THE PHARMACOLOGY OF SOME ANGIOTENSIN ANTAGONISTS

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ABSTRACT

Interactions between tetraethylthiuram disulphide (disulfiram), its metabolite diethyldithiocarbamate (DDC) and the vasoactive polypeptide angiotensin have been studied.

In the pithed rat preparation, both disulfiram and DDC were shown to specifically reduce pressor responses to injected angiotensin. The possible involvement of the sympathetic nervous system in this activity was investigated and the results discussed. It was not possible to reproduce these effects of disulfiram and DDC in the chloralosed, conscious or pithed cat preparations, thus suggesting some form of species specificity. Possible reasons for this are discussed.

Using a variety of test procedures, the effects of disulfiram and DDC on two isolated smooth muscle preparations were determined. In the case of the rat isolated colon preparation, pretreatment with disulfiram was shown to inhibit the spasmogenic activity of 5-hydroxytryptamine and acetylcholine in addition to that of angiotensin. The effect of disulfiram on the contractile responses of the guinea pig isolated ileum was to reduce the fast, cholinergically mediated component of the response to angiotensin without reducing the direct component. In preparations treated with hyoscine, the direct component of the angiotensin response was selectively reduced by DDC.

The effects of prolonged treatment with disulfiram on the development of renal and DOCA/saline hypertension in rats were studied. With the exception of the early stages of renal hypertension, disulfiram did not retard the onset or extent of the hypertension. The reasons for these effects are discussed in the light of published reports concerning renin levels during experimental hypertension.

The mechanism of action of these effects of disulfiram is largely unknown. Results presented in the latter part of

this thesis derived from attempts to mimic these anti-angiotensin effects using a number of unrelated compounds possessing some activity shared with disulfiram or DDC. Dimercaprol, an inhibitor of enzyme -SH groups, produced a non-specific reduction in reactivity to vasoactive agents in the pithed rat. Chelating agents were shown not to inhibit pressor responses to angiotensin in the rat. The results of these experiments are discussed in the light of current knowledge on the metabolism and actions of disulfiram and DDC.

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To Eleanor.

CONTENTS

PART I

HISTORICAL INTRODUCTION

PHYSIOLOGICAL ROLES OF RENIN AND ANGIOTENSIN

Discovery and Identification of Renin and Angiotensin

In an investigation of the possible endocrine function of the kidney, Tigerstedt & Bergman (1898) found that crude saline extracts of rabbit kidney injected into anaesthetised rabbits produced a rise in blood pressure. They named the active substance in their extracts 'renin'. Subsequently, Page & Helmer (1940a) and Braun-Menendez, Fasciolo, Leloir & Munoz (1940) demonstrated that renin was in fact an enzyme which produced its pressor effects by the formation of a small molecule which they called angiotonin and hypertensin respectively. These names were 'merged' to give angiotensin following an agreement by Braun-Menendez and Page in 1957.

A great advance in the study of angiotensin was the determination of its structure (Elliot & Peart, 1956; Lentz, Skeggs, Woods, Kahn & Shumway, 1956) and its subsequent synthesis by Bumpus, Schwarz & Page (1957) and Schwyzer, Iselin, Kappeler, Riniker, Rittel & Zuber (1957). Peart (1956) isolated a pure angiotensin from ox serum which contained eight different amino acids, whereas Skeggs and his colleagues showed that their product was a decapeptide (1955). Peart went on to show that ox angiotensin was also a decapeptide but not identical with that of the horse. Eventually, these difficulties were resolved when the respective structures were elucidated by Elliot & Peart (1956, 1957) and Lentz et al (1956). The critical difference lay in the fifth position from the N-terminal residue. In this position, ox angiotensin has valine and horse isoleucine:-

Ox angiotensin II

Asp-Arg-Val-Tyr-Val-His-Pro-Phe

Horse angiotensin II

Asp-Arg-Val-Tyr-Ileu-His-Pro-Phe

Renin and the formation of Angiotensin I

Renin is formed and stored in the kidney of all mammals and vertebrates so far investigated. It is a proteolytic enzyme which is now known to act on a plasma substrate to release a decapeptide, angiotensin I, which is further metabolised to angiotensin II, the vasoactive octapeptide. The renin substrate is contained in the α_2-globulin fraction of most mammals including man. Of the three factors which partake in the reaction

renin + substrate - angiotensin I

only the reaction product is chemically defined, neither the enzyme nor the substrate being so far available in a pure form. It appears very doubtful whether the reaction is so straightforward, and it would appear that additional factors either affect the enzyme directly or participate in the reaction in an as yet undefined way (Bumpus, 1966; Carretero & Gross, 1967; Smeby et al, 1967).

A number of attempts have been made to obtain a pure renin and these have resulted in some highly active enzyme preparations. Hog renin appears to have a molecular weight of 42,000 to 49,000 (Kemp & Rubin, 1964; Peart, 1965; Warren & Dolinsky, 1966) and human renin a molecular weight of 42,300 (Lubash & Peart, 1966; Warren & Dolinsky, 1966). It has been recently suggested, however, that renin may not be a single substance but consists of a number of components separable by chromatography (Skeggs, Lantz, Kahn & Hochstrasser, 1967).

Structurally, renin would appear to be a protein with a folded configuration cross-linked with disulphide bonds but without critically placed sulphydryl groups (for a review of the evidence see Lee, 1969). Bonds susceptible to endopeptidase activity are present, but these are probably protected by the

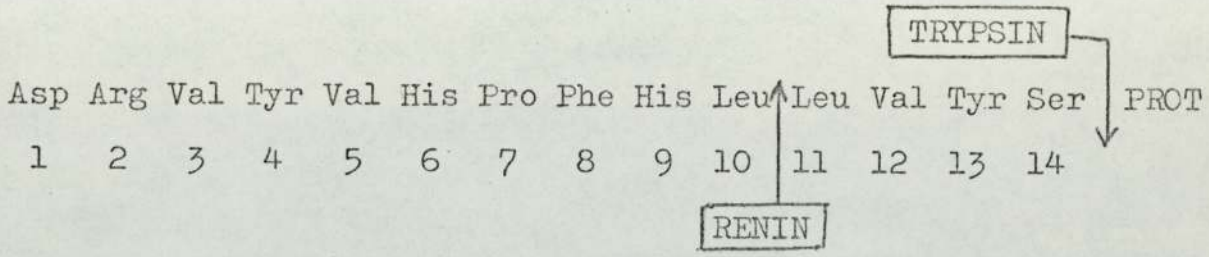
folds of the molecule. Free amino groups and accessible lysine residues also occur. The presence of a metal prosthetic group is unlikely, as is a serine residue in the active centre.

The renin substrate is probably not a single protein, since various substrates characterised as glycoproteins have been isolated from hog plasma (Skeggs, Lentz, Hochstrasser & Kahn, 1964). They have molecular weights of about 57,000 and similar amino acid compositions but differ in their carbohydrate parts. All these substrates yield the same angiotensin I when incubated with renin (Skeggs et al, 1964). An attempt to determine the amino acid composition of the substrate using a tryptic digestion of horse plasma protein yielded a tetradecapeptide (Skeggs, Lentz, Kahn & Shumway, 1958). The first ten amino acids of this peptide were identical with $Ileu^5$-angiotensin I (Fig. 1). Skeggs and his co-workers (1958) assumed that renin acted on the terminal amino acid sequence of the substrate molecule by splitting the bond between the leucines in the 10 and 11 positions to give the decapeptide, angiotensin I.

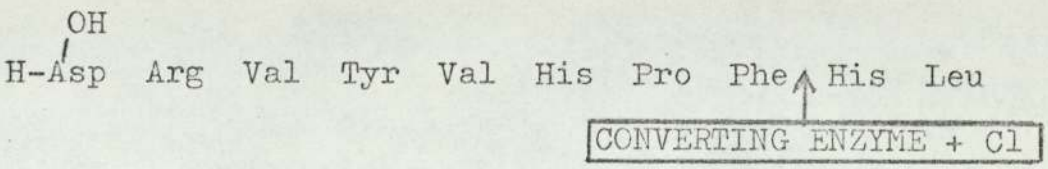
Very little information is available concerning the site of the renin/substrate reaction. It is accepted today that renin is formed and stored in the juxtaglomerular apparatus (Ruyter, 1925; Goormaghtigh, 1939 a,b) probably in the juxtaglomerular or granular cells (for literature see Schloss, 1947; Hartcroft, 1963 a,b; Bing & Kazimierczak, 1964; Hartcroft, Sutherland & Hartcroft, 1964; Bucher & Reidel, 1965) which is mainly located in the wall of the afferent and occasionally also in that of the efferent arteriole (Goormaghtigh, 1932, 1945).

Renin is found in the plasma and in renal lymph (Lever & Peart, 1962; Higgins, Davis & Urquhart, 1962, 1964) but the mechanism of the release of renin from its storage sites into

ANGIOTENSINOGEN (Renin Substrate - α_2- globulin)



ANGIOTENSIN I



ANGIOTENSIN II

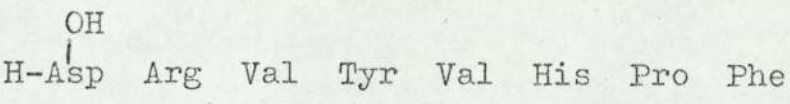


Fig.1. Site of action of renin at the leucyl-leucine bond of the terminal tetradecapeptide isolated by tryptic digestion of renin substrate (an α_2-globulin). Liberation of angiotensin I which is rapidly converted to angiotensin II.

the systemic circulation or into the lymphatic system is not yet fully understood. There does appear to be some correlation between the renin content of the kidney and the concentration of renin in plasma (Gross, Buschor & Zeugin, 1962; Gross, Schaechtelin, Brunner & Peters, 1964; Schaechtelin, Regoli & Gross, 1964). However, whereas variations in plasma renin, caused by acute stimuli, such as haemorrhage or acute reductions in intravascular volume. may occur in a few minutes, variations in the renin content of kidneys develop slowly and its takes several days at least for a change to become detectable. The kidney, therefore, appears to have a secretory capacity for renin that allows it to respond to acute stimuli with increased renin release. This, therefore, leads to discrepancies between the plasma renin content and the renin content of the kidneys. A change in the renin content of the kidneys occurs if the stimulus for renin release is chronic in nature. It follows that alterations in the kidney renin levels are the consequence of a continuous increased or reduced need for renin.

Renin release may be stimulated by various factors which apparently cannot be reduced to a common mechanism. Intravascular volume is, in most cases, inversely correlated with the concentration of renin in plasma. This is true under acute and subacute conditions such as acute sodium deprivation, overtransfusion and water deprivation (Ziegler & Gross, 1964; Gross, Brunner & Ziegler, 1965; Hodge, Lowe & Vane, 1965; Menard, Boucher & Genest, 1966). However, during pregnancy, intravascular volume is increased and plasma renin activitity enhanced (Brown, Davies, Lever & Robertson, 1964; Brown, Davies, Doak, Lever & Robertson, 1966).

Skinner, McCubbin & Page (1964) have suggested the existence of an intrarenal baroreceptor mechanism which is sensitive to the transmural pressure difference in the renal arterial tree. Tobian (1960, 1962) postulated the existence of stretch or volume receptors in the renal arterial system which modulate the secretion of renin by the juxtaglomerular cells. The most likely location of these 'receptors' appears to be the granular cells. Should this 'stretch receptor' hypothesis be, in fact, the case, then reduced stretch of the arteries and arterioles should increase the release of renin, whereas increased stretch should diminish it. The stretch differences involved here could be due to variations in renal perfusion pressure or intravascular volume. Consistent with this mechansim are the situations where an increase in renin output is due to the siting of a clip on one renal artery or acute reduction in intravascular volume following sodium deprivation or haemorrhage. There are, however, exceptions such as the normal release of renin following the application of a clip to the renal artery of the remaining kidney following unilateral nephrectomy.

Stimulation of the renal nerves or infusions of noradrenaline or adrenaline causes release of renin, irrespective of whether pressure in the renal artery is maintained at a normal level or whether both glomerular filtration rate and renal blood flow are reduced (Buñag, Page & McCubbin, 1965; Vander, 1965; Wathen, Kingsbury, Stouder, Schneider & Rostorfer, 1965). In contrast to noradrenaline, angiotensin II infused into the renal artery does not stimulate renin release (Wathen et al, 1965). As a consequence of this observation, it has been suggested that there is a negative feedback system operating between the

level of circulating angiotensin and the release of renin (Vander & Geelhoed, 1965; Czyzewski & Pettinger, 1973). This may be the case in man, where subpressor infusions of angiotensin have been shown to reduce elevated plasma levels induced by a low-sodium diet (de Champlain, Genest, Veyrat & Boucher, 1966).

A number of workers have proposed that renin release is controlled primarily by the macula densa (see review by Vander, 1967). The macula densa is the specialised tubular area which marks the transition from the ascending loop of Henle to the distal tubule. This nephron segment lies in contact with the glomerulus of origin and the cells themselves are closely associated anatomically with the granular cells. The third part of the juxtaglomerular apparatus is the mesangial cells, which appear to be interstitial cells in contact with both the granular cells and the macula densa (Fig. 2).

Over twenty five years ago, Goormaghtigh (1944, 1945) proposed that the glomerular filtration rate is, in some manner, controlled by the macula densa fluid via the juxtaglomerular cells. Even today, some workers still hold that the possibility exists that renin is actually synthesised in the macula densa rather than in the granular cells (Bing & Kazimierczak, 1962). The macula densa theory postulated by Vander (1967) involves renin release varying inversely with the total load rather than the concentration of sodium delivered to the macula densa (Gross, Brunner & Zeigler, 1965; Tobian, Tamboulian & Janacek, 1959; Vander & Miller, 1964). This, Vander suggested, would make renin release highly responsive to changes in body fluid balance and cardiovascular function.

Superimposed on these three main theories of renin-releasing mechanisms is the probability of one or more feedback

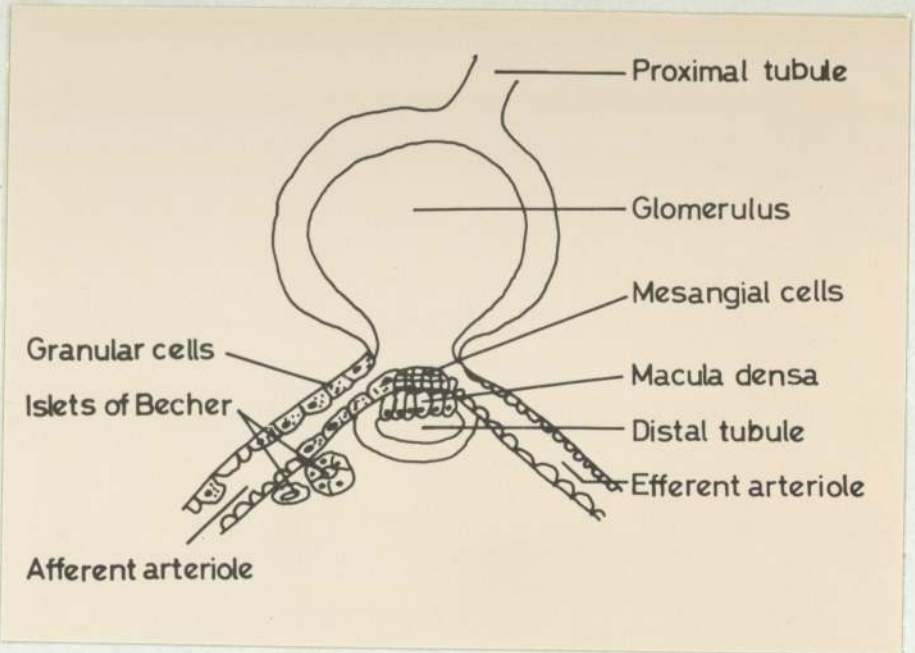


Fig. 2. The juxtaglomerular apparatus (after Cook, 1963). The macula densa is the specialised tubular area which marks the transition from the ascending loop of Henle to the distal tubule. This nephron segment lies in contact with the glomerulus of origin and the cells themselves are closely associated with the granular cells. The third part of the juxtaglomerular apparatus is the mesangial cells which appear to be interstitial cells in contact with the granular cells and the macula densa.

systems. It appears that the plasma angiotensin concentration may play an important role in the control of renin secretion. Vander & Geelhoed (1965) demonstrated that the increased renin release induced in dogs by aortic constriction was supressed by small intravenous infusions of angiotensin. Genest, de Champlain, Boucher, Veyrat & Koiw (1965) found that angiotensin depressed plasma renin levels in salt-depleted human volunteers but aldosterone was unable to lower plasma renin levels in similar experiments.

In attempting to correlate the above postulated systems for the release of renin, particularly with regard to possible physiological functions of the renin/angiotensin system, it may be useful to regard this cautionary statement by Lee (1969):

"A true knowledge of renin release requires careful measurement of the rate of renin release. At the moment, we infer secretion rates from the level of hormone in renal venous or systemic blood. This may be unjustified, as in these situations the half-life of renin could fluctuate markedly, especially when the kidney is manipulated."

Conversion of Angiotensin I to Angiotensin II

The conversion of angiotensin I to angiotensin II was originally thought to occur in the plasma. Skeggs, Kahn & Shumway (1956) claimed that angiotensin I was immediately converted to the octapeptide by a specific enzyme which they called the 'converting enzyme'. More recently, however, doubts have been expressed about the importance of this enzyme in plasma. Using the blood-bathed organ technique, it has been shown that the conversion of angiotensin I to angiotensin II takes place rapidly in the pulmonary circulation (Ng & Vane, 1967, 1968). Thus it would appear that the converting enzyme

is located in the pulmonary cells and not, as previously supposed, in the peripheral blood. In dogs, approximately 50% of injected angiotensin I was converted to angiotensin II in the lung and about 7% to 10% in the kidney (Oparil, Sanders & Haber, 1970). A purified converting enzyme has been prepared from dog lung homogenate (Cushman & Cheung, 1969); it stoichiometrically converts angiotensin I to angiotensin II and histidyl-leucine.

Destruction of Angiotensin II

The use of the term 'angiotensinase' to describe a single enzyme is incorrect, since there is no peptidase which is exclusively responsible for the inactivation of angiotensin (Dengler & Reichel, 1960). Fig. 3 summarises the actions of the various aminopeptidases shown to have a destructive action on angiotensin II. The role of these enzymes in the physiological inactivation of the octapeptide is not clear, but they are unlikely to be very important in the clearance of angiotensin from the plasma. The vasopressor activity of injected angiotensin lasts only a few minutes. This brevity of action may be partially explained by aminopeptidases but mainly by binding at various sites in the body.

Using randomly tritiated angiotensin II, Bumpus and his co-workers (1964) showed that immediately following a large dose of the peptide (4·6 mcg/min for 20 minutes) which produced tachyphylaxis very rapidly after initially raising blood pressure to very high levels, there was high radioactivity in the uterus, adrenal glands and kidneys. In animals killed 30 minutes after infusion, the concentration in the uterus had fallen, that in the brain had risen and the kidneys and adrenals retained substantial activity. The highest activity after

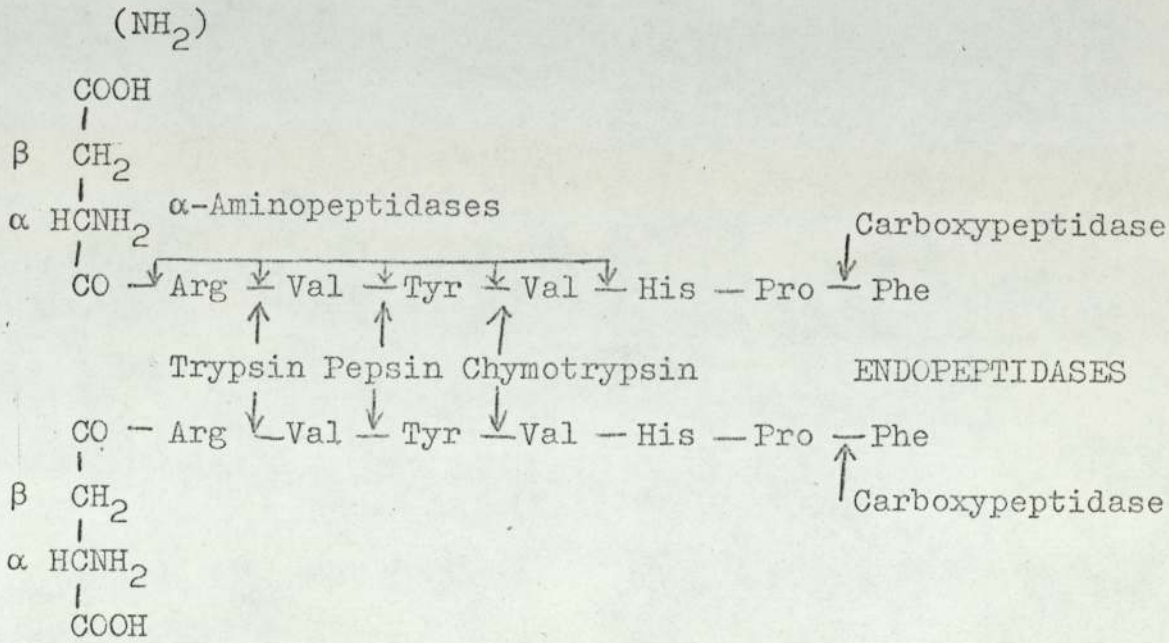


Fig. 3. Degradation of α- and β-angiotensin II-amide by various peptidases. (Gross, 1963).

30 minutes was, however, found in the urine. In contrast, labelled bradykinin did not accumulate to a significant degree in the adrenal gland and there was early excretion in the urine. Nephrectomised rats accumulated more angiotensin in the liver and the blood levels in these animals remained high but the adrenal glands did not accumulate as much peptide. The accumulation of angiotensin II by the adrenal glands may be indicative of a selective uptake mechanism and this could be related to the role angiotensin is thought to play in the control of aldosterone secretion. The adrenal contains little peptidase activity and angiotensin might persist there for a considerable time, not fluctuating directly with blood levels. The same argument also applies to the accumulation of angiotensin II by the central nervous system (Lee, 1969).

Using the multiple organ bath technique (Regoli & Vane, 1964), Hodge, Ng & Vane (1967) demonstrated that the half-life of angiotensin II in circulating blood in the dog varies between 100 and 200 seconds depending on the structure of the angiotensin studied. Destruction of angiotensin in the blood therefore seems to be relatively unimportant. About 50 to 70% of an infusion of angiotensin II disappears in peripheral vascular beds such as the liver, the kidneys and hind legs (Hodge et al, 1967).

From the above evidence, several conclusions may be drawn (Vane, 1969). Firstly, the localisation of converting enzyme in the lungs, but not in other tissues, makes untenable hypotheses (Thurau, 1964; Thurau, Schnermann, Nagel, Horster & Wahl, 1967; Leyssac, 1967) which propose a wholly intrarenal function for the renin-angiotensin system. Secondly, the generation of angiotensin II in the pulmonary circulation

makes it likely that the renin-angiotensin system is designed as a circulating hormone system, the siting of the converting enzyme in the pulmonary vascular bed being a mechanism for protecting the afferent arteriole of the kidney from the higher concentrations of angiotensin II that would otherwise be generated from the high local concentrations of freshly released renin.

THE PHARMACOLOGY OF ANGIOTENSIN

Introduction

Angiotensin II is one of the most pharmacologically-active naturally occuring substances; the minimal concentration which induces contraction of isolated smooth muscle is, on a molar basis, 100 to 1000 times lower than that of acetylcholine or adrenaline. From the pharmacological point of view, angiotensin belongs to the group of polypeptides that affect smooth muscle and blood vessels. Since its vasoconstrictor and hypertensive activity is most pronounced, this has generally been considered in relation to its possible role in the pathogenesis of hypertension and its other qualities have been neglected until relatively recently.

The availability of synthetic angiotensin II and its analogs since the late 1950's has led to a large increase in interest in the pharmacology of the peptide. Most of the work on the pharmacology of angiotensin has in fact involved the use of $Asp(NH_2)^1$-Val^5-angiotensin II (Hypertensin CIBA). There is good reason to assume that the results obtained with the amide do not differ qualitatively from those seen with Asp^1-Val^5-angiotensin II or the corresponding $Ileu^5$-angiotensin II.

Angiotensin I is generally considered to be an inactive intermediate in the formation of the active angiotensin II.

It does however appear to have an oxytocic as well as an intestinal stimulant effect on isolated organs (Carlini, Picarelli & Prado, 1958; Gross & Turrian, 1960). Also, the tetradecapeptide used as a synthetic substrate for renin has been shown to act on the rat isolated uterus and guinea pig ileum (Montague, Riniker, Brunner & Gross, 1966). The possibility cannot be excluded, therefore, that some of the actions of synthetic angiotensin II and its amide are not identical with those of angiotensin liberated endogenously by renin under largely unknown conditions.

Effect on systemic arterial blood pressure

The characteristic response to angiotensin II following intravenous injection is a sharp rise in blood pressure. This type of response may be demonstrated in many species including man. The increase in blood pressure begins 20 to 30 seconds after the injection, the maximum response is reached after 1 to 2 minutes, and within 3 to 5 minutes blood pressure returns to the initial level. Pulse pressure may increase, since the diastolic pressure is raised less than the systolic pressure. This is seen particulary in experiments in unanaesthetised dogs and in man (Bock, Krecke & Kuhn, 1958; Bock & Gross, 1961). The intensity of the pressor response and, to a certain degree, its duration, are dose-dependent.

Intra-arterial injection usually results in a less marked rise of systemic blood pressure than intravenous injection, because angiotensin either is partly destroyed in, or disappears during, its passage through the capillary bed. The onset of the rise is delayed, and the threshold doses are higher, depending on the vascular bed into which angiotensin is injected (Bianchi, de Schaepdryver, de Vleeschhouwer & Preziosi, 1960).

Injection into the renal artery may produce a fall in blood pressure instead of a hypertensive response (Köver, Ellö & Szöcs, 1965; Akinkugbe, Brown & Cranston, 1966 a,b). Infusion of angiotensin into the vertebral arteries does not have a direct vasoconstrictor effect on cerebral vessels, and it was concluded that cerebral vasoconstriction may occur indirectly via autoregulatory responses to increased perfusion pressure (Franklin & Rapela, 1967).

When angiotensin II is infused intravenously, the blood pressure can be maintained at an elevated level for hours, days or even weeks. After the infusion is stopped, the pressure usually reverts rapidly to its preceding value. During the infusion, the responses to additional acute injections of angiotensin are more markedly reduced than those to injections of equipressor doses of noradrenaline (Gross, Bock & Turrian, 1961). The maintenance of a constant elevation of blood pressure is dose-dependent. In the unanaesthetised dog, a sustained increase in pressure was obtained throughout the whole period of the infusion by giving small and medium doses of angiotensin (Page & Olmsted, 1961), whereas higher doses produced initial increases with a subsequent fall to somewhat lower levels. Very high doses (15 µg./kg/min) produced a sharp increase in blood pressure which fell after a few minutes to its preceding level, despite continued infusion of the same dose (Bock & Gross, 1961).

Unlike the dog, the rat responds with a lasting rise in blood pressure to infusions over 8 to 12 days (the infusion was restricted to 12 h per day), and acute hypertensive vascular disease indistinguishable from acute renal hypertension has been observed (Koletsky, Rivera-Velez & Pritchard, 1965).

In man, continuous infusions of angiotensin result in increased sensitivity to angiotensin, and consequently smaller doses are sufficient to maintain blood pressure at the same elevated level (Ames, Borkowski, Sicinski & Laragh, 1965). It was assumed that the increased pressor response to angiotensin in man was due to its action in causing sodium retention.

Angiotensin has a general vasoconstrictor effect on the vasculature of the various organs of the splanchnic region. In dogs, flow in the mesenteric artery is markedly reduced after an intravenous dose of as little as 0·01 µg/kg, the effect being shorter than the increase in blood pressure (Gross & Turrian, 1960). During angiotensin infusion in dogs, total splanchnic blood flow decreased by about 20% (Bashour, Taha & Sellers, 1963) to 32% (Longerbeam & Lillehi, 1963) and there is a marked increase in peripheral resistance in the visceral region. In the cat, angiotensin caused marked vasoconstriction in the spleen and contraction of the capsular smooth muscle (Greenway & Stark, 1970).

Direct effects on renal blood flow, including those occuring after injection or infusion of angiotensin into the renal artery, have been studied by many workers, all of whom observed a reduction in flow. The threshold for the response of the renal arterial bed to angiotensin is lower than that in most other regions (Assali & Westersten, 1961; Barer, 1963). Renal denervation abolishes the vasoconstriction in the renal vascular bed produced by angiotensin, but not when the drug is injected directly into the renal artery. The effect that endogenously liberated angiotensin has on the afferent or efferent arteriole of the glomerulus is still the subject of discussion. A number of workers have presented evidence that

it affects primarily the efferent arteriole (Schmidt, 1962; Gross, Brunner & Ziegler, 1965), whereas others favour the afferent arteriole (Thurau & Schnermann, 1965).

Flow to the adrenal glands when determined by the indicator fractionation technique, increased in response to most doses, whereas adrenal vascular resistance rose somewhat less than total peripheral resistance. It was concluded from this evidence that angiotensin diverts blood to the adrenal glands (Mandel & Sapirstein, 1962).

Studies on flow in the carotid artery have generally revealed little or no increase when high doses were administered. In cats, no effect was observed after intravenous injection, but a decrease in flow took place when angiotensin was injected intra-arterially (Barer, 1963).

Mandel & Sapirstein (1962) showed that at infusion rates of 0·05 to 0·3 mcg/kg/min, angiotensin increased flow by 70% over controls and vascular resistance was consequently reduced.

Effects on the nervous system

(a) Sympathetic system

The initial investigations of the actions of angiotensin on the nervous system were carried out by Braun-Menendez and his colleagues (1940). They showed, by cross-circulation experiments in dogs, that if angiotensin was injected into one dog, then adrenaline was released in the other. This action was preserved even when the adrenal was denervated, suggesting a direct action. More recently, Feldberg & Lewis (1964) have confirmed that the adrenal medulla is extremely sensitive to angiotensin.

There is considerable evidence to suggest that angiotensin specifically increases the responses of tissues to noradrenaline

released from peripheral sympathetic nerves in both whole animals (McCubbin & Page, 1963 a,b) and in isolated tissues (Benelli, Della Bella & Gandini, 1964; Su, 1965; Thoenen, Hurlimann & Haefely, 1965). In some vascular beds the vasoconstrictor action of angiotensin is dependent on an intact sympathetic innervation and is greatly reduced by procedures abolishing sympathetic tone. This has been demonstrated in isolated sympathetically-innervated vascular muscle (Zimmerman, 1962; Laverty, 1963), in whole animals (McGiff & Fasy, 1964, 1965) and in human studies (Johnson, Henning & Ablad, 1965; Scroop & Whelan, 1968; Henning & Johnsson, 1967). Intravenous infusions of noradrenaline or substances which release neuronal noradrenaline (e.g. indirectly acting sympathomimetics or ganglion stimulants)enhance the pressor responses to intravenous injections of angiotensin (Haas & Goldblatt, 1959; Schmitt & Schmitt, 1967; Pals & Fulton, 1968).

The potency of angiotensin as an adreno-medullary stimulant varies from species to species (Vane, 1969). Release of amines from the adrenal medulla was found to make little or no contribution to the pressor response in man (Scroop & Whelan, 1968) or in rats (Hughes, 1968; Schmitt & Schmitt, 1968). In dogs, angiotensin has been variously reported as a very potent adrenal medullary stimulant (Robinson, 1967) and as a weak stimulant (Kaneko, McCubbin & Page, 1961; Staszewska-Barczak & Vane, 1967). The action is not blocked by either hexamethonium or by pempidine (Feldberg & Lewis, 1965; Staszewska-Barczak & Vane, 1967) but is subject to tachyphylaxis (Feldberg & Lewis, 1965).

The contribution of autonomic ganglion stimulation to the

pressor response following intravenous injection of angiotensin is probably insignificant. Drugs which impair the function of the sympathetic nervous system peripheral to the ganglia (e.g. adrenergic neurone blockers) do not decrease the pressor action of intravenous angiotensin (Laurence & Nagle, 1963). In very small doses, angiotensin inhibits transmission across the superior cervical ganglion of the cat (Haefely, Hurlimann & Thoenen, 1965; Panisset & Bourdois, 1968). Larger doses of angiotensin, however, directly stimulate the ganglia and facilitate transmission across both sympathetic and parasympathetic ganglia (Robertson & Rubin, 1962; Lewis & Reit, 1965; Godfraind, Kaba & Polster, 1966).

(b) The central nervous system

The first demonstration that angiotensin had a centrally mediated cardiovascular effect was by Bickerton & Buckley (1961). They showed that when the peptide was infused at 1 to 4 µg/kg into the cerebral circulation of a dog whose head was connected to the body by nerves alone, a pressor response was observed which could be prevented by α-blockade with piperoxan. Intraventricular injections of angiotensin (up to 4 µg) produced similar responses accompanied by tachycardia and contraction of the nictitating membrane (Smookler, Severs, Kinnard & Buckley, 1966). Part of the response was felt to be due to adrenomedullary stimulation and the other part due to stimulation of the midbrain-hypothalamic central sympathetic centre. The bulk of evidence suggests that the central pressor action of angiotensin is mediated via the peripheral sympathetic nervous system although the finding of Scroop & Lowe (1968) that angiotensin exerted its central pressor action by inhibition of vagal tone is of possible importance as this occurs at doses

of the peptide more in keeping with physiological angiotensin levels than those used by other workers.

Anaesthesia may modify the central effects of angiotensin, since Kaneko, McCubbin & Page (1960) and Bianchi et al (1960) found no effect in anaesthetised animals, while Dickinson & Lawrence (1963) and Yu & Dickinson (1965) were able to produce pressor responses by infusing angiotensin into vertebral arteries in unanaesthetised rabbits.

Effect on isolated vascular smooth muscle

Spirally cut rabbit aortae (Furchgott & Bhadrakom, 1953) have been used to assay angiotensin. In other experiments, segments of various arteries (aorta, renal, mesenteric carotid) from different animal species (rabbit, rat) have been compared by isometric recording (Rondell & Gross, 1960). Angiotensin produces a slowly developing contraction, which is followed by a slow relaxation after washing out. The onset of action is delayed for 2 to 4 minutes and maximum contraction may not be reached until after 10 to 20 minutes. This contrasts with the contraction to adrenaline, which begins almost immediately and is complete within 2 to 4 minutes. Most spirally cut arteries develop tachyphylaxis very rapidly with the exception of rabbit and guinea pig aortae (Khairallah, Page, Bumpus & Turker, 1966). Renal arteries from dogs do not respond to angiotensin (Bohr & Uchida, 1967). In the isolated perfused renal artery of the rat, angiotensin has only a small effect and is much less vasoconstrictor than adrenaline, noradrenaline and serotonin (Hrdina, Bonaccorsi & Garattini, 1967). The isolated pulmonary artery of the rabbit is constricted by angiotensin in concentrations of 10^{-9}g/ml or more (Su, 1965).

In concentrations of 5 ng/ml and more, angiotensin II

contracted isolated strips of hepatic, portal, mesenteric and lobar pulmonary veins, but did not affect those of saphenous, femoral or axillary veins and venaecavae even when concentrations were increased by a factor of a hundred (Somlyo & Somlyo, 1966). Tachyphylaxis develops very rapidly in isolated vein preparations. McLeod & Hunter (1967) stated that the rat inferior and superior venae cavae and pulmonary veins did not respond to angiotensin unless the vessels were electrically stimulated, when the peptide had a slight positive inotropic effect. Recently, Blair-West, McKenzie & McKinley (1971) demonstrated that angiotensin was 3 - 10 times as potent as noradrenaline on a weight basis in its ability to contract the rat isolated portal vein. This action they showed to be direct in nature.

Effect on isolated non-vascular smooth muscle

Isolated non-vascular smooth muscle preparations have been used as methods for the assay of angiotensin for a number of years. These include the guinea pig ileum (Collins, 1948; Picarelli, Kupper, Prado, Prado & Valle, 1954), the intestines of the rabbit (Page, 1940) and the toad (Prado, Valle & Picarelli, 1954) and the uterus of rat and rabbit (Luduena, 1940). Of these, the rat uterus is the most sensitive but is not specific. Regoli & Vane (1964) suggested the use of the rat ascending colon for angiotensin assay. This tissue was shown to be directly stimulated by angiotensin. Methysergide, hyoscine, mepyramine, hexamethonium and morphine were each shown to have no effect on the response. Regoli & Vane also suggested that the receptors present in rat colon were similar to those responsible for the pressor response in the same species, as the relative potencies of several analogues of angiotensin were shown to be similar in both cases.

In contrast to its effect on rat colon, angiotensin appears to have a dual action on guinea pig isolated ileum. This tissue responds to low doses of the peptide after a lag period of 20 - 30 seconds and reaches a maximum in 90 - 120 seconds. Response of the ileum could be separated into two components, a fast component due to a rapid rise in tension and subsequent partial relaxation, followed by a slow component, a progressive increase in contraction reaching a maximum in 1·5 - 2 minutes (Godfraind, Kaba & Polster, 1966). The rapid component was due to acetylcholine and could be inhibited by atropine and morphine, the slow component being a direct action of the peptide. The ileum of the mouse and the Mongolian gerbil responds in much the same way as the guinea pig ileum (Goldenberg, 1967). Beleslin (1968), using guinea pig ilea, concluded that angiotensin had three sites of action, postganglionic nerve endings, ganglion cells and intestinal smooth muscle.

Endocrine actions of angiotensin

The relationship between the kidney and the adrenal cortex has been shown to be more than anatomical, particularly with reference to their role in maintenance of sodium and water balance.

In some species, such as the rat, angiotensin causes diuresis and natriuresis over a wide dose range (Peters, 1963). This effect, however, cannot be demonstrated under all conditions. During water diuresis, rats react to angiotensin with anti-diuresis, whereas when hypertonic saline (1·8%) is infused, angiotensin causes diuresis (Schroder, Meyer-Burgdorff, Rott & Brahms, 1961). In other species, such as the rabbit, cat or dog, the response is dose-dependent, low doses having an anti-diuretic and antinatriuretic effect, but higher doses being

diuretic and natriuretic (Gross & Turrian, 1960; Barraclough, 1965). Normal human subjects respond with antidiuresis and sodium retention (Laragh, Cannon, Bentzel, Sicinski & Meltzer, 1963; Brown & Peart, 1962).

In addition to its direct effects, angiotensin has been implicated as a factor promoting sodium retention indirectly via the stimulation of aldosterone release. Several groups have demonstrated that infusion of angiotensin in pressor or even sub-pressor doses increases aldosterone secretion and excretion in various animal species including man. (Laragh, 1962; Davis, 1961; Mulrow & Ganong, 1962). Continuous infusion of angiotensin for several days into intact dogs is followed by initial sodium retention and negative potassium balance which resembles the effect of aldosterone. Probably a continuous increase in aldosterone secretion is responsible for these effects, as they are not seen in adrenalectomised animals treated with maintenance doses of DOCA and cortisone (Urquhart, Davis & Higgins, 1963). Recently it has been shown that by intravenous infusion or chronic administration of high doses of angiotensin an increase in aldosterone and corticosterone secretion is obtained (Dufau & Kliman, 1968). It appears that angiotensin affects the biosynthesis of aldosterone and corticosterone in the adrenal cortex at an early stage in the formation of the hormones (Kaplan, 1963; Davis, 1964; Lommer & Wolff, 1966). Despite various attempts, it was not possible to demonstrate a stimulant effect of angiotensin on aldosterone secretion in rats (Eilers & Peterson, 1964; Cade & Perenich, 1965).

The angiotensin receptor

Very little is known concerning the chemical nature of

the angiotensin receptor. Recently, Lin & Goodfriend (1970) demonstrated specific binding of angiotensin in fragments of rat uterus, colon, trachea, oesophagus, nerve and bladder, rabbit aorta, bovine adrenal and kidney cortex. They suggested that the angiotensin was present in particles sedimenting between 1,000 and 15,000 g in the case of the uterus and the adrenal cortex. Most of the other studies on receptors have been approached indirectly by a study of peptide structure-activity relationships. These are reviewed in Chapter 2 of their book by Page & McCubbin (1968). Amongst others the features of the angiotensin molecule required to either raise rat blood pressure or contract rat uterus are a free carboxyl group at the C-terminus and phenylalanine in position 8. There are other structural requirements of the peptide and it seems likely that its configuration can indirectly characterise the receptor.

The angiotensin receptors on different tissues may not be all identical. Peach, Bumpus & Khairallah (1969) showed that 8-alanine angiotensin was as potent as the parent structure in inhibiting re-uptake of noradrenaline, whereas the pressor and oxytocic activity of the analog was less than 0·1%. Khairallah (1971) suggests that most smooth muscle tissue seems to have identical receptors, whilst receptors on neural tissue differ slightly.

Following its binding to the 'receptor', angiotensin can induce a variety of responses varying from muscle contractions to release of aldosterone. The excitation coupler in each instance would probably be the same and calcium ions would appear to fit the role. Khairallah (1971) tentatively proposed the following scheme: In large doses (about 1 μg), angiotensin

causes depolarisation of smooth muscle cell membranes (Keatinge, 1966). This is associated with a net transfer of calcium from extracellular compartments to a compartment near the myofibrils, leading to contraction. On the other hand, angiotensin in small doses (ng range) probably only mobilises calcium bound on the membrane (Langer, 1968), probably without depolarisation, which finally increases calcium next to the myofilaments. Since the contraction of the myofilament is almost directly proportional to the amount of calcium available (Filo, Bohr & Ruegg, 1965), other drugs which mobilise calcium, such as noradrenaline (Hinke, 1965), may act synergistically, or the two drugs can mutually potentiate one another. This same scheme, Khairallah suggests, could apply to adrenal medullary tissue, since calcium is involved in release of catecholamines by angiotensin (Poisner & Douglas, 1966) and angiotensin depolarises adrenal medullary cells (Douglas, Kanno & Sampson, 1967).

Angiotensin, once bound to receptor sites, also causes flux changes with sodium and potassium ions. Whether these changes are primary events or secondary to calcium movements is as yet unknown.

THE INVOLVEMENT OF ANGIOTENSIN IN THE AETIOLOGY OF HYPERTENSION

From the initial studies of Goldblatt, Braun-Menendez, Page and Pickering, the thesis arose that in renal artery stenosis, a factor was released from the kidney which caused vasoconstriction and accounted for the hypertension resulting from this disorder. This factor was renin. However, elevated renin levels could not be demonstrated in patients with chronic benign hypertension and this led to the view that renin played no part in genesis and maintenance of hypertension. The true

picture appears to be somewhere between the two extremes of thought.

Of the various forms of experimental hypertension, the most elegant and reliable involves stenosis of the renal artery which, in all species so far investigated, is followed by a continuous increase in blood pressure (Wilson & Byrom, 1939; Goldblatt, 1948). Hypertension of similar aetiology is, however, comparatively rare in man, if it exists at all. The various forms of endocrine hypertension permit better comparison of the experimental and clinical features. The hypertension occuring in rats after treatment with desoxycorticosterone (DOCA) or aldosterone and salt resembles the condition of primary aldosteronism in man.

The role of the renin-angiotensin system in the initiation and maintenance of experimental hypertension has been the subject of a considerable amount of study. In particular, attempts have been made to correlate activity of the renin-angiotensin system in the various forms of hypertension. These are best considered under individual headings.

Renal artery stenosis

After a clip is placed on one renal artery, the renin content of that kidney increases within two days, reaching a maximum after about five days and remaining at that level for weeks and months (Gross, Brunner & Ziegler, 1965). In the contra-lateral kidney, the renin content begins to drop within 4 or 5 days, and after three weeks is very low or undetectable. Plasma renin activity increases to three or four times normal and remains high, corresponding to the elevated renin content of the clamped kidney. If, however, the contralateral kidney is removed simultaneously with the placing of the clip on the

renal artery, renin content in the remaining kidney and plasma renin activity do not increase (Regoli, Hess, Brunner, Peters & Gross, 1962). In both cases, hypertension develops and it is more marked in the unilaterally nephrectomised rats with normal plasma renin activity. The presence of the adrenal glands is essential to the development of the hypertension. In adrenalectomised rats maintained with low doses of DOCA, but not given additional saline, clamping of the renal artery is not followed by an increase in blood pressure, but the renin content of the ischaemic kidney reaches even higher levels than in the presence of the adrenal glands (Gross, 1969). It seems difficult, therefore, to correlate the hypertension caused by renal artery stenosis with either high plasma renin activity or with an elevated renin content of the clamped kidney.

Aortic stenosis

Partial constriction of the aorta between the origins of the renal arteries is followed by a more marked increase in the renin content of the kidney distal to the stenosis than that following clamping of the renal artery. In the kidney proximal to the constriction, the renin levels become very low (Masson, Kashii & Panisset, 1964; Masson, Kashii, Panisset, Yagi & Page, 1964). Despite the very high renin content of the ischaemic kidney, the degree of hypertension is the same as after renal artery stenosis, again illustrating the lack of correlation between the height of blood pressure and renin production.

Endocrine hypertension

Experimental hypertension due to high doses of aldosterone or DOCA and salt loading is characterised by marked reduction

in the renin content of the kidneys and a decrease in plasma renin levels to very low or undetectable levels. This process is reversible and after saline is replaced as drinking fluid by water, renin reappears in the kidneys, reaching normal values within three weeks. Simultaneously, hypertension slowly declines, blood pressure returning to normal levels provided the hypertensive state has not persisted for several months. Cortisol-induced hypertension does not produce characteristic changes in either kidney or plasma renin levels.

The most convincing evidence of the contribution of renin towards the pathogenesis of experimental hypertension is the normalisation of blood pressure that promptly follows the removal of the clamped kidney, no matter whether the contralateral kidney is present or absent (Gross, 1968). DOCA-induced hypertension, on the other hand, is not affected if the kidneys are extirpated. The fall in blood pressure following the elimination of the stenosed kidney closely resembles the effect of surgical removal of the diseased kidney in human hypertension due to renal artery disease, when the ischaemic kidney is either revascularised or removed. It therefore appears that the stenosed kidney plays a significant part in the maintenance of the hypertension.

