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Selective inhibition of angiotensin pressor responses in the pithed rat by tetraethylthiuram disulphide (disulfiram) and sodium diethyldithiocarbamate (DDC)

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Summary

1. Pressor responses to sympathetic outflow stimulation, noradrenaline and angiotensin have been recorded in pithed rats.

2. Disulfiram (50 mg/kg) and sodium diethyldithiocarbamate (DDC) (5–100 mg/kg) both caused an initial increase in the pressor response to all three procedures followed by a selective inhibition of the angiotensin responses.

3. Penicillamine (1–100 mg/kg) and ascorbic acid (1–500 mg/kg) increased the pressor responses to all three procedures without any subsequent blocking action.

4. Reserpine (5 mg/kg daily for 3 days) abolished responses to sympathetic outflow stimulation but did not impair angiotensin or noradrenaline responses.

5. In reserpinized rats, the initial enhancement of angiotensin responses after disulfiram and sodium diethyldithiocarbamate was absent or reduced and the onset of the subsequent block was accelerated.

6. Possible mechanisms for the angiotensin-blocking action of disulfiram and sodium diethyldithiocarbamate are discussed.

Introduction

Interest in the search for an antagonist of the contractor action of angiotensin II on smooth muscle centred originally on a series of piperazine derivatives, including cinnarizine (Schaper, Jageneau, Xhonneux, Van Nueten & Janssen, 1963) and lidoflazine (Godfraind, Kaba & Polster, 1966). These compounds were subsequently shown to have a low specificity for angiotensin and the antagonism to be non-competitive (Godfraind, 1968). More recently, Bing & Poulsen (1970) reduced pressor responses to angiotensin II in rats using 'anti-angiotensin II', an immune plasma obtained from rabbits. Marshall, Vine & Needleman (1970) reported that (4-phenylalanine, 8-tyrosine)—angiotensin II was a specific and potent competitive inhibitor of angiotensin II both *in vitro* (rat isolated uterus) and *in vivo* (anaesthetized rat blood pressure). Day & Owen (1970) observed that reserpine reduced by up to 50% the pressor responses to angiotensin in the conscious cat, thus suggesting that neural noradrenaline stores may be involved in the pressor action of angiotensin in this species.

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Schwyzer (1963) reported that angiotensin II formed quantitative amorphous precipitates with copper and zinc ions in aqueous solution. The zinc chelate was shown to be equi-pressor with angiotensin II. Schwyzer suggested that angiotensin II might combine with its 'receptor' in a chelated form and this hypothesis was supported by the observation of Gascon & Walaszek (1966) that osajin, an isoflavone derivative, which also forms complexes with copper and zinc ions, specifically antagonized the contractor action of angiotensin II on guinea-pig isolated ileum. However, osajin was subsequently shown to have no *in vivo* anti-angiotensin activity (Walaszek, personal communication).

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

We have further investigated the actions of sodium diethyldithiocarbamate and penicillamine and have compared them with tetraethylthiuram disulphide (disulfiram), the parent disulphide of the thiol diethyldithiocarbamic acid, and with ascorbic acid and reserpine.

Methods

Pithed rat preparation

Male Wistar rats weighing 180–350 g were anaesthetized with pentobarbitone (Nembutal) (60 mg/kg, i.p.). The trachea was cannulated, artificial respiration started, and the animal pithed by the method of Shipley & Tilden (1947) using a steel pithing rod, 1·5 mm in diameter, prepared as described by Gillespie & Muir (1967). Positive pressure artificial respiration was achieved using a Palmer small animal respirator adjusted to deliver 20 ml/kg body weight in each experiment. The right jugular vein was then cannulated with polythene tubing (Portland Plastics, PP30) previously filled with 0·9% saline containing 10 units/ml heparin. The right common carotid artery was cannulated with polythene tubing (PP30) filled with heparinized saline, and the arterial blood pressure measured by means of a blood pressure transducer (Devices/C.E.C. type 4-327-L221) connected to a Devices M2 recorder. Pressor changes quoted in the text are expressed in terms of changes from the mean pressures (1 mmHg$\equiv$1·333 mbar). In some experiments, heart rate was measured by means of a Devices Instantaneous Ratemeter (Type 2751) triggered by the blood pressure signal.

