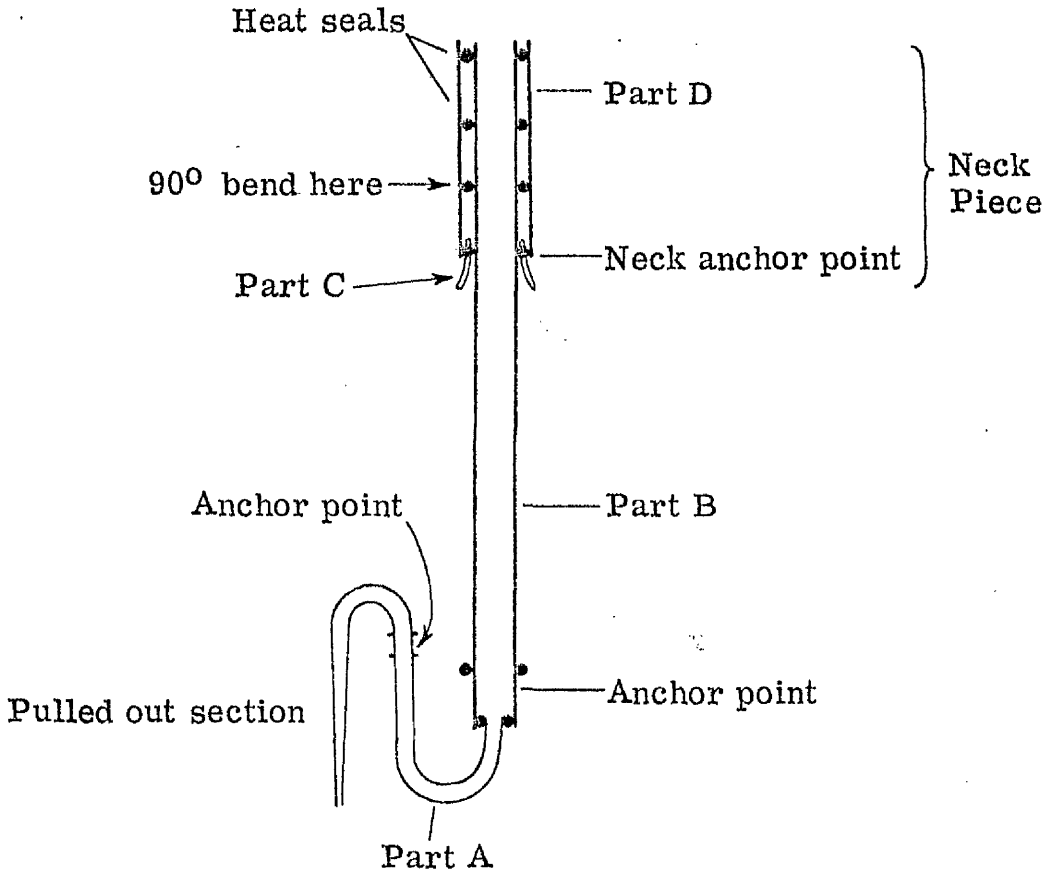


Figure 2 Aortic catheter

Table 2 Parts of the aortic catheter

PART A	10 cm length of PE 10 polythene tubing , internal diameter 0.28mm; external diameter 0.61mm. Clay Adams, Becton, Dickinson and Company.
PART B	15 cm length of polythene tubing 800/100/200, internal diameter 0.58 mm, external diameter 0.96 mm. Portex Ltd., Hythe, Kent, England.
PART C	0.5 cm length of polythene tubing 800/100/280, internal diameter 0.86 mm, external diameter 1.52 mm. Portex Ltd.
PART D	5.0 cm length of polythene tubing 800/100/320, internal diameter 1.14 mm, external diameter 1.5 mm. Portex Ltd.

MATERIALS USED IN THE MAKING OF THE CATHETER.

30 cm length of 35 gauge copper wire.
Silicone Fluid (MS 555, Hopkin and Williams Ltd., Chadwell Heath, Sussex).
20 cm length of 26 gauge stainless steel tubing.
10 cm length of tinned copper wire, 18 gauge
10 cm length of tinned copper wire, 16 gauge.

projected out of the end of the tubing. Part A was threaded onto the wire and Part B threaded over the needle tubing and about 2 mm of part A. The two lengths of polythene were then heat sealed together over a soldering iron. This seal forms one end of the anchor point (Figure 2). Part B was then heated 1 cm from the first seal until pliable. Both ends were pushed slightly together to form a small ridge which was compressed over the needle tubing before the plastic hardened and formed the other end of the anchor point (Figure 2).

The neck piece. Part C was pushed, flared end first, over the top of Part B to about 5 cm from the end. The widened part D tubing was then threaded over part B and onto part C (Figure 2). This join was also heat sealed. The seal and the flange of part C formed the neck anchor point. Further seals between part B and part D at approximately 1 cm intervals were made. The final seal was made and cut through with a sharp scalpel to ensure correct sealing.

