STUDIES ON THE MODE OF ACTION OF HYDRALLAZINE AND RESERPINE

by

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(April, 1959).

Hydrallazine and reserpine are used in the treatment of hypertension and reserpine is also employed in the treatment of certain forms of mental illness. Hypotheses regarding the mode of action of these drugs are numerous. Thus hydrallazine is believed to act on the vasomotor centre, and reserpine to depress central sympathetic tone. Reserpine is also believed to mediate its actions by liberating noradrenaline and 5-hydroxytryptamine from the brain and, in the case of noradrenaline, from the arteries. The work described in this thesis was undertaken to clarify some aspects of the mode of action of these drugs at cellular level.

Experimental evidence has indicated that hydrallazine, dihydrallazine and related compounds did not antagonise certain pressor reflexes - notably the pressor responses due to central vagal stimulation, bilateral carotid occlusion and anoxia. This speaks against their having

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a central site and mechanism of action, because if this were so, all pressor reflexes mediated *via* the central nervous system should be antagonised or depressed. This does not, however, rule out the possibility that hydrallazine acts specifically upon certain cell groups in the central nervous system. Hydrallazine antagonised the pressor effects of adrenaline more than those of *noradrenaline*, and the antagonism of this drug to some pressor reflexes only was explained by assuming that there are quantitative variations in the proportions of the two humoral agents secreted during the initiation of these reflexes. The fact that hypertension caused by constant infusion of a solution of adrenaline was promptly brought back to normal levels by hydrallazine, also favours a peripheral site of action for this drug.

Many of the effects of hydrallazine and reserpine can be explained by assuming that both drugs interfere with the normal energy-yielding mechanisms of the smooth muscle cell. It has been shown that hydrallazine has an effect upon carbohydrate metabolism which underlies its actions upon isolated arterial smooth muscle. Intermediates of carbohydrate metabolism antagonised hydrallazine depression of drug-induced contractions of arterial smooth muscle. The reserpine effect was so persistent that the tissue did not /

not recover. Anoxia and cyanide also antagonised drug-induced contractions of arterial smooth muscle. Intermediates of carbohydrate metabolism gave protection against anoxia and not against cyanide. The use of hydrallazine was, in effect, analogous to rendering the tissue anoxic.

Reserpine did not have marked effects on tissue respiration while hydrallazine depressed it. Reserpine was found to cause in vivo inhibition of oxidative phosphorylation in rat brain and liver, since the ATP/ADP ratio was significantly lowered. Hydrallazine had similar effects in brain and liver. Neither drug influenced the adenosine nucleotide levels in rat skeletal muscle and heart. On the basis of this experimental evidence it has been suggested that hydrallazine may interfere with biological oxidation and reserpine with oxidative phosphorylation.

It has been shown that reserpine depleted the adrenal medulla of catechol amines and ATP in roughly the same proportions. Since the characteristic breakdown products of ATP (ADP and AMP) were not found - as they were found in brain and liver - it is suggested that ATP possesses a specialised function in the storage or release of catechol amines from this gland.

Inhibitors /

Inhibitors of metabolism, such as cyanide and DNP, have marked effects on the transport of sodium and potassium ions in tissues. Reserpine was found to have practically no effect either upon the release of potassium or on the uptake of potassium and sodium. Hydrallazine, however, increased the release of potassium. It decreased potassium retention but increased sodium retention. Cyanide and azide increased potassium release but anoxia and DNP had no effect. On the other hand, cyanide, azide, DNP and anoxia had marked effects on sodium and potassium uptakes which usually varied inversely. Thus a reduction in the efficiency of the enzymes controlling metabolism may reduce the ability of muscle to retain potassium, resulting in the release of this ion.

Hydrallazine formed chelates with different metals, and the iron-hydrallazine chelate was shown to be inert. Hydrallazine in high doses inhibited catalase, depressed the iron-catalysed oxidation of cysteine to cystine and was shown to cause haemolysis.