Electrical stimulation of the sympathetic outflow was carried out as described by Gillespie & Muir (1967). The indifferent electrode, a steel hypodermic needle inserted subcutaneously into the left leg, was connected to one pole of a square wave stimulator (Scientific and Research Instruments Ltd.). The other pole was connected to the pithing rod.

In experiments where stimulation of the sympathetic outflow was performed, 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 ms duration at frequencies of 0·5 or 1·0 Hz applied for periods of 40 s repeated at intervals of 20 or 30 minutes.

Drugs used: Angiotensin II amide (CIBA); ascorbic acid (B.D.H.); atropine sulphate (B.D.H.); disulfiram (Ayerst Labs.); heparin (Evans Medical); (−)-noradrenaline bitartrate (Sigma); penicillamine hydrochloride (Dista); reserpine (injection made by Halewood Chemical Company); sodium diethyldithiocarbamate (B.D.H.); (+)-tubocurarine hydrochloride (Duncan, Flockhart and Evans); vasopressin ('Pitressin', Parke, Davis & Company).

Disulfiram was prepared for injection by suspension in 2% tragacanth in normal saline, using a glass mortar. Intravenous injection volumes were 0·5 ml/kg followed by a flush of 1·0 ml/kg normal saline.

Results

Control experiments

In five preparations, the reproducibility of the pressor responses to sympathetic outflow stimulation (0·5 and 1·0 Hz) and to intravenous injections of angiotensin (50 ng/kg) and noradrenaline (100 ng/kg) was tested for 4 hours. The responses to sympathetic outflow stimulation did not vary by more than ±5% of the initial responses throughout the test period. The responses to angiotensin and noradrenaline increased gradually 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 hours. After 90 min, two of these preparations received an intraperitoneal injection of the vehicle subsequently used in the disulfiram experiments (2% tragacanth, 1 ml/kg); the pattern of responses was not further changed by this procedure. The reason for the progressive increase in the sensitivity to noradrenaline and angiotensin is not clear. A possible explanation is that pentobarbitone, used as an anaesthetic in these experiments, caused an initial decrease in sensitivity to pressor procedures. Gillespie & Muir (1967), who did not report any progressive change in cardiovascular reactivity in their experiments, performed their pithing under ether anaesthesia. In subsequent experiments in which potential angiotensin antagonists were tested, the control responses were repeated for at least 90 min after pithing or until they became constant before the administration of the antagonist.

Tetraethylthiuram disulphide (disulfiram)

Disulfiram produced a dose dependent effect on the pressor responses to sympathetic outflow stimulation, angiotensin and noradrenaline. In the 3 h period after an intraperitoneal dose of 20 mg/kg of disulfiram, the responses to angiotensin remained unchanged, whilst those to sympathetic stimulation and noradrenaline gradually increased by between 10 and 50% of initial responses in different experiments.

Disulfiram (50 mg/kg) 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 a smaller (10–40%) increase in the responses to angiotensin. Subsequently, the responses to angiotensin declined in eight of ten preparations until they were virtually abolished 3–4 h after disulfiram. The responses to noradrenaline and sympathetic stimulation also declined some-

what in the period after the maximal potentiation but remained above control levels at a time when angiotensin responses were abolished (Fig. 1).

At a dose level of 100 mg/kg, disulfiram produced an enhancement of all three control responses similar to that observed after 50 mg/kg. However, in the case of the larger dose, the potentiation was followed by a reduction in the pressor responses to all three control procedures such that in the experiment illustrated in Fig. 2, 150 min after disulfiram, the responses to noradrenaline and sympathetic stimulation were reduced by approximately 50% of the initial responses and the response to angiotensin was abolished (Fig. 2C). Figure 3 summarizes graphically the effects of disulfiram at 3 dose levels on responses to angiotensin, noradrenaline and sympathetic stimulation. Marked and selective inhibition of angiotensin responses occurred 3 h after 50 mg/kg disulfiram (Fig. 3B).