It is also conceivable that the renin-angiotensin system could have an indirect effect on hypertension via, perhaps, the adrenal cortex and involving sodium balance. In the case of immediate fall in blood pressure following the removal of the diseased kidney, this kind of indirect effects seems unlikely from the point of view of the time that would be required to produce a significant alteration in plasma sodium. As a consequence of the lack of evidence to support a correlation

between plasma or renal renin levels and the degree of hypertension the role of angiotensin in the pathogenesis of the condition has been the subject of dispute. However, no more satisfactory alternative has so far been proposed.

Peart (1969) emphasises that a particular level of angiotensin in the plasma gives no indication of rate of production and of disappearance from the blood stream. Once renin is liberated into the blood stream it has to act without interference on its substrate to give the decapeptide and then this has to be converted to the active angiotensin II. This then is subject to binding and peptidases and must act on a vessel wall which may be more or less sensitive under different circumstances. There are obviously a great number of points at which this path could be interfered with and under certain circumstances it would be possible for a higher level of renin to exist with a low final action compared with a lower level of renin in which angiotensin is able to act more efficiently. Perhaps, therefore, studies concerned only with the levels of renin and angiotensin may lead to inaccuracies in the assessment of the importance of their activities in the pathogenesis of hypertension. The use of turnover studies and/or selective inhibitors of angiotensin may shed new light on this problem. The recent observation of Gross, Lazar & Orth (1973) that pepstatin, a polypeptide originally isolated from actinomyces cultures, was a powerful selective inhibitor of renin both in vitro and in vivo may provide another useful means for the investigation of this problem.

FACTORS AFFECTING THE RESPONSE TO ANGIOTENSIN

Tachyphylaxis

Page & Helmer (1940b) first described tachyphylaxis to crude angiotensin and Bock & Gross (1961) and Tetreault (1964), using synthetic l-asparagine angiotensin, found that a gradual decrease in pressor response occured after repeated injections of 0·03 - 2·0 μg/kg in the conscious dog or anaesthetised rat. Onset of tachyphylaxis is more rapid with high doses of angiotensi and cross tachyphylaxis occurs with renin (Page, McCubbin, Schwarz & Bumpus, 1957; Bock & Gross, 1961). Tachyphylaxis has been demonstrated in most tissues which respond to the peptide (see review by Khairallah, 1971).

Nephrectomy

Following nephrectomy, the pressor response to angiotensin increases to reach a maximum 18 to 24 hours after removal of the kidneys (Page & Helmer, 1940b; McCubbin & Page, 1954; Gabelman & Rondell, 1966). This increased responsiveness is specific for angiotensin since responses to noradrenaline, serotonin and vasopressin are variable and much less prominent (McCubbin & Page, 1954). The response was shown by these authors to be independent of changes in sodium intake, intact central nervous system or renal excretory function. The presence of normal kidney tissue prevents the increased responsiveness. Anaesthesia modified the increased responsiveness, pentobarbitone preventing the increase while urethane allowed full increased responsiveness (Sokabe, Shibayama, Mizogami & Sakai, 1965).

Pharmacological potentiation

Drugs that decrease peripheral sympathetic activity by inhibiting ganglionic transmission (tetraethylammonium, hexamethonium, pentolinium, chlorisondamine), by depleting

adrenergic neurotransmitters (reserpine), or by preventing their release (guanethidine, bretylium) generally increase the pressor response to angiotensin. TEA increased not only the vasoconstrictor effect but also the contraction of isolated smooth muscle preparations (guinea pig ileum, rat uterus) and in this respect was more active than hexamethonium or pentolinium (Prado & Carlini, 1959).

In the unanaesthetised dog, atropine increases the arterial pressor response to both angiotensin and noradrenaline, but simultaneously decreases their effect on venous pressure and abolishes the bradycardia that occurs during the pressor phase (Bock & Meier, 1963). This is a result of interference with autonomic reflexes and not a direct effect of atropine. Ganglion-stimulating drugs, such as DMPP (1,1-dimethyl-4-phenylpiperazinium iodide), also increase the pressor response to angiotensin (Haas & Goldblatt, 1959). Guanethidine given once or repeatedly to dogs enhances the pressor action of angiotensin as well as that of noradrenaline (McCubbin, Kaneko & Page, 1961), and similar observations have been made in cats (Miele, 1966). Day & Owen (1970a) showed, however, that adrenergic neurone blockade with bethanidine (3 mg/kg I.V.) produced no significant change in responses to angiotensin in the conscious cat.

In man, bretylium and guanethidine, though they markedly enhanced the response to noradrenaline, had little or no effect on the pressor response to angiotensin (Laurence & Nagle, 1963). Bethanidine has been shown to alter the biphasic response produced by high doses (greater than 200 µg) of angiotensin in the pithed rat to a simple pressor response (Finch & Leach, 1969).

In the rat, high doses of reserpine (1 mg/kg per day) for a period of one week have been shown to enhance the pressor

effect of angiotensin (Formanek, Linder & Selzer, 1966). In dogs treated with reserpine, the response of the hindquarters to intra-arterially injected angiotensin was less than that shown by untreated controls, whereas the response to noradrenaline was increased (Baum, 1963). Reserpine has recently been shown to reduce the pressor responses to angiotensin in the conscious cat by up to 50% while virtually abolishing responses to tyramine and McN-A-343 (Day & Owen, 1970b). These authors concluded that, in the conscious cat, the pressor response to angiotensin was mediated, in part, by release of noradrenaline from peripheral neuronal stores.

Desmethylimipramine (DMI), which also potentiates the angiotensin effect on blood pressure, has been thought to act like guanethidine on postganglionic sympathetic nerves, since it does not potentiate angiotensin in the reserpinised dog (Kaumann, Zuberbuhler & Taquini, 1964). It was postulated that the potentiation of angiotensin by guanethidine or desmethylimipramine is mediated by catecholamines, released from the adrenal medulla by angiotensin since the increase can be abolished by the administration of phentolamine or pronethalol (Miele, 1966). In the pithed rat, DMI markedly potentiated a wide dose range (10 - 500 µg) of angiotensin (Finch & Leach, 1969).

Pharmacological inhibition

There are a number of unrelated drugs that antagonise angiotensin. Some drugs have the opposite biological response and although they may be acting by totally different mechanisms prevent the response to angiotensin. Thus on the isolated ileum, catecholamines caused a relaxation and prevented the contractile response to the peptide (Khairallah & Page, 1962).

Systemic vasodilators such as adenosine triphosphate, theophylline-ethylenediamine and papaverine (Bianchi, de Schaepdryver, de Vleeschhouwer & Preziosi, 1960) and bradykinin (Barer, 1963) also prevented the pressor response to angiotensin.

The indirect actions of angiotensin are inhibited by inhibitors of the mediators released by the peptide. Thus atropine and morphine partially inhibited the response of isolated ileum to angiotensin (Khairallah & Page, 1961; Robertson & Rubin, 1962). Similarly, the pressor responses demonstrated by Bickerton & Buckley (1961) when angiotensin was infused into an isolated head connected to the rest of the body by nerves alone, could be abolished with α- and β-adrenergic blockers.

The direct actions of angiotensin, on the other hand, have been found more resistant to specific inhibition. Cinnarizine, a piperazine derivative with antihistamine and coronary vasodilating activities, has been claimed as a specific angiotensin blocking vasodilator (Schaper, Jageneau, Xhonneux, van Neuten & Janssen, 1963; Kling, Mattila, Pentilla & Jukarainen 1966). Schaper et al (1963) concluded that the in vitro effects of angiotensin on the guinea pig ileum and the papillary muscle of the cat and the in vivo coronary vasoconstriction caused by angiotensin was inhibited by cinnarizine. However, their results seem to suggest that cinnarizine was in fact acting as a non-specific smooth musle relaxant. Lidoflazine (1- 4,4-di-(4-fluorophenyl)-butyl -4- 2,6-dimethyl-(anilino-carbonyl)-methyl piperazine), a long-acting vasodilator (Schaper, Xhonneux, Jageneau & Janssen, 1966) antagonised the slow component of the contractile response produced by angiotensin on the guinea pig ileum by a 'competitive' action

(Godfraind, Kaba & Polster, 1966) at a concentration of 1 mcg/ml. However, in the case of rabbit aortic strips or rat fundic strips, lidoflazine had no effect on the contraction produced by angiotensin or noradrenaline, though it inhibited the action of serotonin (Turker & Kayaalp, 1967).

Gascon & Walaszek (1966) showed that osajin (an isoflavone derivative), specifically blocked the action of angiotensin on guinea pig isolated ileum. They suggested that osajin might be exerting its inhibitory effects by chelation of copper or zinc ions. Walaszek later showed that osajin had no in vivo angiotensin activity (personal communication).

Some monoamine oxidase inhibitors diminish the pressor activity of angiotensin by about 20% (van den Driessche, Trebaul, le Verge, Allain & Eben-Moussi, 1966). Adrenoreceptor blocking agents, such as phentolamine and dibenamine, failed to inhibit the blood pressure response to angiotensin (Meier, Gross, Tripod & Turrian, 1957; Bianchi et al, 1960). Dibenamine, phenoxybenzamine and chloropromazine reduced the contraction of the taenia coli produced by angiotensin, but other adrenolytics, such as dihydroergotamine and phentolamine, were without effect (Shibata & Frankenheim, 1967).

Guancydine (1-cyano-3-T-amylguanidine), given orally (100 mg/kg), reduced the pressor response to angiotensin infusion in rats (Welter & Grace, 1967). This effect could no longer be demonstrated following nephrectomy. In dogs, the vasoconstrictor response to the peptide after guancydine was converted to a biphasic response or to vasodilatation, whereas the response to noradrenaline was unchanged (Cummings, Welter, Grace & Gray, 1969).

Recently, neotetrazolium was said to block the vasopressor

responses to angiotensin in the pentolinium treated, vagotomised rat (Chryssanthou, Nelson, Teichner & Antopol, 1971). These authors suggested that neotetrazolium was inhibiting a noradrenergic component of the angiotensin pressor response which they also succeeded in inhibiting with phentolamine.

Undoubtedly the best inhibitors of angiotensin so far demonstrated have been members of the large group of polypeptide analogues synthesised in recent years. 8-alanine, 8-isoleucine, 8-aminophenyl-isobutyric acid and 8-cyclohexylalanine angiotensin II were all shown to inhibit the musculotropic effects of angiotensin II on rabbit aortic strips (Turker, Yamamoto, Khairallah & Bumpus, 1971; Khairallah, Toth & Bumpus, 1970). These authors also showed good in vivo antagonistic activity in the case of 8-cyclohexylalanine angiotensin II. Marshall, Vine & Needleman (1970) had previously shown that 4-phenyl-8-tyrosine angiotensin II was a specific competitive inhibitor of the parent peptide in vitro (rat isolated uterine strips) and in vivo (rat blood pressure). l-asparigine-8-alanine-angiotensin II has also been shown to be a specific inhibitor of angiotensin II, infusions of the analogue (200 mg/kg min^{-1}) inhibiting the effects of angiotensin in pithed and conscious rats (Pals, Masucci, Sipos & Denning, 1971).

THE BASIS OF THE PROJECT

In 1966, Gascon & Walaszek showed that osajin, a flavonoid extracted from hedge-apples, inhibited the action of angiotensin-amide II on the guinea pig isolated ileum, but was ineffective against bradykinin. It had previously been reported (Schwyzer, 1963) that angiotensin II formed amorphous precipitates

with zinc and copper ions and that the peptide might be acting with its receptors in a chelated state. Gascon & Walaszek therefore suggested that since flavonoids have the property of chelating bivalent ions, osajin could be acting by irreversably complexing a metal which was an integral part of the angiotensin/receptor complex. Subsequently, it was found that osajin had no in vivo anti-angiotensin activity.

In view of the possible dependence of angiotensin II on bivalent metal ions for its spasmogenic and vasoconstrictor actions, it was decided to examine a number of chelating agents for possible anti-angiotensin activity. In a preliminary communication, Day & Owen (1969) showed that the pressor action of angiotensin II was inhibited in the pithed rat and in the adrenalectomised cat by intravenous sodium diethyldithiocarbamate (DDC), a compound known to strongly chelate several bivalent metal ions (Chaberek & Martell, 1959). However, penicillamine, another potent metal ion chelator (Doornbos & Faber, 1964) did not inhibit angiotensin responses.

In this work, it is proposed to further investigate the pharmacology of DDC and its parent disulphide, tetraethylthiuram disulphide (disulfiram), in relation to a number of established actions of angiotensin reviewed above. Much of the work is concerned with attempts at inhibition of the pressor action of angiotensin in the pithed rat, anaesthetised cat and the conscious unrestrained cat. A section of the results deals with attempts at antagonism of the spasmogenic action of angiotensin in the rat isolated colon and guinea pig isolated ileum. The latter part of the work is concerned with the effects of the antagonists on the development and maintenance of renal and steroid hypertension in rats.

TETRAETHYLTHIURAM DISULPHIDE AND DIETHYLDITHIOCARBAMATE

Dithiocarbamates and related compounds, such as thiuram disulphides, are widely used in industry and agriculture (Thorn & Ludwig, 1962). They are mainly used in the rubber industry where they serve as antioxidants and as accelerators for the cross-linking of polymers. In agriculture they are widely used for their fungicidal properties. Dithiocarbamates have also been used in medicated soap products, but medicinal use of these compounds is now relegated to the drug known as Antabuse or disulfiram (tetraethylthiuram disulphide), which is employed in the treatment of alcoholism.

Metabolism

Stromme (1965) studied the metabolism of disulfiram and diethyldithiocarbamate in rats using ^{35}S-labelled compounds. He demonstrated four low molecular weight metabolites of disulfiram:

- diethuldithiocarbamate
- the S-glucuronide of diethyldithiocarbamate
- inorganic sulphate
- carbon disulphide

In addition, a small fraction of the radioactive sulphur was found bound to proteins as mixed disulphides. After I.P. injection of disulfiram (10 mg/kg), at most, 8% of the titratable -SH groups of plasma proteins and 0·1 - 0·2% of the -SH groups of the soluble proteins of the liver were blocked. Free disulfiram was never detected in plasma, liver or in urine, indicating that this compound, in vivo, immediately undergoes reduction to the thiol, diethyldithiocarbamate. The thiol thus formed appears to be metabolised at a high rate along several pathways:

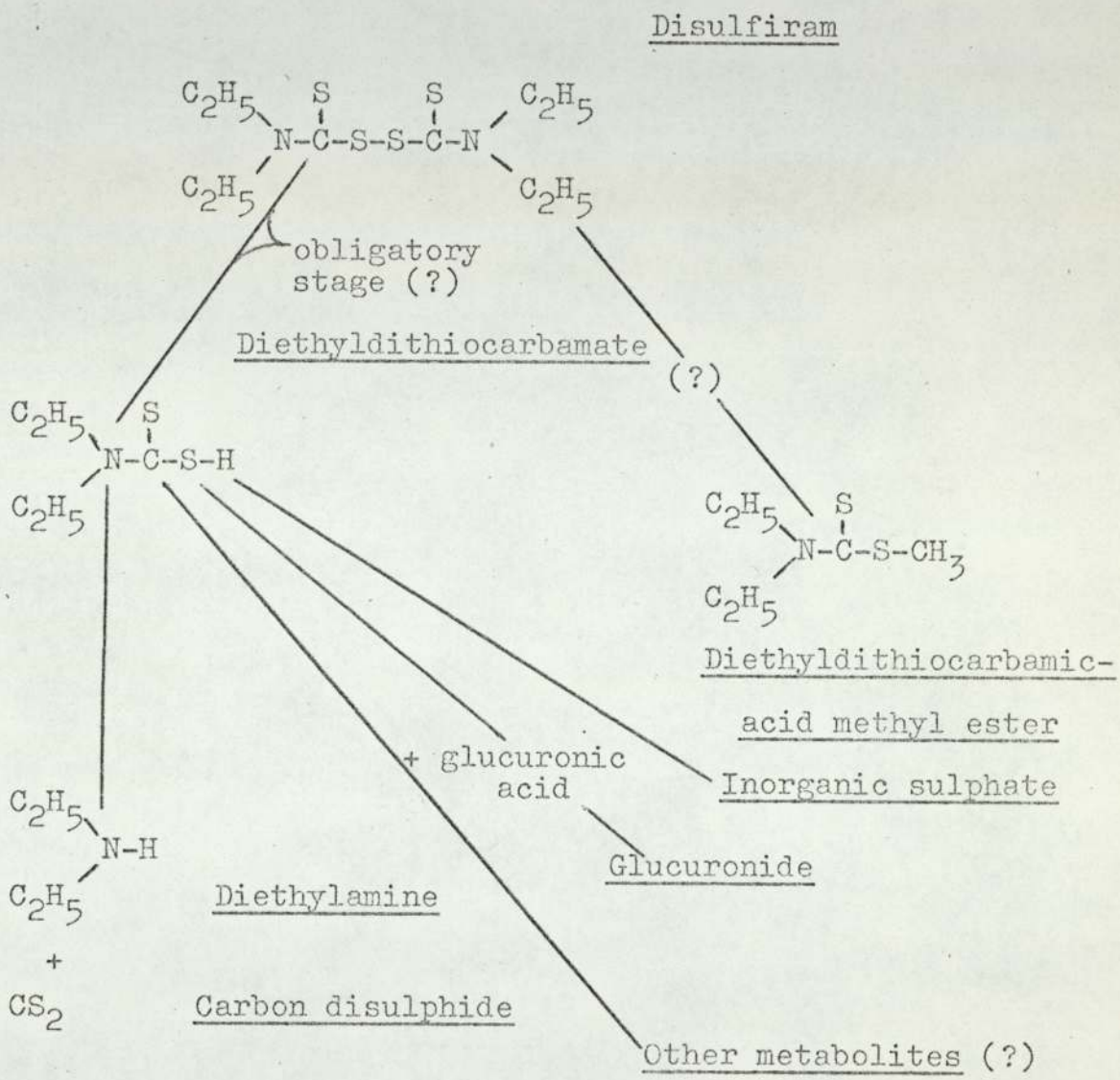


Fig. 4. The metabolism of tetraethylthiuram disulphide (disulfiram) and diethy;dithiocarbamate (Stromme, 1965; Fischer & Brantner, 1967; Gessner & Jakubowski, 1972).

- conjugation with glucuronic acid
- oxidation to sulphate
- breakdown carbon disulphide and diethylamine
- re-oxidation to disulfiram (see below).

The lack of free disulfiram in the plasma reported by Stromme (1965) is in agreement with previous in vitro studies showing that disulfiram has a high reactivity towards protein sulphydryl groups and naturally occuring thiols such as glutathione and co-enzyme A (Johnston, 1953; Stromme, 1963; Stromme, 1965; Eldjarn & Pihl, 1956; Owens & Rubenstein, 1964). Hence it would appear that absorbed disulfiram will at once undergo reactions leading to a complete reduction to the thiol. The rapid appearance of the dithiocarbamate and its S-glucuronide supports this conclusion. Stromme suggests that the fact that the same metabolites are recovered regardless of whether the disulphide or the thiol had been given, indicates that diethyldithiocarbamate is an obligatory intermediate in the metabolism of disulfiram.

Gessner & Jakubowski (1972) recently demonstrated a further metabolite of disulfiram, diethyldithiocarbamic acid methyl ester. This, they suggest, is responsible for some of the previously reported actions of disulfiram which could not be demonstrated with diethyldithiocarbamate. For example, it has recently been reported that disulfiram (200 mg/kg) caused a three-fold increase in rat hexobarbitone sleeping time and an impairing effect on drug metabolism (measured by microsomal ethylmorphine N-demethylase activity) which appeared after 8 hours and lasted up to 72 hours; diethyldithiocarbamate on the other hand, had very little effect on either parameter (Stripp, Greene & Gillette, 1969).

Pharmacology

Williams (1937) reported that he had observed poor tolerance to alcohol in workers exposed to tetramethylthiuram disulphide. This provoked a study of the pharmacology of the thiuram disulphides and led to their introduction in the treatment of alcoholism (Hald & Jacobsen, 1948). Prior to these investigations, similar compounds had been tested, often successfully, for a wide range of clinical and allied usage. Miller & Elson (1949) reported on the structure/activity relationships of 40 dithiocarbamates and thiuram disulphides tested for anti-fungal and anti-bacterial activity (*in vitro*) against human pathogens. The most active against bacteria were tetramethylthiuram disulphide and dimethyldithiocarbamate.

In their search for tuberculostatic agents, Jeney & Zsolnai (1956 a,b) found that among other organic sulphur compounds they tried, sodium and zinc diethyldithiocarbamate and tetraethylthiuram disulphide were especially effective. Powell (1954) observed the effect of dithiocarbamates on sarcoma cells and fibrocytes *in vitro*. Piperidinium and sodium pentamethylene-dithiocarbamate at a concentration of 4×10^{-5}M were toxic to sarcoma cells and embryonic mouse fibrocytes. van Bekkum (1956) found that good radioprotective activity was shown by ammonium dithiocarbamate and by sodium dimethyl- and diethyldithiocarbamates. This action, he considered, was due to protection of essential sulphydryl enzymes by an *in vivo* reversible inhibition.

Disulfiram has been shown to inhibit such enzymes as succinic dehydrogenase (Keilin & Hartree, 1940), liver aldehyde dehydrogenase (Kjeldgaard, 1949), xanthine oxidase (Richert, Vanderlinde & Westerfield, 1950) and glyceraldehyde-3-phosphate

dehydrogenase (Nygaard & Sumner, 1952). This broad enzyme-inhibitory activity of disulfiram is probably due to its high redox potential (Eldjarn & Pihl, 1956). Since blocking of essential -SH groups is probably the common mechanism for the inhibition of these enzymes, it seems reasonable to believe that disulfiram is a general inhibitor of enzymes with active sulphydryl groups. Possibly disulfiram will also interfere with the function of cofactors with essential sulphydryl groups such as Co-enzyme A or thioctic acid.

The principle pharmacological use of disulfiram derives from its dopamine-β-hydroxylase inhibitory activity. This action has been demonstrated both in vitro (Goldstein, Anagnoste, Lauber & McKeregan, 1964) and in vivo (Musacchio, Goldstein, Anagnoste, Poch & Kopin, 1966). The dose used for the in vivo inhibition in these studies was of the order of 400 mg/kg.

PART II

EXPERIMENTAL METHODS

The Pithed Rat Preparation

Male Wistar rats weighing 190-300 g were anaesthetised with pentobarbitone ('Nembutal' - 60 mg/kg I.P.). The trachea was cannulated and the animal pithed via the right orbit by the method of Shipley & Tilden (1947) using a steel pithing rod, 1·5 mm in diameter, prepared as described by Gillespie & Muir (1967) for the stimulation of the sympathetic outflow. Immediately after pithing, positive pressure artificial respiration was commenced using a Palmer small animal respirator adjusted to deliver 20 ml/kg at a rate of 35 inhalations/minute.

The right jugular vein was then cannulated with polythene tubing (Portland Plastics, PP 30) previously filled with 0·9%$^{w}/_{v}$ saline containing 10 units/ml heparin. The right common carotid artery was cannulated with polythene tubing (PP 90, drawn under heat to approximately 0·8 - 1·0 mm o.d. for a terminal length of approximately 20 mm.) filled with heparinised saline, and the arterial blood pressure was measured by means of a blood pressure transducer (Bell & Howell, Type 4-327-A221) connected to a Devices M4 or M2 recorder. In the majority of the experiments, heart rate was measured by means of a Devices Instantaneous Ratemeter (Type 2751) triggered by the blood pressure signal. In many preparations, occlusion of the arterial catheter by blood was prevented by the slow infusion of heparinised saline (0·002 ml/min) into the artery from a slow injection apparatus (Scientific & Research Instruments) connected via the transducer. The slow flow was found to prevent the progressive blockade of the fine catheters without affecting the blood pressure record.

The temperature of the preparation was maintained at approximately 37°C by means of an overhead lamp, the core

temperature being monitored with a rectal thermometer.

Electrical stimulation of the sympathetic outflow of the spinal cord was performed as described by Gillespie & Muir (1967). The indifferent electrode, a steel hypodermic needle, inserted subcutaneously into the left hind limb, was connected to one pole of a square-wave stimulator (Scientific & Research Instruments Ltd.). The other pole was connected to the shaft of the pithing rod. In these experiments, the preparation was injected intravenously with atropine sulphate (1 mg/kg) and (+)-tubocurarine hydrochloride (3 mg/kg). The sympathetic outflow was stimulated with supramaximal strength pulses (80 V) of 1 msec duration at frequencies ranging from 0·25 to 1·0 Hz for periods of 40 sec repeated at intervals of 20 or 30 min. Intravenous injection volumes were 0·5 ml/kg followed by a 'flush' of 1·0 ml/kg of 0·9% saline.

Fig. 5 illustrates the log dose/response relationships for angiotensin and noradrenaline in the pithed rat. The results only cover mean pressure increases of up to 60 mm Hg and therefore do not indicate the entire dose range. It was found that pressor responses in excess of those indicated shortened the life of the pithed rat preparation.

Fig. 6 shows the pressor response/log frequency curve for stimulation of the spinal sympathetic outflow of the pithed rat. This was obtained using a supramaximal stimulation voltage (80 V), a pulse width of 1 msec and a stimulation period of 40 sec. Threshold frequency was found to be 0·25 Hz and a maximal response was obtained with 8·0 - 16·0 Hz. In the majority of the following experiments a frequency of 0·5 or 1·0 Hz was used depending on the sensitivity of the individual preparation.

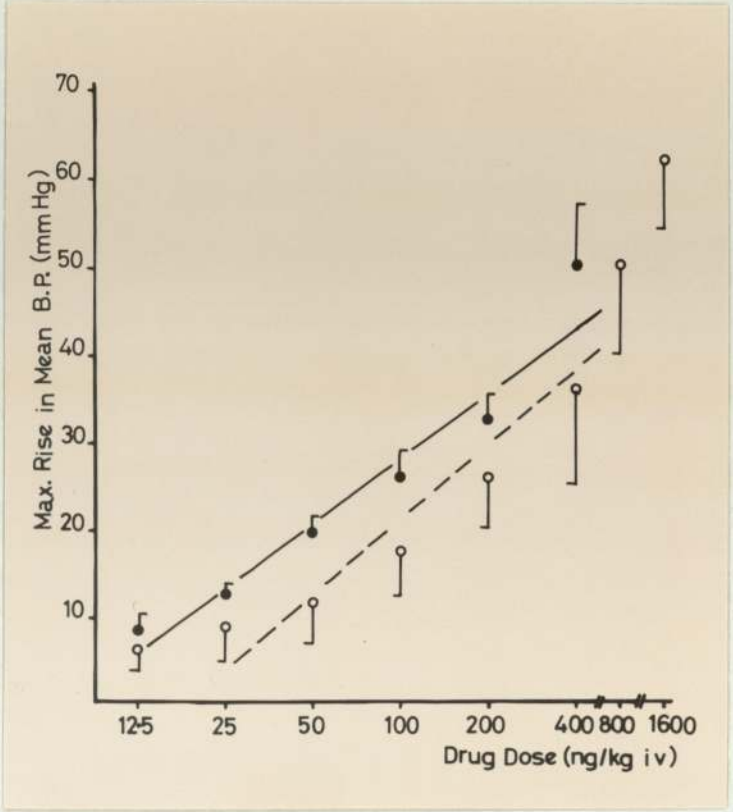


Fig. 5. Pressor responses to intravenous angiotensin (●) and noradrenaline (o) in the pithed rat. Each point is the mean (± SE) of at least 4 readings from different preparations.

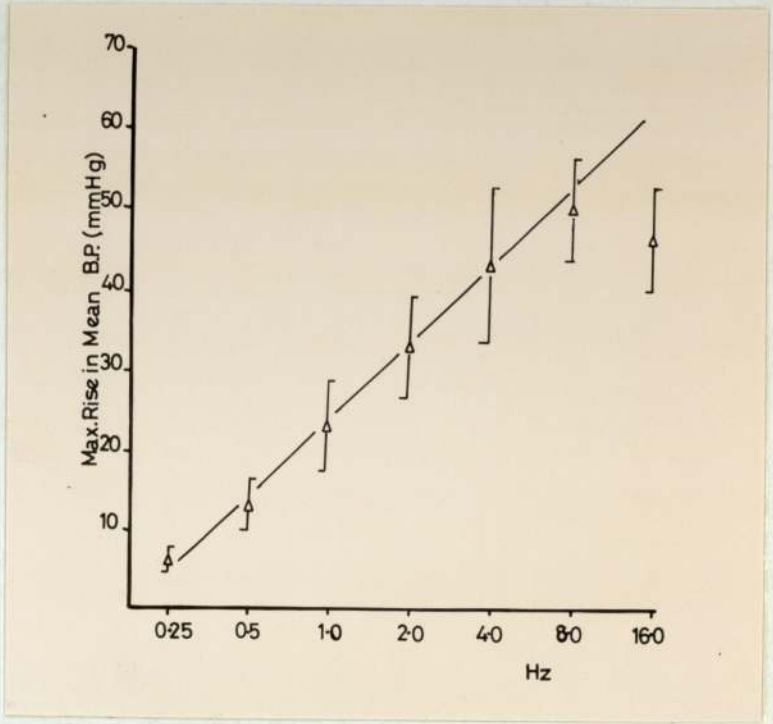


Fig. 6. Pressor responses to spinal sympathetic outflow stimulation in the pithed rat. Stimulation frequency was varied at constant voltage (80V) and pulse width (1 msec) for periods of 40 secs in all cases. Each point is the mean (± SE) of at least 4 readings from different preparations.

Measurement of blood pressure in anaesthetised cats

Anaesthesia was induced with halothane 3·5% in a mixture of oxygen (20%) in nitrous oxide. The right femoral vein was then cannulated (Portland Plastics PP 30) and chloralose 80 mg/kg (as a 1% solution in saline) injected slowly to maintain anaesthesia. During the injection of chloralose, the halothane content of the induction mixture was gradually reduced and when about half the chloralose had been given, the gaseous anaesthesia was withdrawn. The trachea was located and a metal cannula inserted to facilitate respiration and to allow artificial ventilation if necessary. Blood pressure was measured from the right common carotid artery using a Bell & Howell Type 4-327-L221 pressure transducer connected to a Devices M2 or M4 recorder. Heparin (1000 units/kg) was given intravenously. Drugs were administered via the cannulated femoral vein. All drugs were dissolved in saline and given in a volume not exceeding 1 ml and washed in with 2 ml of saline.

Adrenalectomy. In a number of experiments, the adrenal glands were removed, tied off or prepared to allow clamping of the blood supply when required. The glands were located via a mid-line incision in the abdominal muscle wall. The highly vascular surrounding tissue was carefully dissected away and the main body of the gland freed. This allowed removal following the tying off of the diffuse vascular supply of the gland or the application of artery forceps to temporarily occlude the blood supply.

Pithed cat preparation

A pithed cat preparation was used for several experiments. Cats were anaesthetised as described above, the femoral vein cannulated and atropine (1·0 mg/kg) injected. After tracheotomy,

a carotid artery was isolated for blood pressure measurement. The head was flexed forward maximally and a mid-line incision made at the nape of the neck. The superficial platysma muscles directly caudad to the lambdoidal ridge of the cranium were doubly ligated and severed and the underlying occipital muscles penetrated using a blunt dissection technique. The alanto-occipital membrane thus exposed was slit, exposing the spinal cord. Anaesthetic administration was discontinued and artificial respiration commenced. The spinal cord was immediately severed. The brain was destroyed via the foramen magnum using a thin, blunt probe. The spinal cord was destroyed using a flexible steel rod (2 mm diameter). The brain cavity was packed with cotton wool and the wound packed with the same material and sealed with Michell clips.

Measurement of blood pressure and heart rate in conscious, unrestrained cats

The methods used for the recording of aortic blood pressure and for the intravenous injection of drugs in the conscious unrestrained cat were essentially those of Thuransky (1966) and Hall, Gomersall & Heneage (1967) as modified by Day & Owen (1970).

Male crossbred cats, weighing 2·5 - 4·5 kg, were used throughout and were trained for approximately 7 - 10 days to sit quietly for several hours in the cage subsequently used for blood pressure recording.

Implantation of arterial and venous catheters. Anaesthesia was induced with halothane 3·5% in a mixture of 20% oxygen in nitrous oxide and maintained with 1·0 - 1·5% halothane in the same oxygen/nitrous oxide mixture.

A longitudinal incision was made through the previously

shaved ventral surface of the neck and the muscles overlying the trachea parted by blunt dissection to reveal the right carotid artery. A smaller incision was made at the back of the neck. The arterial and venous catheters, filled with heparinised saline, were passed beneath the skin from the dorsal to the ventral incision. The arterial catheter (Portland Plastics SH 90) was superimposed on the cat and a tie fixed to indicate the length to be inserted to place the tip of the cannula in the aorta at the level of the xyphoid cartilage. The artery was ligated craniad and the catheter inserted to the previously marked level. The arterial catheter was connected to a blood pressure transducer (Bell & Howell, Type 4-327-4221) and pen recorder (Devices M2) so that continuous monitoring of blood pressure was possible during the positioning of the catheter The catheter was firmly tied in the artery using an average of 5 or 6 ties.

A branch of the right jugular vein was located (usually the transverse facial vein) and the venous catheter (Portland Plastics PP 30) inserted and fed into the main jugular vein to a depth of about 3 inches. The catheter was then tied tightly into the branch of the vein. Both catheters were tied together for mutual support at the point where they disappeared under the skin.

The ventral incision was ligatured following light dusting with penicillin and sulphathiazole powder. The venous catheter was cut to leave 9 - 14 cms visible at the back of the neck and sealed with a pin. The arterial catheter was closed by means of rubber lined artery forceps placed close to the point of exteriorisation and the tube cut just distally. The cut end was attached to a valve of the type described by Day & Whiting (1972). The base of the valve was placed beneath the skin and

secured loosely at two points anterior and posterior to the body of the valve. These sutures also served to close the incision around the body of the valve. A third suture was used to locate the venous catheter caudad to the valve. The valve was covered with a dust-cap.

The operative sites were well bandaged to avoid disturbance for the first few days after the operation. Subsequently no bandage was found to be necessary as the cat tolerated the valve well. Sodium benzylpenicillin (0·6 mg/kg) was injected intramuscularly immediately after the operation and thereafter daily for 4 days. During the first 7 postoperative days, the arterial catheter was flushed through daily with 2 ml of saline containing heparin (50 iu/ml) and subsequently this was repeated on alternate days and before the start of each recording session. The venous catheter was similarly flushed using 0·5 ml of heparinised saline.

The cats were allowed at least 3 days for recovery after the operation after which they were trained to sit quietly during experiments and to accept intravenous injections without alarm. In most cases, the cats became sufficiently well trained to start experiments after a further 3 days.

Measurement of blood pressure and heart rate. The recording cage was sufficiently large to allow free movement and contained a waste tray and a bedding box. Following a period of exploration lasting up to 20 minutes, the cat settled down and usually remained quiet for periods of up to 4 or 5 hours. Visual disturbances were limited by the use of a cage which was only open on one side. The injection procedures were carried out outside the visual range of the cat. The effect of noise on the animal was, in general, minimal.

For recording blood pressure, the arterial valve cap was replaced by a threaded valve top (Day & Whiting, 1972) which opened the valve and permitted continuous blood pressure measurement to be made. Arterial pressure was recorded by means of a pressure transducer (Bell & Howell, Type 4-327-4221) connected to an electronic recorder (Devices M4). Heart rate was measured by means of a Devices Instantaneous Ratemeter (Type 2751).

Intravenous drug injections. The intravenous catheter was connected via a short polythene sleeve (PP 90) to a 0·5 m length of fine polythene tubing (PP 30). Drugs, usually dissolved in 0·9% saline were administered in a volume not exceeding 0·4 ml and flushed in with a further 1·0 ml of saline. By using a dose volume of 0·4 ml or less, the drug solution was retained in the tubing until flushed in and thus the injection could be delayed on the occasions when the cat became restless at the start of an injection.

Problems associated with the conscious cat preparation. There were two problems encountered during the initial preparation of the cat, both involving the cannulation of the thoracic aorta. The first was easily detected by monitoring the pulse during the passage of the arterial catheter and was due to the diversion of the tip of the cannula into the left ventricle instead of the aorta. When this occured, the pulse pressure became very large as the pressure in the ventricle was recorded. The catheter was withdrawn and reinserted until a 'normal' pulse pressure was seen.

Sometimes, however, the tip of the catheter became caught in the aorta wall and this resulted in a kink in the tubing. Usually this was indicated by a complete loss of the pulse but

occasionally the fault did not become apparent until several weeks later, when it was only possible to obtain an intermittent record due to the kink becoming aggravated by the gradual hardening of the catheter (see below). In these cases, surgical replacement of the arterial catheter was necessary.

The use of P.T.F.E. in the manufacture of the valve reduced the incidence of rejection experienced with the perspex valve of Hall et al (1967) and used by Day & Owen (1970) but did not prevent its occurence. Occasionally the presence of the valve prevented healing of the incision in which it was sited but more usually there was good initial healing followed by a build-up of connective tissue beneath the base of the valve resulting after 2 - 4 months in the emergence of the valve base through the skin. Re-siting the valve was usually successful in these cases.

The most common cause of failure of the preparation was the hardening of the plastic catheters. This was probably due to the leaching-out of plasticisers by the surrounding tissues. This hardening tended to lead to the formation of kinks, particularly where the catheter passed subcutaneously around the neck. Associated with this hardening was the gradual necrosis of the blood vessel. This was apparent in the cases of both the artery and the vein. In animals where the venous catheter had not been inserted as far as the vena cava the necrosis led to occlusion of the jugular vein and prevented intravenous injection.

The Indirect Measurement of Systolic Blood Pressure in the Conscious Restrained Rat

The method was based on the technique described by Friedman & Freed (1949) who employed a microphone to observe

the abolition of a pulse in the caudal artery of the rat when the pressure in a tail cuff was increased above the systolic blood pressure. The present technique also uses an inflatable cuff but the detection of the pulse involves the use of a strain gauge.

Thus the technique involves the measurement of the pressure in the occluding cuff at the point of disappearance or reappearance of the pulse in the caudal arteries. This pressure is approximately equivalent to the systolic blood pressure of the rat (see below).

The detection of the pulse in the caudal arteries was possible at room temperature (20°C) in the case of rats weighing 80 - 90 g or less. This was not possible with rats weighing in excess of this figure. However, this problem was overcome by placing the rats in a warm cabinet maintained at 30°C. 5 to 10 minutes exposure to this temperature caused vasodilatation and increased blood flow in the tail so that a good pulse was obtainable from rats weighing up to 400 g.

Apparatus. The warming cabinet consisted of a wooden cabinet opening at the front with perspex-glazed doors. Heating was provided by three 60 watt light bulbs placed near the base of the cabinet. The rats were protected from direct heat radiation from the bulbs by a galvanised steel sheet. Uniform heat distribution was achieved by a fan mounted in the top of the cabinet and the lights were controlled by a thermostat mounted centrally. The restraining cages were placed on steel mesh shelves. All metal in the assembly was earthed to reduce interference. The cabinet was maintained at 30°C throughout.

The restraining cages were made from galvanised steel and were of the pattern shown in Fig. 7. Access was via a removable

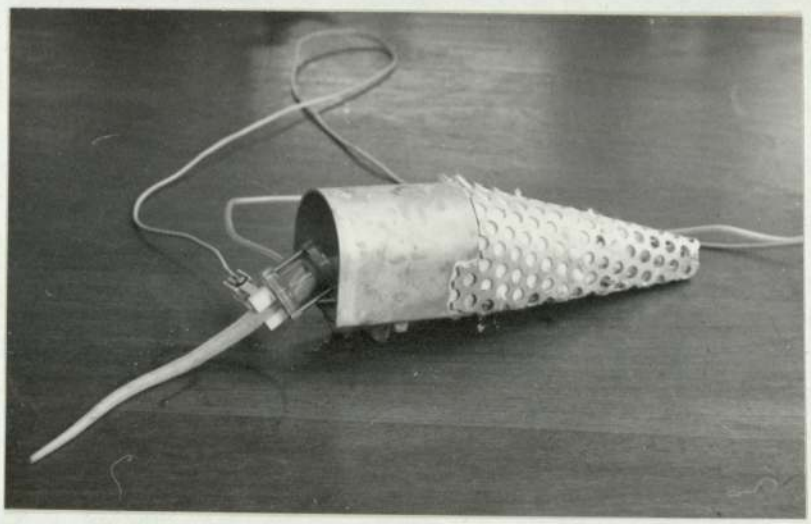


Fig. 7. Restraining cages used for the indirect measurement of rat systolic blood pressure. A strain gauge and cuff is shown in position on the tail of the rat.

rear plate which was cut to allow the exteriorisation of the tail for measurement. The design of the cages allowed for some adjustment to allow for weight variations but a range of three sizes was necessary to cope with rats weighing 80 - 400 g.

The occlusive cuff (Scientific & Research Instruments Ltd.) consisted of a perspex cylinder lined with a thin rubber sheath. This could be inflated to occlude the caudal arteries by means of compressed air controlled by a fine valve. The pressure in this system was measured from a 'T'-piece in the air line using a blood pressure transducer (Bell & Howell, Type 4-327-4221) connected to one channel of a Devices M2 pen recorder (Fig. 8). A short length of rubber tubing fitted with a gate-clip was attached to a further 'T'-piece, the gate-clip being adjusted to provide a 'bleed' which would allow the pressure in the system to return to atmospheric after a period of 10 - 15 seconds.

The strain gauge (Scientific & Research Instruments Ltd.) was arranged as one arm of a Wheatstone Bridge (Fig. 9). Potential for the Bridge was provided by a 4·5 V dry cell in series with a 180Ω resistor acting as a current limiter. The Bridge assembly was mounted in an earthed steel box fitted with a multi-point switch to allow switching between up to six strain gauges.

The signal from the Bridge was fed into a Devices Type 3160 high impedance differential input amplifier (Fig. 8). This first stage of amplification provided a voltage gain of 100. The amplifier incorporated high and low frequency response controls which were adjusted to reduce 'noise' at frequencies beyond the bandwidth required for the recording of the pulse. These controls effectively reduced the interference, particularly high frequency interference, probably electrostatic in origin,

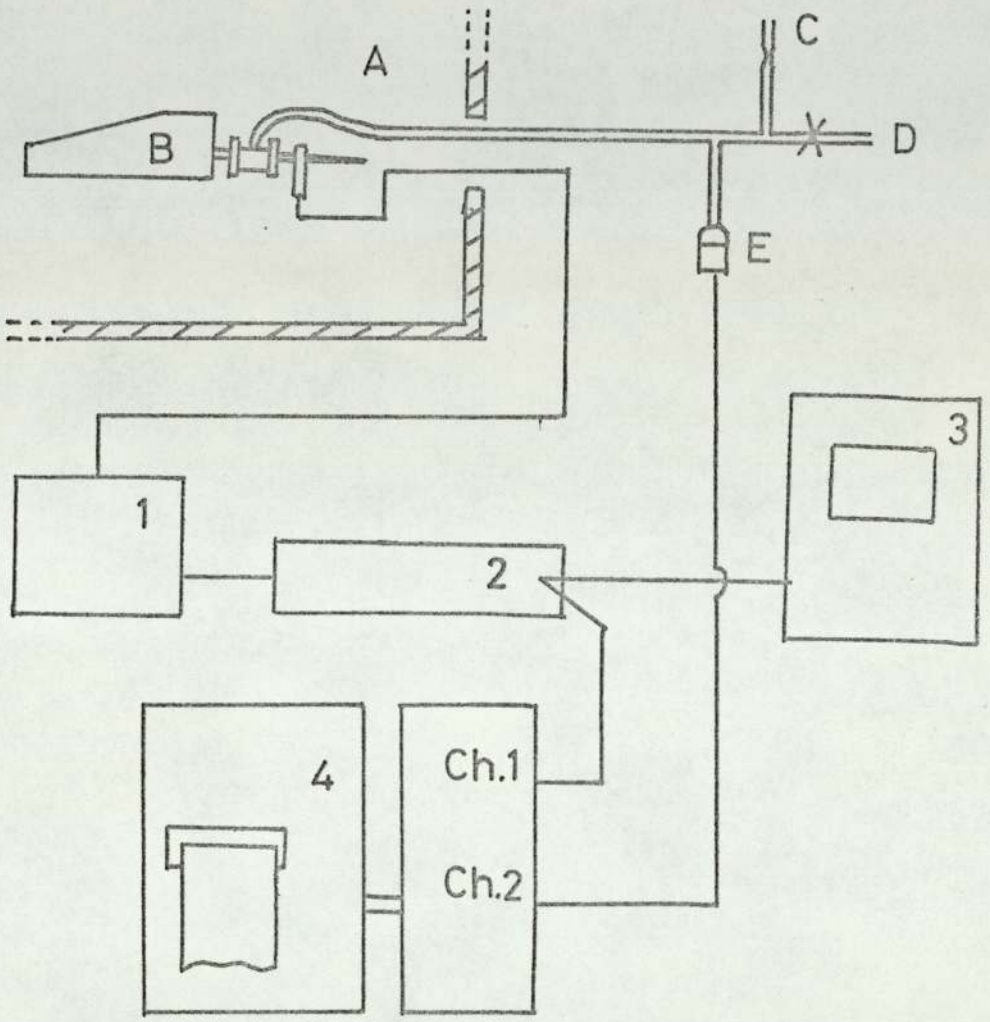


Fig.8. Layout diagram of apparatus used for the indirect measurement of blood pressure in the conscious restrained rat.

A. Warming cabinet at 30°C.
B. Restraining cage. Cuff and strain gauge in position on rat tail.
C. Variable bleed valve.
D. Supply of compressed air.
E. Bell & Howell 4-327-4221 pressure transducer.

1. Wheatstone Bridge assembly (see Fig. 9)
2. Devices Type 3160 preamplifier.
3. Display oscilloscope.
4. Devices M2 recorder (Ch1 - AC7; Ch2 - DC2C).