Doses of reserpine and hydrallazine used in this study are considered by the author to be comparable to those used in man. Since the intact experimental animal or man is more sensitive than the isolated tissue or organ /

organ, the slightly higher doses used in some experiments on isolated tissues do in fact approximate to the therapeutic dose levels. It is finally suggested that interference by these drugs with tissue metabolism may result in a decreased availability of energy for muscular contraction. It is postulated that hydrallazine produces an "anoxia-like" condition in smooth muscle, while in oxidative phosphorylation, reserpine acts as an "uncoupling agent". Such an effect may explain the reduction of inherent tone in vascular smooth muscle and is probably responsible for the lowering of blood pressure.

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A Thesis submitted to the University of Glasgow
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in the
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by

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LIST OF PUBLICATIONS AND COMMUNICATIONS

Publications.

Certain aspects of the work described in this thesis have been published, jointly with J. J. Lewis, with M. S. Zoha and J. J. Lewis, and with G.A.J. Goodlad and J. J. Lewis. The publications are as follows:

(1) Kirpekar, S.M., and Lewis, J. J., (1957), Pharmacological properties of hydrallazine, dihydrallazine and some related compounds.

J. Pharm. Pharmacol., 9, 877.

(2) Kirpekar, S.M., and Lewis, J.J., (1958), Effects of reserpine and hydrallazine on isolated strips of carotid arteries.

J. Pharm. Pharmacol., 10, 255.

(3) /

(3) Kirpekar, S.M., and Lewis, J. J., (1958), Antagonism to the actions of hydrallazine, reserpine, potassium cyanide, sodium azide and anoxia on arterial smooth muscle.

J. Pharm. Pharmacol., 10, 307.

(4) Zoha, M.S., Kirpekar, S.M., and Lewis,J.J., (1958), A note on the pharmacology of rescinnamine and serpentine.

J.,Pharm. Pharmacol., 10, 231T

(5) Kirpekar, S.M., and Lewis, J.J., (1959), Some effects of reserpine and hydrallazine upon tissue respiration and the concentration of adenosine nucleotides in certain tissues.

Brit. J. Pharmacol., 14, 888.

(6) /

(6) Kirpekar, S.M. Goodlad, G.A.J., and Lewis,J.J., (1958), Reserpine depletion of adenosine triphosphate from the rat suprarenal medulla.

Biochem. Pharmacol., 1, 232.

Reprints of the above publications are to be found at the end of the thesis.

The following publication has been submitted to the Editor of the Journal of Pharmacy and Pharmacology:

(1) Kirpekar, S.M., and Lewis, J.J., Some effects of hydrallazine on iron, on red blood corpuscles and iron-containing enzyme system.

Some of the work presented in Chapter VIII, together with some additional studies, will be shortly submitted for publication.

Communications:

Communications:

A part of this work was communicated, jointly with J. J. Lewis, at the following meetings:

(1) British Pharmacological Society meeting in London, January 1957. - Pharmacological properties of hydrallazine and dihydrallazine.

(2) British Pharmacological Society meeting in London, January 1958. - Effect of reserpine and hydrallazine on isolated artery strips.

(3) British Pharmacological Society meeting in Glasgow, July 1958. - Some effects of hydrallazine on iron metabolism.

(4) British Pharmacological Society meeting in Glasgow, July 1958. - Effect of reserpine and hydrallazine on adenosine nucleotides of rat tissues.

(5) /

(5) British Pharmaceutical Society meeting in Bristol, September 1957. - Pharmacological properties of hydrallazine, dihydrallazine and some related compounds.

(6) British Pharmaceutical Society meeting in Llandudno, September 1958. - A note on the pharmacology of reserpinamine and serpentine.