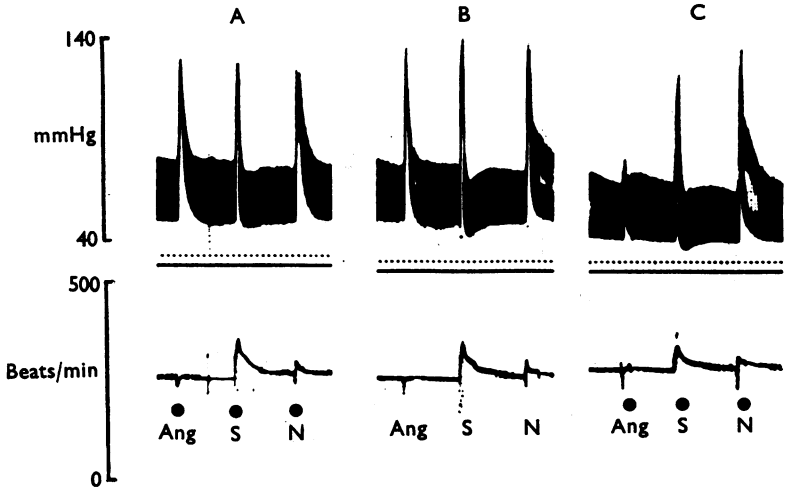

FIG. 1. Pithed rat blood pressure (upper record) and heart rate (lower record). A, Control responses to sympathetic outflow stimulation (0·5 Hz at S), intravenous noradrenaline (100 ng/kg at N) and intravenous angiotensin (50 ng/kg at Ang). B, Responses repeated 2 h and C, 4 h after disulfiram (50 mg/kg i.p.). Time marker in minutes.

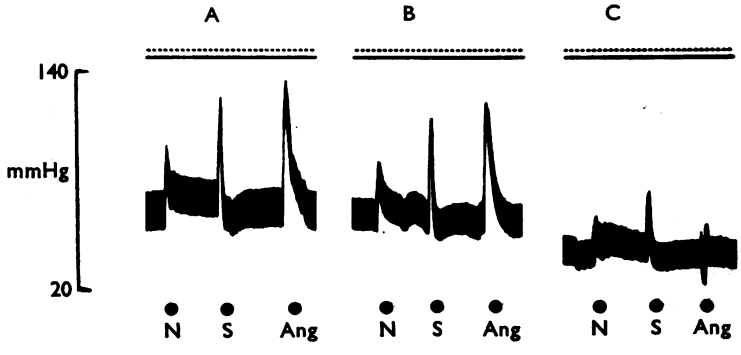

FIG. 2. Pithed rat blood pressure. At N, noradrenaline (100 ng/kg i.v.), at S, sympathetic outflow stimulation (0·5 Hz) and at Ang, angiotensin (50 ng/kg i.v.). In A, control responses; B, 30 min and C, 150 min after disulfiram (100 mg/kg i.p.). Time marker in minutes.

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 course of the next 4–6 h at a similar rate and to a similar extent as in untreated control preparations. In no experiment in which the pressor responses to angiotensin were reduced by disulfiram did the responses recover when observed for periods of up to 6 hours. In three experiments, the selectivity of the blocking action of disulfiram (50 mg/kg) was further tested by comparing its effects on the responses to vasopressin (5 mU/kg) with those to angiotensin (50 ng/kg) and noradrenaline (100 ng/kg). The results of one of these experiments are shown in Fig. 4. Two hours after disulfiram, the responses to angiotensin and noradrenaline