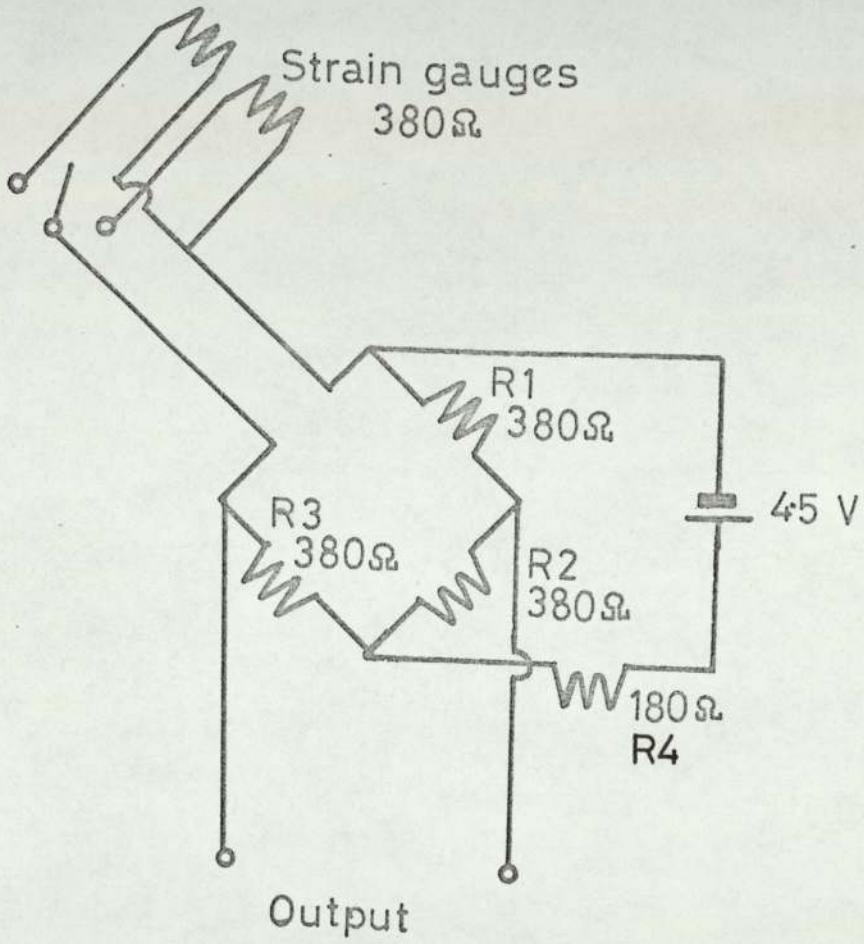


Fig. 9. Wiring diagram for the Wheatstone Bridge arrangement used for the detection of the pulse in the measurement of the blood pressure in conscious restrained rats. The strain gauges form the fourth arm of a bridge consisting of three resistors (R_1, R_2 & R_3). A fourth resistor (R_4) acts as a current limiter on the 4·5 V dry cell which supplies the potential for the circuit.

which appeared to arise from the heating/thermostat system in the warming cabinet.

The amplified signal was finally fed into the remaining channel of the Devices two channel recorder previously mentioned. The recorder was fitted with a Type AC7 pre-amplifier for this purpose. The record of the pulse signal could now be directly compared with the pressure record on the other channel. The signal from the first stage amplifier was also fed into a Cossor Model 1049 oscilloscope which allowed constant monitoring of the pulse during the warm-up phase of the procedure.

Operation. The rat was placed in the restraining cage and the cuff and strain gauge fitted to the tail. Following a period of equilibration in the warming cabinet, usually 5 - 10 minutes, a pulse could be observed on the oscilloscope. The signal was now fed into the recorder by switching the function switch from 'input S.C.' to 'record'. An 80% scale pulse could usually be obtained (Fig.10). With the recorder in operation, the cuff was inflated via the compressed air supply until the pulse disappeared as the systolic pressure was exceeded. With the air supply turned off, the bleed allowed the pressure to return to normal, the pulse reappearing as the pressure in the cuff fell below the systolic pressure. The process was repeated so that the mean of the three values could be taken.

The pressure could be read directly from the record (Fig.10) by relating the reappearance of the pulse to the adjacent pressure record. The value for the systolic blood pressure obtained by this method usually varied by ~~up to~~ a S.E. of approx. ± 5 mm Hg either side of the mean and therefore the systolic blood pressure was taken to be the mean of three readings.

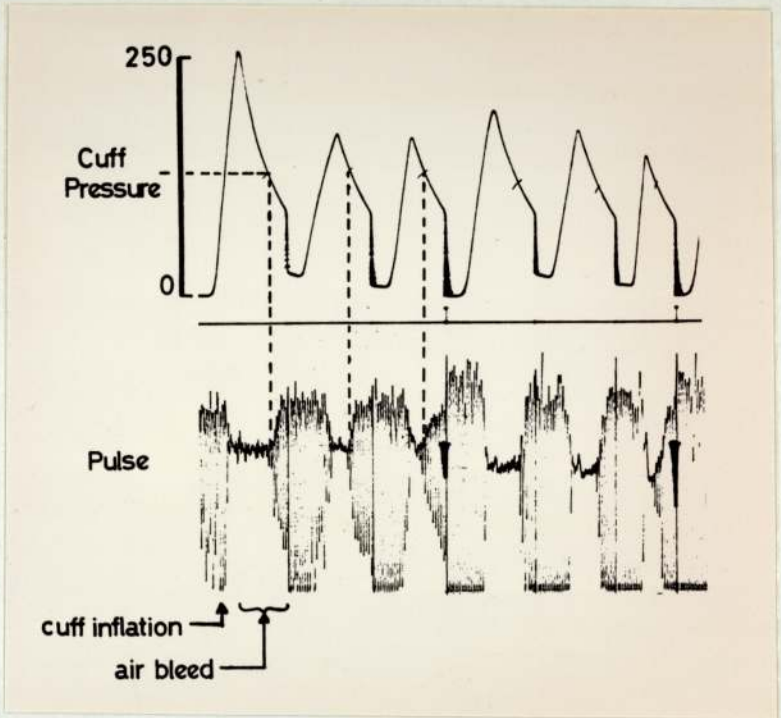


Fig. 10. Record of conscious rat blood pressure recording apparatus showing adjacent pulse and cuff pressure record. The 'pulse' disappears as the cuff pressure exceeds systolic blood pressure.

Production of Hypertension in the Rat

(a) Steroid (DOCA) Hypertension

The method was based on that of Seyle, Hall & Rowley (1943). Male Wistar rats (80-90 g) were anaesthetised with Halothane (3·5% reducing to 1·5%) in nitrous oxide (80%) and oxygen (20%). The lower back was shaved and a longitudinal dorsal incision made approximately 2 cm from the midline. The left kidney was manually exposed and the adrenal gland and adjacent adipose tissue carefully dissected off. The renal vessels and ureter thus exposed were occluded with Mosquito forceps and ligatured proximally with linen thread. The kidney was then removed with a scalpel and the forceps released, allowing the renal attachments to fall back into the peritoneal cavity. A single ligature was tied in the abdominal wall to prevent a hernia and finally the skin was securely ligatured.

A 25 mg desoxycorticosterone acetate (DOCA) implant was placed subcutaneously into the neck via a small incision.

On recovery, the rat was maintained on 1% saline ad lib.

Fig.11 shows the course of the development of the hypertension produced by the above procedure. Following an initial rise in pressure over the first few days, probably due to the operative stress, the blood pressure was maintained at about 120 mm systolic. At the start of the third week there was a rapid rise in pressure until a maximum of 160 - 170 mm Hg was reached at the start of the fourth week. At this time, some animals in the group were exhibiting systolic pressures in excess of 200 mmHg.

(b) 'Grollman' Hypertension

This method was first demonstrated by Grollman in 1944. It involves the placing of a 'figure of eight' ligature around

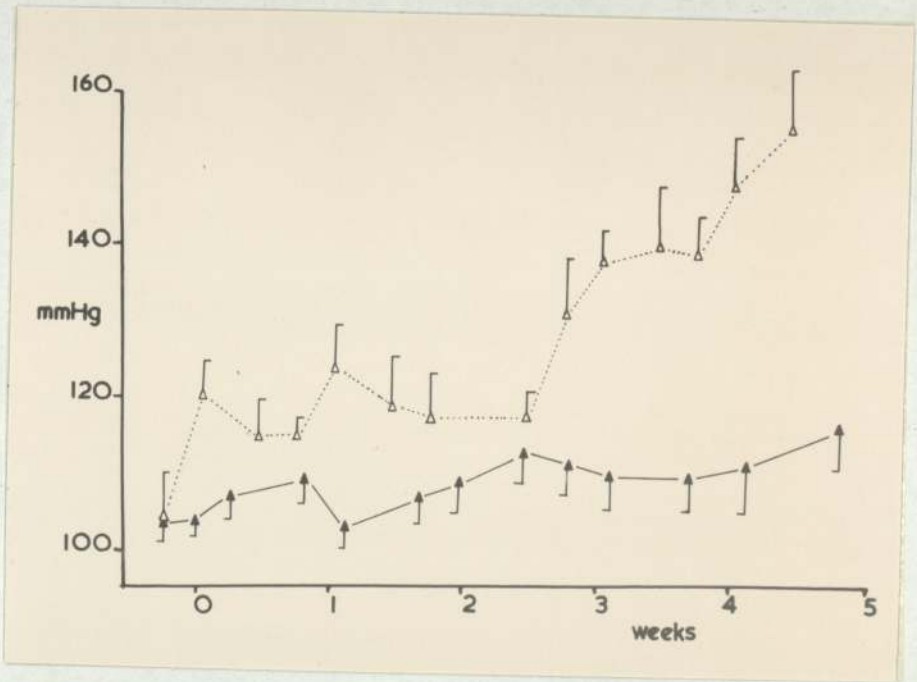


Fig. 11. Graph of systolic blood pressure against time showing development of DOCA/saline hypertension (n = 6). Open symbols indicate hypertensive group, closed symbols normotensive control.

the left kidney with the removal a week later of the contralateral kidney.

Male Wistar rats (80 - 90 g) were anaesthetised with Halothane (3·5% reducing to 1·5%) in nitrous oxide (80%) and oxygen (20%). The lower back was shaved and a dorsal incision made approximately 2 cm from the midline to expose the left kidney. The kidney was carefully dissected free of adipose and connective tissue and a linen 'figure of eight' ligature applied to produce three distinct 'lobes'. The ligatured kidney was reintroduced into the peritoneal cavity and allowed to return to its former position. A single ligature was placed in the abdominal muscle wall and the skin finally securely ligatured.

A week after this first operation, the contralateral kidney was removed as described above in the section dealing with steroid hypertension. The rats were maintained on normal diet and water ad lib.

Fig. 12 shows the course of the development of hypertension in rats prepared in this manner. There was an initial rise in pressure which developed during the week between the two operation This rise was not maintained, however, and the pressure declined until the start of the second week after the removal of the contralateral kidney when the 'true' hypertension began to develop. The pressure continued to rise for the next two weeks until a plateau was attained at 160 mm systolic. This pressure was subsequently maintained for the remaining two weeks observed.

Isolated Intestinal Smooth Muscle Preparations

(a) Guinea pig isolated ileum

Male guinea pigs, weighing 200 - 400 g were killed by cervical dislocation. Pieces of ileum, 15 mm in length, were

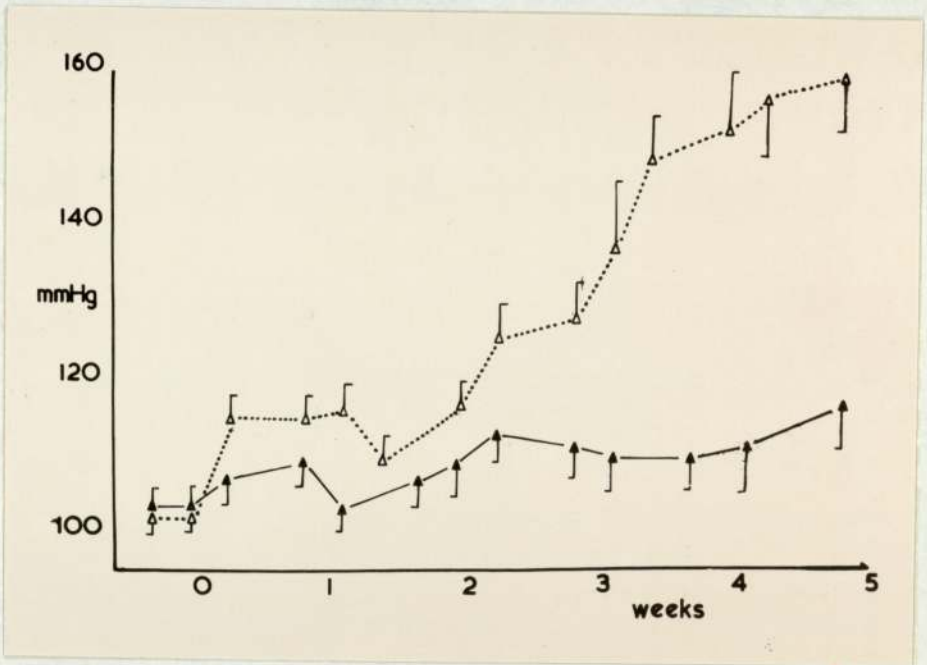


Fig. 12. Graph of systolic blood pressure against time showing the development of 'Grollman' hypertension. 'Figure of eight' ligature applied to left kidney at time '0' and removal of right kidney occured after 1 week (n = 6) Open symbols indicate hypertensive group, closed symbols normotensive control.

removed from about two thirds along its length and immediately were placed into aerated Tyrode's solution (Table 1). The segments were suspended in a 25 ml organ bath at 32°C gassed with air. Longitudinal contractions of the ileum were recorded on a smoked drum using an isotonic frontal writing lever exerting a constant load of approximately 2 g and having a magnification of 6.

Fig. 13 shows control response/log dose curves for the action of the agonists angiotensin, acetylcholine and histamine on the isolated ileum. It is apparent that the preparation is more sensitive to angiotensin than to either acetylcholine or histamine over the dose ranges examined and that this is particularly obvious at lower doses.

In a number of experiments, changes in tension due to the agonists were measured using a Devices Type 2ST02 strain gauge connected to a Devices M2 recorder. The tissue was suspended under an initial load of approximately 1 g in these preparations.

(b) Rat isolated ascending colon

Male rats weighing 180 - 250 g were stunned by a blow on the head and killed by cervical dislocation. Segments of ascending colon (from the region exhibiting diagonal striations immediately adjacent to the caecum) were removed and a piece 15-20 mm in length suspended in Kreb's solution (Table 1) in a 25 ml organ bath at 37°C gassed with 5% carbon dioxide in oxygen. Longitudinal contractions were recorded on a smoked drum by means of a frontal writing lever exerting a load of 1·5 - 2·0 g and having a magnification of 10.

Fig. 14 shows control response/log dose curves for the action of the spasmogens angiotensin, acetylcholine and 5-hydroxytryptamine on the isolated colon. The curves to

Physiological Salt Solutions

Composition in g/litre

	Kreb's*	Tyrode**
NaCl	6·90	8·00
$NaHCO_3$	2·10	1·00
KCL	0·35	0·20
$CaCl_2.2H_2O$	0·37	0·26
KH_2PO_4	0·16	-
$MgSO_4.7H_2O$	0·29	-
$MgCl_2$	-	0·10
NaH_2PO_4	-	0·05
Glucose	1·00	1·00

* Regoli & Vane (1964)

** Tyrode (1910)

Table 1

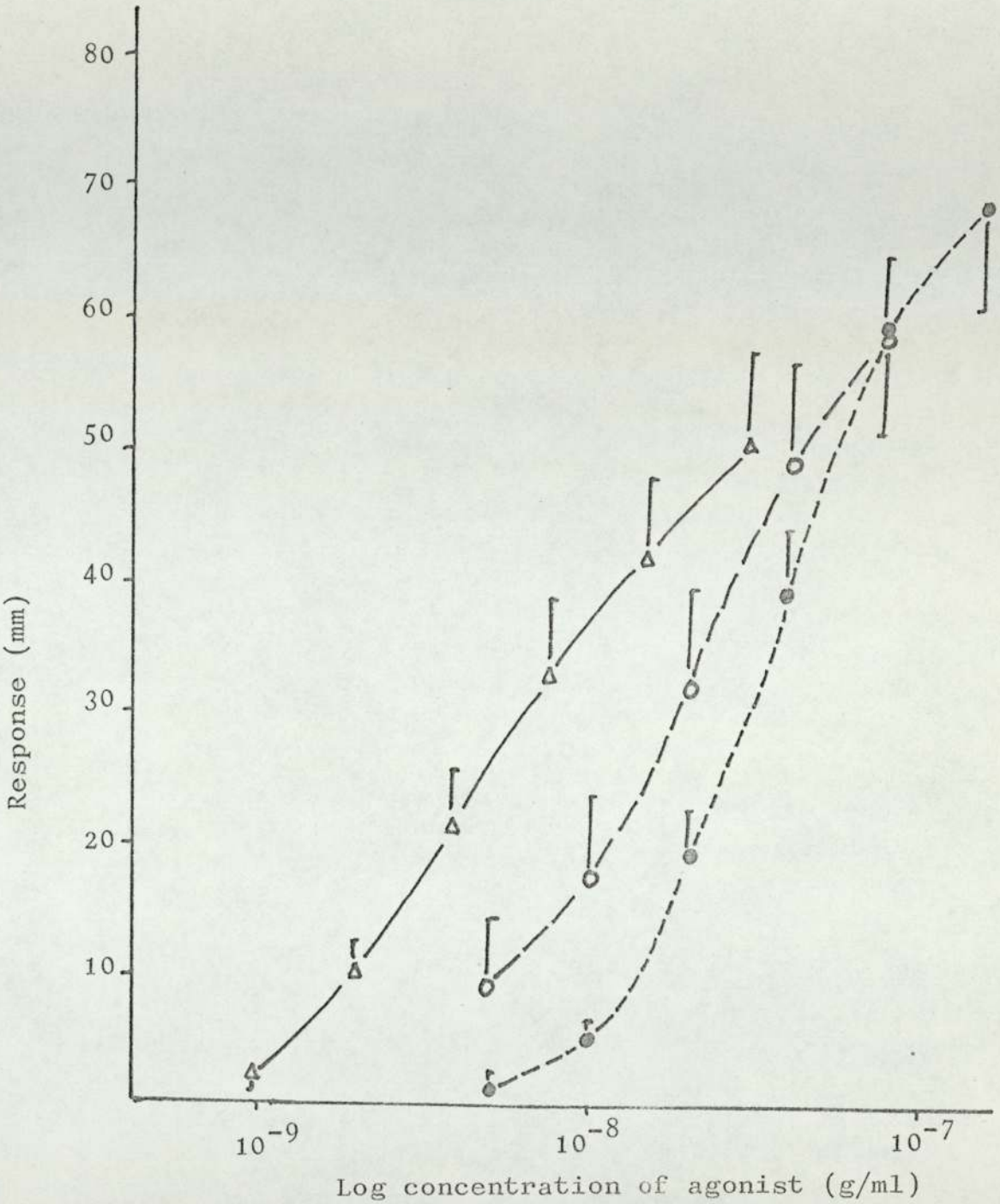


Figure 13. Guinea pig isolated ileum. Control response/ Log dose curves for the action of the agonists angiotensin (—Δ—), acetylcholine (—○—) and histamine (-●-). Responses were measured directly from smoked drum record (Isotonic frontal writing lever, constant load of approximately 2 g; magnification 6x)

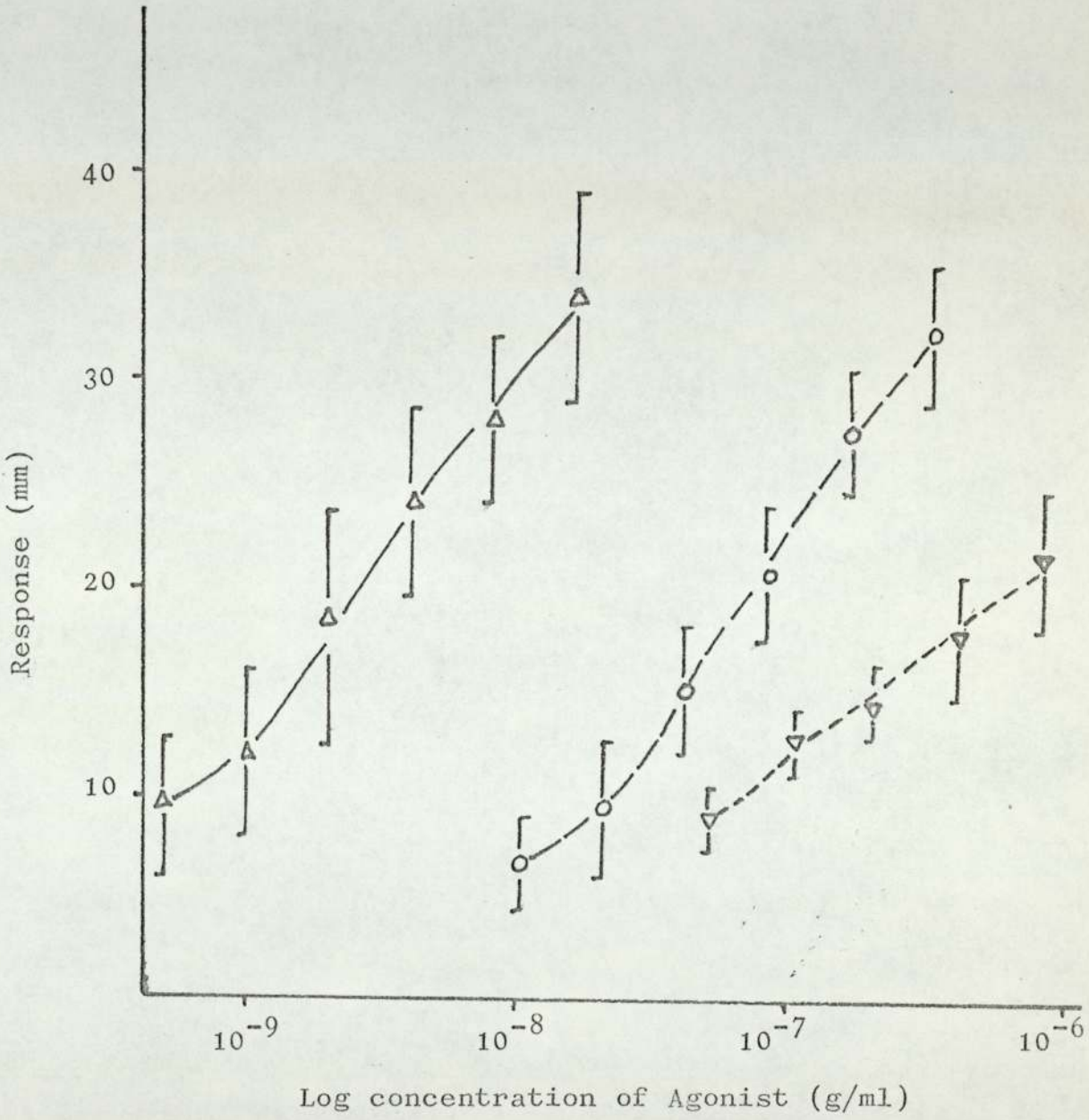


Figure 14. Rat isolated ascending colon. Control response/log dose curves for the action of the agonists angiotensin (—△—), acetylcholine (—○—) and 5-hydroxytryptamine (-▽-). Responses were measured directly from the kymograph record obtained using an isotonic frontal writing lever with a load of approximately 1.5g and a magnification of 10.

angiotensin and acetylcholine were approximately linear over the dose ranges examined. The slopes of these lines were almost parallel whereas that to 5-hydroxytryptamine was noticeably flattened.

Statistical analysis of data

't'-test: Statistical significance was indicated by the use of the student's 't'-test. 't' values were calculated using a Busicom electronic calculator. A probability of < 0.05 was taken as sufficient to confirm significant difference.

Computation of regression: To enable slope comparison of log dose/response curves, simple regression and multiple correlation analysis was computed by the method of least squares (Snecdor, 1946; Speigel, 1961) using an Olivetti Programma desk-top computer and PDP 10 system. Correlation coefficients were considered to be significant if $P < 0.05$.

The slope, standard error of the slope and the fiducial limits of the slope are presented in tabular form where 'b' is the slope, Sb the standard error of the slope and Sb.t the fiducial limits of the slope at the 0·05 level of probability.

DRUGS USED

(o)-Acetylcholine bromide	B.D.H.
Angiotensinamide II	CIBA
Ascorbic acid	B.D.H.
Atropine sulphate	B.D.H.
Bethanidine sulphate	Wellcome
Desmethylimipramine	Geigy
2,3-Dimercapto-1-propanol (Dimercaprol)	Koch-Light
DOCA implants (25 mg)	Organon
Halothane	I.C.I.
Heparin ('Pularin' injection)	Evans Medical
Histamine acid phosphate	B.D.H.
5-Hydroxytryptamine Creatinine Sulphate complex	Sigma
Hyoscine hydrobromide	B.D.H.
Methyldopa (injection)	Merck, Sharpe & Dohme
Neotetrazolium chloride	Koch-Light
(-) Noradrenaline bitartrate	Sigma
Penicillamine hydrochloride	Dista
Pentobarbitone sodium ('Nembutal')	Abbott
Phenoxybenzamine	S. K. & F.
Phentolamine methane sulphonate	CIBA
Propranolol	I.C.I.
Reserpine (injection)	Halewood Chemical Co.
Sodium diethyldithiocarbamate (DDC)	B.D.H.
Spironolactone ('Aldactone')	Searle
Tetraethylthiuram disulphide (disulfiram)	Ayerst Labs.
(+)-Tubocurarine hydrochloride	Duncan, Flockhart & Evans
Tyramine hydrochloride	B.D.H.
Vasopressin ('Pitressin')	Parke, Davis & Co.
P-286	Dow Chemical Corp.

Doses of noradrenaline are expressed throughout in terms of base. All other doses are expressed in terms of the salt described in the list above. All drugs were freshly prepared on the day of the experiment in question and during the experiment were stored on ice. With the exceptions of those mentioned below, all drugs were dissolved in distilled water or normal saline.

Disulfiram was prepared for intraperitoneal and oral injection by trituration with Compound Tragacanth Powder in a glass mortar and subsequent dilution to volume with distilled water.

An emulsion of dimercaprol in water was simply prepared by shaking. Any tendency to 'cream' was easily prevented by further shaking.

PART III

EXPERIMENTAL RESULTS

Chapter 1

THE EFFECT OF SODIUM DIETHYLDITHIOCARBAMATE AND DISULFIRAM ON THE CARDIOVASCULAR RESPONSES TO ANGIOTENSIN II IN THE PITHED RAT

Day & Owen (1969) demonstrated in pithed rats that injection of sodium diethyldithiocarbamate (DDC) (5-25 mg/kg intravenously) caused a small pressor response and initially enhanced the pressor response to angiotensin (2-50 ng/kg) and to noradrenaline (5-50 ng/kg). The pressor responses to angiotensin were subsequently abolished at a time when responses to noradrenaline did not differ greatly from control. They further reported that pretreatment of the rats with reserpine (5 mg/kg i.p.) 18 h before pithing accelerated the development of the DDC-induced reduction of angiotensin pressor responses.

This inhibition has been further investigated and confirmed in this work. The study has been extended to include tetraethylthiuram disulphide (disulfiram), the oxidation product of DDC. Disulfiram was expected to reproduce the effects seen with DDC since DDC has been long thought to be the active metabolite of disulfiram and indeed may be an obligatory metabolite of the disulphide (Stromme, 1965).

The first part of this chapter describes experiments to determine the time course and intensity of the anti-angiotensin effects of DDC and disulfiram in the pithed rat. The chapter goes on to describe experiments involving the use of a number of other vasoactive agents in order to determine the specificity of the antagonism of angiotensin by both the inhibitors. The pressor agents used were noradrenaline, tyramine, 5-hydroxytryptamine (serotonin) and the octapeptide vasopressin. Disulfiram and DDC are both recognised as inhibitors of dopamine-β-hydroxylase

(Goldstein et al, 1964; Musacchio et al, 1966) and it was therefore considered that they might possibly alter the noradrenergic sympathetic transmission in some way. Consequently, in addition to demonstrating the effects of disulfiram and DDC on the pressor agents referred to above, their effects on the pressor responses to sympathetic outflow stimulation (Gillespie & Muir, 1967) were also observed.

The final part of the chapter describes experiments designed to indicate the nature of the inhibition, that is, competitive or non-competitive, produced by disulfiram. Dose/response curves to angiotensin were compared before and after partial blockade with disulfiram administered acutely.

RESULTS

The Reproducibility of Pressor Responses in the Pithed Rat

In 5 preparations, the reproducibility of the pressor responses to sympathetic stimulation (0·5 & 1·0 Hz) and to intravenous injections of angiotensin (50 ng/kg) and noradrenaline (100 ng/kg) was tested at 10 min intervals for 4 h. The responses to sympathetic outflow stimulation did not vary by more than ± 5% of the initial responses throughout the test period as can be seen from Fig. 15. The responses to angiotensin and noradrenaline increased by up to 40% of the initial responses during the first hour after pithing and thereafter they remained virtually unchanged or in some preparations slowly increased by a further 10-20% over the next 3 h. (Fig. 15). After 90 min two of these preparations received an intraperitoneal injection of the vehicle subsequently used in the disulfiram experiments (2% tragacanth in water, 1 ml/kg); the pattern of responses was not changed by this procedure. The reason for the

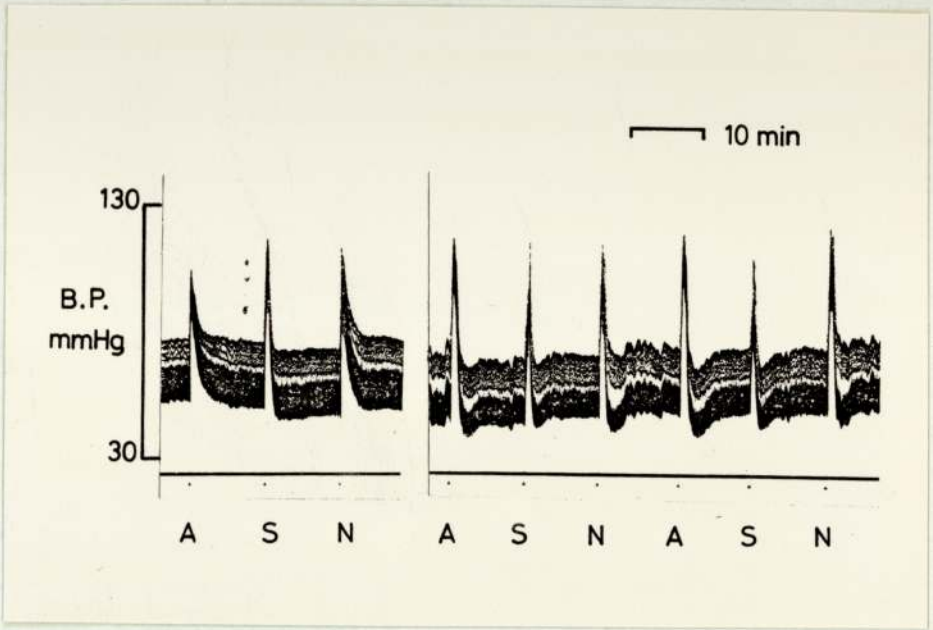


Fig. 15. Pithed rat blood pressure. First panel shows initial responses to angiotensin (A - 50 ng/kg), sympathetic outflow stimulation (S - 0·5 Hz) and noradrenaline (N - 100 ng/kg). The second panel shows the reproducibility of responses 60 minutes later. This reproducibility was maintained for a further 5 - 6 h in the majority of preparations.

progressive increase in the sensitivity of the preparation to noradrenaline and angiotensin is not clear. A possible explanation is that pentobarbitone ('Nembutal'), used as an anaesthetic in these experiments, caused an initial decrease in the sensitivity to the pressor procedures. Gillespie & Muir (1967), who did not report any changes in the vascular reactivity of their preparations, performed their pithing under ether anaesthesia. In the following experiments control responses were observed for about 90 min or until the responses became reproducible, before administration of blocking drugs.

Inhibition of Angiotensin Pressor Responses with Sodium Diethyldithiocarbamate (DDC) and Disulfiram

As shown by Day & Owen (1969), sodium diethyldithiocarbamate (DDC) inhibited the pressor responses to angiotensin in the pithed rat. However, in 15 acute experiments, there was no obvious relationship between the dose of DDC and the degree of reduction of the angiotensin responses. An initial potentiation of responses to angiotensin (10-50 ng/kg), noradrenaline (20-200 ng/kg) and sympathetic outflow stimulation (0·5 Hz) was recorded after injection of DDC (5-100 mg/kg i.v.). Subsequently, in 11 of the 15 experiments, the responses to angiotensin declined more rapidly than did those to noradrenaline and sympathetic outflow stimulation. In these preparations, 3 - 4 h after DDC, the responses to angiotensin were abolished or considerably reduced whilst those to noradrenaline and sympathetic outflow stimulation were within 20% of control levels. This pattern of response was seen over the whole range of DDC doses used. In the remainder of the experiments, the responses to angiotensin returned to control levels after potentiation for 1 - 2 h.

Disulfiram (20-100 mg/kg i.p.) produced a dose-dependent effect on the pressor responses to angiotensin, sympathetic outflow stimulation and noradrenaline (Fig.16). In the 3 h period following the injection of a low dose of disulfiram (20 mg/kg i.p.) the responses to angiotensin remained unchanged whilst those to sympathetic stimulation and to noradrenaline gradually increased by between 10 and 50% of the initial responses in different experiments (n = 4).

Higher doses of disulfiram (50-100 mg/kg i.p.) caused a greater initial potentiation (30-60%) of the responses to noradrenaline and sympathetic stimulation which reached a plateau about 2 h after the injection. There was also an enhancement of the responses to angiotensin (10-40%) in experiments where 50 mg/kg disulfiram was used. The enhancement was less marked at higher doses (100 mg/kg), as shown in Fig.16. In 12 of the 14 experiments involving these higher doses, the responses to angiotensin declined until they were virtually abolished 3-4 h after disulfiram. The responses to noradrenaline and sympathetic outflow stimulation were also substantially reduced at the highest dose used (100 mg/kg i.p.) as illustrated by Fig.16. However, disulfiram (50 mg/kg i.p.) did not reduce the responses to noradrenaline or sympathetic stimulation significantly; the responses were usually enhanced by 10-20% but were occasionally at control levels or reduced by up to 10%.

Disulfiram (20-100 mg/kg) itself produced only a small transient fall in blood pressure after intraperitoneal injection. The basal pressure of disulfiram-treated rats declined over the following 4-6 h at a similar rate and to a similar degree to that seen in control preparations.

In no experiments where reponses to angiotensin were

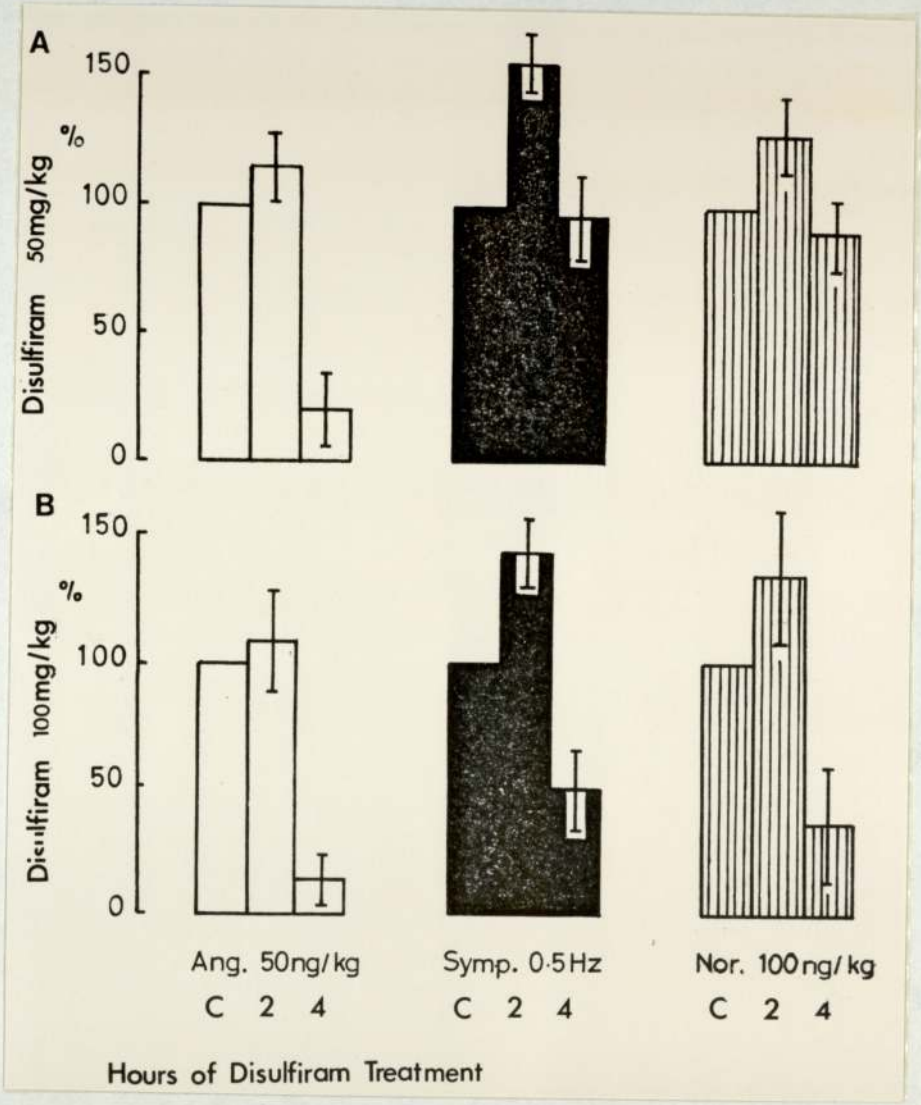


Fig. 16. The effect of intraperitoneal disulfiram (50 and 100 mg/kg) on responses to angiotensin (Ang), sympathetic outflow stimulation (Symp) and noradrenaline (Nor). Responses 2 and 4 h following disulfiram treatment are expressed as mean (±SE) and are shown as a percentage of the control responses obtained immediately before disulfiram treatment. Group sizes are 10 (Panel A) and 4 (Panel B).

selectively reduced by disulfiram (50 mg/kg i.p.) did the responses recover when observed for periods of up to 6 h. An attempt was made to determine the duration of the angiotensin-blocking action of disulfiram by pretreating 5 rats with 50 mg/kg i.p. 3.5 h before pithing and then recording the responses to angiotensin (50 ng/kg), noradrenaline (100 ng/kg) and sympathetic outflow stimulation (0·5 Hz) for periods of up to 8·5 h after the injection. The responses obtained were compared with those obtained from 4 control rats treated with the vehicle alone. The basal pressures of both groups of rats were similar. The results of these experiments are shown graphically in Fig.17. The responses to angiotensin were reduced by approximately 50% ($2P < 0.05$) by the disulfiram treatment, the responses remaining depressed for the duration of the experiments.

There was also a smaller reduction of the responses to noradrenaline and to sympathetic stimulation which was significant ($2P < 0.05$) at 4 h after disulfiram but which had returned to control levels by 6 h and did not subsequently change for the remaining 2 h of the experiments.

DDC (10-50 mg/kg i.p.) pretreatment 3 h before pithing produced no marked effect on the basal blood pressure or pressor responses to angiotensin (50 ng/kg), noradrenaline (100 ng/kg) or sympathetic outflow stimulation (0·5 Hz); the responses remained constant during 3 experiments (up to 7·5 h after DDC). Similarly, in 2 experiments, DDC (100 mg/kg i.p.) 18 h before pithing had no effect on the responses to angiotensin (50 ng/kg) or noradrenaline (100 ng/kg).

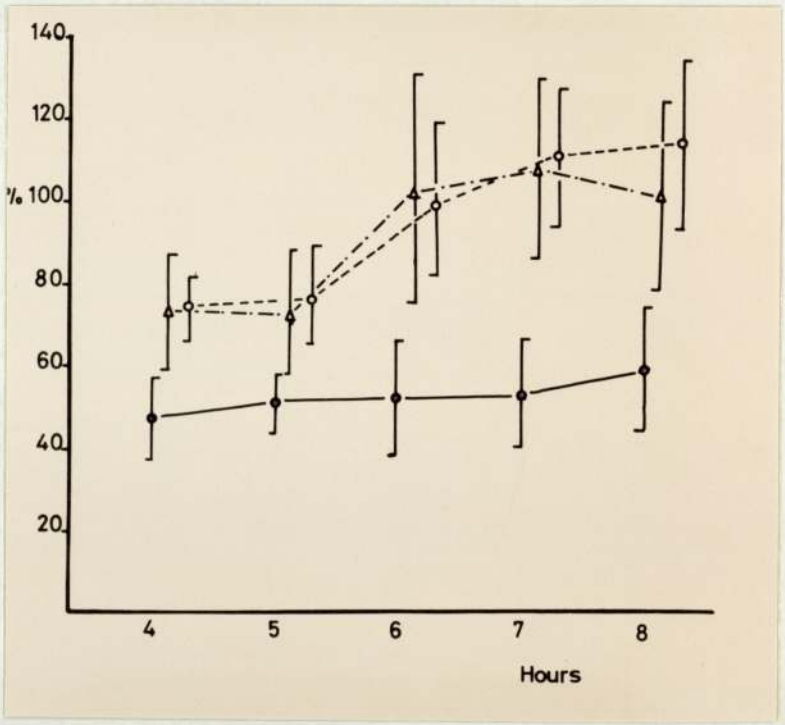


Fig. 17. Mean responses to 50 ng/kg angiotensin (●), sympathetic outflow stimulation at 0·5 Hz (Δ) and 100 ng/kg noradrenaline (o) in five pithed rats pretreated with disulfiram (50 mg/kg i.p.) at time 0 hours. Values are expressed as percentage of mean control responses taken from four untreated preparations. Vertical bars indicate standard errors of the mean.

The Specificity of the Disulfiram Inhibition of Angiotensin responses

Disulfiram, in doses ranging from 50 to 100 mg/kg consistently inhibited the acute pressor responses to intravenous angiotensin (above). The pressor responses to other vasoactive stimuli have been compared with those to angiotensin in the presence of disulfiram (Fig.18).

The effect of disulfiram (50-100 mg/kg i.p.) on the responses to noradrenaline and sympathetic outflow stimulation have been described above. At a dose of 50 mg/kg, disulfiram enhanced (by 30 - 60%) the responses to noradrenaline and sympathetic stimulation, the enhancement becoming maximal after about 2 h. Subsequently, the responses declined to within ± 15% of control levels at a time when responses to angiotensin were abolished by the disulfiram.

At a dose of 100 mg/kg, disulfiram produced a similar potentiation of the responses to noradrenaline and sympathetic stimulation. However, rate of decline of the responses subsequent to their becoming maximal after 2 h was greater than that seen in the presence of the lower dose. Thus, as shown in Fig.16 above, the responses to noradrenaline and sympathetic stimulation were reduced to between 10 and 30% of control values in 4 experiments.

In 3 experiments, the selectivity of the blocking action of disulfiram (50 mg/kg i.p.) was further tested by comparing its effects on the pressor responses to vasopressin ('Pitressin') (5 mU/kg) with those of angiotensin (50 ng/kg) and noradrenaline (100 ng/kg). The results of one of these experiments are shown in Fig. 19. Two hours after disulfiram, the responses to angiotensin and noradrenaline were enhanced whereas the

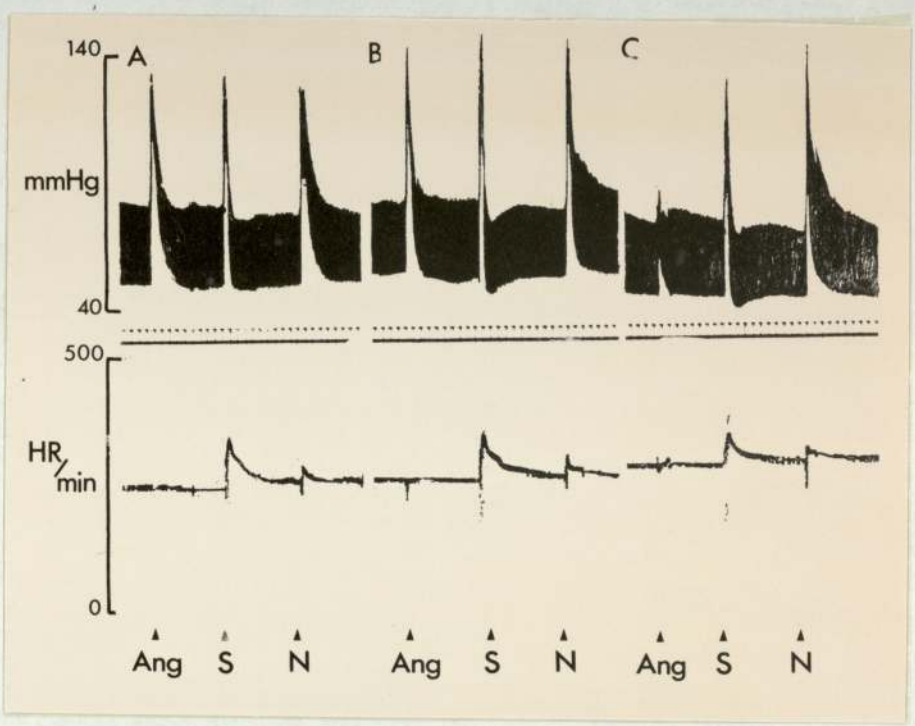


Fig. 18. Pithed rat blood pressure (upper record) and heart rate (lower record). A - control responses to sympathetic outflow stimulation (0·5 Hz at 'S'), noradrenaline (100 ng/kg i.v. at 'N') and angiotensin (50 ng/kg i.v. at 'Ang'). B - responses repeated 2 h and C - 4 h after disulfiram (50 mg/kg i.p.). Time marker in minutes.