C O N T E N T S

STUDIES ON THE MODE OF ACTION OF HYDRALLAZINE AND RESERPINE

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C O N T E N T S

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C O N T E N T S

C O N T E N T S

CHAPTER I

CHAPTER I

I N T R O D U C T I O N

Although considerable effort has been expended in seeking the mechanisms which underlie human essential hypertension, we still lack knowledge of the cause of the generalised increase in peripheral vascular resistance which appears to be the fundamental abnormality in this condition. Consequently, attempts at therapy with drugs represent treatment of the symptoms rather than the underlying disease; a situation which is similar to that of treating hyperpyrexia without knowing the cause. However, just as lowering fever intermittently may permit the patient to tolerate the toxic process more successfully, so reduction of excessive hypertension - even partially - may help the patient to withstand the ravages of the disease, provided the blood flow to vital organs remains adequate. Therefore, reduction of extremely elevated pressure in the vascular system - even if only for short periods of time - seems rational in an attempt to reduce cardiac work, prevent retinal and cerebral haemorrhages, and possibly to prevent progressive damage to the arteriolar wall[1,2]. Some hesitate to accept this view, for they regard high blood pressure as a sort of compensatory process by which tissue nutrition is secured during the presence of constricted blood vessels.

Perhaps /

Perhaps the view most widely held is that blood pressure reduction is desirable, but that to take this too far is to render the patient more susceptible to cerebral and coronary accidents. The clinical improvement which has followed effective hypotensive therapy does not support the view that high blood pressure serves ordinarily as a compensatory process. This school of thought believes that "whatever the underlying disease, the course of hypertension is likely to be of the malignant type if the arterial pressure is sufficiently raised, and of the benign type if the rise is less". In short, high blood pressure is regarded as a symptom and not as a disease[3]. Fibrinoid arteriolar necrosis and papilloedema are thought to be due to high intra-arterial and cerebrospinal fluid pressure respectively. Pickering[1,3] and Smirk[2] feel that hypotensive agents can cause regression of these lesions and improve prognosis. On the other hand, acute hypotension may precipitate such conditions as cerebral thrombosis, renal failure and coronary thrombosis if regional circulations cannot adapt to lowered perfusion pressure. That such accidents do not often happen during hypotensive therapy is probably the result of the intrinsic ability of the regional circulations to adjust to lowered perfusion pressure.

These /

These differences of vascular behaviour are important from the physiological point of view because they can be correlated with differences in the functions of the individual organs. They are also of particular interest because they may provide information as to the nature of the agent by which arterial pressure is raised.

An ideal antihypertensive drug should produce a prolonged reduction of blood pressure in a large percentage of patients through generalised peripheral vasodilatation without side effects or development of tolerance during prolonged administration. A drug of limited practical value for prolonged administration should not cause the development of marked tolerance; side effects should not be severe enough to interfere with the patient's normal way of life; diastolic blood pressure should be reduced by 20 mm. Hg. or more for one fourth to one half of each day. Cardiac output, and renal and coronary blood flow should not be reduced significantly.

Unfortunately such ideal antihypertensive drugs are not so far available. The existing drugs fail to meet the requirements listed above, and in this sense are incomplete. The progress of experimental pharmacology in the last few years can be judged from the fact that the medical

treatment of hypertension in 1948-49 was confined almost wholly to thiocyanate, papaverine and nitrite therapy, while recent therapy included at least five other antihypertensive drugs which, for the most part, are under clinical evaluation and are accepted as superior to thiocyanates etc. Hoobler and Dontas[4] have suggested the following classification of the antihypertensive drugs according to their modes of action.

I. Drugs which interfere with sympathetic vasomotor activity.

A. Adrenergic blocking agents:

β-haloalkylamines (dibenamine, phenoxybenzamine)

imidazolines (tolazoline, phentolamine)

benzodioxanes (benodaine)

yohimbine

the alkaloids of ergot.

B. Ganglion blocking agents:

Quaternary ammonium compounds (tetraethyl-ammonium, bismethonium compounds, piperidium compounds)

thiophanium derivatives (trimetaphan).