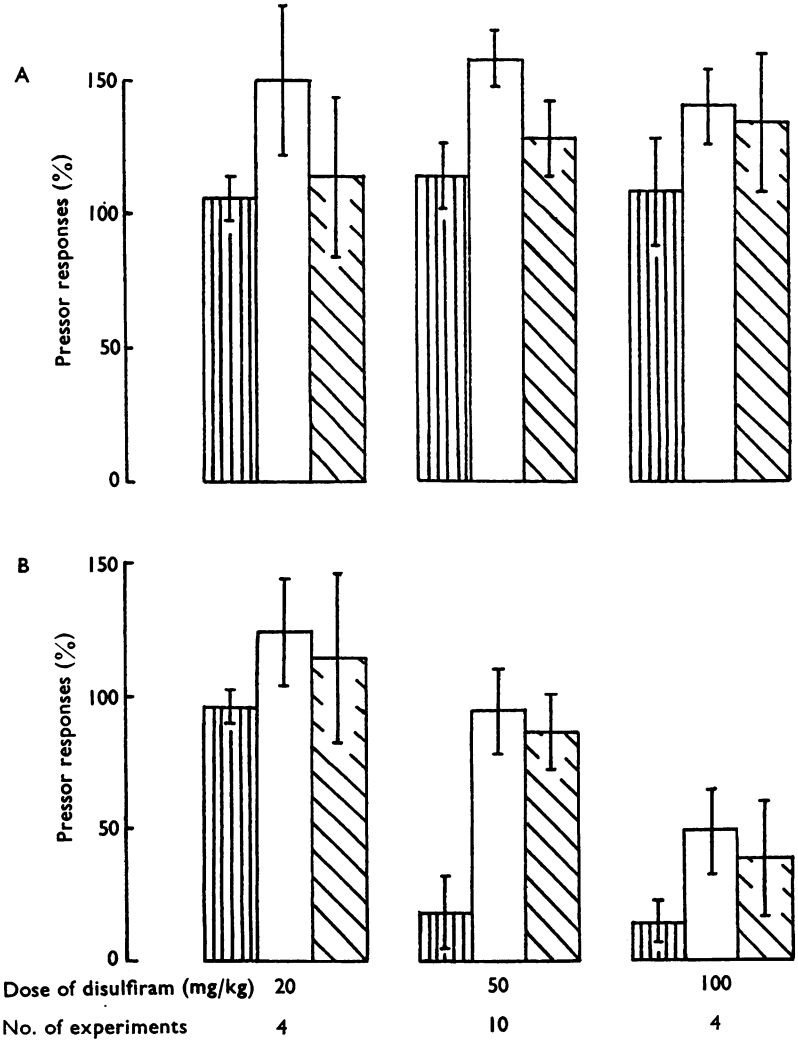

FIG. 3. Effect of disulfiram at 20, 50 and 100 mg/kg on responses expressed as a percentage of mean control responses to 50 ng/kg angiotensin (vertical hatched columns), sympathetic outflow stimulation at 0·5 Hz (open columns) and 100 ng/kg noradrenaline (cross-hatched columns). Responses in A are 1 h, and in B 3 h after disulfiram administration.

were enhanced whereas the response to vasopressin was unaffected (Fig. 4B). However, at 4 h, the response to noradrenaline had returned to control level, angiotensin was markedly reduced whilst the vasopressin response was unaltered (Fig. 4C).

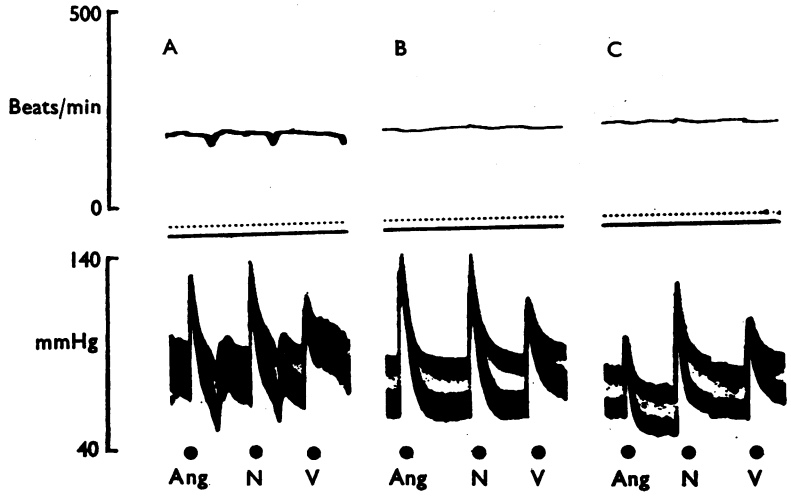

FIG. 4. Pithed rat blood pressure (lower record) and heart rate (upper record). In A, control responses to angiotensin (50 ng/kg at Ang), noradrenaline (100 ng/kg at N) and vasopressin (5 mu/kg at V). B and C, responses repeated 130 and 250 min after disulfiram (50 mg/kg i.p.). Time marker in minutes.