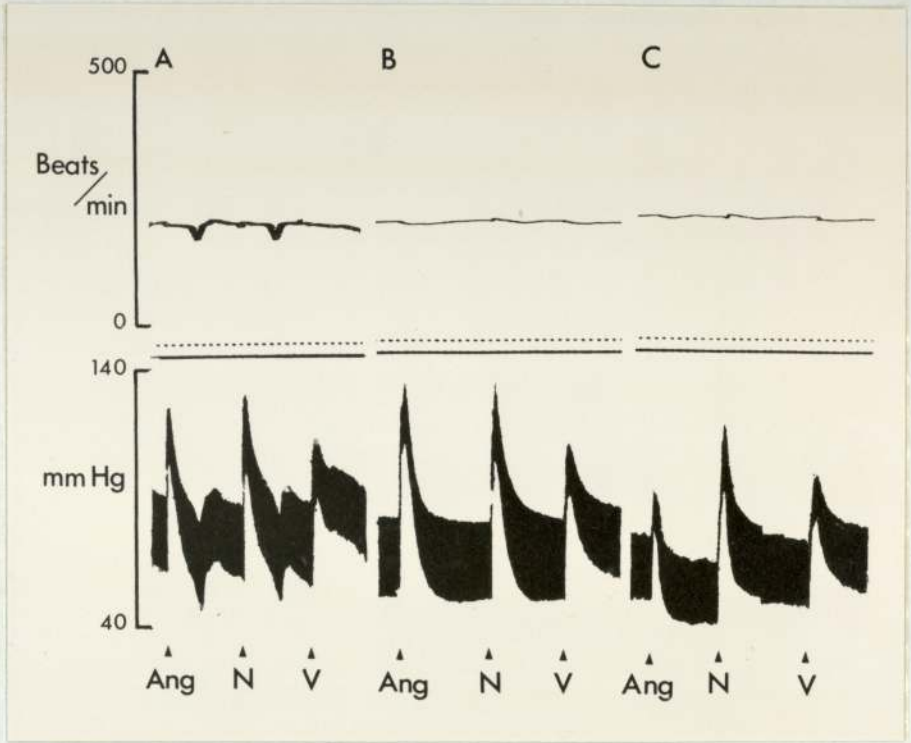


Fig.19. Pithed rat blood pressure (lower record) and heart rate (upper record). A - control responses to angiotensin (50 ng/kg at 'Ang'), noradrenaline (100 ng/kg at 'N') and vasopressin ('Pitressin') (5 mU/kg at 'V'). B and C - responses repeated 130 and 250 min after i.p. disulfiram (50 mg/kg). Time marker in minutes.

responses to vasopressin were unaffected. Furthermore, after 4 h, the responses to noradrenaline had returned to control levels and the responses to vasopressin were unaltered at a time when those to angiotensin were markedly reduced.

The vasopressor response to 5-hydroxytryptamine (5-HT) in the pithed rat appears to be due to a direct effect on vascular smooth muscle. In a preliminary experiment, phentolamine (2·0 mg/kg i.v.) and the ganglion blocking agent pempidine (2·5 mg/kg i.v.) had no effect on the pressor response to 5-HT (20 µg/kg i.v.) confirming the direct nature of the pressor response to the amine in the pithed rat.

In 3 experiments, the effect of disulfiram (50 mg/kg i.p.) on the pressor actions of angiotensin (50 ng/kg) and sympathetic outflow stimulation (0·25 & 0·5 Hz) were compared with its effect on the pressor responses to 5-HT (20 µg/kg). Fig. 20 shows the result of a typical experiment. Two hours after treatment with disulfiram, the responses to angiotensin declined as expected and the responses to sympathetic outflow stimulation had returned to control levels. 5-HT, at this time, was enhanced by about 15% and this enhancement increased slightly over the next 3 h, during which time the responses to angiotensin were considerably diminished.

The use of 100 mg/kg disulfiram in two further similar experiments virtually abolished pressor responses to angiotensin and noradrenaline but did not reduce responses to 5-HT; in fact, the responses to 5-HT usually increased in magnitude by 15 - 45% over the course of the experiments.

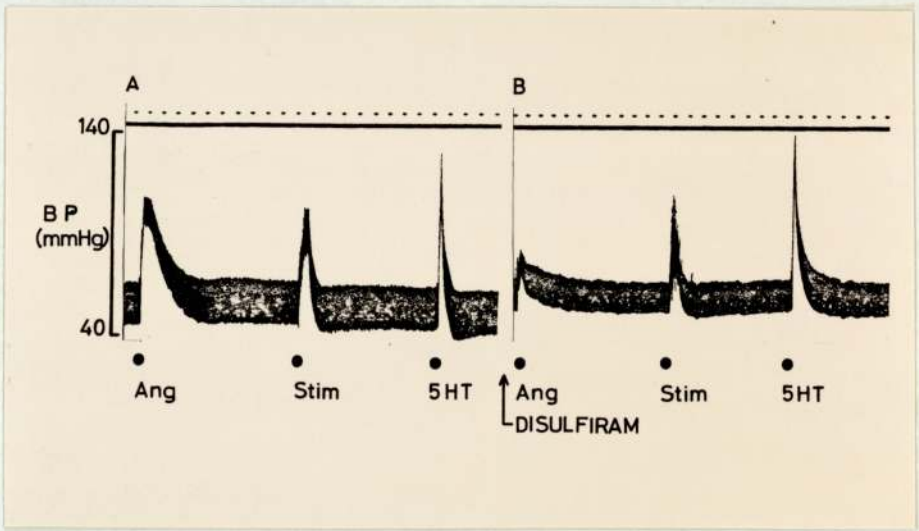


Fig. 20. Pithed rat blood pressure. Panel A shows responses to angiotensin (A - 50 ng/kg), sympathetic outflow stimulation (S - 0·5 Hz) and 5-hydroxytryptamine (20 µg/kg - 5HT). Panel B shows responses to the same agonists 3·5 h after treatment with disulfiram (50 mg/kg i.p.). Time marker - minutes.

The Effect of Disulfiram Inhibition on the Dose-Response Curve for Angiotensin in the Pithed Rat

The disulfiram-induced displacement of Log dose/response curves for angiotensin in the pithed rat was observed in 7 preparations. Fig. 21 illustrates the results of one of these experiments. Plots of Log dose/response curves obtained 1·5 - 2 h and 4 - 4·5 h following disulfiram (50 mg/kg i.p.) were compared with a previously obtained control curve. As can be seen from the figure, during the 'potentiation' stage occuring between 1 and 2 h following the disulfiram, there is an approximately uniform displacement of the curve to the left. However, there appeared an appreciable flattening of the curve 4 h after the disulfiram. The responses to the lower doses (12·5 - 100 ng/kg i.v.) of angiotensin were virtually indistinguishable from the injection artefact caused by the administration of an equal volume of saline. From the graph (Fig. 21) there appears to be a superficial parallelism developing between the responses due to angiotensin in doses in excess of 100 ng/kg and the control curve. These responses were, however, atypical in that they were biphasic in nature. The mechanism of this biphasic response is the subject of work discussed in Chapter 2.

DISCUSSION

Following the observations of Schwyzer (1963) that angiotensin II formed amorphous chelates with copper and zinc ions in aqueous solution, Gascon & Walaszek (1966) showed that osajin, an isoflavone derivative also known to form complexes with bivalent metal ions, specifically antagonised the contractor action of angiotensin II on guinea pig isolated ileum. Subsequently, osajin was shown to have no *in vivo* anti-angiotensin action (Walaszek, personal communication).

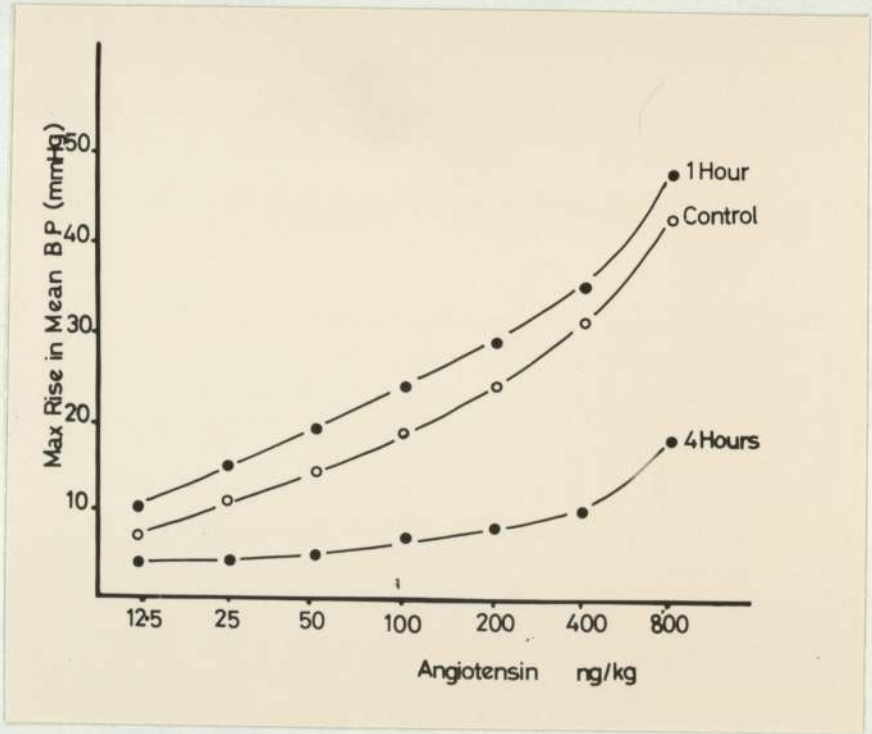


Fig. 21. Pressor responses to angiotensin in one pithed rat (165g ♂) showing dose response curves obtained before (Control), and 1 and 4 h after disulfiram (50 mg/kg i.p.). Injections of angiotensin were given when the pressor response to the previous injection had been completed.

Day & Owen (1969) examined a number of unrelated chelating agents for possible in vivo angiotensin inhibitory activity, and reported that the pressor effect of angiotensin II in the pithed rat was inhibited by the metal chelating agent sodium diethyldithiocarbamate (DDC). The present experiments confirm this observation and further demonstrate a similar activity in tetraethylthiuram disulphide (disulfiram).

In the experiments described, disulfiram and DDC exhibited two, apparently distinct, actions. There was an initial enhancement of the responses to angiotensin which was maximal after about 90 min and persisted some 2 - 3 h. However, in each experiment, this enhancement was always less than that seen in the cases of the responses to sympathetic outflow stimulation and noradrenaline. The enhancement of these responses was observed following all doses of disulfiram (20-100 mg/kg i.p.) and DDC (5-100 mg/kg i.v.) investigated, even when no subsequent inhibition of angiotensin responses were observed. Approximately 3 - 4 h following treatment with disulfiram (50-100 mg/kg i.p.) or DDC (10-100 mg/kg i.v.) pressor responses to angiotensin were observed to decline such that they were abolished or considerably reduced. A similar decline, albeit at a lesser rate was seen in the cases of the responses to noradrenaline and sympathetic outflow stimulation when high doses of disulfiram (100 mg/kg) and DDC (100 mg/kg) were used. In experiments where lower doses of the antagonists were used, however, the responses to noradrenaline and sympathetic outflow stimulation were potentiated by up to 30% or were reduced to up to 5% below control responses at a time when the responses to angiotensin were abolished.

The similarity of the effects observed following disulfiram and DDC was to be expected when it is considered that DDC has

been thought to be the active metabolite of disulfiram. Stromme (1965a) showed that the same metabolites (i.e. diethyldithiocarbamate, the S-glucuronide of diethyldithiocarbamate, inorganic sulphate and carbon disulphide) were recovered from rats regardless of whether DDC or disulfiram was injected. Furthermore, Stromme (1965a) failed to demonstrate the presence of disulfiram in plasma and suggested that the thiol was an obligatory metabolite of the disulphide. The results obtained from the experiments described in this section indicated, however, that there were some differences observed between the two compounds. The inhibition produced by disulfiram was more predictable than that produced by DDC; there was a marked dose-effect relationship in the case of the former whereas the effect of a given dose of DDC was variable, no dose-effect relationship being demonstrated. The lack of predictability associated with the anti-angiotensin effect of DDC may be due to its rapid metabolism relative to that of disulfiram although there appears to be little difference in the time-course of the inhibition when it occurs. The administration of disulfiram in suspension i.p. probably leads to a depot effect. Post-mortem examinations 5 - 6 h after the injection of disulfiram shows traces of the compound adhering to the intestines. On the other hand it seems unlikely that significant concentrations of an intravenous injection of DDC would remain in circulation at this time. The possibility also exists that the active metabolite may be neither disulfiram nor DDC. A number of reports have suggested that some actions of disulfiram could not be demonstrated with DDC. For example, Stripp and his co-workers (1969) showed that disulfiram (200 mg/kg) caused a three-fold increase in rat hexobarbital sleeping time and had

an impairing effect on drug metabolism. Using DDC, however, they were unable to show any significant effect on either functio Gessner & Jakubowski (1972) demonstrated a metabolite of disulfiram, diethyldithiocarbamic acid methyl ester, which they suggest is reponsible for this type of discrepancy.

At the 50 mg/kg dose level, disulfiram appears to specifically inhibit angiotensin. Experiments outlined above showed that at this dose pressor responses to noradrenaline, sympathetic outflow stimulation and 5-hydroxytryptamine were not reduced at times when the pressor responses to angiotensin were abolished or considerably reduced. The initial enhancement of angiotensin responses caused by disulfiram and DDC was, however, also observed in the case of the responses to noradrenaline and sympathetic outflow stimulation. The enhancement seen in the latter cases was, in fact, always greater than that recorded for angiotensin. Responses to 5-hydroxytryptamine, on the other hand, were never enhanced following disulfiram. The fact that 5-hydroxytryptamine pressor responses were not enhanced by disulfiram rules out the possibility that the potentiation of the other agonists was due to a general increase in the vascular reactivity of the preparation caused by the disulfiram. The enhancement of the responses to noradrenaline and sympathetic stimulation could be explained by a chelation mechanism. Copper chelation, which has been reported as a property of DDC (Thorn & Ludwig, 1962), has been previously reported to enhance pressor responses to adrenaline (Fischer, Lecomte & Delandtsheere, 1950). The potentiation of the responses to angiotensin was thought to be due to a similar enhancement of a possible sympathetic component of the response but this does not seem very likely in the light of evidence to be presented in Chapter 2.

The non-parallel shift of the angiotensin Log dose/response curve to the right 4 h after disulfiram appears to indicate that the inhibition is non-competitive in nature. The failure of a large dose of angiotensin to promote a reversal of the block together with the fact that the inhibition persists for at least 8 h with no sign of recovery seems to indicate that the block is irreversible. The development of the uncharacteristic biphasic responses to high doses of angiotensin (in excess of 100 ng/kg) following disulfiram was further investigated in experiments described in Chapter 2.

The possible mechanism or mechanisms of the anti-angiotensin action of disulfiram and DDC are the subject of work described in later chapters. From the experiments described in this chapter, several possibilities were apparent. As was outlined above, disulfiram and DDC were first examined as possible angiotensin antagonists on the basis of their ability, or at least the ability of DDC, to chelate bivalent metal ions. To establish or otherwise the validity of this possibility, a number of unrelated drugs with similar chelating properties have been examined for possible activity (Chapter 6).

In the light of the ability of disulfiram and DDC to inhibit dopamine-β-hydroxylase and hence interfere with sympathetic transmission, the role of the sympathetic nervous system in the pressor response to angiotensin in the absence and presence of disulfiram has been examined (Chapter 2).

A third possibility involves the inhibition of sulphydryl groups on enzymes necessary for the pressor action of angiotensin. Disulfiram had been previously shown to have a high reactivity towards protein -SH groups and naturally occuring thiols such as glutathione and co-enzyme A (Johnston, 1953;

Stromme, 1963; Stromme, 1965b; Owens & Rubenstein, 1964). This suggestion was supported by the findings of Page & Green (1949) who showed that dimercaprol, a well known sulphydryl inhibitor, produced a 'refractoriness' to angiotensin in dogs. The action of dimercaprol in pithed rats has therefore been studied (Chapter 6).

Chapter 2

THE ROLE OF THE SYMPATHETIC NERVOUS SYSTEM IN THE DISULFIRAM-INDUCED INHIBITION OF ANGIOTENSIN IN THE PITHED RAT

The sympathetic nervous system has been implicated in the mechanism of the pressor response to intravenous angiotensin in a number of ways. For example McCubbin & Page (1963 a,b) showed that infusions of angiotensin enhanced pressor responses to endogenous noradrenaline released by tyramine, pharmacological stimulation of sympathetic ganglia and by reflex activation of the sympathetic nervous system by carotid occlusion. It has also been shown that the peptide releases catecholamines from the adrenal medulla (Feldberg & Lewis, 1964) and from the peripheral adrenergic nerves (Benelli, Della Bella & Gandini, 1964; Distler, Leibau & Wolff, 1965) and stimulates sympathetic ganglia (Lewis & Reit, 1965; Trendelenburg, 1966).

The pressor response to intravenous angiotensin would therefore appear to be the sum of a number of its actions including direct vasoconstriction, release or facilitation of release of endogenous noradrenaline from sympathetic nerve endings, release of catecholamines from the adrenal medulla and sympathetic ganglion stimulation. Consequently, any modification of the pressor responses to angiotensin might be expected to involve one or several of the component factors of the response. For example, in the cat, the responses to angiotensin has been shown to be biphasic (Farr & Grupp, 1967; Ross & White, 1966) and that the second pressor phase may be modified by adrenalectomy (Ross & White, 1966) thus implicating the adrenal gland in this response. The importance of a functional sympathetic nervous system for the pressor response to angiotensin in the cat was confirmed by Day & Owen (1970b) who

demonstrated that treatment with reserpine (50 µg/kg per day) reduced the pressor response to the peptide by about 50%, the inhibition being reversed following treatment with α-methyldopa or a combination of monoamine oxidase inhibitor and noradrenaline.

The role of the sympathetic nervous system in the pressor response to angiotensin in the pithed rat is less well defined, however. Schmitt & Schmitt (1968) reported that the pressor response to the peptide in the pithed rat was not mediated via the release of noradrenaline nor altered by adrenalectomy. Furthermore, Pals, Fulton & Masucci (1968) showed that angiotensin had no effect on the pressor responses to procedures causing noradrenaline release from sympathetic stores.

Day & Owen (1969), however, demonstrated an enhancement of pressor responses due to electrical stimulation of the sympathetic outflow, tyramine and ganglion stimulation with tetramethylammonium, by infusions of angiotensin (200-500 ng/kg/min).

Finch & Leach (1969) showed that high doses of angiotensin (200-500 ng/kg) produced a biphasic pressor response in pentobarbital anaesthetised and pithed rats. Acute or chronic adrenalectomy did not alter the form of the response to the peptide in these preparations. These authors, however, showed that adrenergic neurone blockade with bethanidine or amine depletion with reserpine altered the response to a simple rise. They concluded that large doses of angiotensin were capable of indirectly stimulating sympathetic nerves.

In the present experiments, the acute effects of α-blockade with phentolamine or phenoxybenzamine, with or without accompanying β-blockade with propranolol have been observed in connection with the pressor responses to angiotensin in the

pithed rat. The effects of reserpine pretreatment acute adrenalectomy and adrenergic neurone blockade on the responses to angiotensin in the pithed rat have also been established. Furthermore, the effect of interference with the sympathetic nervous system by these agents has been examined with a view to ascertaining the involvement or otherwise of the sympathetic nervous system in the disulfiram-induced inhibition of angiotensin. This it was felt important to establish, since disulfiram and its metabolite sodium diethyldithiocarbamate have been used for a number of years as dopamine-β-hydroxylase inhibitors, albeit at a dose usually eight times that used in this study (e.g. Musacchio et al, 1966).

The chapter continues to describe experiments showing the effect of disulfiram on the enhancement of the pressor responses to sympathetic outflow stimulation induced by an intravenous infusion of angiotensin (Day & Owen, 1969). In this way it was hoped to compare the effect of disulfiram on the acute pressor responses to angiotensin (described in Chapter 1) with its effect on the indirect action of the peptide.

Finally, experiments are described involving the 'reversal' of the disulfiram-induced inhibition of acute pressor responses to angiotensin by infusions of such agents as noradrenaline and α-methyldopa. The properties of the responses thus obtained are described and compared with the properties of control responses to the peptide.

RESULTS

The Effect of Interference with the Sympathetic Nervous System on the Acute Pressor Response to Angiotensin

The effects on the pressor response to angiotensin of a number of drugs which interfere with sympathetic transmission

have been reported for a number of species. It was decided, therefore, to examine the effects of a number of these agents acting at different stages in the sympathetic transmission process on the pressor responses to angiotensin in the pithed rat. The experiments were controlled using pressor responses to noradrenaline (100-200 ng/kg i.v.) and sympathetic outflow stimulation (80 V; 40 sec; 1 msec pulse width; 0·25 - 1·0 Hz).

Reserpine: Eight rats were pretreated with reserpine (2·5 mg/kg daily for 3 days) and the effects of sympathetic stimulation (0·5 Hz), noradrenaline (100 ng/kg) and angiotensin (50 ng/kg) recorded. In these preparations, the resting blood pressure was higher (mean systolic $70 \pm_{S.E.M.} 10$ mm Hg) than that in control untreated rats (mean systolic $55 \pm_{S.E.M.} 6$ mm Hg). Responses to sympathetic stimulation were abolished whilst those to noradrenaline and angiotensin were unaltered from control or, more commonly, increased by up to 20% as shown in Fig. 22.

Adrenoceptor blockers: The effect on pressor responses to angiotensin of α-adrenergic inhibition by phentolamine and phenoxybenzamine was observed in 5 and 3 preparations respectively. Cline, Goldstein & Bunker (1969) had reported that phenoxybenzamine (10 mg/kg) but not phentolamine (1 mg/kg) shifted the dose response curve to angiotensin in the dog to the right. However, in the present experiments in pithed rats, both phentolamine (1 mg/kg) and phenoxybenzamine (2 mg/kg) enhanced pressor responses to the peptide by 10 - 20%. Concurrently, these inhibitors both produced a decrease in responses to noradrenaline (100 ng/kg) and sympathetic stimulation (0·5 & 1·0 Hz) of 50 - 80% with responses to sympathetic stimulation being generally more susceptible to α-blockade by both agents. Fig.23 illustrates the effect of

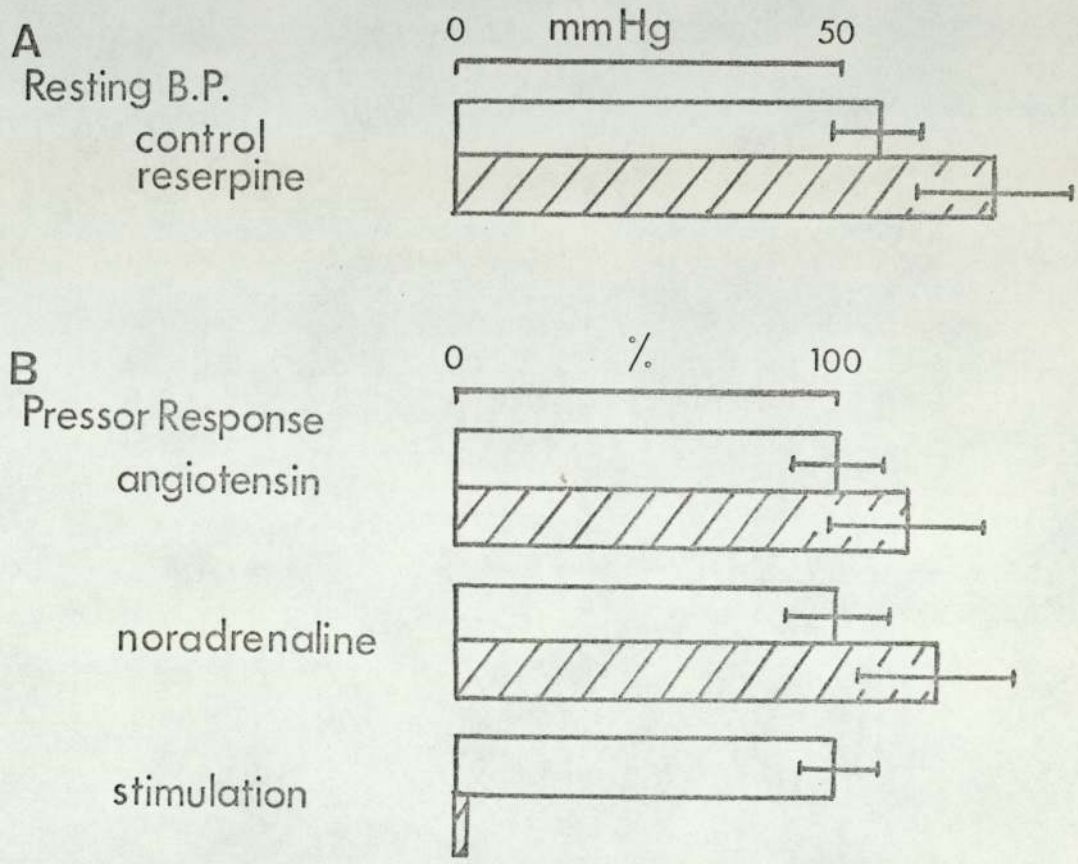


Fig. 22. The effect of reserpine pretreatment (2·5 mg/kg daily for 3 days) on the resting systolic blood pressure of the pithed rat (Fig.22A) and the pressor responses to intravenous injections of angiotensin (50 ng/kg) and noradrenaline (100 ng/kg), and spinal sympathetic outflow stimulation (0·5Hz) (Fig.22B). Recordings were made in each case between 1 and 2 hours after pithing. The results are compared with those from untreated control preparations (n=10) under similar conditions.

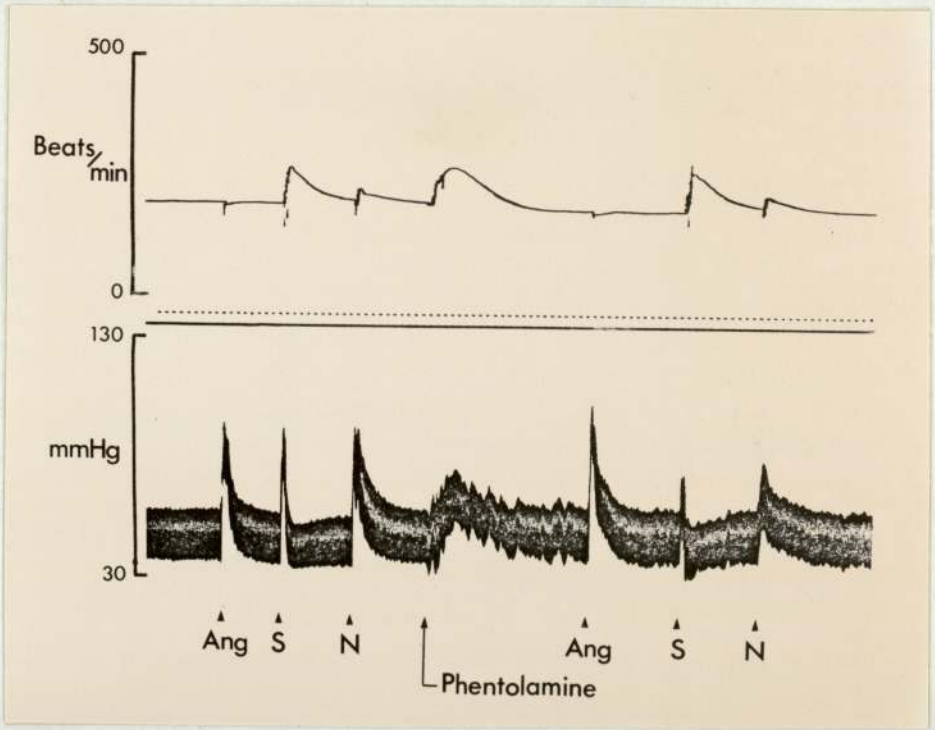


Fig. 23. Pithed rat blood pressure (lower trace) and heart rate (upper trace) showing the effect of phentolamine (1 mg/kg i.v.) on pressor responses to angiotensin (50 ng/kg - Ang), sympathetic outflow stimulation (0·5 Hz - S) and noradrenaline (100 ng/kg - N). Time marker - minutes.

phentolamine on the pressor responses to angiotensin, noradrenaline and sympathetic stimulation.

Propranolol (1 mg/kg i.v.) produced no marked alterations in responses to angiotensin or noradrenaline but abolished the tachycardia produced by sympathetic outflow stimulation in 4 preparations. Phentolamine (1 mg/kg i.v.) given after propranolol produced its usual effect.

Adrenergic neurone blockers: The administration of the adrenergic neurone blocking agent bethanidine (1 mg/kg i.v.) produced after 30 - 60 min a similar effect to that observed following the reserpine pretreatment reported above. Responses to angiotensin (50-100 ng/kg) were enhanced by up to 20% as were responses to noradrenaline (100-200 ng/kg). As expected, responses to sympathetic outflow stimulation were reduced by approximately 50%.

Adrenalectomy: In 4 experiments, acute adrenalectomy subsequent to pithing, reduced the resting blood pressure by about 5-10 mm Hg Responses to angiotensin and noradrenaline were immediately reduced by up to 20% and these continued to decline throughout the limited life (up to 3 h) of the preparations.

P-286: N,N-di-isopropyl-N'-isoamyl-N'-diethylaminoethylurea (P-286) has been reported to specifically inhibit release of catecholamines from the dog adrenal medulla in vivo (Gardier, Abreu, Richards & Herrlich, 1960). It was decided to briefly investigate the possible effects of this compound on the pressor response to angiotensin in the pithed rat. In 3 preparations, P-286 (5 mg/kg i.v.) produced a widening in pulse pressure of 30-40 mm Hg accompanied by a marked bradycardia. The pulse pressure returned to control levels after 20 min. Following

this treatment, pressor responses to angiotensin and noradrenaline were enhanced by 20 - 40% but responses to sympathetic outflow stimulation were little affected. As a result of subsequent α-blockade with phentolamine (1 mg/kg) responses to noradrenaline and stimulation were reduced by 50 - 60% but the enhanced pressor responses to angiotensin were unaffected by Increasing the dose of P-286 to a total of 10 mg/kg reduced the secondary phase of the angiotensin response.

The biphasic pressor responses elicited by high doses of angiotensin reported by Finch & Leach (1969) were demonstrated in work described in Chapter 1. However, it was noted during the course of the inhibition of responses by disulfiram treatment that biphasic responses were produced by lower doses of angiotensin than those usually required to elicit such a response. In a number of experiments, at a time when responses to lower doses were totally abolished by disulfiram treatment, responses to high doses of the peptide were of the form shown in Fig. 24.

In an investigation of the possible sympathetic involvement in the mechanism of these compound responses to high doses of angiotensin, phentolamine (1 mg/kg) and bethanidine (1 mg/kg) were shown to have little effect. The biphasic response to angiotensin under these circumstances would therefore appear to be independent of an adrenergic mechanism.

The Effect of Inhibition of Sympathetic Nervous System Function on the Action of Disulfiram on Responses to Angiotensin

The anti-angiotensin actions of disulfiram (50 mg/kg i.p.) (five experiments) and DDC (50 mg/kg i.v.) (three experiments) were examined in rats which had been previously treated with reserpine (2·5 mg/kg for 3 days). The results following both substances were similar; the initial enhancement of noradrenaline

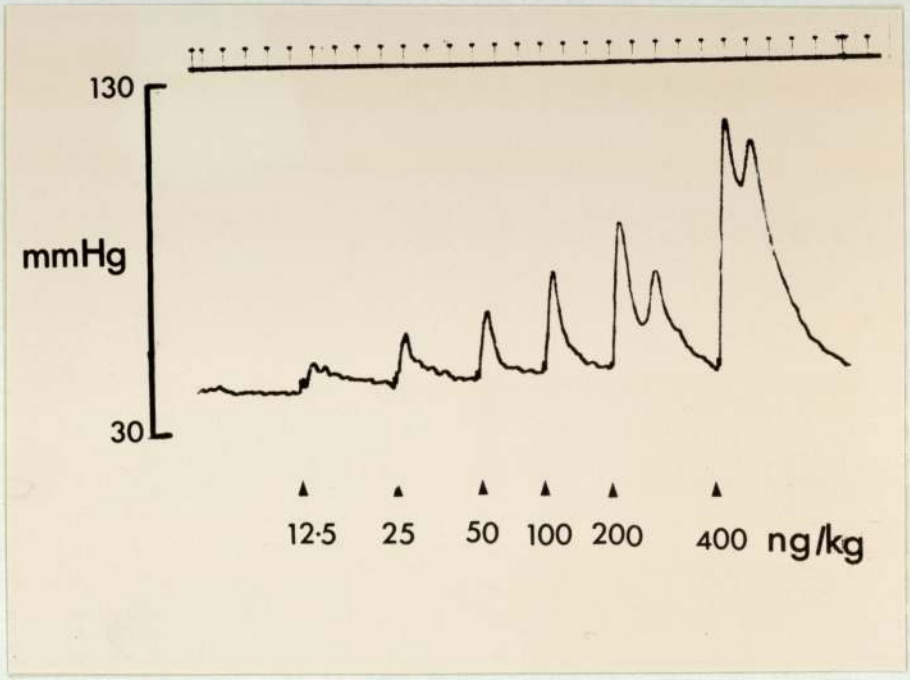


Fig. 24. Pithed rat. Mean blood pressure record showing the pressor responses to angiotensin (12·5 - 400 ng/kg) obtained 2½ - 3 h following treatment with disulfiram (50 mg/kg i.p.). Note the biphasic responses following the higher doses of the peptide. Time marker - minutes.

and angiotensin responses usually seen after disulfiram or DDC (Chapter 1) was either greatly reduced or in most experiments absent as shown in Fig.25B. The rate of decline of responses to angiotensin after disulfiram or DDC was slightly greater in reserpine-treated preparations (Fig.25) than in untreated preparations (Fig.18).

A similar modification in the pattern of the inhibition of responses to angiotensin following disulfiram was seen after acute pretreatment with bethanidine (1 mg/kg i.v.) in 3 experiments.

Treatment with phentolamine (1 mg/kg i.v.) or phenoxybenzamine (2-3 mg/kg) produced no alteration in the subsequent inhibition of disulfiram (50 mg/kg i.p.) of pressor responses to angiotensin. The initial potentiation of responses to angiotensin, noradrenaline or sympathetic outflow stimulation usually occuring during the first hour after disulfiram administration (Chapter 1) were absent following α-blockade.

Mixed α and β adrenoceptor blockade with pentolamine (1 mg/kg i.v.) and propranolol (1 mg/kg i.v.) in four experiments produced effects similar to those seen following phentolamine alone.

The Effect of Disulfiram on the Potentiation of Pressor Responses to Sympathetic Outflow Stimulation by Infusions of Angiotensin

Intravenous infusions of angiotensin (50-200 ng/kg/min for 30 mins) produced a dose-dependent, sustained rise in blood pressure. After the 30 min infusion period, the blood pressure returned rapidly to pre-infusion levels or slightly below. Re-starting the infusion following an interval in excess of 30 min produced a similar sustained rise in blood pressure in 5 of 6 experiments. In the remaining experiment, the blood

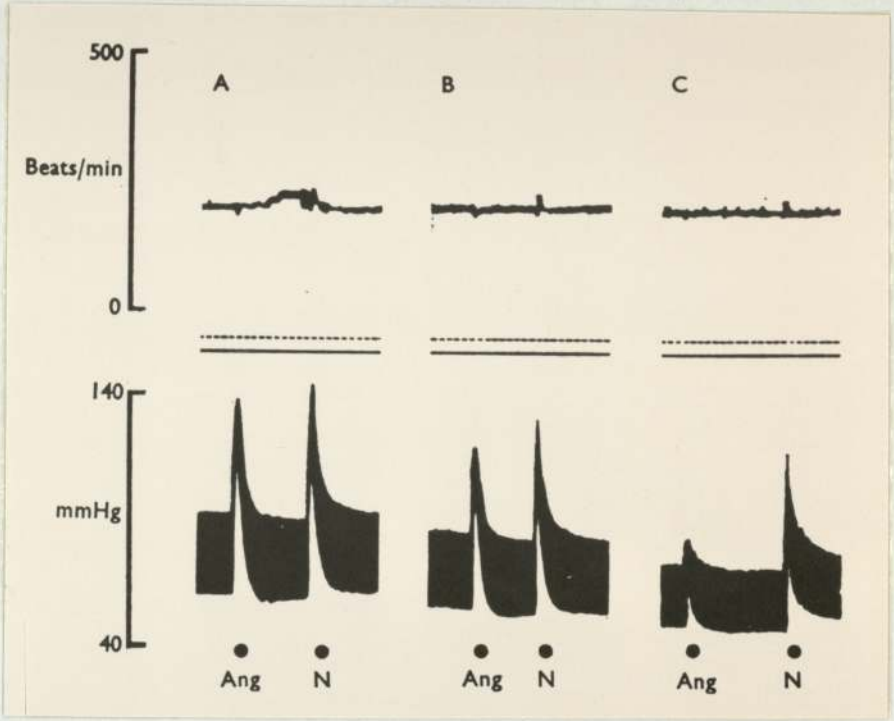


Fig.25. Pithed rat blood pressure. The effect of disulfiram (50 mg/kg i.p.) on the pressor responses to angiotensin (50 ng/kg -Ang) and noradrenaline (100 ng/kg - N) in a rat treated with reserpine (2·5 mg/kg daily for 3 days). Time marker - minutes. Panel A shows control responses, panels B and C responses 2 and 4 h after disulfiram.

pressure had fallen below pre-infusion level following the cessation of the first infusion and the second infusion resulted in a markedly reduced pressor response.

Following an initial settling period of about 60 min following the setting-up, the pithed rat preparation reacts to electrical stimulation (0·5 Hz) of the spinal outflow with reproducible pressor responses for periods of up to several hours (Chapter 1). Infusions of angiotensin (200 ng/kg/min for 30 min) markedly increased responses to sympathetic stimulation (0·5 Hz) in five experiments (Fig.26A). The enhancement of these responses was seen as soon as 5 min after the start of the infusions and was maximal after 10-15 min. Upon cessation of the infusion, the first pressor response to sympathetic stimulation was always reduced by up to 50% of control. Subsequent responses returned to control levels. This enhancement of responses was repeated in each case at hourly intervals for up to 4 hours. By this time, the enhancement was up to 30% less than that seen following the initial infusion. Treatment with disulfiram (50 mg/kg i.p.) produced, after 4 h, an almost total reduction in the pressor response to an infusion of angiotensin (200 ng/kg/min). In conjunction with this, the enhancement of responses to sympathetic stimulation was considerably reduced by the disulfiram treatment as may be seen in Fig.26B

The Role of α-methyldopa in the 'Reversal' of the Disulfiram-induced Inhibition of Pressor Responses to Angiotensin

During an experiment concerned with the reactivity of the disulfiram treated rat to noradrenaline, it was noticed that responses to a previously ineffective dose of angiotensin were partially restored following a large dose of noradrenaline. This situation was successfully repeated in four experiments,

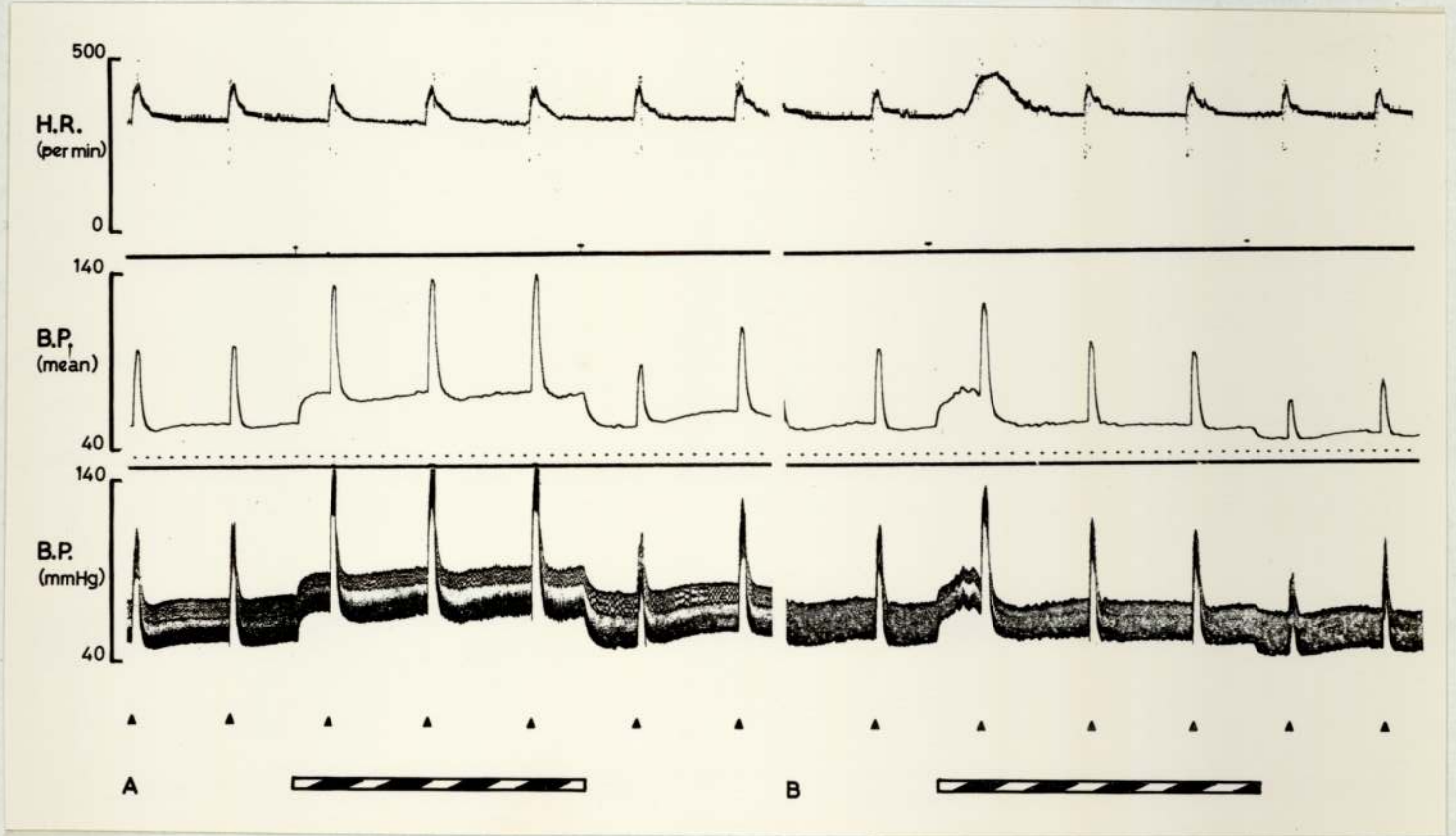


Fig. 26. Pithed rat blood pressure and heart rate. The effect of angiotensin infusion (200 ng/kg/min - indicated by hatched bar) on pressor responses to sympathetic outflow stimulation A - before, and B - 4 hours after disulfiram (50 mg/kg i.p.). Time marker - minutes.

the results of one of which are shown in Fig.27A. Responses to angiotensin (100 ng/kg i.v.) were abolished following 4 h treatment with disulfiram (50 mg/kg i.p.). However, as can be seen in Fig.27A during the recovery phase of the pressor response to noradrenaline (2 µg/kg i.v.) responses to angiotensin were partially restored. This restoration of responses was short lived, the responses to subsequent injections of angiotensin falling to pre-noradrenaline levels. The possibility of the temporary restoration of responses being due to the elevated blood pressure per se was ruled out following the absence of responses to angiotensin during the recovery phase of a pressor response to 5-hydroxytryptamine.

Following the rather tenuous analogy of the α-methyldopa reversal of the reserpine-induced inhibition of tyramine, it was decided to investigate the possible action of α-methyldopa on the disulfiram-induced inhibition of angiotensin.

In the untreated preparation, intravenous injection of 10-50 mg/kg of α-methyldopa (Aldomet-commercial injection, M.S.D.) characteristically produced an initial transient fall in blood pressure coupled with a fall in heart rate, followed immediately by an elevation in pressure becoming maximal after 5 min and lasting 15-20 min.. Responses to angiotensin, noradrenaline and sympathetic outflow stimulation were slightly enhanced ($< 20\%$) over a period of up to 3 h after the methyldopa.

In 6 experiments, pithed rats were treated with disulfiram and responses to angiotensin (12·5-400 ng/kg) observed until they were abolished (Fig. 28 B). The injection of α-methyldopa (50 mg/kg i.v.) produced a characteristic acute response (Fig.27B) similar to that seen in the control preparations. 30 min following the α-methyldopa, responses to angiotensin were

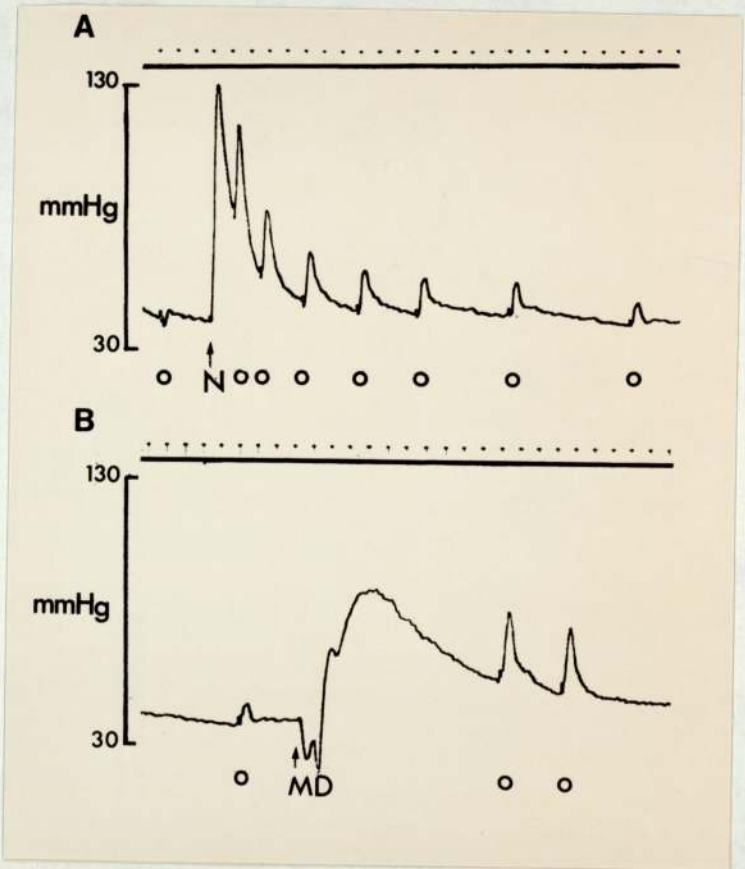


Fig. 27. The acute 'reversal' of the disulfiram-induced inhibition of pressor responses to angiotensin (100 ng/kg at 'O') by noradrenaline and methyldopa. Panel A shows the effect of noradrenaline (2 mg/kg at N) on the responses to a series of injections of angiotensin (100 ng/kg). Panel B shows the acute effect of intravenous methyldopa (50 mg/kg at MD) on responses to angiotensin under the same conditions. Time marker - minutes.