C. Centrally acting inhibitors of sympathetic vasomotor activity:

Dihydrogenated alkaloids of ergot

pentaquine

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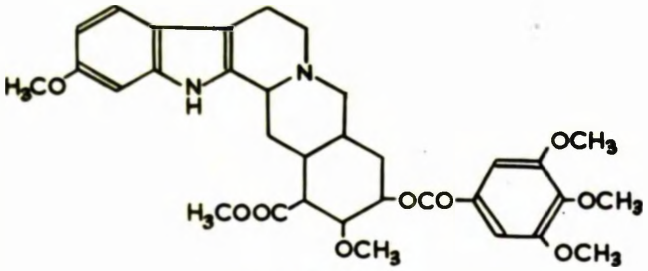
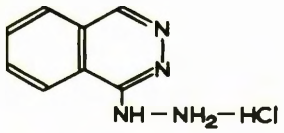


Reserpine



Hydrallazine

Fig. 1.1

Structural formulae of reserpine and hydrallazine.

C. (continued)

hydrallazine.

D. Drugs activating afferent vascular reflexes

Veratrum alkaloids.

II. Drugs acting directly on vascular smooth muscle.

Papaverine, nitrites, adenylic acid.

III. Drugs exerting unknown or mixed effects.

Hydrallazine, thiocyanates, sodium nitroprusside, Rauwolfia serpentina, vitamins, dimercaprol, pyrogens, bismuth and cobalt salts, elemental iodine, caffeine.

The alkaloids of R. serpentina (of which reserpine is the most important) and hydrallazine - a hydrazinophthalazine - are used in the treatment of hypertension. Both reserpine and hydrallazine are believed to act on the vasomotor centre in the brain but some recent reports question the truth of this assumption, and it is suggested that they may also have a peripheral component. The work presented in this thesis is an attempt to explain, at least in part, the modes of action of reserpine and hydrallazine (Fig. 1.1). It may be useful therefore to give a brief review of some of the more relevant aspects of /

of the subject, and to attempt to assess the present position of these drugs.

Of all the Rauwolfia alkaloids so far isolated, the one which has been most extensively studied both in laboratory and clinic is reserpine - the first alkaloid to display the peculiar tranquillising and hypotensive effects associated with Rauwolfia preparations.

Reserpine causes a slow and relatively prolonged reduction in blood pressure which is greater in hypertensive than in normotensive human subjects(5). Reserpine has very little effect on the blood pressure of experimental animals if this is already low. Thus no depressor response can be demonstrated in spinal or decerebrate cats(6). The reduction in blood pressure is accompanied by a distinct bradycardia which is unaffected by atropine(6 to 9). Since cardiac function in vivo is generally unaffected in man(10) and in animals(11) during a simultaneous reduction of peripheral resistance, it has been concluded(12) that the fall in blood pressure is due to peripheral vasodilatation.

Respiration is depressed by reserpine in all animal species /

species studied[7,13]. However, the vagal respiratory reflex and the sensitivity to electrical stimulation of the medullary areas, which are associated with respiratory reflexes, are uninfluenced by reserpine[14].

The suggestion has been made that the autonomic actions of reserpine are due to effects on the hypothalamus and that changes in the temperament and activity of animals, with somnolence and lessened aggression, may be due wholly or in part to effects on this part of the brain. Schneider[13] notes the suppression of the manifestations of sham rage in cats. The characteristic increase in appetite and gain of weight may also be of hypothalamic origin.

The pattern of effects seen after administration of reserpine has been compared[15] to a syndrome reported by Hess[16] following electrical stimulation of certain diencephalic structures in the cat. This similarity of the actions of reserpine to those of hypothalamic stimulation, coupled with the absence of a peripheral antisympathetic action, ganglionic blockade or peripheral vagal stimulation, led Bein and his colleagues[4,6,7] and other workers[8 to 11,13,17] to suggest that the action of /