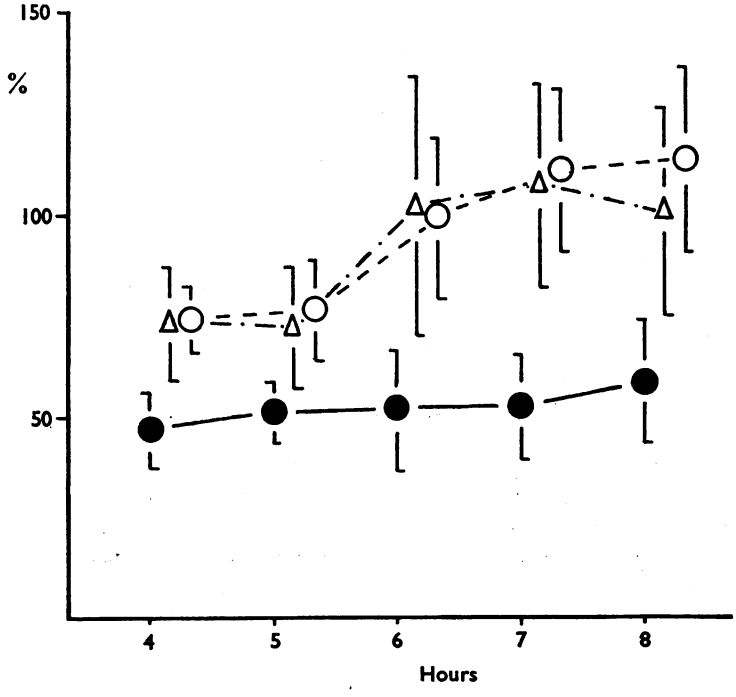

FIG. 5. Mean responses to 50 ng/kg angiotensin (●), sympathetic outflow stimulation at 0·5 Hz (△) and 100 ng/kg noradrenaline (○) in five pithed rats pretreated with disulfiram (50 mg/kg i.p.) at time 0 hours. Values are expressed as percentage of mean control responses taken from four untreated preparations.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

An attempt was made to determine the duration of the angiotensin blocking action of disulfiram by pretreating five rats with 50 mg/kg 3·5 h before pithing and then recording the responses to angiotensin (50 ng/kg), noradrenaline (100 ng/kg) and sympathetic stimulation (0·5 Hz) for periods of up to 8·5 h after the injection. The responses obtained were compared with those obtained from four control rats. The basal blood pressure of rats pretreated with disulfiram was no different from that of the untreated group. The results are illustrated graphically in Fig. 5. The responses to angiotensin were reduced by approximately 50% ($2P<0·05$) throughout 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 but which had returned to control levels by 6 h and did not subsequently change for the remaining 2 h of the experiments.

Sodium diethyldithiocarbamate

This compound, like disulfiram, selectively inhibited the pressor responses to angiotensin. However, in fifteen acute experiments there was no obvious relationship between the dose of sodium diethyldithiocarabamate and the degree of reduction of the angiotensin responses. An initial potentiation of responses to angiotensin (10–40 ng/kg), noradrenaline (20–200 ng/kg) and sympathetic outflow stimulation (0·5 Hz) was observed after intravenous injection of sodium diethyldithiocarbamate (5–100 mg/kg). Subsequently, in 11 of 15 experiments, the responses to angiotensin declined more rapidly than did those to noradrenaline and sympathetic stimulation. In these experiments, 2–3 h after sodium diethyldithiocarbamate, the responses to angiotensin were abolished or considerably diminished whilst those to noradrenaline and sympathetic stimulation were within 20% of their control levels. This pattern of response was observed over the whole range of diethyldithiocarbamate doses. In the remainder of the experiments, the responses to angiotensin returned to control levels after potentiation for 2–3 hours.

After intraperitoneal injection of sodium diethyldithiocarbamate (10 mg/kg) 3 h before pithing, there was no marked effect on the basal blood pressure, or responses to angiotensin, noradrenaline and sympathetic outflow stimulation; the responses remained constant during the three experiments (up to 7·5 h after sodium diethyldithiocarbamate). Similarly, in two experiments, sodium diethyldithiocarbamate (100 mg/kg) 18 h before pithing had no effect on the responses to angiotensin (50 ng/kg) or to noradrenaline (100 ng/kg).

Penicillamine

Penicillamine (1–100 mg/kg, i.v.), caused an increase in the pressor responses to both angiotensin (20–100 ng/kg) and noradrenaline (40–200 ng/kg). This enhancement was immediate in onset and varied in extent from experiment to experiment but was usually of the order of 50–100% in height and a smaller increase in duration. The enhancement of the responses persisted for approximately 2 h and then returned to their control level.