The S-bend Part A was bent on a plastic former, dipped into boiling water and then set by cooling rapidly under the cold tap. The S-bend was such that the A-B junction occurred just before the first bend, the thin walled portion of part A occurring just before the second bend (Figure 2).

A 90 degree bend on the neck piece. A bend was made at the third heat seal on the neck piece as shown in Figure 2. The neck piece was threaded over a length of 26 gauge needle tubing. The tubing and hence the catheter was bent into a right angle, so that it protruded from the skin surface at right angles when in position. The needle tubing and neck piece were dipped into boiling water and set by cooling in cold water. On removing the needle tubing the 90^{0} bend remained.

APPENDIX 3 CONSTRUCTION OF VENA CAVAL CATHETER

The vena caval catheter, like the aortic catheter, also consisted of 4 pieces of polythene, welded together by heating. The materials used are identical to those used in the aortic catheter (Table 2) with one exception, 17 cm of polythene tubing was used as part B of the venous catheter instead of 15 cm as in the aortic catheter. Figure 3 shows a constructed catheter.

Preparation of catheter parts before assembly.

Straightening part A

10cm of polythene was threaded over the 35 gauge copper wire, which had been siliconised. The tubing was clamped to the wire at both ends using artery forceps. The polythene and wire were dipped into boiling water and then rapidly cooled in water. The wire was removed and the polythene was now straight.

The remaining parts of the catheter were prepared as before for the aortic catheter.

Assembly of the catheter.

The straightened length of polythene (part A) was threaded onto 35 gauge wire needle tubing formerly described in the construction of the aortic catheter. Part B was threaded onto the tubing and pushed over part A to give an overlap of 3-5 mm. The junction was heat sealed. An anchor point was formed 2 cm from this seal by heating an area of the tubing until a ridge had formed

The remaining parts were assembled as for the aortic catheter.

The U-bend.

A U-bend was made in the catheter by bending the part of catheter distal to the anchor point on part B round the plastic former used in the construction of the aortic catheter and dipping it into boiling water followed by rapid cooling under the cold tap.

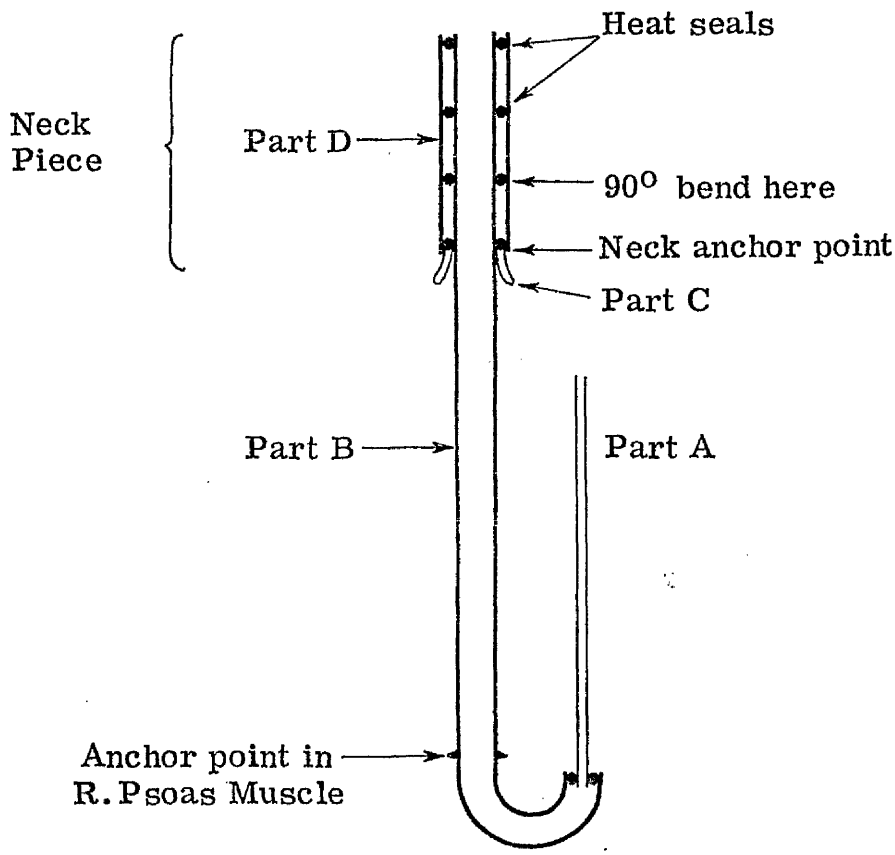


Figure 3. Vena caval catheter

A 90^{0} bend was made on the third seal on the neck piece, as in the aortic catheter. Catheters were tested as before.

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