of reserpine was predominantly central . The exact site of the action has not yet been demonstrated conclusively. It has been pointed out [6,14], however, that many of the affects following administration of the drug can be explained by assuming a partial suppression of sympathetic tone. This hypothesis is supported by the finding that relatively small doses of reserpine block the reflex pressor response to carotid sinus occlusion in dogs[9,14] and cats[6,9,14]. There is no direct effect upon the stretch receptors of the carotid sinus. The drug does not affect the pressor response to afferent vagal stimulation in the dog[9] or cat[6,7] or to stimulation [6,18] of the sciatic or tibial nerves in cats; and the pressor response to intracranial pressure in dogs is not prevented[11]. It has, therefore, been suggested that reserpine probably acts at a point higher than the medulla, possibly on the afferent inflow which normally stimulates sympathetic activity in the hypothalamus. Bein suggested[14], (following his demonstration of a partial reversal of the carotid sinus pressor reflex block by section of the brain stem) that reserpine stimulated certain normally inhibitory "substances" in the brain. Dasgupta et al.[18] found a greatly augmented response to a solution of total /

total Rauwolfia alkaloids when these were given intracisternally rather than intravenously. Bhargava and Borison[19] and Horwitz and Wang[20] demonstrated that reserpine depresses the pressor responses from brain stem vasomotor centres, and that the medullary vasomotor centres are susceptible to reserpine even after midcollicular decerebration.

The effects of reserpine on the E.E.G. are usually slight and variable e.g. the responses in the electrocorticogram obtained by direct stimulation or by peripheral stimulation of the reticular activating system are not altered significantly by reserpine. Killam and Killam[21] failed to show a change in the threshold of this area or in the thalamocortical recruiting circuits in cats. In view of the alleged central mode of action of reserpine, one might expect a change in threshold of the reticular activating system after administration of reserpine. In the absence of any change, it is rather difficult to support this assumption.

That reserpine may not act *per se* is suggested by the long latency in its action and by the fact that its actions *in vivo* are seen long after its presence can no longer be /

be detected[22]. The extreme insolubility of reserpine may be responsible for the latency of action. On the other hand, much attention has recently been focused upon certain observations by Brodie and his colleagues[22 to 2] and others[27 to 29] who found that reserpine liberated 5-hydroxytryptamine from the brain, intestinal tract, spleen, mast cells and platelets of various animal species. Brodie and his co-workers[30] have postulated that the primary action of the active Rauwolfia alkaloids is to impair 5-hydroxytryptamine binding sites in the body. In consequence the 5-hydroxytryptamine in various depots (including the brain) is released and metabolised by the action of mono-amine oxidase. The 5-hydroxy-tryptamine that continues to be formed presents a persistently low concentration of free 5-hydroxytryptamine to brain tissue. It is this free 5-hydroxytryptamine that is considered to exert the central actions attributed to reserpine. These actions persist until the binding sites have recovered or until new ones are formed. Hence the possibility exists that reserpine acts by impairing the capacity of the cells to bind 5-hydroxytryptamine[31]. The effect of reserpine on the /

the central nervous system appears to be related more to the 5-hydroxytryptamine content of the brain than to the concentration of reserpine[22].

Brodie and Shore[32] put forward an hypothesis that implicates 5-hydroxytryptamine and noradrenaline as chemical mediators of mutually antagonistic centres in the brain. They attempt to explain the actions of the tranquillizing agents, reserpine and chlorpromazine, and the hallucinogenic agents, lysergic acid diethylamide (LSD) and mescaline, in terms of interactions with 5-hydroxytryptamine and noradrenaline in the central nervous system. It is suggested that 5-hydroxytryptamine acts as a chemical mediator in brain. The amine is unevenly distributed in brain, its concentration being highest in the hypothalamus and lowest in the cortex, with only traces in the cerebellum[33]. Relatively larger quantities of 5-hydroxytryptamine are found in the hypothalamus than in any other part of the brain, and since the hypothalamus controls many of the fundamental processes of the body e.g. vasomotor tone, body temperature, fat, carbohydrate and water metabolism etc., it has been suggested that this substance may have

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a physiological significance in the specialised functions of this region. Support for the suggestion that 5-hydroxytryptamine plays a part in nerve transmission is provided by studies of the distribution of monoamine oxidase, the enzyme which metabolises it, and 5-hydroxytryptophan decarboxylase, the enzyme which catalyses its synthesis from tryptophan. Both of these enzymes have been reported by Gaddum and Giarman[34] to be present in highest concentrations in the hypothalamic areas. Welsh[35] in his work with molluscs strongly argues for a neurohumoral role for 5-hydroxytryptamine in certain invertebrates.