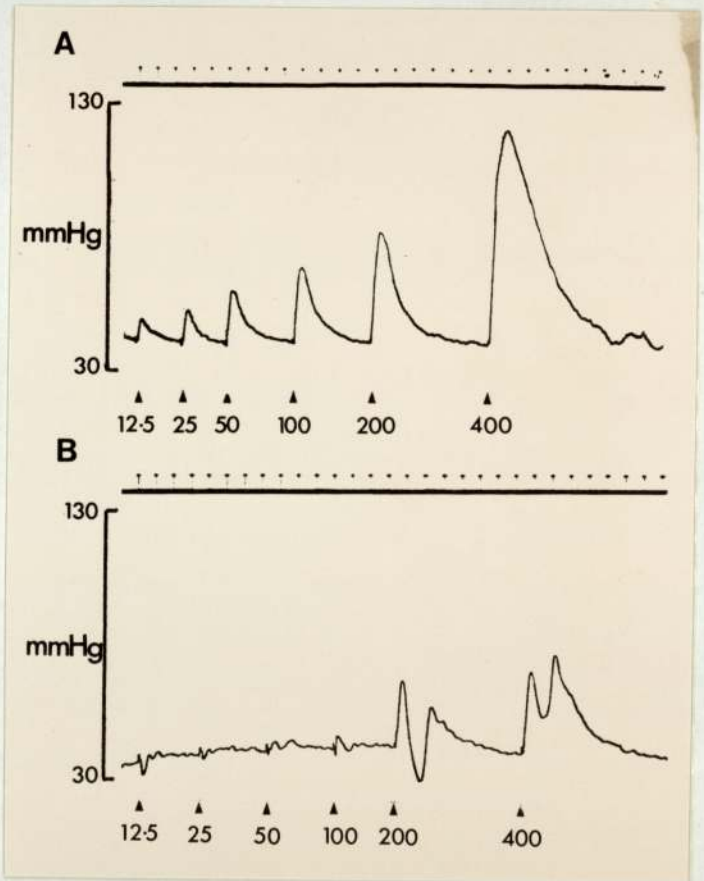


Fig.28(i). The action of methyldopa in its 'reversal' of the disulfiram inhibition of pressor responses to angiotensin in a pithed rat (180g♂). Panel A shows control responses to increasing doses of angiotensin (▲). Panel B shows responses to the same doses of the peptide 4 h after treatment with disulfiram (50 mg/kg i.p.)

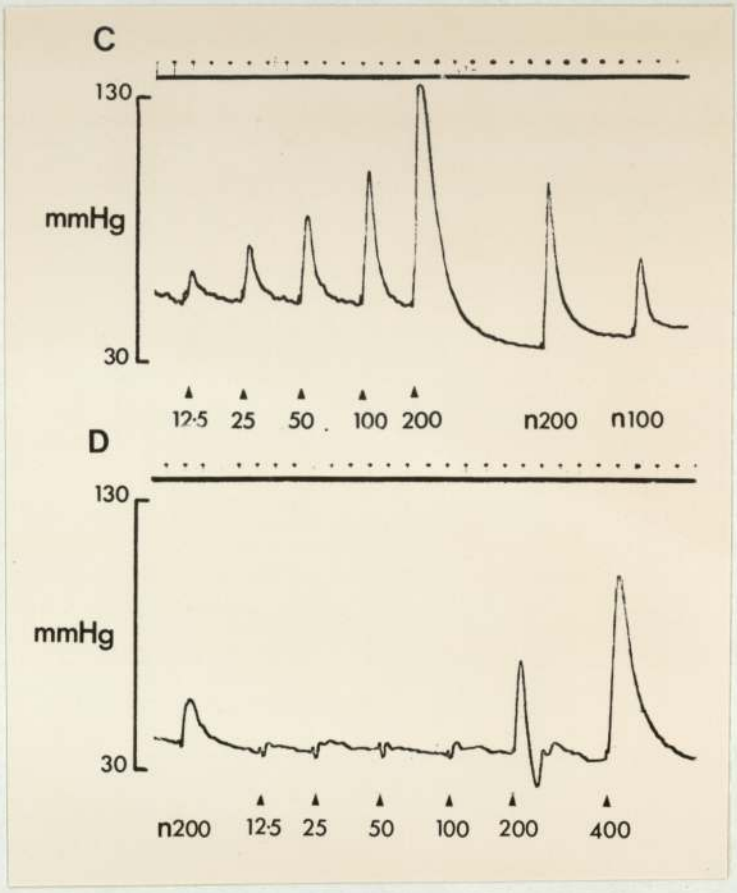


Fig.28(ii). Panel C shows pressor responses to angiotensin 30 mins after treatment with methyldopa (50 mg/kg i.v.). The 'restored' responses are similar in height but shorter in duration than control responses (Panel A). Subsequent acute treatment with phentolamine (1 mg/kg i.v.) reduced the pressor responses to noradrenaline (n) by about 60-70% and totally abolished responses to lower doses of angiotensin (12·5 - 100 ng/kg) and reduced responses to higher doses (200-400 ng/kg).

restored to approximately control levels as illustrated by Fig.28C. However, the subsequent addition of phentolamine (1 mg/kg i.v.) in three experiments completely abolished the restored response to angiotensin, as shown in Fig. 28D. This was in contrast with the previously observed effect of phentolamine on responses to angiotensin in untreated rats which was one of enhancement. It would appear, therefore, that the restored responses to angiotensin following α-methyldopa differ qualitatively from those observed in untreated pithed rats.

DISCUSSION

The role of the sympathetic nervous system in the vasopressor response of the pithed rat to angiotensin has been examined using a number of drugs known to interfere with sympathetic function. Schmitt & Schmitt (1968) concluded that the pressor responses to the peptide in the pithed rat did not involve the release of noradrenaline and the results presented in this chapter to some degree support this conclusion.

Reserpinisation and acute treatment with bethanidine produced qualitatively similar results. Responses to sympathetic outflow stimulation were largely abolished whilst responses to both noradrenaline and angiotensin were enhanced. The vascular hypersensitivity to noradrenaline following treatment with reserpine is well documented but that to angiotensin is somewhat more difficult to explain.

The results presented in the first part of this Chapter confirm the observations of Formanek, Linder & Seltzer (1966) who reported that reserpine pretreatment (1 mg/kg daily for 1 week) enhanced the vasopressor effect of angiotensin in the rat. There does, however, appear to be some species variation

in the effect of reserpine on the pressor response to angiotensin. Day & Owen (1970b) reported a reduction in responses to angiotensin of up to 50% following reserpine (50 µg/kg per day for 3 days) in the conscious cat. In dogs treated with reserpine (1 mg/kg daily for 7 days) the responses of the perfused hind-quarters to intra-arterial angiotensin was less than that shown by untreated controls (Baum, 1963). Using experiments involving the use of rabbit isolated atria and aortic strips, as well as the blood pressure of the pithed rabbit, Quevedo & Perez-Olea (1972) demonstrated that the effects of angiotensin were significantly enhanced following reserpine pretreatment. In the isolated preparations, refilling of neuronal stores with noradrenaline restored responses to angiotensin to control levels. These authors concluded that there exists a non-specific reserpine induced supersensitivity which is reversible by restoration of sympathetic adrenergic catecholamines.

The enhancement of responses to angiotensin by the α-blocking agents phentolamine and phenoxybenzamine but not by the β-blocking agent propranolol would appear to indicate some 'receptor specificity' for the enhancement activity of adreno-ceptor blocking substances. This could well apply to the angiotensin-enhancing effects of reserpine and bethanidine which are primarily the result of interference with noradrenaline release, either indirectly by depletion of catecholamines or directly by an action on the neuronal membrane. The possibility thus exists that, assuming the mechanism of the angiotensin potentiation is common, the magnitude of the vasopressor responses to angiotensin is to some degree inversely proportional to the patency of noradrenergic transmission in vascular tissues. It would perhaps be of interest to attempt to correlate the

angiotensin-potentiating properties of a number of noradrenergic inhibitors with their α-inhibitory activity.

The adrenal gland appears to play a relatively minor role in the vasopressor responses to angiotensin in the pithed rat. Finch & Leach (1969) demonstrated that acute or chronic adrenalectomy produced no marked alteration in the response to angiotensin. The current experiments showed a non-specific decline in responses to noradrenaline and angiotensin following acute adrenalectomy but this was accompanied by a 5-10 mm Hg fall in resting blood pressure which continued to decline during the experiments.

The results of the experiments involving P-286 (N,N-di-isopropyl-N'-isoamyl-N'-diethylaminoethylurea) were rather unexpected and do not appear to shed much light on the role of the adrenal gland in the vasopressor response to angiotensin. P-286 was claimed to specifically inhibit the release of catecholamines from the dog adrenal gland (Gardier et al, 1960). These authors deduced this activity from the ability of P-286 to reverse the pressor response to acetylcholine in atropinised dogs. In experiments described above, a total dose of 10 mg/kg intravenously of P-286 was required to inhibit the secondary adrenal-mediated response to tetramethylammonium bromide. At this dose P-286 enhanced pressor responses to angiotensin, noradrenaline and sympathetic outflow stimulation by up to 40%. Subsequent α-blockade with phentolamine (1 mg/kg i.v.) reduced responses to noradrenaline and sympathetic stimulation but did not alter responses to angiotensin. P-286 appears, therefore, to produce a non-specific increase in vascular reactivity in the pithed rat. Its lack of effect on the secondary responses to TMA at doses below 10 mg/kg would seem to indicate the limited

activity of P-286 in blocking release of catecholamines from the adrenal medulla of the pithed rat.

Interference with the function of the sympathetic nervous system by reserpine, bethanidine or α-blockers had little effect on the subsequent inhibition of pressor responses to angiotensin by disulfiram. The rate of decline of responses to the peptide following disulfiram was increased following reserpine and bethanidine but this appeared to be a consequence of the initially enhanced responses to angiotensin. The time required for the abolition of responses to angiotensin by disulfiram does not appear to differ between treated and untreated groups.

Intravenous infusions of angiotensin (200 ng/kg/min for 3 min) have been shown to reproducibly cause an enhancement of responses to sympathetic outflow stimulation. Day & Owen (1969) showed that this enhancement was specific to angiotensin since equipressor infusions of noradrenaline failed to increase responses to sympathetic stimulation. A similar angiotensin-induced enhancement of sympathetic vasoconstriction was demonstrated in the dog hind limb (Zimmerman & Whitmore, 1967) and in the dog renal artery (Zimmerman & Gisslin, 1968). These enhancements were said to be associated with a small increase in the neural output of noradrenaline suggesting a pre-synaptic site of action for angiotensin. Whether or not an increased output of noradrenaline is involved in the action of angiotensin infusions in the pithed rat is open to question. Certainly, as was shown above, interference with sympathetic transmission at any of several stages does not reduce the acute response to angiotensin in the pithed rat.

It was interesting to note, therefore, that disulfiram, in addition to abolishing the acute pressor responses to

angiotensin, reduced both the vasoconstrictor effect and the enhancement of responses to sympathetic outflow stimulation caused by an infusion of the peptide. This suggests that the acute pressor response to angiotensin and its facilitatory effect on noradrenergic transmission involve a common, disulfiram-sensitive mechanism.

The requirement of a model to explain the angiotensin-sympathetic synergism, illustrated in a number of ways by the experiments discussed above, are partly fulfilled by that proposed by Pals & Fulton (1968). These authors postulated the existence in the vascular resistance vessels of the rat of α-adrenergic receptors and angiotensin receptors on the same smooth muscle cells. These cells, it was suggested, are composed of series contractile and elastic components (for a review of the evidence for this concept see Sonneblick, 1966). Thus, it was said, stimulation by one agonist (noradrenaline in the case of Fig.29(2)) would result in an overall shortening of the muscle which would be due to an active shortening of the contractile element of some of the cells coupled with an increase in the tension in the elastic element of those cells and a passive shortening of the elastic component of non-stimulated cells. Fig.29(3) shows the effect of stimulating the angiotensin receptors of the previously α-stimulated cells. The already tensioned elastic components provide optimum conditions for the contraction induced by the second agonist (in this case, angiotensin). There is a lesser contribution to the overall contraction of the muscle by the previously non-stimulated cells when they are stimulated by the second agonist since part of their contractile effort is spent in overcoming the series elastic component.

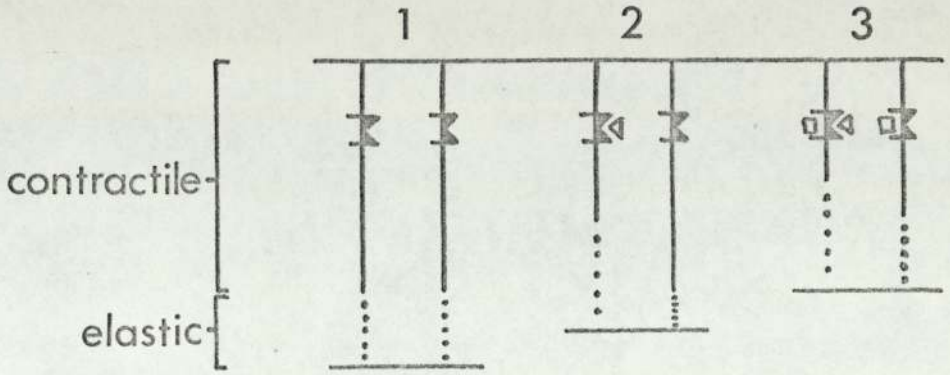


Fig.29. Model of the possible sequence of events involved in the synergism of angiotensin and noradrenaline at the vascular smooth muscle cell. Section 1 represents a pair of smooth muscle cells comprising series contractile and elastic components and possessing an angiotensin and a noradrenaline receptor. Sections 2 and 3 show respectively the effect of stimulation by noradrenaline (◁) of one of the cells and the effect of further stimulation by angiotensin (□).

Adapted from Pals & Fulton (1968).

This hypothesis is consistent with the observed synergism between noradrenaline and angiotensin reported above. Furthermore, the actions of disulfiram on both the acute pressor effect of angiotensin and the enhancement of responses to neuronally-released noradrenaline by infusions of the peptide can be seen to be related to an action of disulfiram in the region of the angiotensin receptor. The angiotensin-enhancing effects of phentolamine and phenoxybenzamine may be due to a partial agonist effect, the α-blocking agents having sufficient agonist activity to raise the tension of the elastic component of the smooth muscle cells and thus facilitating the contractile actions of subsequently added angiotensin.

The effects of drugs mainly acting pre-synaptically, i.e. bethanidine and reserpine, are a little more difficult to reconcile with the Pals & Fulton model. Pals (1968) demonstrated an enhancement of responses to angiotensin in the pithed rat following pretreatment with the adrenergic neurone blocking agent bretylium. This facilitation, he suggested, was involved with α-receptor stimulation due to an initial sympathomimetic effect of bretylium, probably involving displacement of neuronal noradrenaline. Should this, in fact, be the case, then it seems likely that bethanidine would behave in a similar manner. The enhancement of responses to angiotensin following reserpine pretreatment has been discussed previously in this Chapter. It would appear that this action of reserpine is not directly related to the relationship between the α-adrenergic and angiotensin receptors.

In view of the apparent lack of involvement of such agents as bethanidine and reserpine on the disulfiram-induced inhibition of responses to angiotensin, the acute 'reversal' of

the inhibition by a large dose of noradrenaline was of great interest. The possibility that the 'reversal' was due to the temporary elevation of blood pressure during the noradrenaline pressor response seemed unlikely when it was considered that an equipressor dose of 5-hydroxytryptamine failed to enhance the responses to angiotensin. In subsequent experiments, it was noted that intravenous methyldopa also caused a 'reversal' of the disulfiram inhibited angiotensin pressor responses. In this case, however, the enhancement of the responses was seen for at least an hour after the injection of methyldopa, by which time the basal pressure was at pre-methyldopa levels. Although the responses to angiotensin following methyldopa were quantitatively similar to those seen prior to the disulfiram-induced inhibition, they were qualitatively dissimilar. This was shown by the action of phentolamine which abolished the 'restored' responses to angiotensin. In earlier experiments it had been noted that phentolamine enhanced pressor responses to angiotensin in otherwise untreated rats.

The most obvious explanation for this novel noradrenergically mediated response to angiotensin would appear to involve a synergism between the peptide and circulating catecholamines. The presence of noradrenaline in the blood following a high dose of the amine is obvious and it would be expected that following the intravenous injection of methyldopa there would be circulating metabolites of methyldopa as well as noradrenaline displaced from adrenergic terminals by the more avidly bound α-methylated analogs. Thus injections of angiotensin which have no direct pressor activity as a result of disulfiram pretreatment may facilitate the actions of the circulating amines leading to a pressor response which in turn would be susceptible to an

α-blocking drug. If this were, in fact, the case then there would appear to exist a paradoxical situation where disulfiram is capable of preventing the synergism between angiotensin and endogenously released amines (due to sympathetic outflow stimulation) and unable to prevent a synergism between the peptide and exogenous noradrenaline. To resolve this situation it would appear that two sites of action would be required for the noradrenaline activities. Though speculative, this situation is a possibility, particularly when it is considered that experiments reported in this chapter indicated that responses to endogenously released noradrenaline were more susceptible to the α-blocking agents phentolamine and phenoxybenzamine than were responses to injected noradrenaline.

Chapter 3

THE EFFECTS OF DDC AND DISULFIRAM ON THE VASOPRESSOR RESPONSES TO ANGIOTENSIN IN THE CAT

In order to try to determine the species specificity of the anti-angiotensin activity of disulfiram, its effects, together with those of DDC, were determined in cats. In a preliminary communication, Day & Owen (1969) reported that intravenous DDC caused an enhancement of responses to angiotensin and noradrenaline in the intact anaesthetised cat, but no subsequent inhibition of responses to the peptide was noted. However, following acute adrenalectomy or complete bilateral occlusion of the blood supply to the adrenal glands, responses to angiotensin but not those to noradrenaline were reduced in the presence of DDC. Restoration of the blood supply to the adrenal glands was said to reverse this reduction. It was apparent from this result that there was an adrenal gland involvement in the acute pressor response to angiotensin in the cat which was not present in pithed rats. This difference is consistent with the report of Vane (1969) noting the species variation of the adrenomedullary stimulant activity of angiotensin

In view, therefore, of the possible complication of adrenomedullary involvement in the angiotensin pressor response, efforts have been made to repeat the work of Day & Owen cited above and to extend it to include disulfiram. The effects of reducing the actions of endogenously released amines by administration of mixtures of α and β adrenoceptor blocking agents were also studied.

The importance of pithing on the anti-angiotensin actions of disulfiram in the rat was not established. However, in view of the results reported by Day & Owen (1969) regarding the

differences in the actions of DDC in the chloralosed cat and pithed rat, it was decided to investigate the effect of pithing on the vasopressor actions of the cat to angiotensin and their possible inhibition by disulfiram.

The conscious cat preparation described by Day & Owen (1969) afforded a third model for these experiments. The good day to day reproducibility of pressor responses to angiotensin and other pressor agents allowed the effects of catecholamine depletion by reserpine and antagonism of the renal actions of aldosterone by spironolactone to be determined.

RESULTS

The Vasopressor Action of Angiotensin in the Cat

Fig. 30 shows pressor responses to angiotensin in the three preparations used in the current work, namely, the chloralose anaesthetised, conscious and pithed cats. These traces illustrate several qualitative differences in the responses to the peptide of the preparations. A feature of the responses to angiotensin in the conscious cat was the marked reflex bradycardia which served to limit the magnitude of the responses. A consequence of this was a very 'flat' dose-response curve and an apparent lack of reactivity to the pressor agents angiotensin, noradrenaline and tyramine. At the other extreme, as was expected, no reflex bradycardia was produced by angiotensin or noradrenaline in the pithed cat. In the chloralose cats, a reflex bradycardia was usually observed during a pressor response to either angiotensin or noradrenaline but it was never as great as that seen in the conscious animals.

In the pithed cat, doses of angiotensin up to 100 ng/kg (i.v.

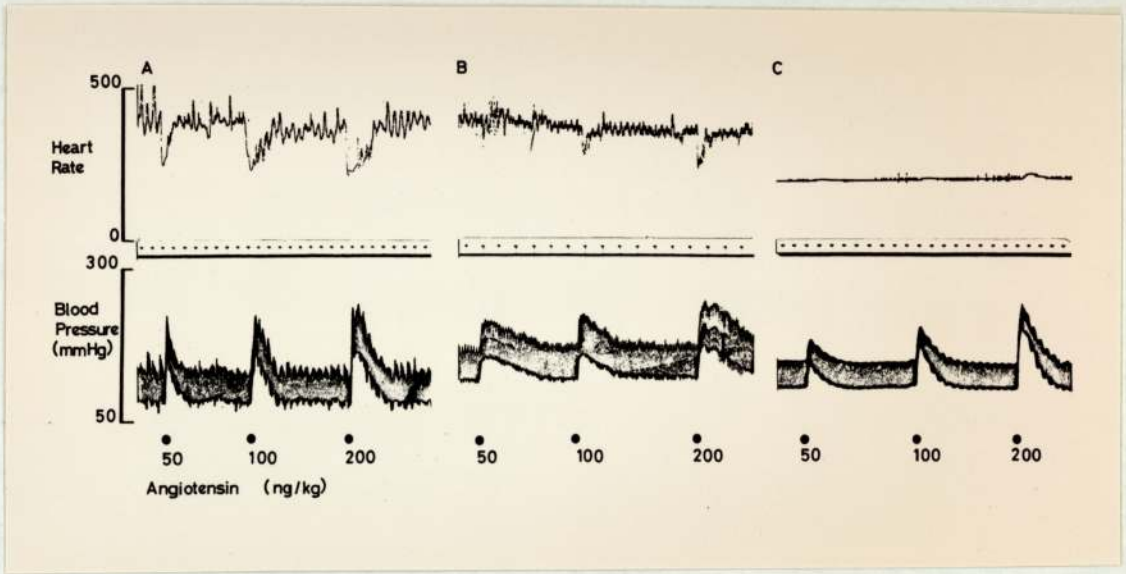


Fig. 30. Pressor and heart rate responses to angiotensin in the cat. Panels A, B and C show responses in conscious, chloralosed and pithed cats respectively. Time marker - minutes.

produced a monophasic pressor response. Higher doses were not tested. In both anaesthetised and conscious preparations, large doses of angiotensin (> 300 ng/kg) produced distinctly biphasic responses, both phases being of similar magnitude but the second being prolonged over 60-90 secs. To try to characterise this biphasic response, the effect of α and β adrenoceptor blocking agents were examined.

In the chloralosed cat, phentolamine (2 mg/kg i.v.) alone (Fig.31) or in combination with propranolol (3 mg/kg i.v.) had no effect on the responses to angiotensin (100-400 ng/kg) whilst those to noradrenaline (400 ng/kg) were reduced by 50-60%. Treatment with the ganglion blocking agent, pempidine reduced the reflex bradycardia resulting from pressor responses to angiotensin (100-400 ng/kg) and noradrenaline (200-400 ng/kg) in two experiments. Subsequent injections of high doses of angiotensin (> 300 ng/kg) did not, however, produce the tachycardia which might have been expected had adrenal catecholamine release been involved in the secondary response to angiotensin.

In the conscious cat, results were obtained similar to those seen in the chloralosed cat when phentolamine (3 mg/kg i.v.) was administered (n=2). The effect of pempidine was not determined.

The acute effects of DDC and disulfiram

In Chapter 1 it was shown that disulfiram (50 mg/kg i.p.) and DDC (10-100 mg/kg i.v.) produced a selective inhibition of pressor responses to angiotensin in the pithed rat. This reduction was evident from 3-4 h after the injection of the inhibitors. Attempts to repeat this observation in cats have met with limited success.

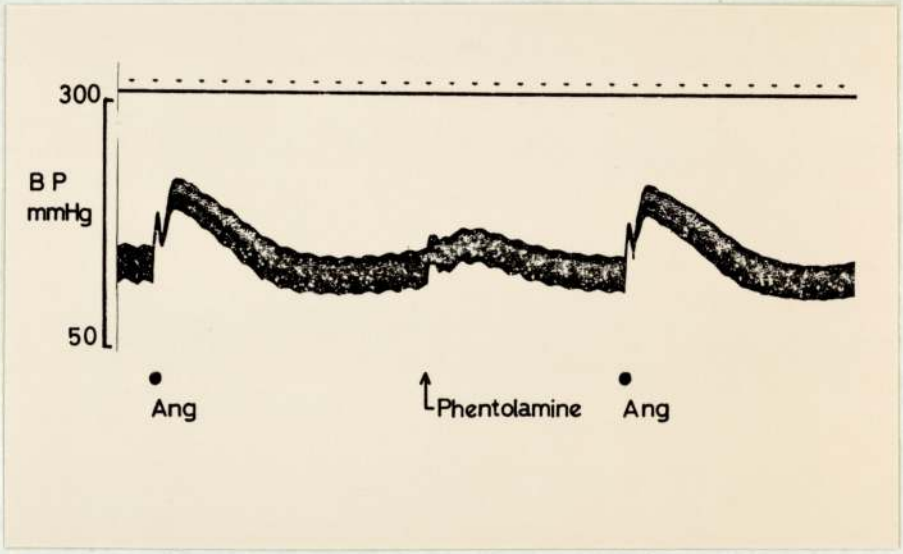


Fig.31. Chloralosed cat blood pressure recording showing the lack of effect of phentolamine (3 mg/kg i.v.) on the biphasic pressor response to angiotensin (100 ng/kg). Time marker - minutes.

Intravenous DDC (50-200 mg/kg) enhanced responses to angiotensin (100-200 ng/kg) and noradrenaline (200-400 ng/kg) in the chloralosed cat (n=4). This enhancement was maximal after 30-60 min and was dose-dependent, 200 mg/kg DDC producing a 30 - 50% increase in height and duration of pressor responses compared to 0-15% for 50 mg/kg. Three to four hours following DDC the enhancement was rarely apparent but no significant decrease in responses to angiotensin was noted.

Attempts to repeat the observations of Day & Owen (1969) relating to the effect of acute bilateral adrenalectomy on the anti-angiotensin activity of DDC were unsuccessful. In 4 preparations, acute adrenalectomy by a ventral midline approach led to deterioration of the animal to such a degree that meaningful comparisons of pressor responses were impossible.

In the pithed cat preparation, DDC (100 mg/kg i.v.) itself produced a transient rise in blood pressure. There was little enhancement of pressor responses to angiotensin (50 ng/kg) or noradrenaline (150 ng/kg) observed in two preparations. At the same time, it should be noted that the resting blood pressure was declining slowly (approximately 10 mm Hg/h).

In the conscious cat preparation, DDC (100-200 mg/kg i.p.) enhanced responses to angiotensin (50-125 ng/kg) and noradrenaline (100-200 ng/kg) in a manner similar to that seen in chloralosed preparations.

In two chloralosed cats, disulfiram (50 mg/kg i.p.) had little effect on resting blood pressure or on pressor responses to angiotensin (50 ng/kg) or noradrenaline (100-200 ng/kg). In one preparation, a higher dose of disulfiram (150 mg/kg i.p.) reduced responses to both agonists. This reduction in responses was never greater than 20% and recovery was complete within

2 - 2·5 h of the disulfiram injection. There was no initial enhancement of responses noted.

In the conscious cat (4 experiments) disulfiram (50-200 mg/kg)i.p.) had no observed effect on pressor responses to angiotensin, tyramine, noradrenaline or McN-A-343.

The effects of disulfiram and DDC on the pressor responses to angiotensin in the reserpinised conscious cat

The effect of reserpine (50 µg/kg daily) on pressor responses to angiotensin (25-50 ng/kg), noradrenaline (100-200 ng/kg), tyramine (50 µg/kg) and McN-A-343 (15-30 µg/kg) was studied over period of 3 (2 experiments) and 4 days (2 experiments). Responses to tyramine were, in all cases, abolished after 2 days treatment. In two experiments, responses to McN-A-343 were similarly abolished after 2 days but in the remainder, responses were only reduced by up to 50%. Pressor responses to angiotensin and noradrenaline proved to be very variable from day to day in each cat but indicated an overall reduction, after 3 days, of about 50% in each case.

Treatment of reserpinised animals with disulfiram had little effect. The only consistently observed feature was an enhancement of responses to angiotensin over a 2-3 h period following the disulfiram. Pressor responses to the other agonists were little affected.

The effect of disulfiram on the pressor responses to angiotensin in the spironolactone-treated conscious cat

Fig. 32 illustrates the results obtained from one experiment These results were similar to those obtained in a further two experiments in other cats. As may be seen from the figure, treatment of the cat with the aldosterone antagonist spironolactone (3 mg/kg p.o.) twice daily for a period of 6 days was

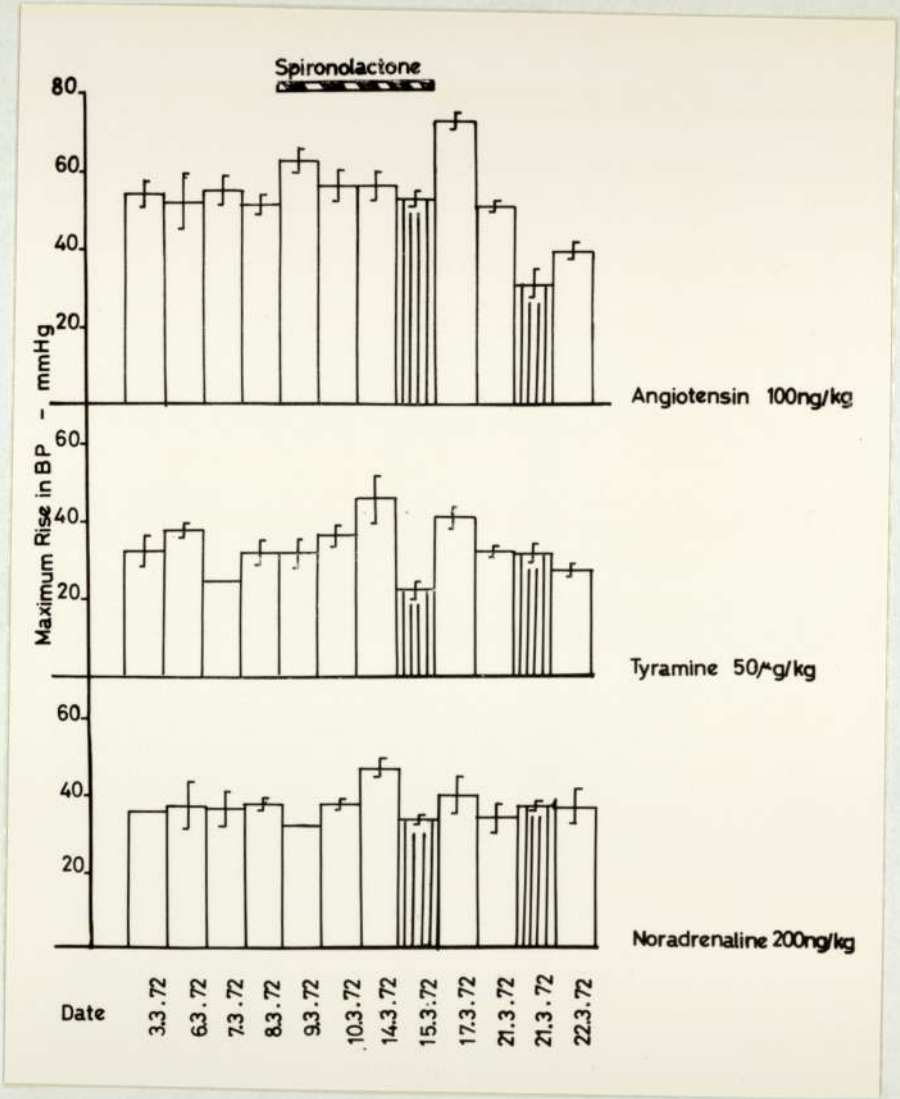


Fig. 32. Conscious cat blood pressure. Effect of spironolactone (3 mg/kg p.o. twice daily) on pressor responses to angiotensin, tyramine and noradrenaline. Hatched bars show responses 3 h after treatment with disulfiram (100 mg/kg i.p.)

without effect on pressor responses to angiotensin (100 ng/kg), tyramine (50 µg/kg) and noradrenaline (200 ng/kg). Withdrawal of the spironolactone appeared to selectively sensitise the cat to angiotensin for up to 2 days after the treatment. Disulfiram (100 mg/kg i.p.) injected during the course of the spironolactone treatment appeared to reduce responses to tyramine but not to angiotensin or noradrenaline. Six days after the withdrawal of the aldosterone antagonist, 3h pretreatment with disulfiram caused a reduction in the pressor responses to angiotensin (15-20%) but not those to the other agonists.

DISCUSSION

The literature has suggested a number of differences in the responses of the rat and those of other species to the various actions of angiotensin. Chapter 2 of the current work was particularly concerned with the possible involvement of the sympathetic nervous system in the pressor responses to angiotensin of the pithed rat. It was interesting to note that drugs such as reserpine, bethanidine and phentolamine, all of which interfere in some way with normal sympathetic transmission, enhanced responses to angiotensin. This observation confirmed the work of others, notably Schmitt & Schmitt (1968) and Formanek et al (1966). Furthermore, it was concluded that the biphasic response to high doses of angiotensin did not appear to directly involve a sympathetic component.

Since the pressor response of the pithed rat to angiotensin was shown to be reduced by treatment with DDC or disulfiram, it was considered of interest to determine whether or not this activity could also be demonstrated in the cat. Day & Owen (1969) in a preliminary communication, showed that DDC did not reduce

responses to the peptide in the intact chloralosed cat but that it was effective in acutely bilaterally adrenalectomised preparations. Day & Owen (1970b) had also shown that reserpine treatment (50 µg/kg daily) reduced responses to angiotensin in the conscious cat, thus indicating a sympathetic component to the response.

Work reported in the early part of this chapter suggests that release of endogenous noradrenaline plays little part in the pressor response to angiotensin in the chloralosed or conscious cat. This is in agreement with the results of Day & Owen (1970b) who came to a similar conclusion. These authors also showed that injections of combinations of α and β adrenoceptor blocking drugs reduced the second part of the biphasic response to high doses of the peptide, suggesting that this part of the response was mediated by adrenal catecholamines. The present work, however, suggests that this may not be the case. Following treatment with the ganglion blocker, pempidine, high doses of angiotensin produced a typical biphasic response but without the tachycardia that might have been expected had adrenal catecholamines been released into the circulation.

If, therefore, the pressor actions of angiotensin in the cat are independent of endogenously released noradrenaline or adrenal catecholamines then a number of questions arise. The observations of Day & Owen (1970b) that reserpine treatment reduced responses to the peptide in the conscious cat, repeated with limited success in the present work, and the report of the same authors regarding the effect of acute adrenalectomy on the anti-angiotensin effect of DDC appear to be something of a paradox. Day & Owen (1970a) concluded that the anti-angiotensin effect of reserpine might be due to an action unrelated to its

amine-depleting effects. Inhibition of the actions of angiotensin by reserpine seems to be restricted to the cat. In the rat (Chapter 2) and rabbit (Quevedo & Perez-Olea, 1972), reserpine treatment led to a sensitisation to the peptide.

Attempts to determine the effect of acute adrenalectomy on the pressor responses to angiotensin in the chloralosed cat were unsuccessful. It was therefore not possible to repeat the work of Day & Owen (1969) regarding the consequences of this procedure on the anti-angiotensin effects of DDC. However, since neither reserpinisation nor treatment with α and β adrenoceptor blockers uncovered an anti-angiotensin activity of DDC in the cat, it would seem that the effects of adrenalectomy reported by these authors did not concern the adrenal medulla. Furthermore, use of the aldosterone antagonist spironolactone failed to allow the demonstration of an anti-angiotensin effect of disulfiram in the conscious cat. The reason for the angiotensin blocking action of DDC being demonstrated in the adrenalectomised cat but not the intact animal remains a mystery. Possibly the effect noted by Day & Owen was the result of a non-specific reduction in reactivity following the adrenalectomy. This type of reaction has been noted in rats by Drew & Leach (1970) and Carpi & Cartoni (1968) who showed that immediate replacement therapy with both mineralocorticoids and glucocorticoids was necessary to restore reactivity to injected pressor agents.

Little success was achieved with the pithed cat preparation. Following pithing, there was a decline in resting blood pressure which was sufficiently slow to allow short term experiments but prevented the longer term experiments required to investigate the effects of disulfiram.

Chapter 4

AN INVESTIGATION OF THE POSSIBLE ANTAGONISM OF THE SPASMOGENIC ACTIVITY OF ANGIOTENSIN II BY SODIUM DIETHYLDITHIOCARBAMATE AND DISULFIRAM IN INTESTINAL SMOOTH MUSCLE PREPARATIONS

The vasopressor response to angiotensin in the pithed rat is generally thought to be due to a direct musculotropic effect on vascular smooth muscle (for evidence see Chapter 2). This action of angiotensin on vascular smooth muscle in the rat was, however, selectively inhibited by sodium diethyldithiocarbamate and disulfiram. It was decided, therefore, to determine if these compounds exerted this inhibition of the spasmogenic activity of angiotensin on isolated non-vascular smooth muscle. The preparations used in this study were the rat isolated ascending colon and the guinea pig isolated ileum, both tissues having features which led to their use.

The rat colon preparation has been widely used as an assay procedure for angiotensin, particularly with reference to determinations of the peptide in body fluids (Regoli & Vane, 1964). The colon was shown to be very sensitive to the direct effect of angiotensin and methysergide, hyoscine, mepyramine, hexamethonium and morphine were shown to have no effect on these responses (Regoli & Vane, 1964). From a comparative study of the actions of a number of angiotensin analogs on the rat colon and rat blood pressure preparations, these authors suggested that the receptors in both cases were similar.

In contrast to the purely direct spasmogenic action of angiotensin on the rat colon, the peptide has been shown to have a more complicated action on the guinea pig ileum preparation. A number of authors have reported the partial (50-70%) inhibition of the contractile response of the ileum to angiotensin by

atropine, nicotine and morphine (Ross, Ludden & Stone, 1960; Khairallah & Page, 1961; Robertson & Rubin, 1962). It appears that angiotensin acts in this preparation in two ways. There is an indirect neurogenic component probably due to stimulation of the neural cells in Auerbach's and Meissner's plexi and a non-neuronally mediated direct spasmogenic action (Khairallah & Page, 1961). The two components may be separated by isometric recording, the indirect action being typically a rapid rise in tension followed by a partial relaxation and the direct component being a slow progressive increase in tension reaching a maximum in 1·5 - 2·0 min (Godfraind, Kaba & Polster, 1966).

A number of substances have been claimed as antagonists of the direct contractile actions of angiotensin in these preparations. Cinnarizine, in high doses, has been shown to inhibit the contractile actions of angiotensin on the guinea pig ileum (Schaper et al, 1963; Klinge et al, 1966). The specificity of this inhibition is, however, in doubt since these authors also reported an intense coronary vasodilatation and antihistaminic activity. The data seem to support the view that cinnarizine is a non-specific smooth muscle relaxant. Lidoflazine, a long acting vasodilator (Schaper et al, 1966), has been shown to antagonise the slow component of the angiotensin action on the guinea pig ileum by a competitive mechanism (Godfraind, Kaba & Polster, 1966). However, in strips of rabbit aorta or rat fundus, lidoflazine had no effect on the contractions produced by angiotensin or noradrenaline, though it inhibited the action of 5-hydroxytryptamine (Turker & Kayaalp, 1967). More recently, Ellis & Reit (1969) reported a non-specific lidoflazine-induced inhibition of the contractile response of the rat isolated colon to angiotensin. They observed that

exposure to lidoflazine (10^{-6}M) also inhibited the contractile responses to acetylcholine, 5-hydroxytryptamine and barium ions, and suggested that the antagonism was similar to that seen with papaverine (10^{-5}M).

In 1966, Gascon & Walaszek showed that the flavonoid compound osajin could antagonise the spasmogenic action of angiotensin on the guinea pig ileum without affecting the actions of bradykinin, eledoisin, 5-hydroxytryptamine, acetylcholine or histamine. Subsequent work, however, indicated that osajin had no in vivo activity (Walaszek, personal communication). Later, Leme & Walaszek (1967) observed similar effects of the flavonoids apiin and hesperidin as antagonists for bradykinin and eledoisin. More recently, a number of other flavonoids such as homoeriodicyol and quercitin have been shown to possess anti-angiotensin activity in the guinea pig ileum (Chau & Haley, 1969). Gascon & Walaszek (1966) suggested that the anti-angiotensin action of flavonoids might be due to zinc or copper chelation.

A number of angiotensin analogs have been synthesised which have no spasmogenic action per se but act as competitive inhibitors of the parent peptide. Khairallah, Toth & Bumpus (1970) reported that 8-ala-angiotensin II in concentrations in excess of 500 ng/kg effectively blocked the contractile response to angiotensin II without affecting the response to vasopressin or 5-hydroxytryptamine on the guinea pig isolated ileum. Turker and his co-workers (1971) further showed that this analog, at low concentrations ($7{\cdot}75 \times 10^{-9}$M) competitively antagonised the myotropic effect of angiotensins I and II on the rabbit isolated aortic strip and rat colon but had no effect on pressor responses to angiotensin II in the cat.

Marshall, Vine & Needleman (1970) demonstrated that

4-phenyl-8-tyrosine-angiotensin II was a competitive inhibitor of the spasmogenic action of angiotensin II on rat isolated uterus strips.

The present work is an attempt to examine disulfiram and its metabolite sodium diethyldithiocarbamate as possible antagonists of the spasmogenic action of angiotensin II on the rat isolated colon and guinea pig isolated ileum. The acute effects of DDC on the preparations together with those due to pretreatment of the animals with DDC and disulfiram are described. In the guinea pig isolated ileum experiments, acetylcholine and histamine were used as control agonists. In the rat isolated colon experiments, acetylcholine was used together with 5-hydroxytryptamine.

RESULTS

(i) The Action of DDC on the Responses of the Rat Isolated Colon to Angiotensin, Acetylcholine and 5-hydroxytryptamine

Control responses to angiotensin (0·5 - 16·0 ng/ml), acetylcholine (10·0 - 320·0 ng/ml) and 5-HT (50 - 1600 ng/ml) were recorded using a five minute dose-cycle. DDC was then added to the bathing solution at a concentration of 40 mg/L and the responses were again observed.

The results of these experiments (n=5) are illustrated graphically in Fig.33. As may be seen from Fig.33, there was no significant inhibition of the responses of the colon to any of the three agonists. There was, however, a slight decrease in the frequency of the spontaneous contractions of the tissue in the presence of DDC.

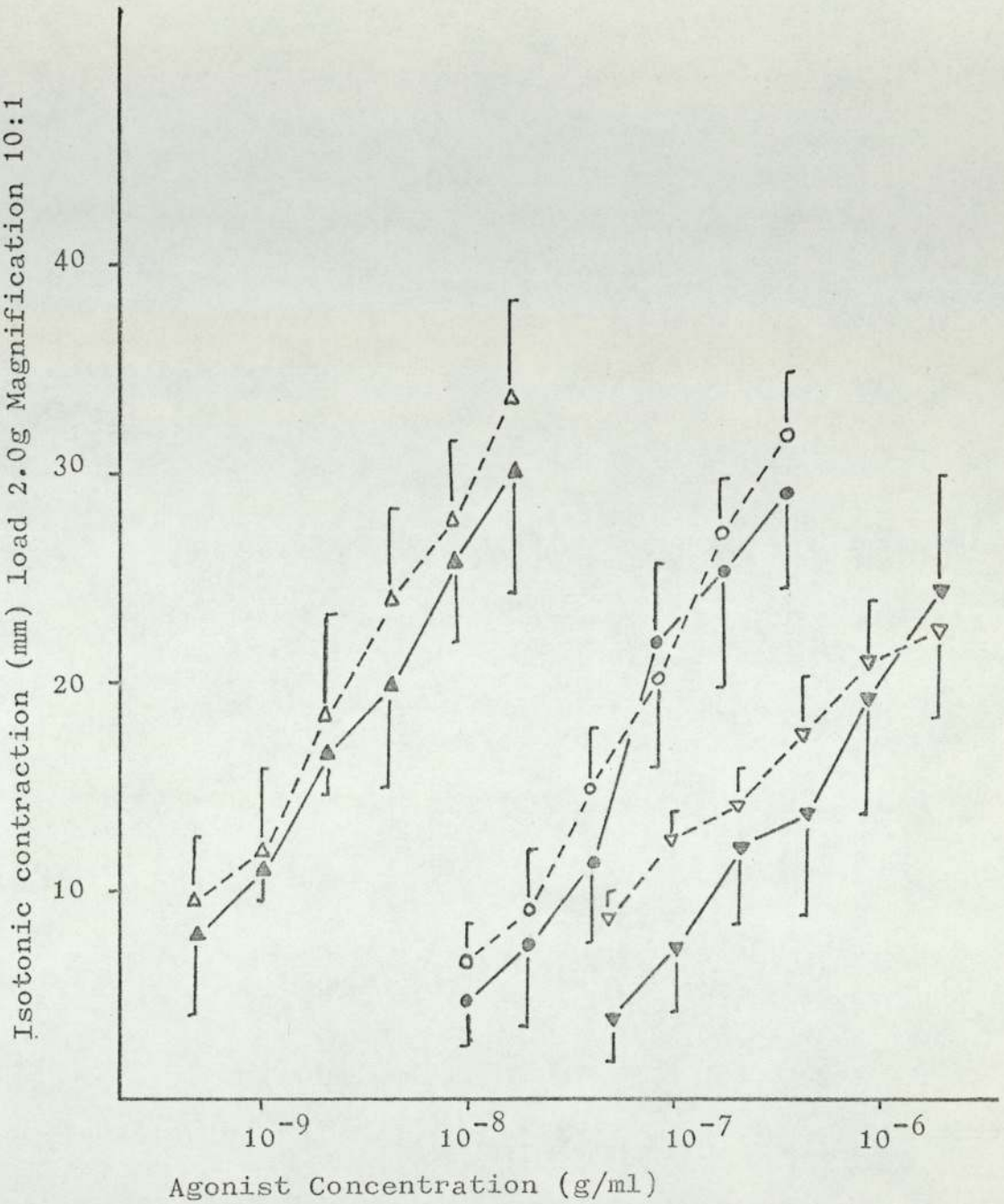


Figure 33. Rat isolated colon. The acute effect of DDC (40 mg/L) on responses to angiotensin (Δ), acetylcholine (o) and 5 hydroxytryptamine (▽). Responses obtained in the presence of DDC are indicated by the closed symbols. No significant difference ($2p > 0.05$) between control (– – –) and 'treated' responses (——) was observed (n=5).