Ascorbic acid

In seven experiments, ascorbic acid (1–500 mg/kg, i.v.) caused an increase in the pulse pressure by increasing the systolic pressure and also caused a dose-dependent

enhancement of the responses to angiotensin (50 ng/kg), noradrenaline (100 ng/kg) and sympathetic outflow stimulation (0·5 Hz). The enhancement was rapid in onset and when maximal, following a dose of 500 mg/kg was of the order of a 5-fold increase in height and duration. This potentiation of the responses to angiotensin and noradrenaline persisted for the duration of all experiments (up to 5 h).

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 animals, the basal pressure was higher (mean 70 ± 10 mmHg) than that in control rats (mean 55 ± 6 mmHg); the responses to sympathetic stimulation were abolished whilst those to noradrenaline and angiotensin were either unaltered or, more commonly, increased by up to 20%. These rats received 50 mg/kg of either disulfiram (five experiments) or sodium diethyldithiocarbamate (three experiments). The results following both substances were similar; the initial enhancement of noradrenaline and angiotensin responses usually seen after disulfiram (Fig. 1) and sodium diethyldithiocarbamate was either greatly reduced or in most experiments absent (Fig. 6B). The rate of decline in the responses to angiotensin after disulfiram or sodium diethyldithiocarbamate was slightly greater in reserpine treated preparations as shown in Fig. 6.

Changes in heart rate

The changes in heart rate induced by the pressor procedures recorded in some of these experiments were, in general, small. Angiotensin was virtually without effect,

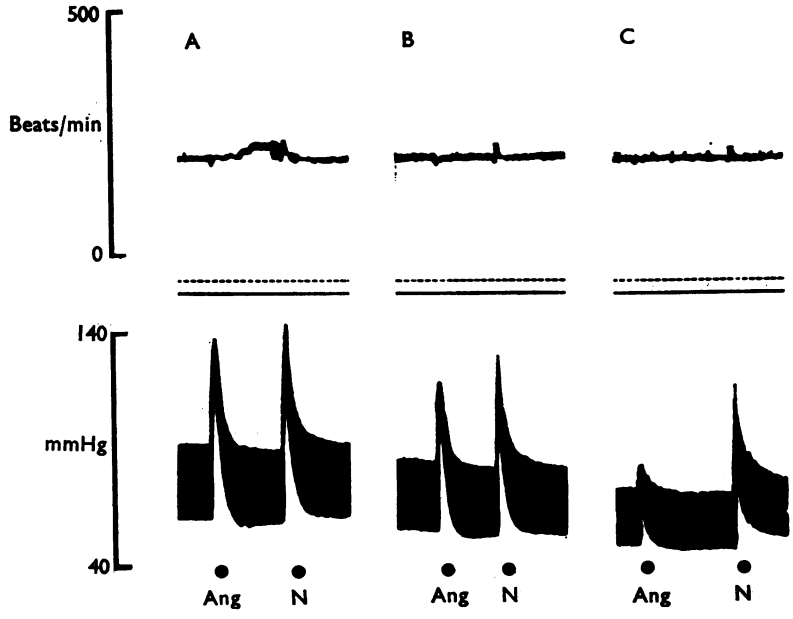

FIG. 6. Pithed rat blood pressure (lower record) and heart rate (upper record) from rat pretreated with reserpine (2·5 mg/kg daily for 3 days). In A, control responses to 50 ng/kg angiotensin (Ang) and 100 ng/kg noradrenaline (N). B is 2 h and C, 4 h after disulfiram (50 mg/kg i.p.). Time marker in minutes.

whilst sympathetic stimulation and noradrenaline produced tachycardia ranging from 10 to 100 beats/minute. The tachycardias to noradrenaline and sympathetic stimulation tended to be smaller at the end of each experiment in control as well as treated preparations and no clear effect could be attributed to any drug treatment.

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 isolflavone derivative also known to form complexes with bivalent metal ions, specifically antagonized the contractor action of angiotensin II on guinea-pig isolated ileum. Subsequently, osajin was shown to have no *in vivo* anti-angiotensin activity (Walaszek, personal communication).