(ii) The Effect of Pretreatment with Disulfiram upon the Responses of the Rat Isolated Colon to Angiotensin, Acetylcholine and 5-hydroxytryptamine

In Chapter 1 it was demonstrated that the injection of disulfiram (50 mg/kg i.p.) led to the inhibition of pressor responses to angiotensin after a delay of approximately three hours. Following the failure of DDC to inhibit the spasmogenic action of angiotensin on the rat colon it was decided to examine the reactivity of colon taken from rats pretreated for 4 h with disulfiram (50-200 mg/kg i.p.). It had been previously shown that disulfiram pretreatment (50 mg/kg i.p.) for 3·5 h significantly ($2P < 0.05$) inhibited pressor responses to angiotensin in the pithed rat. Since the myotropic action of angiotensin on vascular smooth muscle was thus inhibited following this pretreatment, it was considered of interest to determine whether or not the anti-angiotensin action of disulfiram extended to include non-vascular smooth muscle directly stimulated by angiotensin i.e. the rat colon.

The effect of disulfiram pretreatment on responses to angiotensin (0·5 - 16·0 ng/ml), acetylcholine (10-320 ng/kg) and 5-hydroxytryptamine (50-1600 ng/kg) was examined. Three doses of the disulphide were used, 50 mg/kg (n=9), 100 mg/kg (n=5) and 200 mg/kg (n=5). The drug was injected in suspension in 2% tragacanth in water (2 ml/kg) 4 h prior to sacrifice. At post mortem, disulfiram was detectable in the peritoneal cavity at all dose levels. Following the highest dose of disulfiram, the spontaneous activity of the colons was reduced both in frequency and magnitude.

The results of these experiments are shown in Table 2. Regression lines computed from these data are plotted in Figs. 34, 35, 36 & 37. Comparison of the slopes of these regression

TREATMENT	Angiotensin (ng/ml)						Acetylcholine (ng/ml)						5-HT (ng/ml)					
	0.5	1.0	2.0	4.0	8.0	16.0	10	20	40	80	180	320	50	100	200	400	800	1600
CONTROL $\bar{x}$	9.5	12.1	18.7	24.1	28.1	34.3	6.7	9.1	15.0	20.4	27.0	31.8	8.3	12.3	14.1	17.4	21.1	22.7
(Vehicle) ±SE	3.2	4.1	5.1	4.8	4.2	5.3	1.5	1.4	1.9	2.7	3.3	4.6	2.3	2.9	2.9	3.4	2.9	3.0
DISULFIRAM $\bar{x}$ 4h	6.8	11.6	17.2	24.4	31.4	34.4	5.4	9.7	14.5	18.6	21.5	24.5	4.4	7.6	10.4	13.1	13.9	15.6
(50 mg/kg)±SE	3.3	4.8	6.3	6.5	8.3	7.5	1.1	2.4	3.4	4.0	4.2	4.3	1.2	2.1	2.6	2.7	3.0	2.7
DISULFIRAM $\bar{x}$ 4h	4.7	9.6	13.3	17.6	21.7	25.9	5.8	11.6	18.2	21.2	23.8	31.8	6.0	9.8	11.3	14.0	16.0	18.0
(100 mg/kg)+SE	0.9	0.9	2.0	2.4	2.3	2.6	1.0	2.3	2.4	2.4	2.6	5.7	1.3	1.4	2.3	1.3	2.0	1.7
DISULFIRAM $\bar{x}$ 4h	0.3 *	1.3 *	3.7 *	6.3 *	8.7 *	13.7 *	2.7 *	3.7 *	8.6	10.0	13.3	14.3	2.0 *	4.3 *	7.0 *	9.3 *	10.7 *	10.7 *
(200 mg/kg)±SE	0.3	0.7	1.9	3.2	4.8	6.7	1.2	2.2	5.7	6.0	6.9	7.0	2.0	1.9	2.5	3.5	4.3	4.3
DDC $\bar{x}$ 4h	7.3	10.7	13.0	19.7	23.0	25.0	6.3	10.3	16.0	22.3	25.0	28.0	6.0	9.0	11.0	14.7	17.7	18.3
(50 mg/kg)±SE	4.5	3.8	3.6	4.7	5.6	6.8	1.2	3.3	4.0	2.6	2.5	3.6	0.6	0.7	2.0	4.1	3.3	2.0
DISULFIRAM $\bar{x}$ 18h	6.0	8.8	13.3	17.0	21.0	25.0	6.3	10.0	13.3	17.0	18.8	20.8	6.5	9.0	11.8	13.3	14.5	15.5
(50 mg/kg)±SE	1.0	0.9	2.9	3.2	3.5	4.0	0.9	1.7	2.8	2.1	2.5	2.5	1.2	2.1	2.5	2.5	3.4	3.0
DDC $\bar{x}$ 18h	6.7	8.7	11.7	16.7	21.7	25.0	6.7	10.0	15.0	19.7	23.7	26.0	8.7	9.0	11.7	12.3	16.3	18.3
(50 mg/kg)±SE	2.2	3.2	2.8	2.9	3.5	2.5	0.9	0.0	1.0	3.7	4.7	5.0	4.8	4.6	5.4	5.2	6.4	6.4

The effect of disulfiram and DDC pretreatment (4h) on the responses of the rat isolated colon to angiotensin, acetylcholine and 5-hydroxytryptamine (5-HT). Following 200 mg/kg disulfiram, spontaneous contractions of the colon were reduced in magnitude and frequency. From the data, regression lines were computed and are plotted in Figs. 34, 35, 36 & 37.

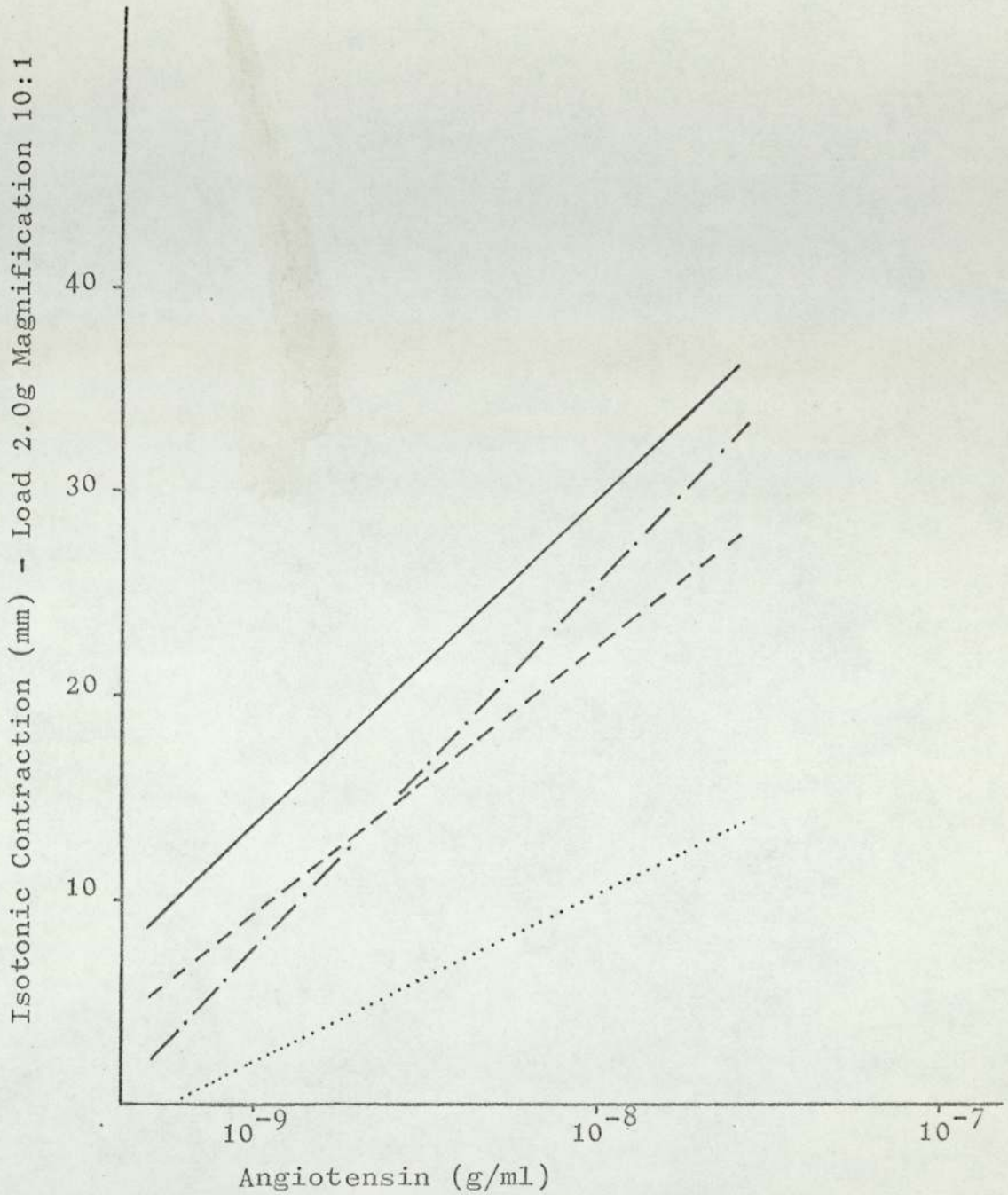


Figure 34 Regression lines computed for the Log. dose/ relationship for the myotropic action of angiotensin on the rat isolated colon. Donor rats were pretreated with one of 3 doses of disulfiram 50 mg/Kg (– – –), 100mg/Kg (— · —) or 200 mg/Kg (·····) 4 h prior to sacrifice. The control group (———) received vehicle only.

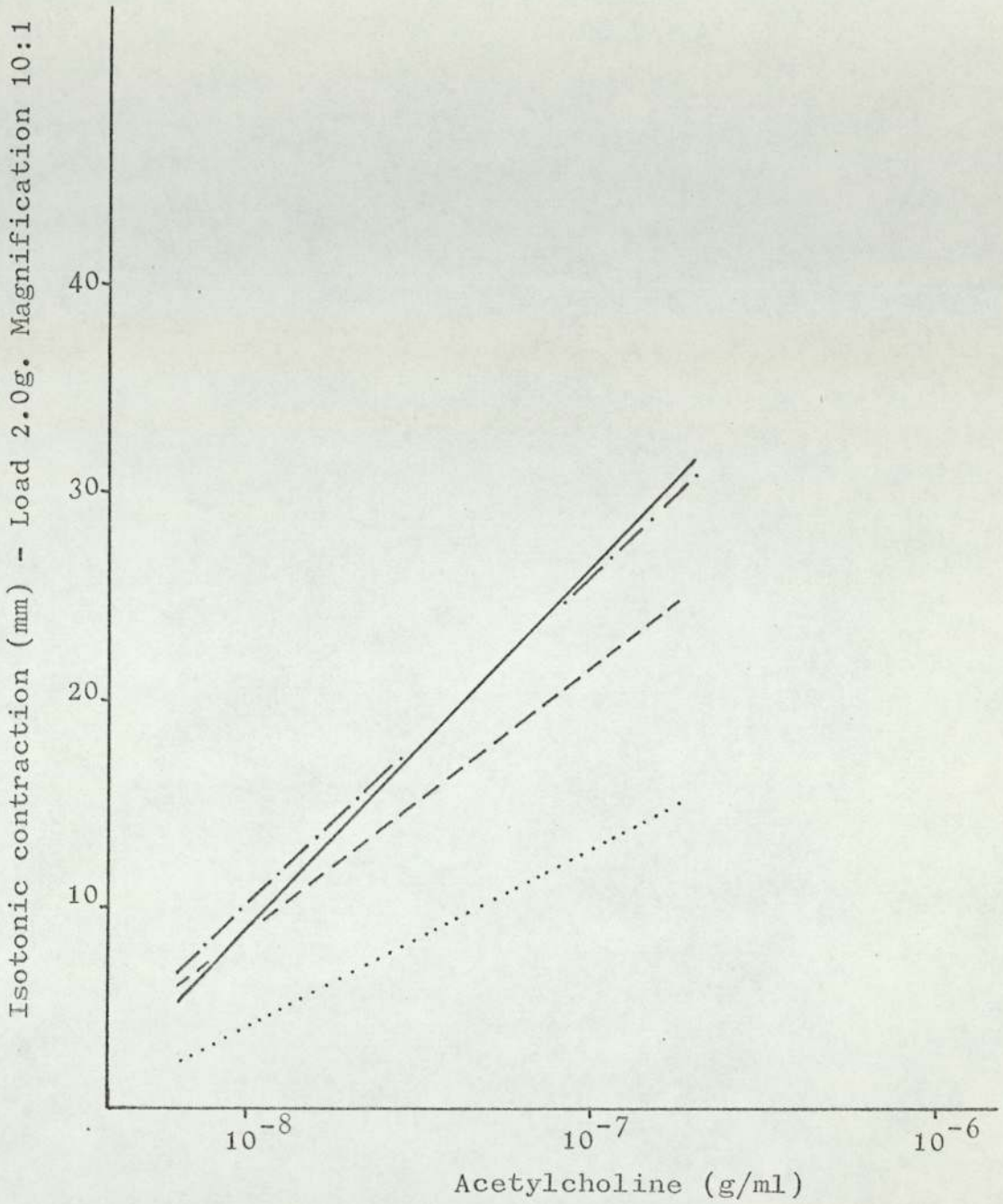


Figure 35. Regression lines computed for the log dose/response relationship for the myotropic action of acetylcholine on the rat isolated colon. Donor rats were pretreated with one of 3 doses of disulfiram - 50 mg/kg (– – –), 100 mg/Kg (— · —) or 200 mg/Kg (·····) 4 h prior to sacrifice. The control group (——) received vehicle only.

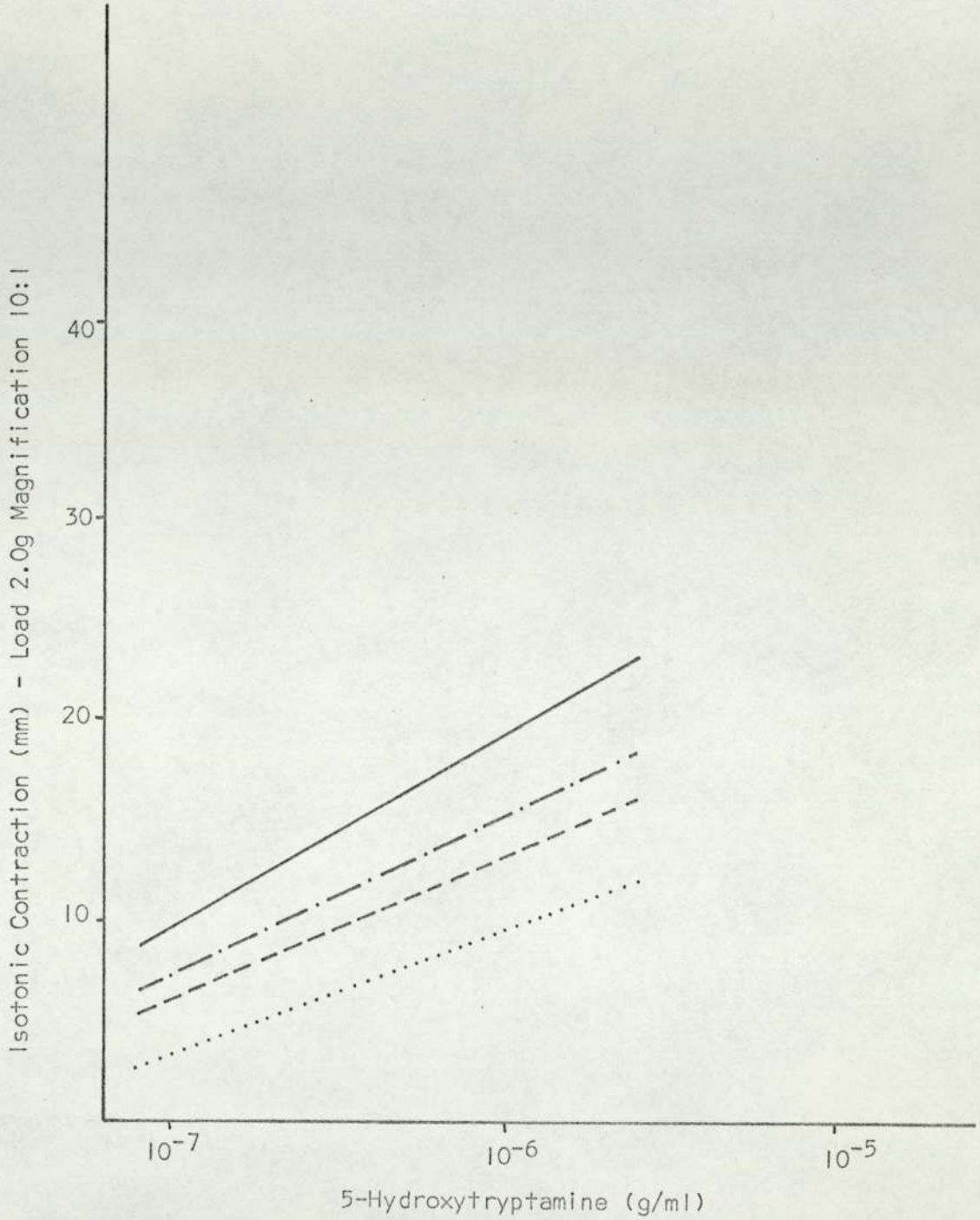


Figure 36 Regression lines computed for the Log.Dose/response relationship for the myotropic action of 5-HT on the rat isolated colon. Donor rats were pretreated with one of 3 doses of disulfiram - 50mg/kg (– – –) 100mg/kg (—·—) or 200mg/kg (·····) 4h prior to sacrifice. The control group (——) received vehicle only.

lines appears in Table 2. Regression lines were considered to be 'parallel' when there was no significant difference between the computed slopes. The slopes (± S.E.) were compared using the 't'-test and the results are indicated in Table 2. Significance was taken at the $2P < 0.05$ level.

Thus disulfiram (50 & 100 mg/kg i.p.) pretreatment was shown to cause a slight, non-significant decrease in the responses to all doses of angiotensin. The highest dose of disulfiram (200 mg/kg), however, considerably reduced angiotensin responses, with a significant reduction in the slope of the regression line, indicating a non-competitive inhibition.

The effect of disulfiram on contractile responses to acetylcholine was shown to be similar to its effects on responses to angiotensin. Inhibition of the spasmogenic action of acetylcholine occurred only following pretreatment with the highest dose of disulfiram (200 mg/kg) and was again characterised by a significant reduction in the slope of the regression line.

In the case of 5-hydroxytryptamine, a slightly different pattern of responses was noted. At the 50 and 100 mg/kg dose level, disulfiram pretreatment caused a slight, non-significant inhibition of responses to 5-hydroxytryptamine. Following 200 mg/kg disulfiram, however, the responses to 5-hydroxytryptamine were significantly reduced from control but there was no significant change in the slope of the regression line.

Thus it appears that following pretreatment with high doses of disulfiram, there is a non-selective inhibition of the spasmogenic activities of angiotensin, acetylcholine and 5-hydroxytryptamine on the rat colon. The inhibition of 5-hydroxytryptamine by disulfiram may, however, involve a different mechanism to that operating in the cases of angiotensin and acetylcholine.

(iii) Comparison of the Effects of Pretreatment with Disulfiram and DDC on the Responses of the Rat Isolated Colon to Angiotensin, Acetylcholine and 5-hydroxytryptamine.

In Chapter 1 it was shown that although the anti-angiotensin action of disulfiram and DDC in the pithed rat blood pressure preparation were similar, there were quantitative differences observed. It was considered desirable, therefore, to compare the action of these two compounds on the rat colon preparation. The spasmogenic actions of angiotensin (0·5-16 ng/ml), acetylcholine (10-320 ng/ml) and 5-hydroxytryptamine (50-1600 ng/ml) were thus compared on segments of colon taken from groups of rats which had been subjected to 4 or 18 h pretreatment with disulfiram (50 mg/kg i.p.) or DDC (50 mg/kg i.p.). The results of these experiments are included in Table 2. Regression lines were computed from this data and plotted in Fig. 37 (4 h pretreatment) and Fig. 38 (18 h pretreatment). Criteria for assessing parallelism were as above. The computed slopes (± S.E.) and their limits are included in Table 3.

Following the 4 h pretreatment, no significant differences were observed between the log dose-response curve following either disulfiram or DDC. Following either compound, there was a non-significant reduction in the slopes of the regression lines for angiotensin and acetylcholine when compared with control (Table 3; Fig.38). In the case of 5-hydroxytryptamine, there was a non-significant parallel shift to the right following DDC or disulfiram.

Increasing the disulfiram (50 mg/kg) and DDC (50 mg/kg) pretreatment time to 18 h produced a similar slight inhibition to that seen following 4 h pretreatment (Table 3; Fig.38). Regression lines for the log dose-response relationship for 5-hydroxytryptamine and angiotensin were very similar following

Table 3

DRUG TREATMENT(mg/kg)	n	Angiotensin			Acetylcholine			5-Hydroxytryptamine		
		b	±Sb	Sbt	b	±Sb	Sbt	b	±So	±Sbt
Control	7	16.9	3.4	6.9	17.6	1.8	3.8	9.7	2.2	4.4
50 mg Disulf (4h)	9	13.9	1.5	3.2	12.8	2.5	5.0	7.3	1.8	3.8
100 mg Disulf (4h)	5	19.0	1.9	3.9	16.1	2.2	4.5	7.7	1.1	2.3
200 mg Disulf (4h)	5	8.6*	2.6	5.5	8.4*	3.7	4.8	6.1	2.2	7.8
50 mg DDC (4h)	3	12.5	3.4	7.3	15.1	2.1	3.1	8.6	1.4	4.5
50 mg DDC (18h)	4	12.9	2.0	4.3	13.5	2.2	4.7	6.7	3.8	8.1
50 mg Disulf(18h)	4	12.9	2.1	4.3	9.7*	1.5	3.2	6.0	1.8	3.8

The effect of pretreatment with disulfiram and DDC on the responses of the rat colon to angiotensin (0·5-16·0 ng/ml), acetylcholine (10-320 ng/ml) and 5-hydroxytryptamine (50-1600 ng/ml). Regression data computed from results shown in Table 2 showing slope of regression (b), ± standard error of the slope (±Sb) and fiducial limits of the regression calculated at the 0·05 probability level (Sbt). Comparison of regression slopes involved use of 't'-test, significance ($2P < 0.05$) indicated thus (*).

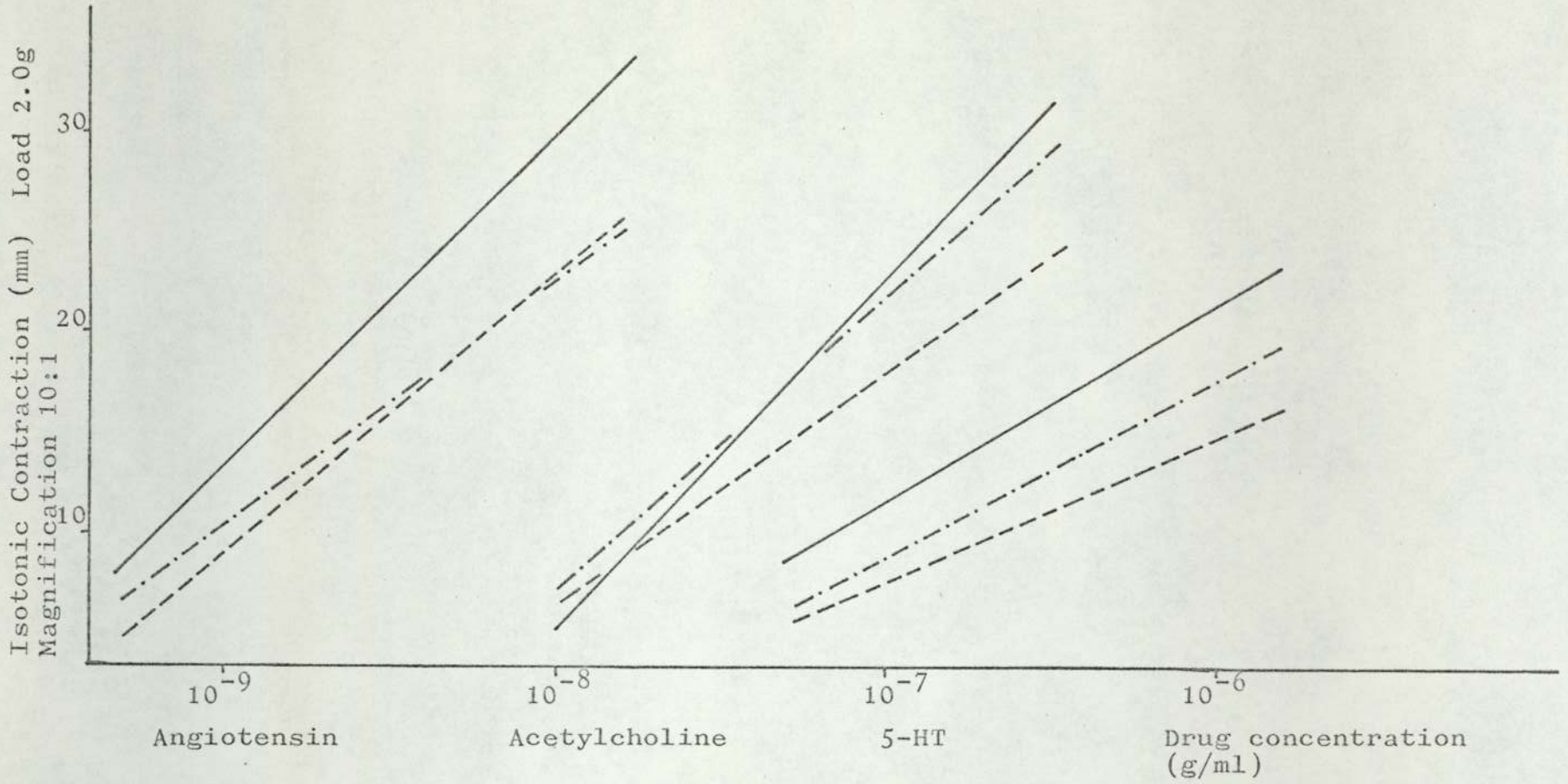


Figure 37. Comparison of the effects of 4h pretreatment with disulfiram (---) and DDC (-·-) (50mg/kg i.p.) the log dose/response relationship of angiotensin, acetylcholine and 5-HT on the rat colon. Regression lines computed from data in Table 2. The control group (——) received vehicle only.

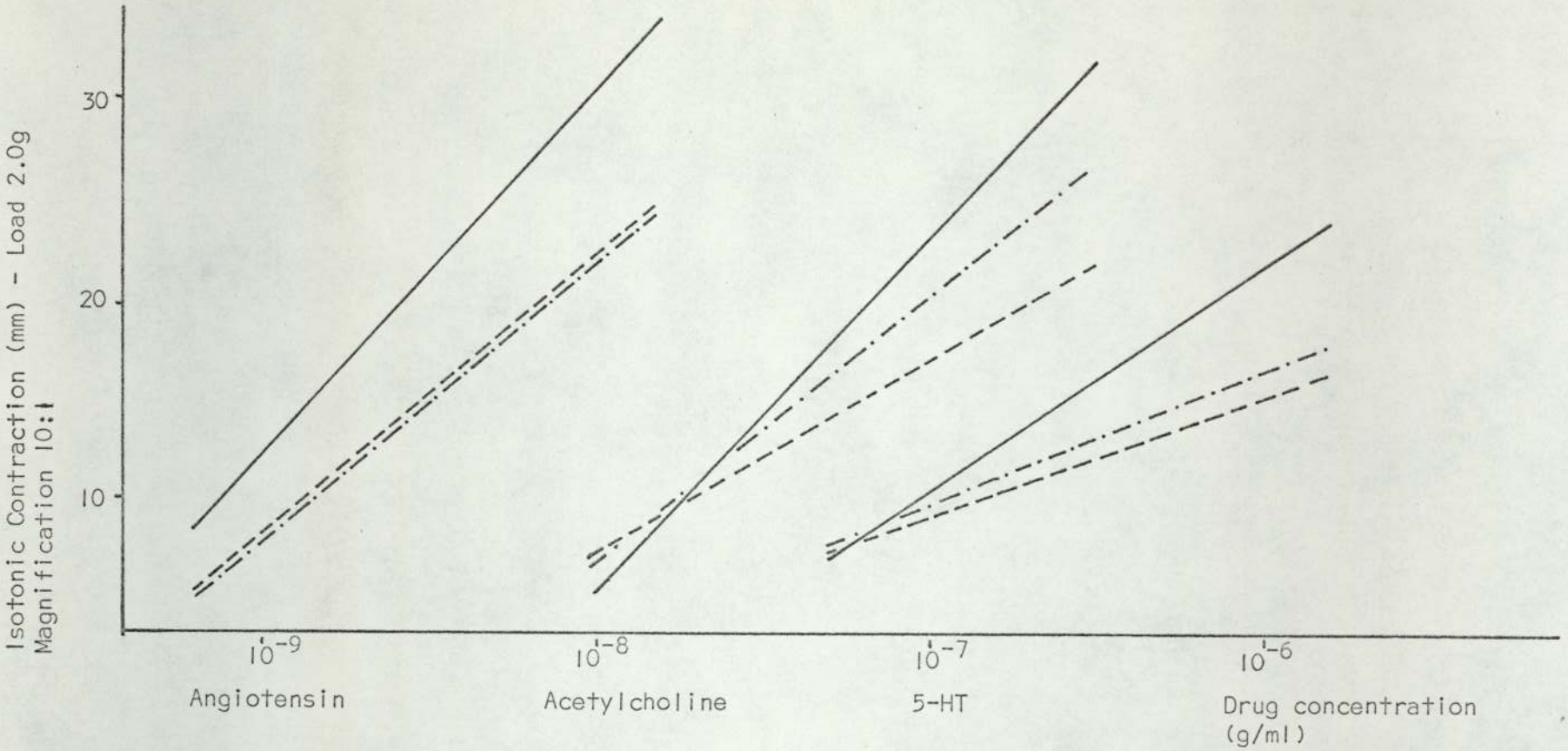


Figure 38. Effect of 18h pretreatment with disulfiram (---) and DDC (—·—) (50mg/kg i.p.) on the Log. dose/response relationships of angiotensin, acetylcholine and 5-HT on the rat colon. Regression lines computed from data summarized in Table 3. The control group (——) received vehicle only

either inhibitor (Fig.38). An interesting feature of the regression lines for 5-hydroxytryptamine was the marked, though not statistically significant, decrease in slope. This was in contrast to the parallel shifts seen following 4 h pretreatment and parallel shifts seen following pretreatment (4 h) with higher doses of disulfiram. In the case of responses to acetylcholine, there was a decrease in slope of the regression lines following DDC or disulfiram. This was significant ($2P < 0.05$) following disulfiram but not DDC (Table 3).

(iv) Acute Effect of DDC on the Myotropic Responses of the Guinea Pig isolated Ileum to Angiotensin, Acetylcholine and Histamine in the Absence and Presence of Hyoscine.

There appear to be two components involved in the spasmogenic action of angiotensin on the guinea pig ileum, a direct action and an indirect action mediated via the parasympathetic innervation (Khairallah & Page, 1961). There are thus two sites at where an antagonist may act. The indirect action of angiotensin may be readily inhibited by a cholinergic inhibitor such as hyoscine. Experiments are described in which the acute effect of DDC (1 µg/ml) on responses to angiotensin, histamine and acetylcholine is examined in the presence and absence of hyoscine (100 ng/ml).

From the results of these experiments, regression lines were computed and data derived from these included in Table 4. As above, significance was taken at the 0·05 level and parallelism compared by 't'-test.

Figs. 39 and 40 illustrate the effects of hyoscine (100 ng/ml) and the subsequent addition of DDC (1 µg/ml) in the presence of the hyoscine on the contractile response of the guinea pig ileum to angiotensin (1 - 32 ng/ml), histamine

Table 4

TREATMENT	n	Angiotensin b	Angiotensin ±Sb	Angiotensin Sbt	Acetylcholine b	Acetylcholine ±Sb	Acetylcholine Sbt	Histamine b	Histamine ±Sb	Histamine Sbt
Control	10	27.1	7.3	15.2	50.7	8.8	18.2	36.7	9.1	18.3
+Hyoscine	10	22.7	4.5	9.2	-	-	-	42.2	9.1	18.3
+Hyoscine+DDC	10	12.2*	4.5	9.3	-	-	-	47.6	8.2	16.6
Control	12	37.1	7.3	15.0	43.4	11.2	22.6	61.3	9.3	18.8
+DDC	12	32.4	6.1	12.4	42.0	10.1	20.4	63.5	7.5	15.3
+DDC+Hysocine	12	19.2*	4.5	9.3	-	-	-	52.1	8.6	17.5

Acute effects of DDC and hyoscine alone and in combination on the Log dose/response curves produced by the action of angiotensin (1 - 32 ng/ml), acetylcholine (5-160 ng/ml) and histamine (5-160 ng/ml) on the isolated guinea pig ileum. Data indicates the computed slope (b) standard error of the slope (±Sb) and the fiducial limits of the slope at the $2P < 0.05$ probability level (Sbt) of the regression line. Plots of the regression lines are shown in Fig.39, 40 & 41. *Indicates significant difference from control.

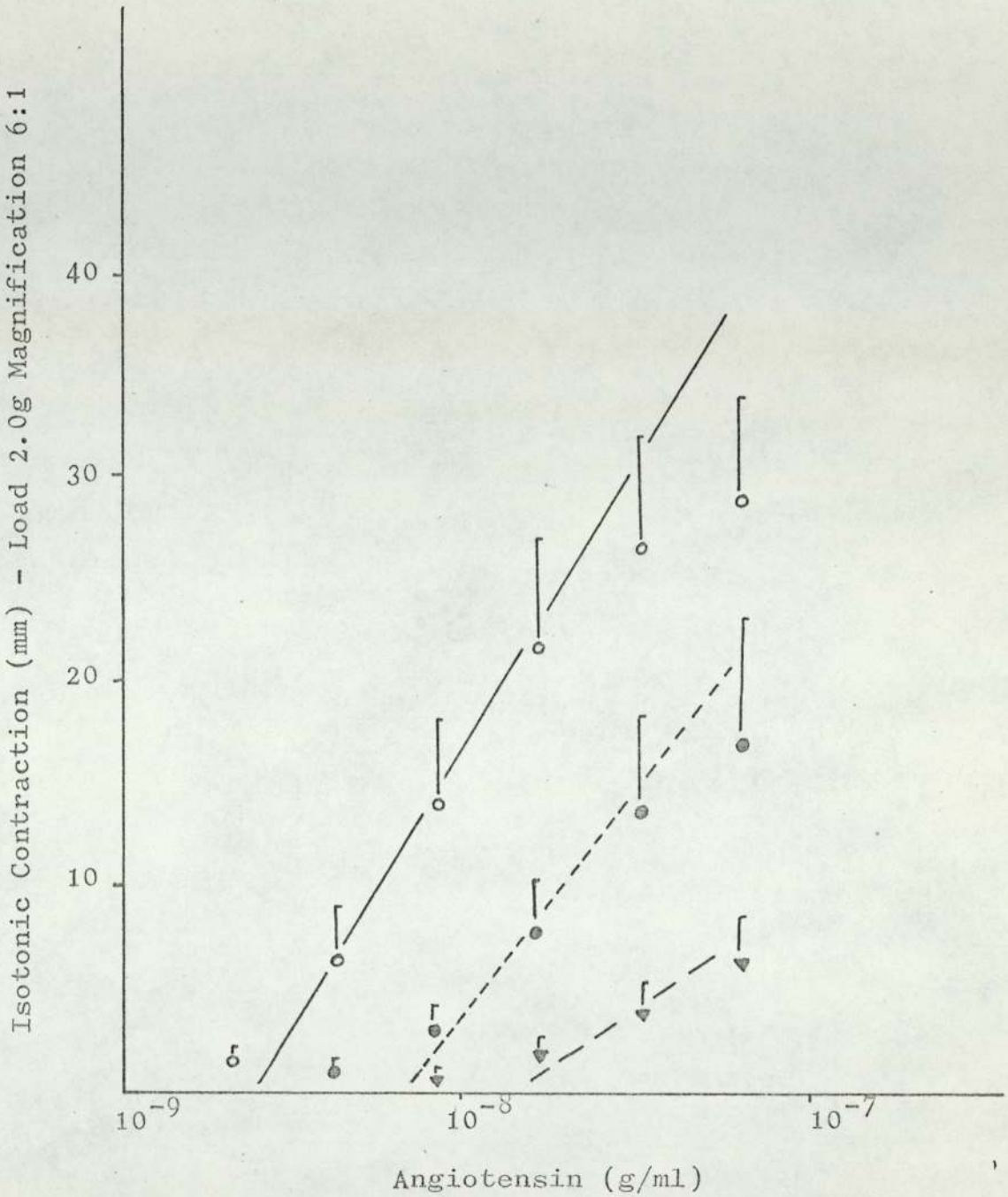


Figure 39 Guinea pig isolated ileum. Contractions to angiotensin, controls (—o—), in the presence of 0.1µg/ml hyoscine (- • -) and in the presence of 0.1µg/ml hyoscine and 1µg/ml DDC (—▼—). Regression lines were computed.

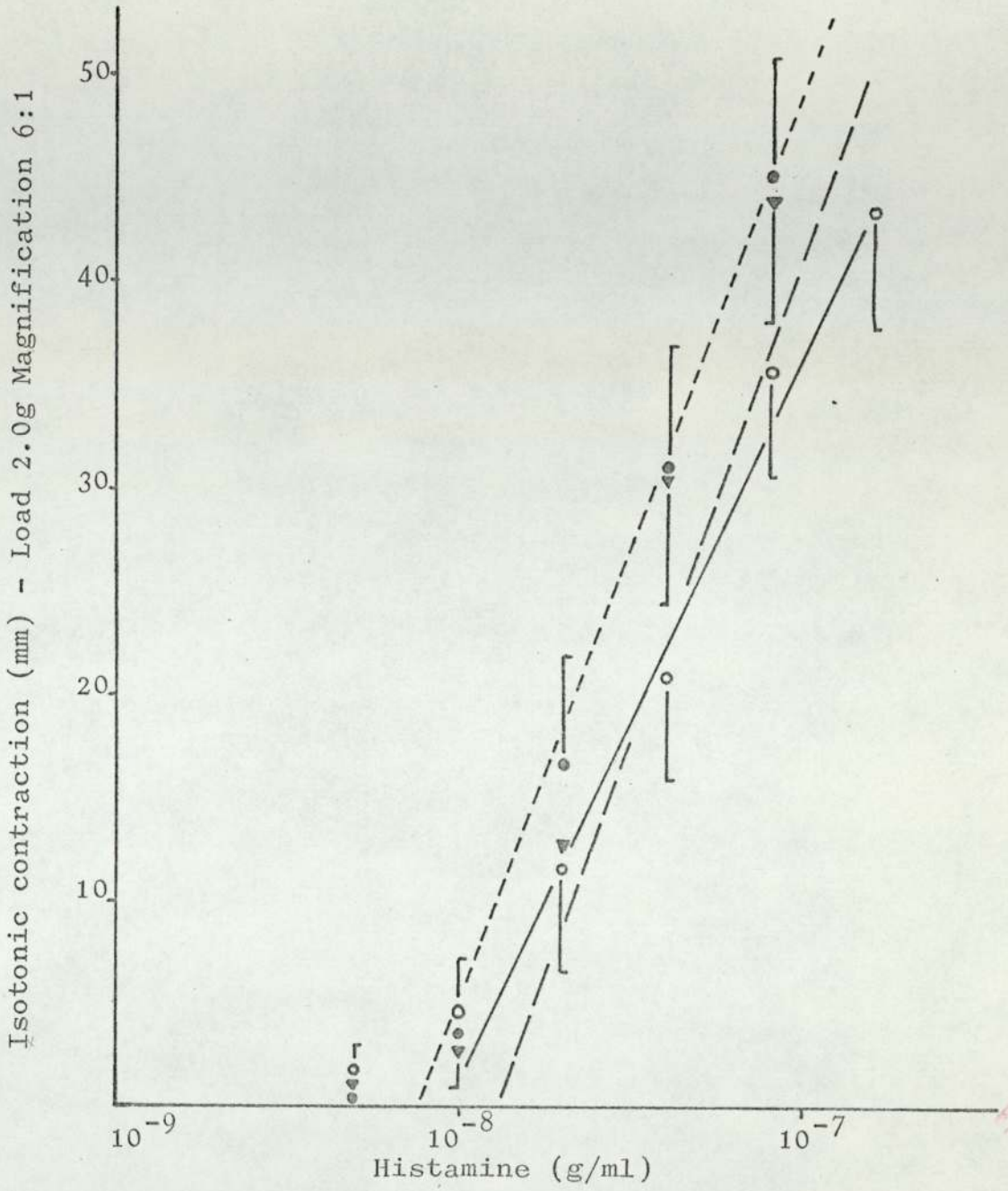


Figure 40 Guinea pig isolated ileum. Contractions to histamine, controls (—o—), in the presence of 0.1 µg/ml hyoscine (- ● -) and in the presence of 0.1 µg/ml hyoscine and 1 µg/ml DDC (—▼—). Regression lines were computed.

(5 - 160 ng/ml) and acetylcholine (5 - 160 ng/ml). As expected, hysocine completely abolished responses to acetylcholine. Responses to angiotensin were reduced by approximately 50% with a parallel shift of the regression line. Contractile responses to histamine were unaltered by the hyoscine. Addition of DDC (1 µg/kg) to the bathing medium in the presence of hyoscine produced no alteration in responses to histamine. DDC did, on the other hand, cause a significant decrease in the slope of the regression line of the log dose/response curve for angiotensin. Responses to individual doses of angiotensin were reduced to about 30% of those recorded in the presence of hyoscine alone.

Reversal of the order in which hyoscine and DDC were added to the preparation produced similar results (summarised in Table 4). DDC alone did not cause a significant alteration in the slope and displacement of the regression lines computed for the log dose/response curves for angiotensin (Fig. 41), histamine (Fig. 42) or acetylcholine (Fig. 43). The subsequent addition of hyoscine (0·1 µg/ml) abolished responses to acetylcholine (Fig. 43) whilst contractile responses to histamine were unaffected (Fig. 42). Under the same conditions, responses to angiotensin were reduced to above 50% of control with a significant reduction in the slope of the regression line from that of control and from that obtained in the presence of hyoscine alone (Fig. 41; Table 4).

These results suggest that whereas DDC itself produces no significant alteration in the responses of the guinea pig isolated ileum to angiotensin, in the presence of hyoscine, the thiol is capable of exerting a non-competitive inhibition. This combination does not appear to alter responses to histamine, and DDC alone, at the concentration used, does not appear to have any anti-cholinergic activity.

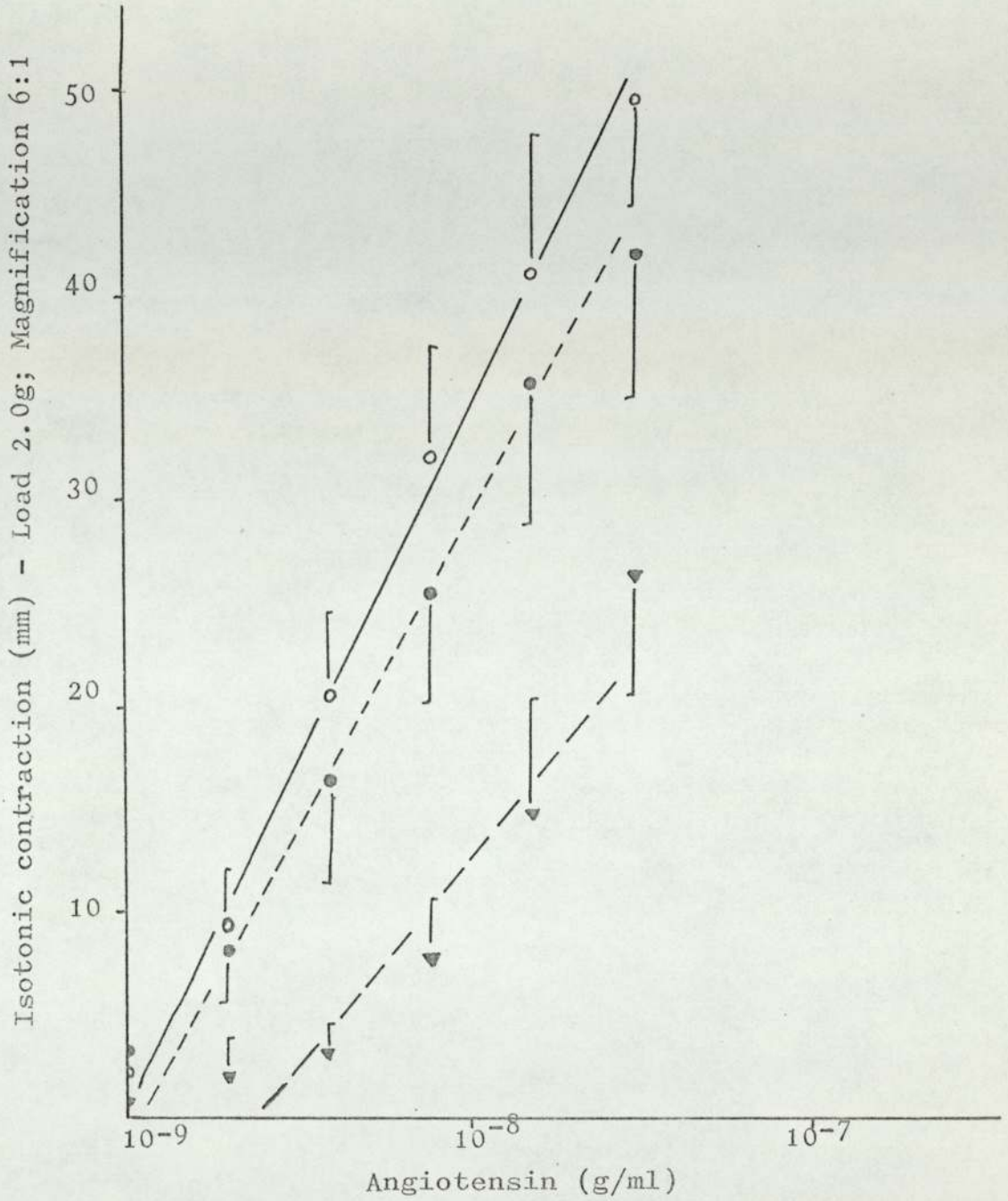


Figure 41. Guinea pig isolated ileum. Contractions to angiotensin, controls (—o—), in the presence of 1 µg/ml DDC (- o -) and in the presence of 1µg/ml DDC and 0.1 µg/ml hyoscine (—▼—). Regression lines were computed.

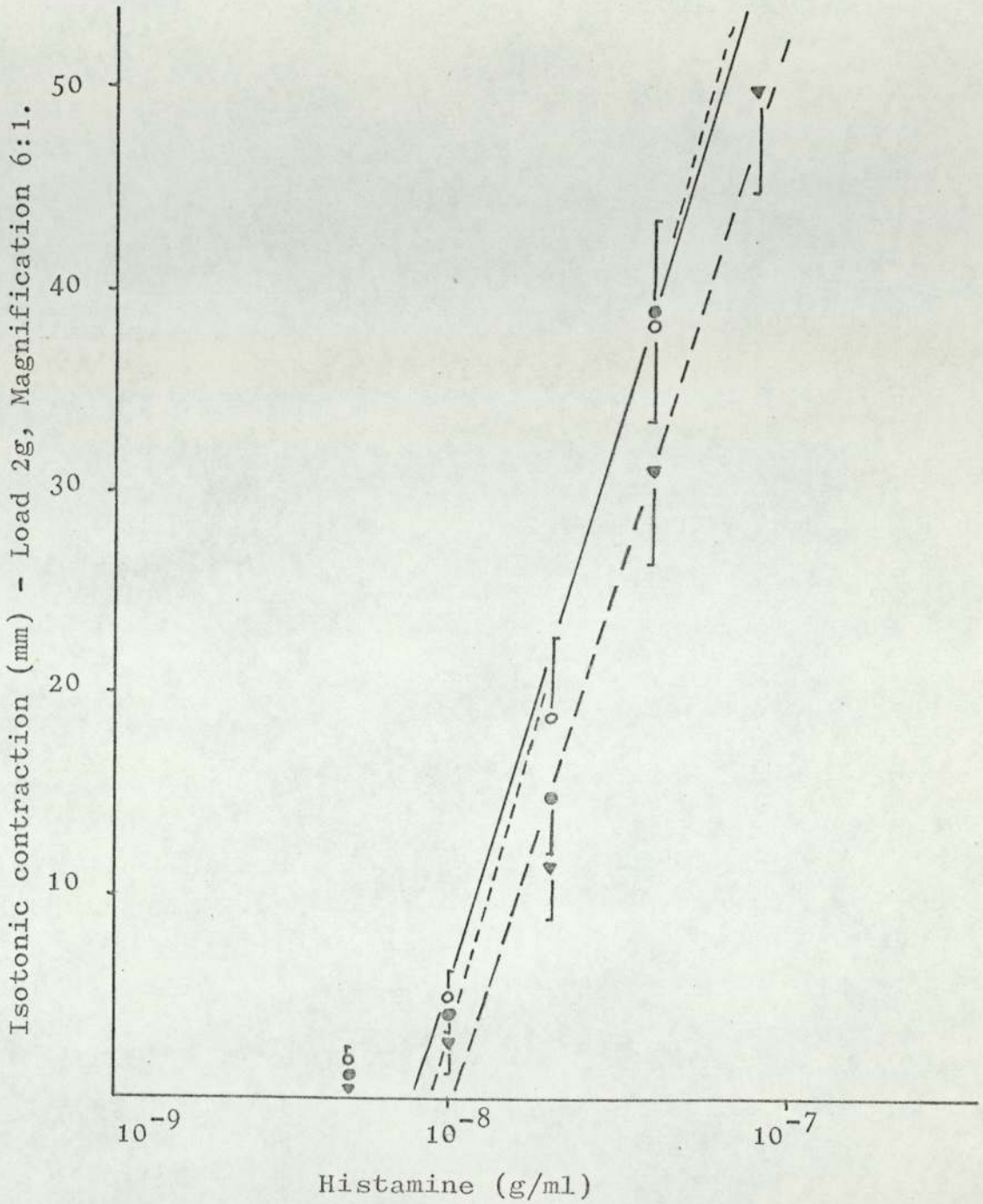


Figure 42. Guinea pig isolated ileum. Contractions to histamine, controls (—o—), in the presence of 1µg/ml DDC (-●-) and in the presence of 1µg/ml DDC and 0.1µg/ml hyoscine (—▼—). Regression lines were computed.

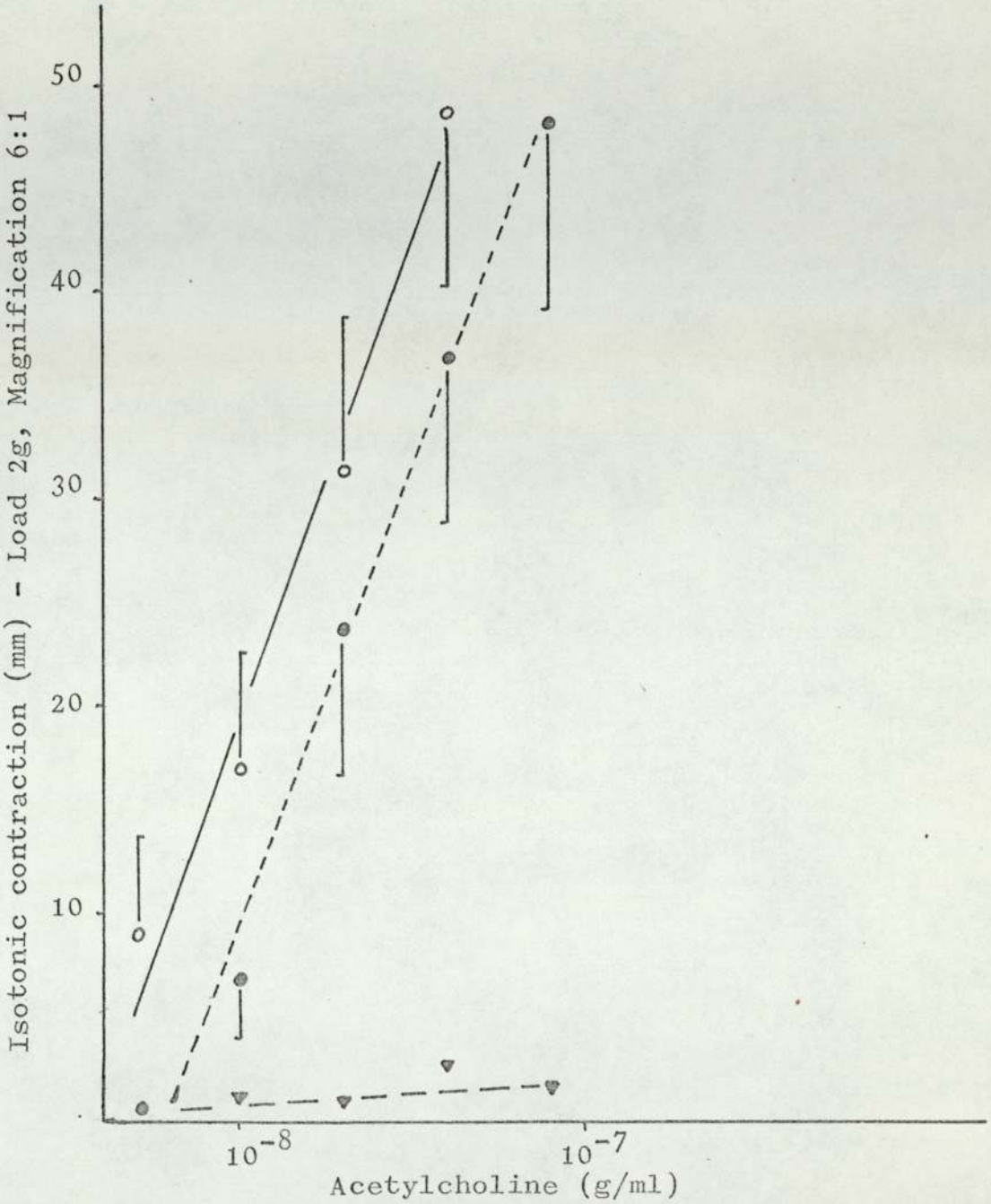


Figure 43. Guinea pig isolated ileum. Contractions to acetylcholine, controls (—o—), in the presence of 1 µg/ml DDC (-•-) and in the presence of 1µg/ml DDC and 0.1 µg/ml hyoscine (—▾—). Regression lines were computed.

(v) The Effect of Pretreatment with Disulfiram upon the Responses of the Guinea Pig Isolated Ileum to Angiotensin, Acetylcholine and Histamine

Contractile responses of isolated ilea obtained from guinea pigs pretreated with disulfiram (50 mg/kg i.p.) 3·5 h prior to sacrifice were compared with those of ilea from controls which had received the vehicle alone. The agonists used in this experiment were those in the acute experiments reported above, i.e. angiotensin (1-32 ng/ml), acetylcholine (5-160 mg/ml) and histamine (5-160 ng/ml). As was observed in the rats which had been similarly pretreated with disulfiram, residues of the disulfiram were seen at the time of removal of the gut segments. The effect of hyoscine (0·1 µg/ml) on the responses of the test ilea to the agonists was observed. Regression lines for the log dose/response curves were computed and criteria for parallelism were as before.

The results of these experiments are summarised in Table 5 and plots for the responses to angiotensin, histamine and acetylcholine are shown in Figs. 44, 45 and 46 respectively.

Disulfiram pretreatment produced no significant alteration in the size of contractions to angiotensin, histamine or acetylcholine. The addition of hyoscine (0·1 µg/ml) to these treated ileal segments completely abolished responses to acetylcholine and had no significant effect on responses to histamine. A surprising feature was, however, the lack of effect of hyoscine on responses to angiotensin. Previously (Fig. 39), it had been shown that there was a reduction in responses to angiotensin of about 50% following hyoscine. This was accompanied by a parallel shift in the regression line. However, as may be seen in Fig. 44 hyoscine had no effect on the responses of the disulfiram-treated ileum to angiotensin even though equi-effective

Table 5

TREATMENT	n	Angiotensin b	±Sb	Sbt	Acetylcholine b	±Sb	Sbt	Histamine b	±Sb	Sbt
Control	16	37.1	7.3	15.0	43.4	11.2	22.6	61.3	9.3	18.8
Disulf	16	38.0	6.6	13.5	42.1	6.6	13.4	44.1	7.1	14.4
Disulf + Hyosc.	16	38.8	7.1	14.8	-	-	-	41.6	6.3	12.9

Effect of disulfiram pretreatment (50 mg/kg i.p. 4 h prior to sacrifice) on the contractile response of the guinea pig ileum to angiotensin, acetylcholine and histamine. The effect of subsequent addition of hyoscine (0·1 mg/ml) to the bath was also recorded. The data shows the computed slope (b), standard error of the slope (Sb) and the fiducial limits of the slope at the $2P < 0{\cdot}05$ probability level (Sbt) of the regression lines. Plots of the regression lines are shown in Figs. 44, 45 & 46. No significant differences from control recorded.

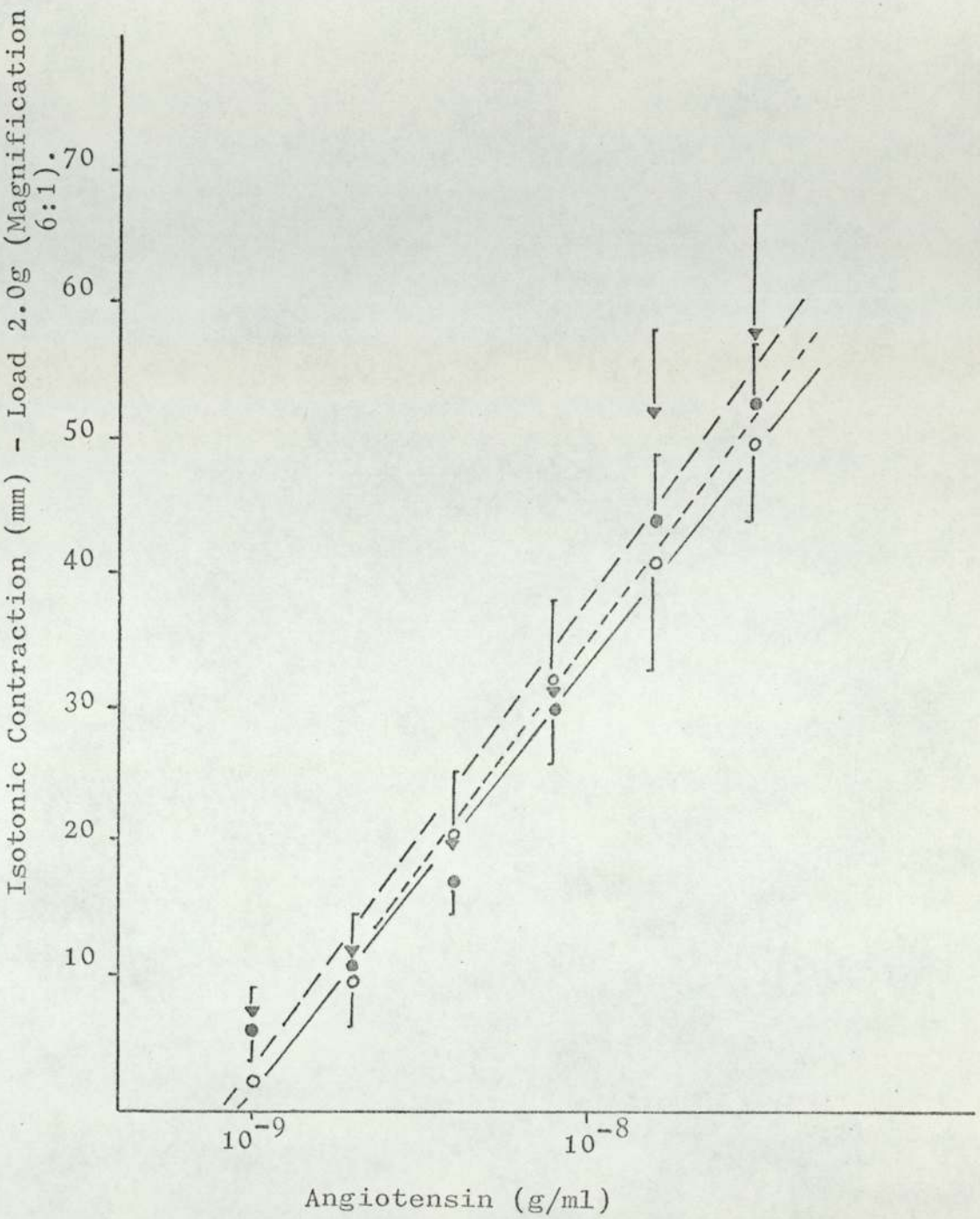


Figure 44. The effect of disulfiram pretreatment (50 mg/Kg i.p. 4h prior to sacrifice) on the contractile responses of the guinea pig ileum to angiotensin in the absence (-●-) and presence (—▼—) of hyoscine (0.1µg/ml). Regression lines for the dose/response curves were computed. The control group (—o—) received vehicle only.

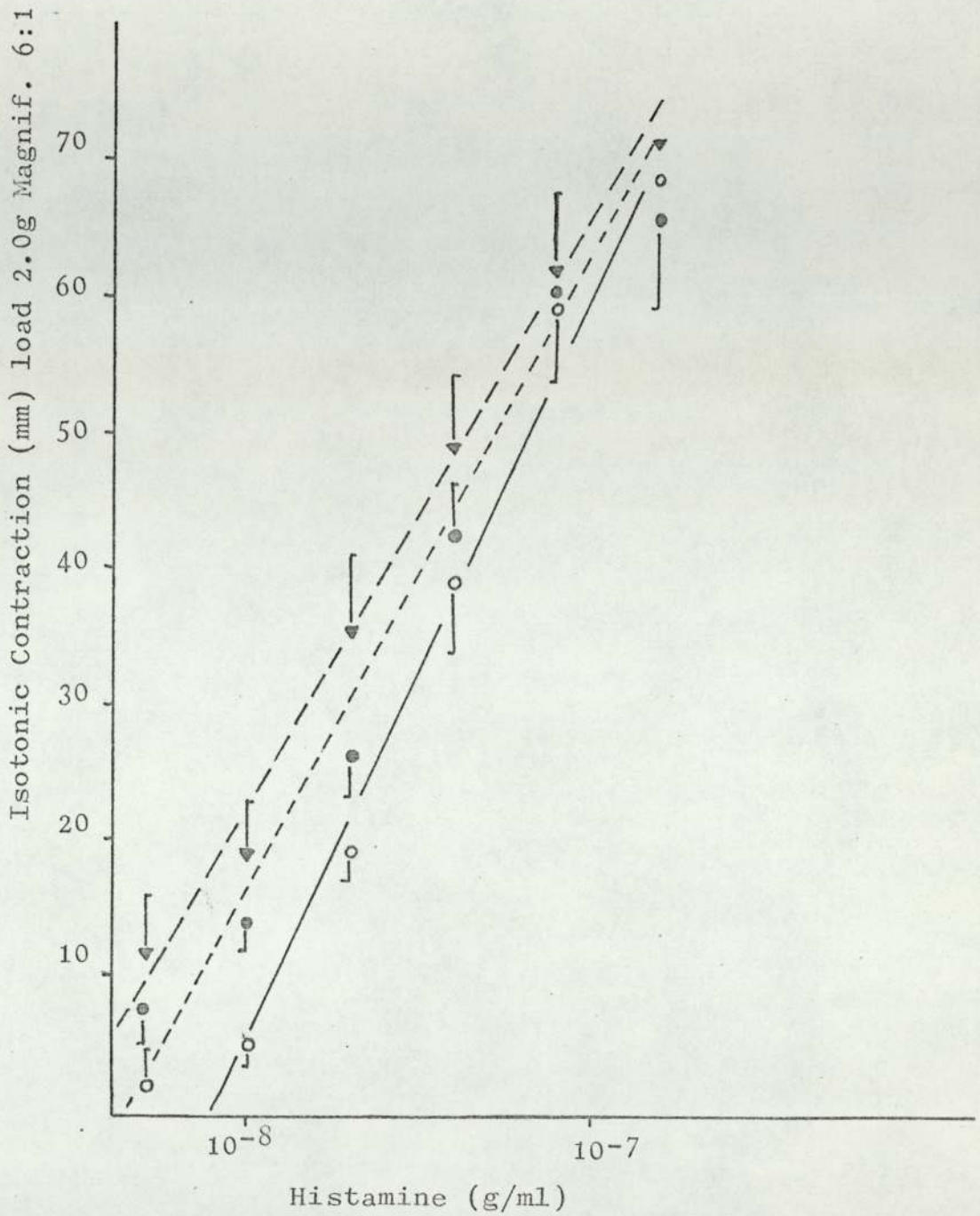


Figure 45. The effect of disulfiram pretreatment (50 mg/Kg i.p. 4h prior to sacrifice) on the contractile responses of the guinea pig ileum to histamine in the absence (-●-) and presence (—▼—) of hyoscine (0.1µg/ml). Regression lines were computed. The control group (—o—) received vehicle only .

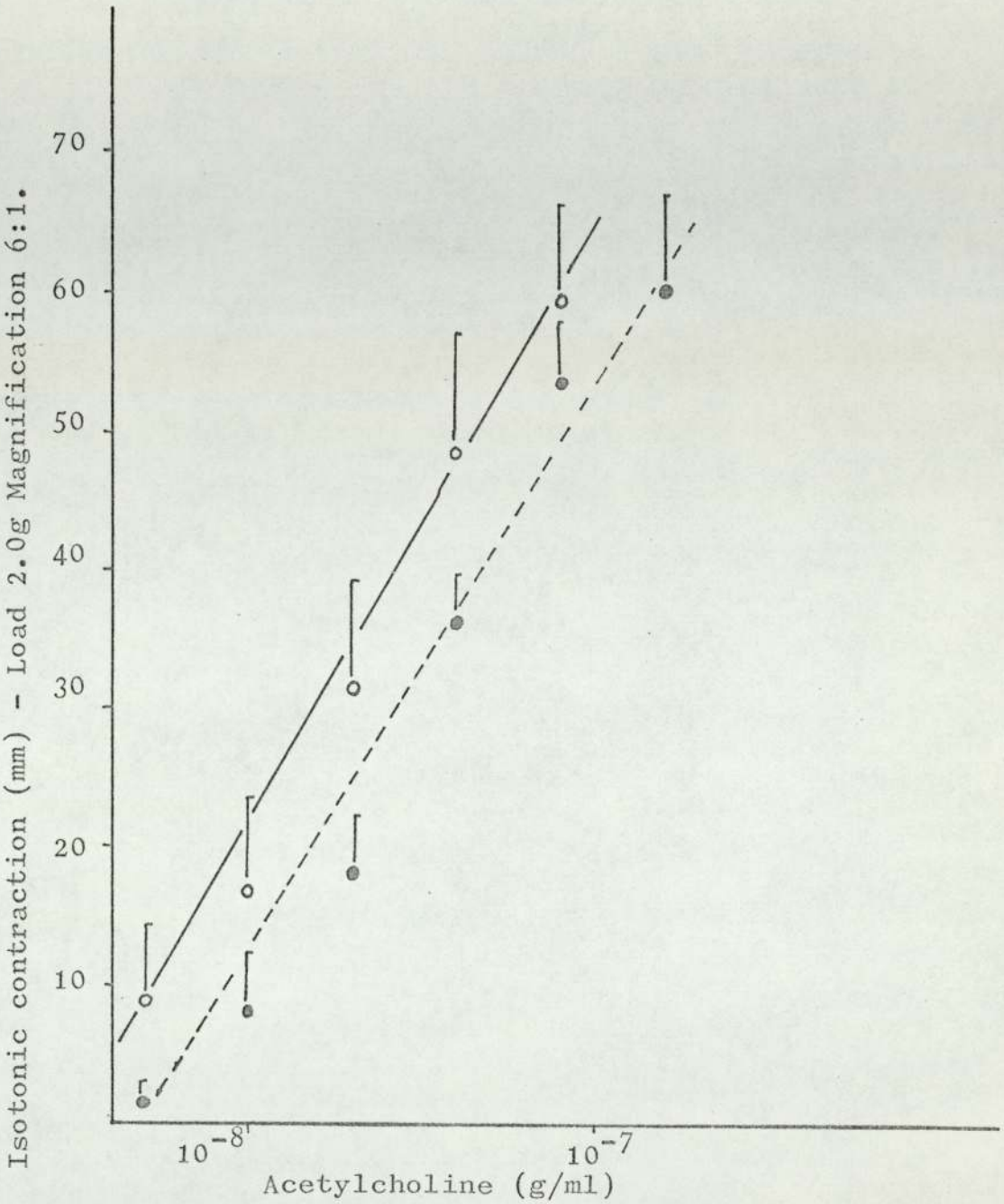


Figure 46. The effect of disulfiram pretreatment (50mg/Kg i.p. 4h prior to sacrifice) on the contractile responses of the guinea pig ileum to acetylcholine (-●-). Subsequent addition of hyoscine (0.1 µg/ml) totally abolished responses at all dose levels. Regression lines were computed. The control group (—o—) received vehicle only.

concentrations of acetylcholine were antagonised by the hyoscine in the same preparations.

It would appear, therefore, that disulfiram pretreatment abolishes the indirect cholinergic component of the angiotensin action on the ileum. In order to confirm or deny this possibility, it was decided to repeat the experiment using isometric strain gauge recording with which the two phases of the angiotensin myotropic action could be distinguished. Fig. 47 consists of isometric recordings obtained from control and disulfiram pretreated (50 mg/kg i.p. - 3·5 h) preparations. It may be seen that the initial rapid rise in tension due to the indirect component of the peptide action is absent in the treated preparation.

DISCUSSION

Other than those relating to analogues of angiotensin, reports concerning the inhibition of the spasmogenic actions of the peptide on the rat colon and guinea pig ileum preparations have involved a relatively small number of compounds. Cinnarizine and the related compound lidoflazine were claimed to selectively inhibit the direct actions of angiotensin on the guinea pig ileum, the latter being proposed as a competitive antagonist (Schaper et al, 1963; Klinge et al, 1966; Godfraind et al, 1966). Lidoflazine was also shown to produce a non-specific inhibition of the responses of the rat colon to angiotensin (Ellis & Reit, 1969). However, in view of the widespread range of activity of these compounds, they appear to behave as non-specific smooth muscle relaxants. Ellis & Reit (1969) likened the action of lidoflazine to that of papaverine. Osajin was shown by Gascon & Walaszek (1966) to antagonise the direct

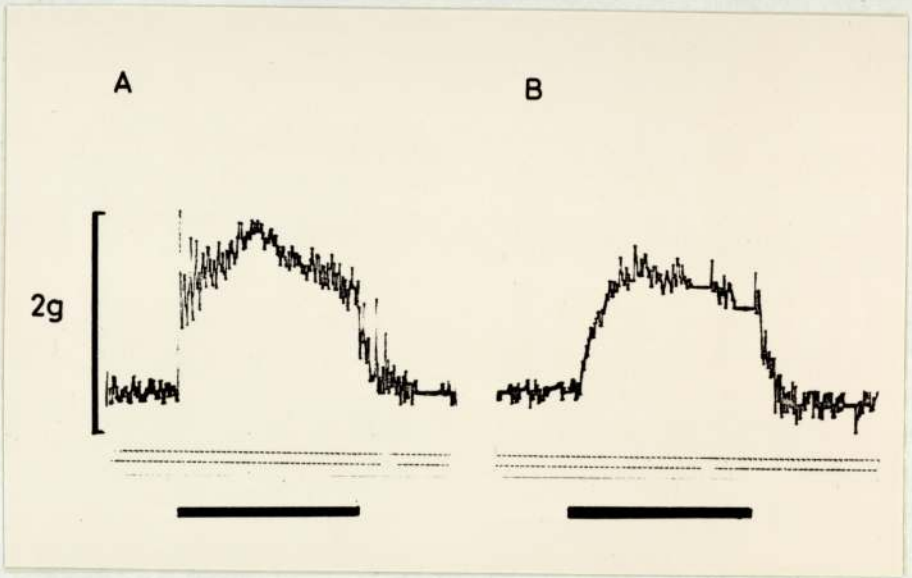


Fig. 47. Isometric recordings of contractions of guinea pig isolated ileal segments showing the response to angiotensin (·20 ng/ml - horozontal bar). Panel A shows the response of a segment taken from a control animal and Panel B shows the response of a segment taken from a guinea pig treated with disulfiram (50 mg/kg i.p.) 3·5 h previously.

action of angiotensin on the guinea pig ileum but Walaszek (personal communication) later showed that this anti-angiotensin action was not extended to the in vivo state.

The observation that disulfiram and DDC inhibited the pressor action of angiotensin in the pithed rat suggested that these inhibitors might successfully inhibit the spasmogenic activity of the peptide in isolated smooth muscle preparations. The experiments described here show that the anti-angiotensin activity of disulfiram and DDC on vascular smooth muscle was, in general, not extended to non-vascular smooth muscle.

At a concentration of 40 µg/ml, DDC was shown to have no effect on responses of the rat isolated colon to angiotensin, acetylcholine or 5-HT. Rather than pursue the possible effects of DDC added to the bathing medium, it was decided to study the effects of 3·5 h pretreatment with both disulfiram and DDC on subsequently isolated rat colon preparations. Following this pretreatment, it had been established that pressor responses to angiotensin were abolished (Chapter 1). Experiments described above were performed to compare the effects of three dose levels of disulfiram (50, 100 & 200 mg/kg i.p.) given 3·5 h prior to sacrifice on the responses of the rat colon to angiotensin, acetylcholine and 5-HT. Only following the highest dose of disulfiram was any reduction in responses to the spasmogens observed and this took the form of a general depression in responsiveness to all three agonists. This was accompanied by a significant decrease in the slopes of the regression lines calculated for the log dose/response curves for angiotensin and acetylcholine. Of possible importance is the fact that there appeared a parallel shift in the regression line established for 5-HT. This may indicate an involvement of a different mechanism

in the inhibition of 5-HT by disulfiram to that operating in the antagonism of angiotensin and acetylcholine. It is of interest to note at this point that Mansner and his co-workers (1968) reported that prolonged treatment of rats with disulfiram (250 mg/kg p.o. daily for 21 days) diminished responses of isolated ileum to 5-HT but not to acetylcholine. These authors suggested that this selective inhibition was due to impairment of function of cholinergic nerves, the 5-HT action on the rat isolated ileum being indirect in nature.

A comparison of the effects of administering equal doses (50 mg/kg i.p.) of disulfiram and DDC to rats 3·5 and 18 h before sacrifice demonstrated little, if any, differences in the activity of the compounds. This was to be expected if the concept of DDC as an obligatory metabolite of disulfiram is accepted (Stromme, 1965a). However, the actions of the two compounds do not wholly coincide and it was considered of interest to investigate their possible differences in activity. This was thought to be of importance in the case of the experiments involving the 18 h pretreatment when considering the relative rates of metabolism of these compounds (Stromme, 1965a). An interesting consequence of this more prolonged study was the decrease in the slope of the log dose/response regression line for 5-HT (Fig. 38) when compared with the parallel displacement previously observed following 4 h pretreatment (Fig. 37).

From the results outlined above it would appear, therefore, that under the conditions described, disulfiram and DDC are not capable of a selective inhibition of the contractile responses of the rat isolated colon to angiotensin. This was disappointing in view of the alleged similarity between the angiotensin 'receptors' in the rat vascular smooth muscle and the rat colon

established by a comparison of the effects of a series of angiotensin analogues (Regoli & Vane, 1964).

The acute effect of DDC (1·0 µg/ml) on the contractile responses of the guinea pig isolated ileum was investigated in the presence and absence of hyoscine (0·1 µg/kg). Hyoscine alone, in a concentration which abolished responses to previously equi-effective concentrations of acetylcholine, reduced responses to angiotensin by approximately 50% with a parallel shift in the log dose/response regression line. Histamine, under the same conditions, was not significantly affected. This partial inhibition of responses to angiotensin was expected in view of the previously reported dual action of the peptide on guinea pig ileum (Khairallah & Page, 1961; Godfraind et al, 1966).

DDC (1·0 µg/ml) alone produced no significant reduction in the responses to angiotensin, acetylcholine or histamine. However, in the presence of hyoscine, at which stage the remaining contractile activity of the peptide on the ileum is wholly direct, DDC produced a reduction in responses to angiotensin of the order of 50% with a significant reduction in the slope of the regression line from that of control and that obtained in the presence of hyoscine alone. Responses to histamine on the other hand were not reduced by the combination of hyoscine and DDC. Furthermore, DDC alone does not appear to have any anticholinergic activity. The lack of effect of DDC alone in inhibiting the directly mediated responses of the guinea pig ileum to angiotensin may be due to the masking of this effect in the isotonic recording system used, by the indirectly mediated phase of the peptide action. This theory could possibly be confirmed by establishing the duration of response in an isotonic system; inhibition of the second 'direct'

phase leading to an increased rate of recovery. Alternatively, an indication of the effect of DDC on the components of the biphasic response distinguished by isometric recording could be obtained.

In the light of the acute effect of DDC on the 'direct' component of the response of guinea pig ileum to angiotensin, the results of 3·5 h pretreatment with disulfiram (50 mg/kg i.p.) was something of a paradox. The disulfiram pretreatment did not produce any detectable alteration in the magnitudes of the responses to angiotensin, histamine or acetylcholine. The subsequent addition of hyoscine (0·1 μg/ml) abolished responses to acetylcholine but did not reduce responses to histamine. However, hyoscine did not produce the expected significant reduction in the responses of the ileum to angiotensin (Fig. 44). This apparent absence of an indirectly mediated component to the response did not result in a significant decrease in the magnitude of the isotonically recorded response. These results suggest that disulfiram is interfering with the post-ganglionic parasympathetic transmission. The absence of this 'indirect' component could possibly have been masked by a marginal increase in the rate of onset of the direct component which would have brought the maximum directly induced contraction in the 45 sec contact time used in these experiments.

The suggestion of possible disulfiram interference with the parasympathetic nervous system is supported by the report of Mansner and his co-workers who showed that prolonged treatment with disulfiram inhibited the indirect actions of 5-HT on the rat isolated ileum. Further support came from an examination of isometric recordings of responses of control and disulfiram-pretreated guinea pig ilea to angiotensin. These seem to indicate the absence of the fast, cholinergically-mediated component of the response as a result of the pretreatment.

Chapter 5

THE ROLE OF ANGIOTENSIN IN THE DEVELOPMENT AND MAINTENANCE OF HYPERTENSION IN THE RAT EXAMINED BY THE USE OF DISULFIRAM AS AN INHIBITOR OF THE PRESSOR ACTIONS OF THE PEPTIDE

The renin-angiotensin system has been implicated in the aetiology of renal hypertension since the original observation of Goldblatt and his co-workers that a pressor factor was released following renal artery stenosis. This involvement of the renin-angiotensin system in hypertension has yet to be confirmed or denied.

A major approach to the problem has involved the use of determination, by a variety of methods, of tissue renin levels, particularly plasma renin levels and the subsequent establishment or otherwise of a correlation with the degree of hypertension present. This method loses some of its validity, however, when the complexity of the renin-angiotensin system is appreciated. Peart (1969) points out the general unreliability of plasma renin for angiotensin levels as such, noting that it would be quite possible for a low concentration of angiotensin to be present in the plasma as a result, for example, of active uptake at smooth muscle receptor sites so that its activity could be very marked. It is evident, therefore, that studies involving 'levels' of renin or angiotensin would be relatively meaningless in the absence of information concerning reactivity to and the rates of production, release and removal of the substances involved.

An alternative approach involves the use of a selective inhibitor of the biological activity of angiotensin. There is little question that a number of angiotensin analogues are the most specific competitive inhibitors available (Marshall et al,

1970; Pals et al, 1971; Turker, Yamamoto, Khairallah & Bumpus, 1971). However, limited availability of these synthetic analogues has thus far restricted their use in long term studies relating to the role of angiotensin in the development of hypertension.

In Chapter 1 it was shown that the acute pressor response to angiotensin in the pithed rat was reduced by the administration of disulfiram (50 mg/kg i.p.) or DDC (10-50 mg/kg i.v.) This action could be demonstrated from about 3·5 h after the injection of the inhibitor and there was no sign of recovery of responses to the peptide after 8·5 h. The existence of traces of disulfiram in the peritoneum at this stage suggested that the duration of action could be even longer. In view of this anti-angiotensin activity in the pithed rat it was decided to investigate the action of disulfiram in experimental hypertension in the rat.

Two experimental hypertension models were used. The first, originally described by Grollman (1944) involved the tying of a 'figure-of-eight' ligature around the left kidney followed by the removal of the contralateral kidney one week later. The perinephritis induced by the ligature has been shown by a number of authors to lead to a high level of renin in the ligatured kidney coupled with a low renin level in the contralateral kidney. Removal of the contralateral kidney leads to a 'normalisation' of the renin level in the ligatured kidney (Tobian, 1960; Gross, Brunner & Ziegler, 1965).

The second model used was a steroid hypertension produced by desoxycorticosterone acetate (DOCA) and saline administration following unilateral nephrectomy. This procedure has been shown to lead to a virtual depletion of renin in the remaining kidney (Tobian, Tomboullian & Janeck, 1959).

The effects of prolonged administration of disulfiram has been examined in both types of experimental hypertension and the results are given in this chapter.

RESULTS

The Effect of Disulfiram on the blood pressure of Normotensive Rats

The effects of prolonged administration of disulfiram to normotensive rats is shown in Fig. 48. The drug was injected intraperitoneally on alternate days for three weeks. There was a progressive rise in blood pressure of the control rats and this rise was indistinguishable from the increase in blood pressure of the treated rats.

Disulfiram at both dose levels (50 & 100 mg/kg i.p.) reduced the increase in body weight of the treated groups compared to that of the control group (Table 6). On withdrawal of the disulfiram treatment there was an immediate weight gain of approximately 40g/rat over the following week. However body weights of the animals in the treated groups remained significantly below those of the control animals for the 7 weeks duration of the experiment.

The Effect of Disulfiram on the Development of DOCA/saline Hypertension in the Rat

The effects of two dose levels of disulfiram (50 & 100 mg/kg) on the development of DOCA/saline hypertension in the rat were examined. Groups of 8 male Wistar rats were used, the animals receiving disulfiram at 2 days intervals starting the day prior to unilateral nephrectomy and implantation of the DOCA (25 mg) implant. In addition to examining two doses of disulfiram the effect of the route of administration was observed groups receiving the drug intraperitoneally or per os.

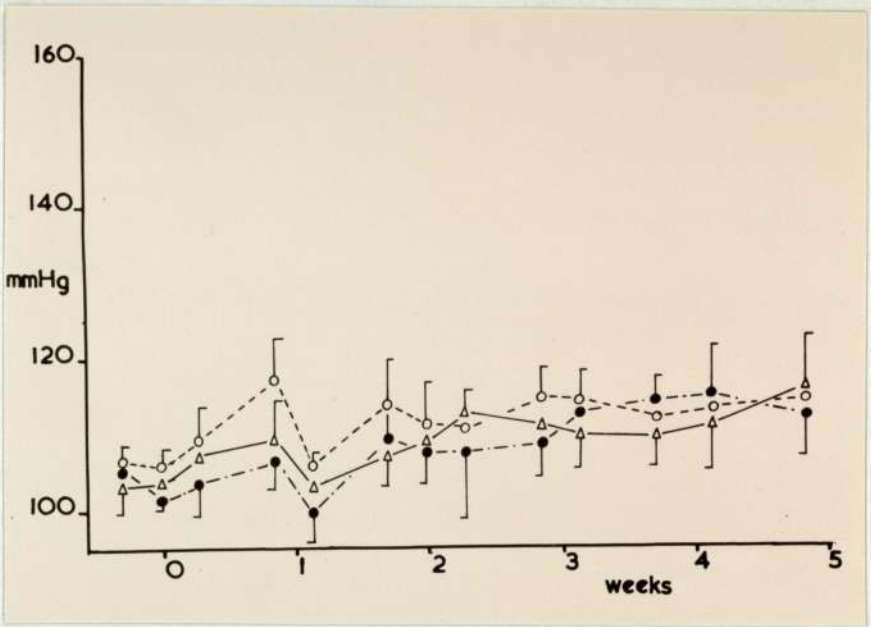


Fig. 48. The effect of disulfiram on the systolic blood pressure of the conscious normotensive rat. Disulfiram was administered at two dose levels, 50 (o) and 100 mg/kg (●) intraperitoneally in suspension for the first three weeks of the above record.
The blood pressures (mean ±SE) of the treated groups (n=8) are compared with those of a control group treated with the vehicle alone (mean ±SE indicated by the solid lines).
Blood pressure readings were obtained indirectly from the caudal artery.

Week	0	1	2	3	4	5	6	7
Control Normotensive	108 ± 4	132 ± 6	170 ± 7	201 ± 4	206 ± 7	239 ± 7	262 ±10	290 ±12
Normotensive + Dis. 50	118 ±11	124 ±13	147 * ±11	159 ± 7	197 ± 5	219 ± 6	232 ± 7	239 ± 9
Normotensive + Dis. 100	107 ± 5	109 ± 4	131 * ± 6	142 ±20	186 ±21	199 ±16	211 ±20	202 ± 9
Control Hypertensive	122 ± 2	131 ± 2	158 ± 3	183 ± 3	218 ± 5	234 ± 4	258 ± 5	269 ± 5
Hypertensive + Dis. 50(2)	113 ± 4	122 ± 5	144 ± 6	146 ± 6	201 ± 7	208 ± 9	213 ±10	223 ± 9
Hypertensive + Dis. 100(2)	114 ± 3	131 ± 3	159 ± 4	165 ± 2	187 ± 5	190 ±11	200 ±13	198 ±16
Hypertensive + Dis. 50(1)	120 * ± 5	130 ± 6	148 ± 7	160 ± 9	186 ±20	240 ± 6	261 ± 7	277 ±14
Hypertensive + Dis. 100(1)	109 * ± 4	113 ± 2	136 ± 7	155 ± 8	189 ± 9	214 ± 9	232 ±13	257 ±13

Table 6.

Body weight changes as a result of the development of Grollman hypertension and the effects of treatment with disulfiram (50 & 100 mg/kg i.p.) over the first week of the hypertension (1) and subsequent to the first week of the hypertension (2). Normotensive animals were treated with disulfiram for the initial three weeks of the experiment. *indicates the point of withdrawal of the disulfiram.

The results of these experiments are shown in Figs. 49 & 50. As can be seen from these figures, disulfiram, at the dose and routes used did not significantly alter the rate of development or the extent of the hypertension produced by the DOCA/saline treatment. On the contrary, disulfiram (50 & 100 mg/kg) administered orally appeared to produce a slight, and during weeks 2-3 significant, increase in the hypertension (Fig. 50).

The Effect of Disulfiram on the Development of Renal (Grollman) Hypertension in the Rat

A number of authors have demonstrated elevated rat kidney renin levels during the week following the application of a Grollman ligature to one kidney and prior to the removal of the contralateral kidney (Tobian, 1960; Gross et al, 1965). It was decided, therefore, to examine the effect of inhibition of the pressor actions of angiotensin during two periods in the induction of 'Grollman' renal hypertension. Thus disulfiram (50 & 100 mg/kg i.p.) was injected on alternate days during (a) the week following the application of the 'figure-of-eight' ligature, and (b) four weeks after the removal of the contralateral kidney.

Groups of 8 male Wistar rats were used. The systolic blood pressures were measured indirectly by a tail-cuff method. The results of these experiments are shown in Figs. 51 & 52.

A feature of the developing Grollman hypertension was a transient rise in blood pressure occuring during the week following the tying of the 'figure-of-eight' ligature, coinciding with the previously reported increased kidney renin levels. Disulfiram (50 & 100 mg/kg i.p.) treatment during this period abolished the initial rise but did not significantly alter the subsequent development of hypertension (Fig. 51).

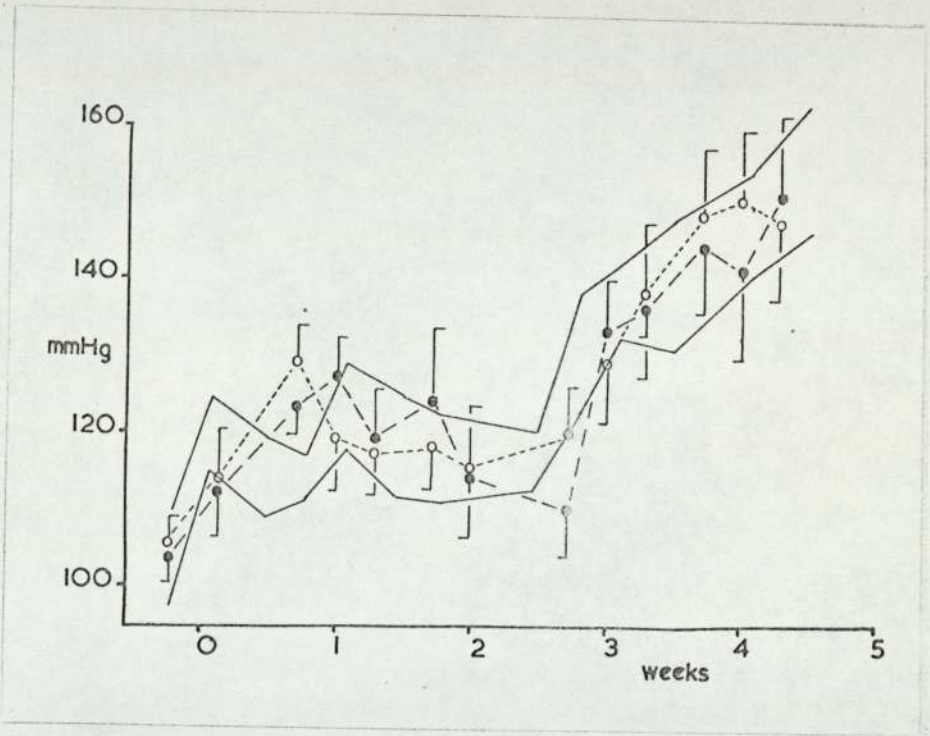


Fig. 49. The effect of disulfiram on the development of DOCA/saline hypertension in the rat. Disulfiram at two dose levels, 50 (o) and 100 mg/kg (●) was injected intraperitoneally for the duration of the experiment, starting at time zero. The blood pressures (mean ±SE) measured indirectly from the caudal artery, are compared with those of the control 'DOCA hypertensive' group (n=8) indicated by the solid lines.

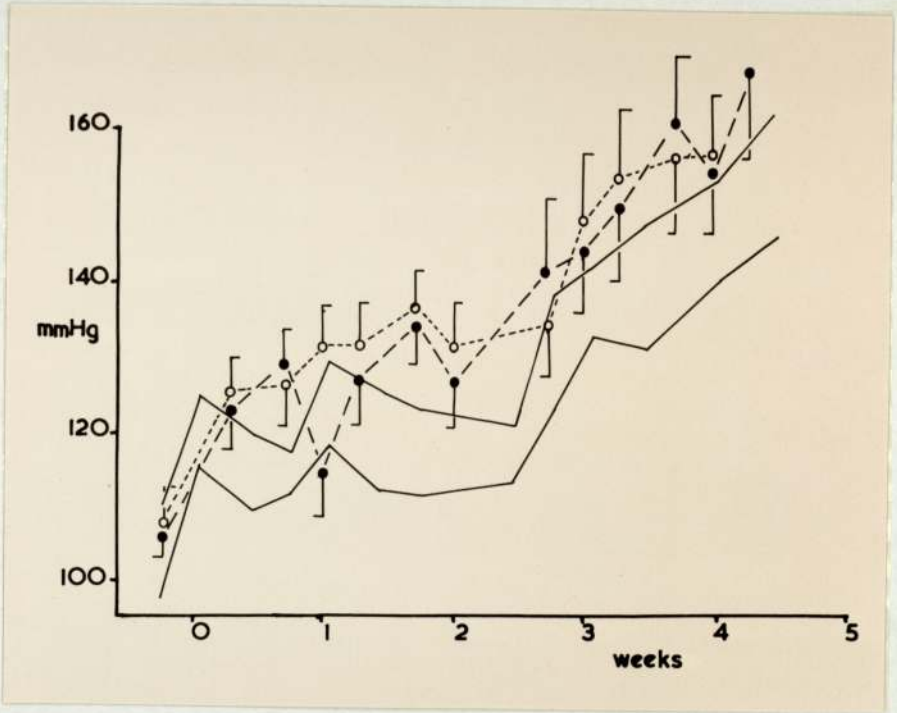


Fig. 50. The effect of disulfiram on the development of DOCA/saline hypertension in the rat. Disulfiram at two dose levels, 50 (o) and 100 mg/kg (●) was injected per os for the duration of the experiment, starting at time zero. The blood pressures (mean ±SE) measured indirectly from the caudal artery, are compared with those of the control 'DOCA hypertensive' group (n=8) indicated by the solid lines.