Day & Owen (1969) showed that the pressor effect of angiotensin II in the pithed rat was inhibited by the bivalent metal chelating agent sodium diethyldithiocarbamate. The present work confirms this observation and further demonstrates a similar activity in tetraethylthiuram disulphide (disulfiram), the parent disulphide of the thiol sodium diethyldithiocarbamate. This selective antagonism could not be demonstrated using structurally dissimilar compounds known to be chelating agents (Chaberek & Martell, 1959; Dwyer & Mellor, 1964) namely penicillamine and ascorbic acid.

Reserpine, which produces a 50% inhibition of pressor responses to angiotensin in conscious cats (Day & Owen, 1970) did not reduce, and in most experiments produced up to a 20% enhancement of angiotensin pressor responses in the pithed rat. However, the rate of decline of responses to angiotensin after administration of disulfiram or sodium diethyldithiocarbamate was slightly greater in reserpinized preparations than that observed in untreated rats, thus suggesting a possible involvement of neural noradrenaline stores in the pressor response to angiotensin.

In the experiments described, disulfiram and sodium diethyldithiocarbamate exhibited two distinct actions. There was an initial enhancement of the responses to angiotensin which was maximal after about 90 min and lasted some 2–3 hours. However, in each experiment, this enhancement was always less than that seen in the cases of the responses to sympathetic stimulation and noradrenaline. A similar effect was observed after the administration of penicillamine and ascorbic acid. A subsequent inhibition of responses to angiotensin was only observed after sodium diethyldithiocarbamate or disulfiram. This inhibition became apparent 3–4 h after the injection of these compounds and was specific to angiotensin. There was no significant inhibition of the pressor responses to noradrenaline, sympathetic stimulation or the octapeptide vasopressin. Indeed, in many experiments, responses to noradrenaline and sympathetic stimulation were enhanced by up to 30% of control values at a time when responses to angiotensin were abolished.

The enhancement of the responses to noradrenaline and sympathetic stimulation after penicillamine, ascorbic acid, disulfiram and sodium diethyldithiocarbamate may well be due to copper chelation. Both penicillamine (Doornbos & Faber, 1964) and DDC (Thorn & Ludwig, 1962) complex strongly with copper and other bivalent metal ions. Similarly, ascorbic acid is known to chelate bivalent metal ions (Chaberek & Martell, 1959). Disulfiram, though not itself a chelating agent, is

reduced *in vitro* and *in vivo* to sodium diethyldithiocarbamate (Stromme, 1965a ; Goldstein, Anagnoste, Lauber & McKereghan, 1964).

Copper chelation has been previously reported to enhance pressor responses to adrenaline (Fischer, Lecomte & Delandtsheere, 1950). The potentiation of the responses to angiotensin may be due to a similar enhancement of a possible sympathetic component of the response.

The specific inhibition of angiotensin by disulfiram or sodium diethyldithiocarbamate appears to be independent of a chelation mechanism, since ascorbic acid and penicillamine produced no similar activity. The results of chelation may, in fact, have been responsible for delaying the inhibition of the angiotensin pressor responses produced by sodium diethyldithiocarbamate or disulfiram since the initial potentiation was reduced after reserpine and the development of the angiotensin inhibition was accelerated in these preparations.

The inhibition would further appear to be independent of dopamine-β-hydroxylase inhibition since angiotensin responses were often abolished at a time when responses to sympathetic stimulation were relatively unaltered, indicating unimpaired sympathetic function. Further, for satisfactory dopamine-β-hydroxylase inhibition in rats it is usual to employ a dose of disulfiram of the order of 400 mg/kg intraperitoneally (Porter & Torchiana, 1971), an 8-fold increase on the effective dose used in this study.

The most likely mechanism for the angiotensin inhibition produced by disulfiram involves blockage of active sulphydryl groups. Disulfiram has a high reactivity towards protein sulphydryl groups and naturally occurring thiols such as glutathione and coenzyme-A (Johnston, 1953 ; Stromme, 1965b ; Stromme, 1963 ; Owens & Rubenstein, 1964). It is thus possible that both disulfiram and sodium diethyldithiocarbamate, these structures being readily interconvertible (Stromme, 1965a ; Thorn & Ludwig, 1962), inhibit a process necessary for the pressor activity of angiotensin II and which is dependent on the availability of proteins or other substances containing active sulphydryl groups. This suggestion is supported by the findings of Page & Green (1949) who showed that dimercaprol, a well known sulphydryl inhibitor, produces a ' refractoriness ' to angiotensin in anaesthetized dogs.

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