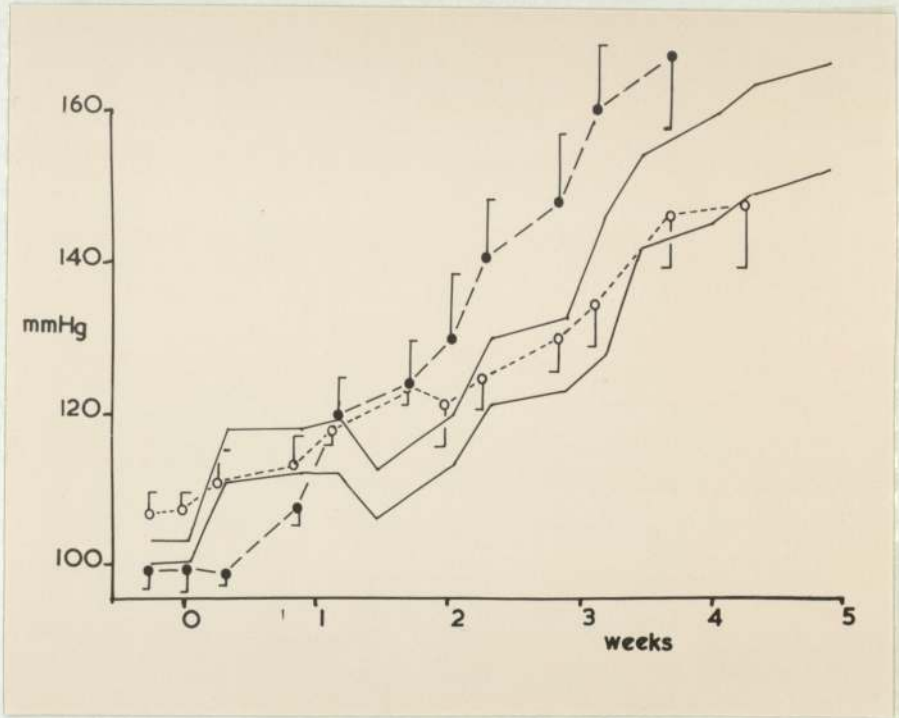


Fig. 51. The effect of disulfiram on the development of Grollman hypertension in the rat. The 'figure-of-eight' ligature was applied at Time 0 and the contralateral kidney removed after 1 week.
Disulfiram (50 (o) and 100 mg/kg (●) i.p.) was injected during the first week of the experiment.

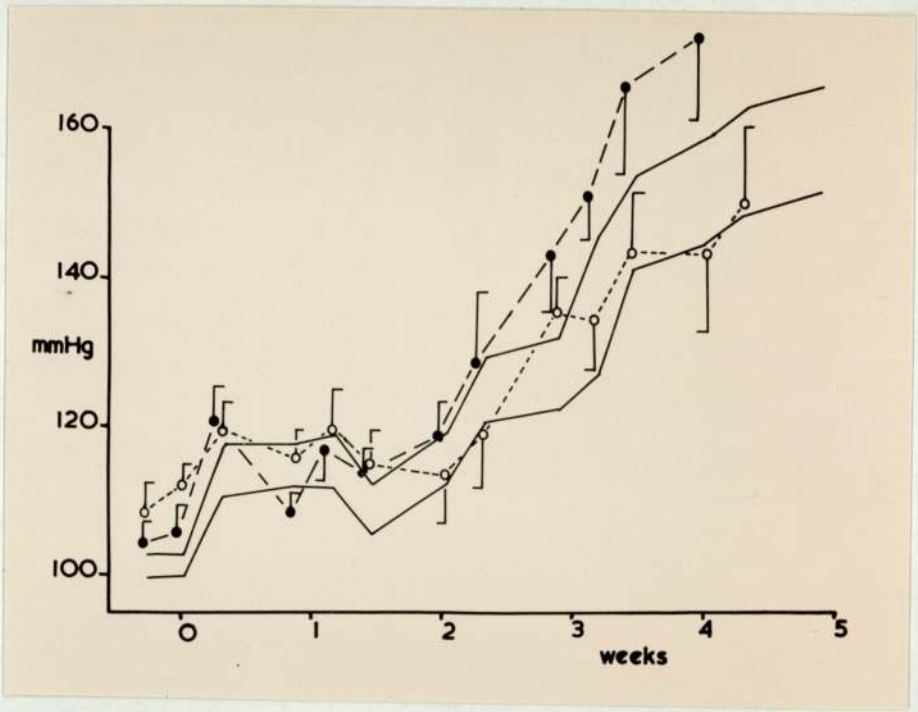


Fig. 52. The effect of disulfiram on the development of Grollman hypertension in the rat. Disulfiram (50 (o) and 100 mg/kg (●) i.p.) was injected for 4 weeks following the removal of the contralateral kidney at week 1. Blood pressure was recorded indirectly from the caudal artery.

Prolonged administration of disulfiram for 4 weeks subsequent to the unilateral nephrectomy did not inhibit the development of the hypertension and may even have exacerbated it (Fig. 52).

As was the case with the disulfiram-treated normotensive animals, disulfiram at both dose levels, reduced the rate of weight gain of the hypertensive rats compared to that noted for the hypertensive controls (Table 6). This was only significant, however, following the prolonged treatment of several weeks following nephrectomy, the groups subjected to only one week's dosing suffering no reduction in weight gain.

The Acute Effects of Disulfiram on the Blood Pressure of the Conscious Hypertensive Rat

The time course of the inhibitory action of disulfiram on the pressor responses to angiotensin in the pithed rat was outlined in Chapter 1. It was considered of interest in the light of these results to establish what effect disulfiram, at doses known to inhibit responses to angiotensin in the pithed rat, had on the blood pressure of the conscious hypertensive rat.

Fig.53 shows the effect of a single dose of disulfiram (50 mg/kg i.p.) on the systolic blood pressure of the conscious restrained rat. The blood pressures were measured indirectly from the caudal artery. As may be seen from the figure, disulfiram produced a hypotensive effect in both Grollman and DOCA/saline hypertensive rats. In the case of the Grollman rats, the hypotensive effect of the disulfiram was maximal 2 h after the injection and recovery was virtually complete after 8 h. The peak effect occurred somewhat later, after 5 h in the case of the DOCA/saline rats but again there was almost complete recovery after 8-9 h. The degree of hypotension produced in each case was similar, being approximately 30 mmHg at its maximal.

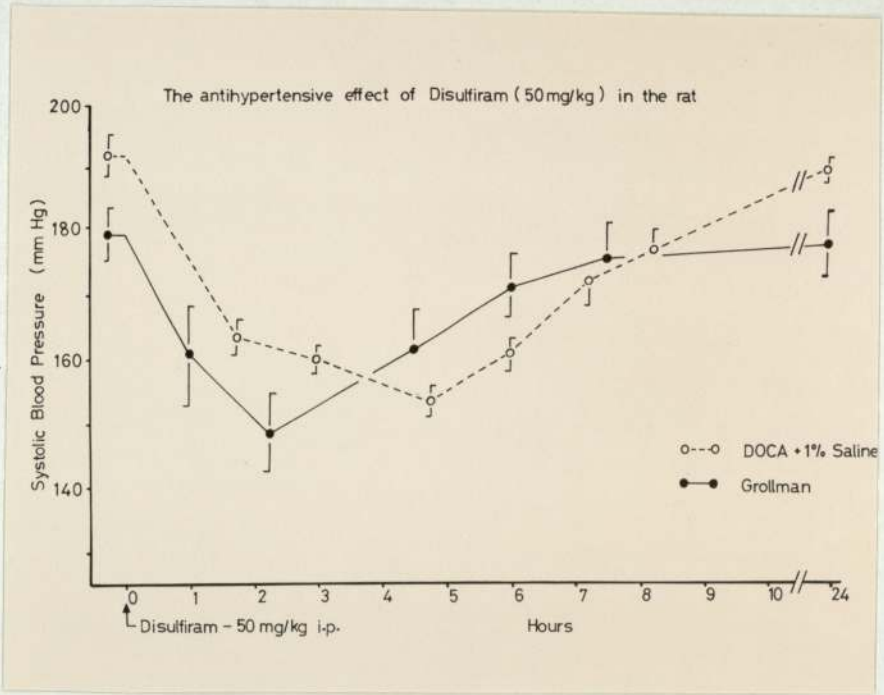


Fig. 53. The effect of a single injection of disulfiram (50 mg/kg i.p.) on the blood pressure of DOCA/saline and Grollman hypertensive rats (Group size - 5). Blood pressures were recorded indirectly from the caudal artery.

DISCUSSION

With the possible exception of the early stages of Grollman hypertension, the disulfiram treatments described did not retard the development of either DOCA or renal hypertension in the rat. This suggests one of two alternatives. Either the treatments described were ineffective in their desired inhibition of the pressor actions of endogenous angiotensin or the treatments were effective but the peptide was playing no significant part in the development of the hypertension.

Contrary to these findings, Crossley, Defeo & Defianti (1969) reported that the metabolite of disulfiram, DDC (66·5 mg/kg on alternate days) caused a significant reduction in the blood pressure of unilaterally nephrectomised or Goldblatt hypertensive rats. This fall in blood pressure they correlated with increased dopamine levels in the urine. The report of these results did not state the time interval which elapsed between injection of the DDC and the indirect measurement of blood pressure. A possible explanation of the 'anti-hypertensive' effect of DDC reported by these workers, not repeated in the present work, appears to present itself. Should Crossley et al. have recorded blood pressures within 2-4 h following injection of the DDC then they would have observed the acute lowering of blood pressure noted in the present chapter.

In Chapter 1 it was shown that the anti-angiotensin activity of disulfiram (50 mg/kg i.p.) persisted for at least 8 hours. It was not established, however, whether this effect persisted for periods of the order of 24 hours. It would appear, therefore, that the experimental design used in the work described here, where the drug administration and blood pressure measurements were carried out on alternate days,

might not have been ideal for establishing an anti-hypertensive action of disulfiram. It was felt, nevertheless, that it was desirable to avoid the confusion in the interpretation of the blood pressure readings that would have occured had these readings been taken at a time when the acute hypotensive activity of disulfiram (Fig. 53) would be apparent. Since the time course of this hypotensive action of disulfiram appeared to be unrelated to that of the anti-angiotensin activity of the disulphide albeit in the pithed rats the two events would appear to be unrelated. There is, in fact, a good correlation between the acute hypotensive effect of disulfiram with the behavioural depression (locomotor activity) observed by Moor & Rech (1969). These authors showed that the depression was associated with a specific depletion of noradrenaline in the brain. The possibility thus exists that the acute fall in blood pressure in hypertensive rats due to disulfiram may be due to a similar depletion.

In the absence of direct evidence to suggest that the anti-angiotensin effect of disulfiram would be present at the time of the blood pressure recording it is felt that even an inhibition of the peptide for 8 to 12 h in a 48 h period might produce a retardation in the development of hypertension should angiotensin be playing a significant role. Since it has been shown that disulfiram treatment did not, in fact, retard the development of either DOCA/saline or Grollman hypertension it would appear that the pressor actions of angiotensin are of limited importance in the aetiologies of these states.

This finding is not at variance with work concerned with renin/angiotensin levels in these experimental hypertensions in rats. DOCA/saline treatment has been shown to produce a considerable reduction in renal and plasma renin levels (Tobian

et al, 1959; Pettinger, Marchelle & Augusto, 1971). Renal hypertension produced by the Grollman method produces a more complicated renin picture. A number of workers have shown that increased renin concentrations develop in the ligatured kidney and this is associated with a fall in the renin level in the contralateral 'normal' kidney (Tobian, 1960; Gross et al, 1965). The elevated renin levels reported to occur in the ligatured kidney were said to be at their greatest 3-5 days after the placing of the ligature and return to pre-ligature levels at the end of 7 days. This may have been the cause of the transient rise in blood pressure observed during the first week of the Grollman hypertension described in this chapter. This rise was prevented by treatment with disulfiram (50 & 100 mg/kg) possibly due to an inhibition of the pressor actions of angiotensin by the disulphide. Inhibition of this early phase did not, however, subsequently retard the development of the hypertension.

From the results presented in this chapter, though they are by no means conclusive, it would appear that the pressor action of angiotensin per se does not contribute significantly to the development of the hypertensions described. Recently, however, a hypothesis has been presented linking once again the renin/angiotensin system with hypertension. Czyzewski & Pettinger (1973) have shown that in the spontaneously hypertensive rat, but not the normotensive rat, that at the age of 40 weeks DOCA/saline treatment completely fails to suppress renin release. This situation, the authors suggested, was analagous to that occuring in human malignant hypertension. The inference is, therefore, that the hypertension occuring in the human malignant state and the spontaneously hypertensive rat is due to increased renin/angiotensin activity as a result of a decrease in feedback control.

Chapter 6

AN INVESTIGATION OF A NUMBER OF UNRELATED COMPOUNDS FOR POSSIBLE ANTI-ANGIOTENSIN ACTIVITY IN THE PITHED RAT

The mechanism involved in the inhibition of pressor responses to angiotensin in the pithed rat by disulfiram is largely unknown. The original investigation of the possible anti-angiotensin action of the chelating agent DDC by Day & Owen (1969) was based on the hypothesis proposed by Gascon & Walaszek (1966) that the peptide acted on smooth muscle receptors in a chelated form with zinc or copper ions. This proposal arose from an observation by Gascon & Walaszek that osajin, an isoflavone derivation known to chelate these ions, specifically antagonised the contractile action of angiotensin on the guinea pig isolated ileum. However, following work by Day & Owen (1969) the likelihood of chelation being involved in the inhibition of the acute cardiovascular effects of angiotensin in the pithed rat by DDC seemed more remote. These authors demonstrated the lack of anti-angiotensin activity amongst the structurally unrelated compounds penicillamine, ascorbic acid and colchicine, all three of which were known to be chelators of zinc and copper (Chaberek & Martell, 1959; Doornbos & Faber, 1964).

The demonstration of the anti-angiotensin properties of disulfiram was no surprise when it is considered that DDC is said to be a major metabolite of the disulphide (Stromme, 1965a). This activity of disulfiram was shown to be more reproducible than that of DDC (Chapter 1) and it was consequently considered that disulfiram might be the active structure. If this was the case, then it would be necessary for DDC to be oxidised in vivo to disulfiram. A possible pathway for this oxidation involving

cytochrome-C has been suggested by Stromme (1963).

Disulfiram has been reported as inhibiting a wide range of enzymes including succinic dehydrogenase (Keilin & Hartree, 1940), liver aldehyde oxidase (Kjeldgaard, 1949) and glyceraldehyde-3-phosphate dehydrogenase (Graham, 1951). This broad enzyme-inhibitory activity is probably due to the high redox potential shown by disulfiram (Eldjarn & Pihl, 1960). Since the blocking of essential sulphydryl groups is probably the common mechanism for the inhibition of all these enzymes, it seems reasonable to assume that disulfiram is an inhibitor of all 'sulphydryl enzymes'. Possibly, disulfiram also interferes with the function of cofactors with essential sulphydryl groups such as co-enzyme-A and thioctic acid.

The possibility therefore exists that the anti-angiotensin activity of disulfiram and, indirectly that of DDC (via conversion to disulfiram), might be due to its ability to block sulphydryl groups. This hypothesis was supported by the report of Page & Green (1949) who showed that dimercaprol (British Anti Lewisite), a very active inhibitor of sulphydryl groups, produced what they termed a 'refractoriness' to angiotensin in the anaesthetised dog.

The first part of this chapter is thus concerned with experiments designed to confirm or otherwise the lack of anti-angiotensin activity in the pithed rat of the chelating agents penicillamine and ascorbic acid. This is followed by a description of the effects of the sulphydryl inhibitor dimercaprol on responses to angiotensin, noradrenaline and sympathetic outflow stimulation in the pithed rat.

The final part of the chapter involves a brief investigation of the actions of neotetrazolium in the pithed rat. This

compound had been recently reported as being capable of inhibiting the vasopressor actions of angiotensin in the pentolinium treated vagotomised rat (Chryssanthou et al, 1971).

RESULTS

Penicillamine

Penicillamine (25-500 mg/kg i.v.) produced an enhancement of responses to angiotensin (50-100 ng/kg), noradrenaline (100-150 ng/kg) and sympathetic outflow stimulation (0·5 Hz) in five pithed rats. As shown in Fig. 54 the enhancement appeared as an increase in the height of the responses with a similar increase in duration. Penicillamine in doses in excess of 100 mg/kg also produced a slight (5-10 mm Hg) rise in mean blood pressure principally due to an increase in systolic pressure. These effects were seen for periods of 1-3 h after which time, responses returned to pre-treatment levels. Subsequent injections of penicillamine at this stage produced further increases in the magnitude of responses to all three pressor procedures. In no experiment was a reduction in responses to angiotensin recorded.

Ascorbic Acid

Ascorbic acid (25-200 mg/kg i.v.) produced an enhancement of responses to angiotensin (50 ng/kg), noradrenaline (100 ng/kg) and sympathetic outflow stimulation (0·5 Hz) similar to that seen following treatment with penicillamine. The ascorbic acid generally caused an increase in systolic blood pressure as can be seen in Fig. 55. The enhancement of responses to the three pressor challenges was dose-dependent and was of the order of a 150-200% increase following 200 mg/kg. The effects of the ascorbic acid were observed for up to 4 h following its injection.

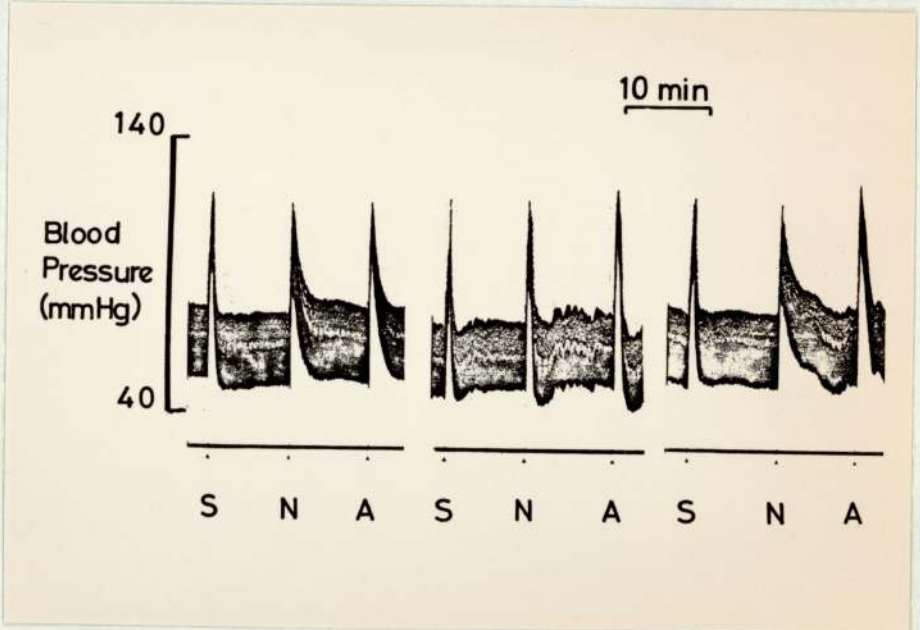


Fig. 54. Pithed rat blood pressure showing responses to sympathetic outflow stimulation (0·5 Hz at S), noradrenaline (100 ng/kg at N) and angiotensin (50 ng/kg at A). The first panel shows control responses and the centre and final panels show responses 1 and 4 hours respectively after penicillamine (50 mg/kg i.v.)

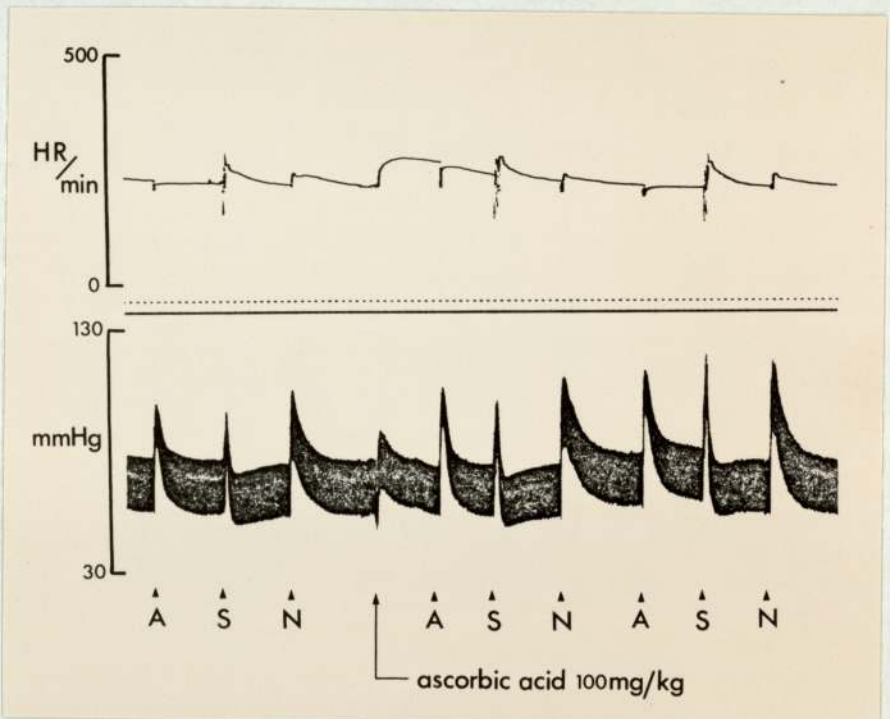


Fig. 55. Pithed rat blood pressure (lower record) and heart rate (upper record). The effect of ascorbic acid (100 mg/kg i.v.) on pressor responses to angiotensin (50 ng/kg at A), sympathetic outflow stimulation (0·5 Hz at S) and noradrenaline (100 ng/kg at N). Time marker - minutes.

Dimercaprol

In six experiments, dimercaprol (2,3-Dimercapto-1-propanol) produced a dose-dependent hypotensive effect illustrated in Fig. 56. Following 10 mg/kg (aqueous emulsion, i.p.) there was a slight (8-12 mm Hg) fall in blood pressure becoming maximal after 15-30 min. This was accompanied by a decrease in responses to angiotensin (50 ng/kg), noradrenaline (100 ng/kg) and sympathetic outflow stimulation (0·5 Hz). The blood pressure and the vasopressor responses returned to control levels after approximately 90 min, after which time responses to angiotensin were enhanced by 5-15% (Fig. 56) and those to noradrenaline and sympathetic stimulation by 20-50% for the duration of the remainder of the experiments.

A similar pattern of response was seen following a higher dose of dimercaprol (20 mg/kg i.p.). However, following 40 mg/kg there was a marked fall in resting blood pressure becoming apparent within a few minutes of the injection and persisting for up to 5 h (Fig. 56). This was accompanied by an approximately 50% reduction in responses to angiotensin, noradrenaline and sympathethic outflow stimulation.

Neotetrazolium

The action of neotetrazolium (0·5 - 4·0 mg/kg i.v.) on blood pressure and pressor responses to angiotensin (50 ng/kg), noradrenaline (100 ng/kg) and sympathetic outflow stimulation (0·5 Hz) was studied in 6 pithed rats. Intravenous neotetrazolium appeared to behave as a weak α-adrenergic agonist, a dose response curve obtained in one preparation being shown in Fig. 57. The pressor responses to neotetrazolium were abolished by phentolamine (1 mg/kg i.v.). In addition to this agonist activity, neotetrazolium produced at low doses (0·5-1·0 mg/kg)

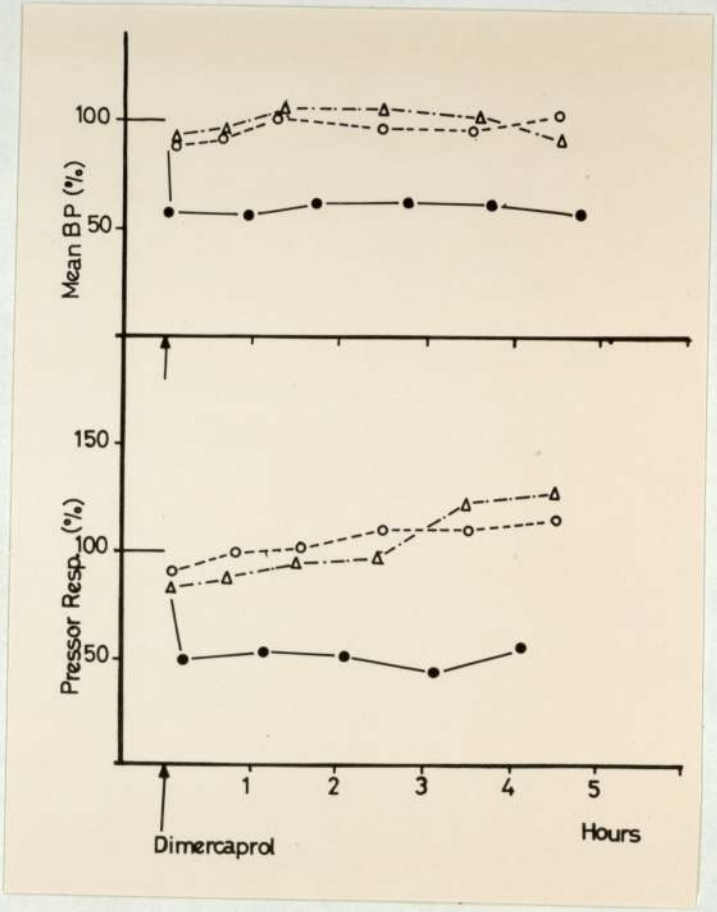


Fig. 56. Pithed rat blood pressure and pressor response to angiotensin (50 ng/kg) expressed as percentage of pre-treatment responses. The effect of three dose levels of dimercaprol (aqueous emulsion - i.p.) is shown. (o) represents the effect of 10 mg/kg; (Δ) the effect of 20 mg/kg and (●) the effect of 40 mg/kg.

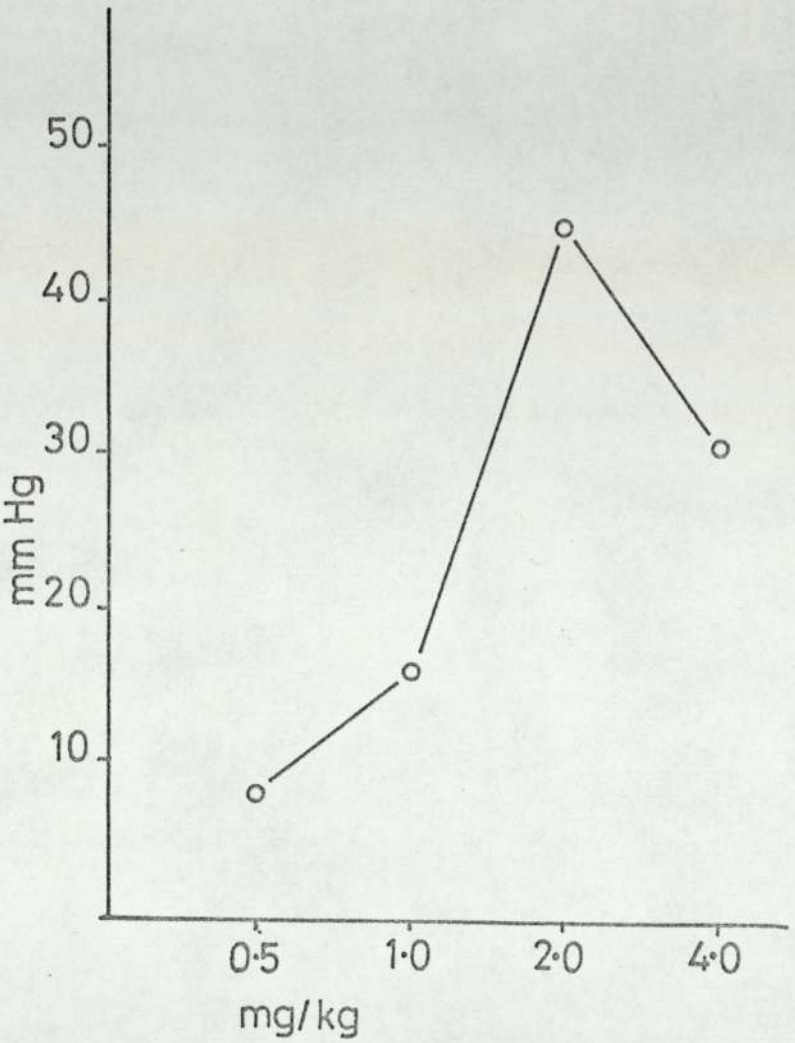


Fig. 57. Pithed rat blood pressure. Vasopressor responses to increasing doses of neotetrazolium. Time intervals between doses - 30 minutes.

a relatively brief (30-60 min) reduction (20%) in responses to sympathetic outflow stimulation whereas at higher doses (2·0-4·0 mg/kg) the inhibitory activity extended to include responses to noradrenaline and angiotensin, the inhibition persisting in excess of 2 h.

DISCUSSION

The results presented in the first parts of this chapter were obtained in an effort to elucidate the anti-angiotensin activity of disulfiram and its metabolite DDC. Two aspects were investigated. The first was concerned with the chelating activity of DDC (Thorn & Ludwig, 1962) and the second, the blocking of protein sulphydryl groups due to the formation of mixed double sulphides by disulfiram (Stromme, 1963). The chelating activity shown by DDC has been reported as being a property of the thiocarbamate and not shown by the disulphide, disulfiram (Thorn & Ludwig, 1962). Conversely, the ability to combine with active sulphydryl groups appears to be a facet of the activity of disulfiram but not DDC (Stromme, 1963). It was hoped therefore that the work described above would indicate the mechanism of the angiotensin inhibition and as a corollary, the active form of the antagonist. Results presented in Chapter 1 indicated that although both DDC and disulfiram had antagonist activity towards the pressor actions of angiotensin in the rat, the antagonism was more readily demonstrated in the case of disulfiram. This was in spite of the fact that Stromme (1965a) had suggested that free disulfiram *per se* did not exist in vivo and therefore DDC was an obligatory and rapidly produced metabolite. Two reasons for the differences in activity of equivalent doses of DDC and disulfiram were considered. The first involved the relative rates of elimination of the two forms.

Stromme (1965a) had shown using ^{35}S DDC and disulfiram in rats that 47% of an intraperitoneal dose of DDC was excreted via the kidneys and lungs (CS_2) in 4 h whereas the value for disulfiram under the same conditions was 23%. The second possibility involved the mechanism of action of DDC/disulfiram, it being suggested that disulfiram might be the active form. This would then require the formation of disulfiram from DDC which could possibly explain the variability of the anti-angiotensin action of the latter compound.

The inability to demonstrate any inhibition of pressor responses to angiotensin in the pithed rat by such structurally dissimilar chelating agents as penicillamine and ascorbic acid would seem to confirm that the chelating properties of DDC were not responsible for its inhibitory activity. All three agents, however, produced an enhancement of responses to angiotensin, noradrenaline and sympathetic outflow stimulation. Copper chelation, in particular, has been reported to enhance pressor responses to adrenaline (Fischer, Lecomte & Delandtsheere, 1950). It may be not unreasonable to suggest, therefore, that the enhancement of responses to the pressor challenges by the three chelating agents may be due to a common mechanism, possibly involving the complexing of copper or a similar ion.

The non-specific reduction in blood pressure and responses to pressor agents due to dimercaprol bore little resemblance to the specific reduction of responses to angiotensin by disulfiram described in Chapter 1. This would seem to cast doubt on the possibility that inhibition of active sulphydryl groups by disulfiram is involved in its anti-angiotensin actions. Nevertheless, the possibility cannot be ruled out completely on this evidence. The fact that dimercaprol was

shown to inhibit pressor responses to angiotensin, admittedly non-specifically, is perhaps still significant as this sort of activity is rarely seen. Perhaps the problem could be solved by experiments designed to estimate the degree of blockade of free sulphydryl groups in vivo during the course of a disulfiram-induced inhibition of pressor responses to angiotensin in the pithed rat.

Somewhat unrelated to work discussed so far are the experiments described in this chapter relating to the vascular effects of neotetrazolium. This compound has been described as having the ability to block α-adrenergic receptors and to inhibit vasopressor responses to angiotensin in the pentolinium-treated, vagotomised rat (Chryssanthou et al, 1971; Nelson, Chryssanthou & Antopol, 1969). The inhibition of responses to angiotensin, these authors suggested, was due in part to an inhibition of an adrenergic component of the response and due in part to a direct inhibitory action of neotetrazolium on the angiotensin receptor. They further showed that treatment with phentolamine partially reduced responses to angiotensin in their rats. This last observation was at variance with results obtained in the pithed rat by Schmitt & Schmitt (1968) and confirmed in the present work (Chapter 2) that the vasopressor response to angiotensin was devoid of an adrenergic component under most conditions. It was decided, therefore, to briefly investigate the properties of neotetrazolium in the pithed rat. The compound was subsequently shown to behave as a weak partial α-adrenergic antagonist. Allowing sufficient intervals between doses (in excess of 30 min) it was possible to demonstrate a dose response relationship when neotetrazolium was used as an agonist. The pressor responses to neotetrazolium were totally abolished by

phentolamine treatment indicating their adrenergic nature. Neotetrazolium also exhibited weak α-adrenergic blocking activity. At high doses, however, there appeared a non-specific depression of responses to angiotensin, noradrenaline and sympathetic outflow stimulation. It was felt, however, that this was not significant and no further investigation was warranted.

PART IV

GENERAL DISCUSSION

GENERAL DISCUSSION

For many years, a specific inhibitor of the pharmacological and physiological actions of angiotensin has been sought. A number of compounds have been said to fulfil the necessary requirements for such an inhibitor but which have been subsequently found wanting. It was, therefore, of great interest when Day & Owen (1969) reported that sodium diethyldithiocarbamate (DDC) 'specifically' inhibited the acute pressor response to angiotensin in the pithed rat. It was further stated that this effect could be demonstrated in the anaesthetised adrenalectomised cat but not in the intact anaesthetised cat. The present work was undertaken in the hope of characterising the anti-angiotensin effects of DDC and to elucidate its mechanism of action.

The period covered by the course of these investigations was one of great activity in the peptide field resulting in a number of angiotensin analogues which have been shown to be competitive antagonists of the parent compound in several test situations. Although it would appear, therefore, that the objects of the work which forms the subject of this thesis has been eclipsed by the development of these analogue antagonists, a number of intrinsically interesting observations have been made.

Experiments were designed with two aims in mind. The first objective was to determine whether or not the anti-angiotensin activity of DDC reported by Day & Owen (1969) and repeated in the present work. extended into other systems where angiotensin was active. Thus, the effects of the inhibitors were examined on the vasopressor actions of the peptide in the cat, its spasmogenic actions on intestinal smooth muscle and on

the possible involvement of angiotensin in the development of hypertension in the rat. The second aim was to elucidate, if possible, the mechanism involved in the inhibition in the pithed rat. In order to achieve this, the effects of structurally dissimilar compounds with shared properties with DDC were examined. Furthermore, the possible involvement of the sympathetic nervous system was studied.

The inhibitor effect of DDC on the pressor responses to angiotensin in the pithed rat was rather variable and no dose-response relationship was established. Following the observation that disulfiram exhibited similar anti-angiotensin properties to those of DDC it became evident that the activity of disulfiram was more reproducible and a dose-response relationship was established. In the course of this work, it was shown that the initial enhancement of pressor responses to angiotensin and noradrenaline or sympathetic outflow stimulation was produced by doses of disulfiram which did not subsequently produce a reduction in responses to the peptide. Furthermore, penicillamine and ascorbic acid were found to mimic the enhancement produced by low doses of disulfiram. Since the common property of these two compounds and DDC, produced as a metabolite of disulfiram (Stromme, 1963), was one of chelation, this appeared to be the mechanism involved in the enhancement. This suggestion was supported by published work showing the enhancement of pressor responses to adrenaline following the chelation of copper ions (Fischer, Lecomte & Delandtsheere, 1964). If this were, in fact, the case it would be necessary for the vasopressor actions of angiotensin to involve a sympathetic or adrenergic component, a possibility not borne out by other work described in this thesis. Alternatively, the effects of angiotensin on vascular

smooth muscle may require a similar mechanism to that required for noradrenaline.

The latent period that elapses before the reduction of pressor responses to angiotensin in the rat appears to be the same for DDC and disulfiram. This indicates that metabolism and/or transport of DDC or disulfiram is not likely to be the onset limiting factor. The reason for the 3 h latent period remains a mystery. It may well be that disulfiram, DDC or an active metabolite has to affect some system necessary for the vasopressor effect of angiotensin before inhibition becomes apparent. The long duration of the effect and its apparent lack of reversibility appear to support this concept. The high degree of specificity of the inhibition indicates that the system is independent of the stimulus-contraction coupling mechanims required for the majority of vasoactive hormones such as noradrenaline.

The specificity of action of disulfiram and DDC was rather surprising when considering the wide range of activities of these compounds. At high doses, disulfiram was shown to reduce pressor responses to noradrenaline and sympathetic outflow stimulation in addition to those of angiotensin. At lower doses only responses to the peptide were reduced. Furthermore, pressor responses to 5-hydroxytryptamine and the peptide, vasopressin, were similarly unaltered by disulfiram treatment at times when pressor responses to angiotensin were considerably reduced. The reduction of pressor responses to sympathetic stimulation following the higher doses of disulfiram was to be expected when considering the well documented depletion of noradrenaline following such treatment (e.g. Thoenen et al, 1966). That this reduction in responses was paralleled by a similar effect on responses to noradrenaline was, however, less expected.

The possibility exists, therefore, that the common reduction in responses was due to an effect on the noradrenaline 'receptor' rather than an pre-synaptic effect on transmitter synthesis or storage. There was no general reduction in reactivity at this dose (100 mg/kg) since responses to 5-hydroxytryptamine were unaffected.

The effect of disulfiram on pressor responses to 5-HT in the pithed rat was of particular interest in view of the report of Mansner et al (1968) describing the lack of reactivity to 5-HT of isolated colons taken from rats treated with disulfiram. This observation was confirmed in the present work. In addition, responses of the colons to angiotensin and acetylcholine were affected by similar pretreatment. This would seem to question the conclusions of Regoli & Vane (1964) who suggested that vascular and colonic receptors to angiotensin were very similar.

On the guinea pig ileum, the direct response to angiotensin after hyoscine treatment was inhibited by DDC added to the organ bath.

Of further interest was the fact that the responses to angiotensin of ilea taken from guinea pigs pretreated with disulfiram appeared to be totally 'direct' in origin. In other words, the indirect cholinergically mediated 'fast' response was missing. There was no apparent difference in overall response between control and treated preparations but whereas responses of the former to the peptide were reduced by hyoscine, those to the latter were not affected. Isometric recording experiments appeared to confirm this observation. The reason for this effect is difficult to imagine, particularly when it is remembered that responses to acetylcholine were unaffected by disulfiram treatment.

Attempts to demonstrate an in vivo inhibition of pressor responses to angiotensin in the cat were unsuccessful. It was not possible to repeat the work of Day & Owen (1969) concerning the effects of acute adrenalectomy on vasopressor responses of the chloralosed cat to the peptide following treatment with DDC. It was hoped to elucidate the role of the adrenal gland in this phenomenon but this remains one of the least satisfactory areas of the current work. Difficulties were experienced in performing adrenalectomies so that the survival time of the preparations was, as a result, rather short. Where the procedure was apparently successful, the resting blood pressure was very low and vascular reactivity to noradrenaline was considerably reduced making definitive studies of any possible reduction in pressor responses to angiotensin impossible.

In the conscious cat, administration of the aldosterone antagonist spironolactone had no effect on responses to angiotensin, tyramine or noradrenaline. The effects of disulfiram in these cats were similar to those seen in untreated cats. The combined administration of α and β adrenoceptor blocking agents to prevent any actions of adrenaline release from the adrenal medulla by angiotensin was similarly ineffectual in 'uncovering' any anti-angiotensin activity of disulfiram. These results would appear to indicate that disulfiram/DDC does not exhibit an anti-angiotensin effect in the cat. No data is available covering the metabolism of these compounds by this species. It may well be that variations in their elimination rates or routes of metabolism may account for this apparent species difference. It may be argued that the vasopressor responses of the cat to angiotensin might differ in origin to those in the rat. Certainly these two species vary in the aldosterone

releasing response of the adrenal cortex to the peptide (Peach, verbal communication, 1972).

The involvement of the renin-angiotensin system in hypertension has been the subject of discussion for many years. The effect of prolonged administration of disulfiram on the development of two models of experimental hypertension was therefore considered of interest. These experiments were complicated by the fact that disulfiram and DDC exert an acute anti-hypertensive effect. From its time-course, this effect appeared to bear no relation to the previously established anti-angiotensin activity of disulfiram. In order to avoid spurious anti-hypertensive effects it was necessary to measure blood pressure on days when no drug administration was performed.

One of the models of hypertension was induced by desoxycorticosterone acetate (DOCA) and saline treatment. The development of DOCA hypertension was not retarded by disulfiram treatment as might be expected in view of the fact that low renal and plasma renin activity has been reported as being a feature of this type of experimental hypertension. More interesting was the similar lack of effect seen in the case of Grollman hypertension. It appeared, however, that the transient hypertension seen to occur during the week following the tying of the 'figure-of-eight' ligature and prior to the extirpation of the contralateral kidney was reduced by disulfiram. This period has been shown to be characterised by a high renin activity, notably in the intact kidney, and that the transient hypertension may have been caused by this activity is borne out by the effect of disulfiram. It is interesting to note, however, that subsequent development of hypertension was unimpaired in these groups.

From the results discussed above, it seems likely that the anti-angiotensin effects of disulfiram and DDC are confined to the pithed rat. It is to be regretted that experiments were not performed to establish the importance of pithing the rat. This procedure may have contributed to the apparent species variation noted above. Successful experiments with the pithed cats might also have been helpful in this context. The mechanism of the anti-angiotensin actions of disulfiram/DDC in the pithed rat nevertheless remains of interest.

Day & Owen (1969) reported that colchicine, ascorbic acid and penicillamine did not selectively inhibit responses to angiotensin in the pithed rat. These results have been confirmed with respect to ascorbic acid and penicillamine. Colchicine was not investigated. The enhancement of pressor responses to angiotensin, noradrenaline and sympathetic outflow stimulation produced by these chelating agents has been discussed previously. Since DDC and disulfiram were unique in subsequently inhibiting responses to angiotensin, it would appear that this activity is independent of chelation.

Early work by Page & Green (1949) showed that vasopressor responses to angiotensin in the dog were reduced by dimercaprol, a chelating agent with powerful sulphydryl binding activity. Since disulfiram also possessed the capability of binding to form mixed double sulphides with sulphydryl groups, it was considered that this might be a possible mechanism for the anti-angiotensin actions of disulfiram. In the pithed rat, dimercaprol produced a dose-dependent fall in resting blood pressure with an associated reduction in pressor responses to angiotensin, noradrenaline and sympathetic outflow stimulation. The contribution of the sulphydryl binding by disulfiram to

its anti-angiotensin activity should not, therefore, be ignored. The lack of specificity of dimercaprol might be due to factors not common to disulfiram.

Investigations of the role of the sympathetic nervous system in the pressor response to angiotensin before and after disulfiram treatment produced possibly the most interesting results of the work reported in this thesis. Several workers, notably Schmitt & Schmitt (1968) have shown that the pressor response of the pithed rat to angiotensin does not involve the release of noradrenaline. This observation has been confirmed by work described in the early part of Chapter 2. A number of drugs which impair adrenergic transmission such as bethanidine, reserpine and phentolamine were shown to enhance pressor responses to the peptide. However, the pressor response to angiotensin appears to be related to, if not directly involve, noradrenergic transmission. The enhancement of responses to noradrenaline by infusions of low doses of the peptide confirms this interaction. The model proposed by Pals & Fulton (1968) appears to go some way in explaining the link. It was interesting to note, therefore, the inhibition of the angiotensin-induced enhancement of pressor responses to sympathetic outflow stimulation by disulfiram. This would seem to support the Pals & Fulton double receptor hypothesis; the same receptor being responsible for the pressor response to angiotensin and its facilitation of noradrenaline responses.

The 'reversal' of the disulfiram-induced inhibition of pressor responses to angiotensin by large intravenous doses of noradrenaline and intravenous α-methyldopa was a little unexpected. However, the nature of the pressor responses to angiotensin following this 'reversal' was different from that

of responses in untreated preparations. This was shown by the fact that phentolamine completely inhibited the former but had previously been shown to enhance the latter. The responses after the 'reversal' appeared to be entirely mediated by noradrenaline. It was not possible to investigate this phenomenon any further but it would have been interesting to determine the effects of an adrenergic neurone blocking agent on the response since it appears to involve a presynaptic action of angiotensin which is not normally evident. A receptor facilitation effect seems unlikely in view of the results obtained with the angiotensin-induced enhancement of responses to noradrenaline and its prevention by disulfiram. Possibly angiotensin is able, in the disulfiram treated rat, to cause a 'leak' of loaded adrenergic stores (following high doses of noradrenaline or methyldopa). The reason this may not be seen in rats to which disulfiram has not been given may be that the circulating angiotensin is avidly taken up by its receptors, a process which is reduced by disulfiram enabling higher concentrations of the peptide to exist at the noradrenergic terminals.

In conclusion, although the need for a specific competitive inhibitor for the many biological activities of angiotensin has been fulfilled to a very great extent by the proliferation of peptide analog antagonists, the work in this thesis remains interesting from the point of view of the apparently unique interaction between disulfiram/DDC and angiotensin in the rat. Of particular importance is the role of disulfiram in the relationship between angiotensin and the sympathetic nervous system in the rat. Hopefully, many of the questions unanswered by this thesis will be answered in the future by the increasing use of the peptide inhibitors.

PART V

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