5-hydroxytryptamine may be released in minute amounts at synaptic junctions where it may act as a chemical mediator initiating nerve impulses in postsynaptic fibres. Reserpine, by impairing the sites which hold 5-hydroxytryptamine in a bound form without blocking the synthesis of the amine, would present a low but persistent concentration of free 5-hydroxytryptamine to the brain. In consequence, a continuous volley of impulses would bombard certain areas of the brain, resulting in the usual manifestations of reserpine action. Brodie /

Brodie et al.[32] explain the differences in the modes of action of reserpine and chlorpromazine by assuming that they act on physiologically antagonistic systems in the brain stem which are involved in wakefulness, regulation of temperature, control of blood pressure, and other autonomic functions. Drug-induced paralysis of one system would release the opposite system and allow it to predominate. In support of this concept, it has been demonstrated that there are both parasympathetic and sympathetic areas in the brain stem. From a consideration of the pharmacological properties of reserpine it may be supposed that 5-hydroxytryptamine is the transmitter of nerve impulses to the centres of the parasympathetic division. By blocking these fibres, LSD would unmask the action of opposing sympathetic symptom, and produce its typical sympathomimetic responses. Reserpine can be considered to invoke its parasympathomimetic effects by virtue of releasing free 5-hydroxytryptamine which activates the parasympathetic centres, while chlopromazine may be postulated to block nervous impulses activating central sympathetic centres and thus augment the activity of parasympathetic system.

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This attractive and ingenious suggestion that reserpine acts through the mediation of 5-hydroxytryptamine is not supported by all the experimental evidence available. It is reasonable to expect 5-hydroxytryptamine to possess most of the properties of reserpine if, in fact, it is the actual active substance. That is not so. Thus 5-hydroxytryptamine does not potentiate camphor or leptazol convulsions in mice[36]. Parenteral administration of 5-hydroxytryptamine produces effects on respiration, heart rate, blood pressure and the nictitating membrane which differ from those seen following reserpine[37,38].

Gaddum and Vogt[39] have shown that 5-hydroxytryptamine administered intraventricularly does not cause the miosis, hypotension or relaxation of the nictitating membrane in cats which are characteristic of the actions of reserpine. They also show that reserpine effects can be blocked by LSD but not by bromlysergic acid diethylamide (brom LSD), both of which are powerful antagonists of 5-hydroxytryptamine, and suggest that the antagonisms are unspecific. It must be remembered that there is no evidence that the effects of intraventricular 5-hydroxytryptamine /

5-hydroxytryptamine are exerted directly on neurones and are not mediated through a constriction of cerebral blood vessels in regions near the ventricles. The depression produced by injecting large doses of adrenaline into the ventricles[40] bears much resemblance to the effect of intraventricular 5-hydroxytryptamine, and vascular effects may play some part at least in the response to both drugs when they are administered by these routes. Shaw and Woolley[41] observe that LSD may exhibit 5-hydroxytryptamine-like properties when acting upon the heart of the clam or the dog, and that it is 1 to 3 times more active in raising the blood pressure of the dog. In the brain, LSD appears to antagonise the effects of 5-hydroxytryptamine. They suggest that substances chemically related to 5-hydroxy-tryptamine may combine with receptors either to induce or block 5-hydroxytryptamine-like actions. Thus one and the same compound can induce some 5-hydroxytryptamine-like actions yet block others. It has been shown[41] that BAS (1:benzyl 2:methyl 5:methoxy tryptamine) - a potent "antimetabolite" of 5-hydroxytryptamine - antagonises the pressor effect not only of 5-hydroxytryptamine but also of LSD on the blood pressure of the dog, thus adding

to /

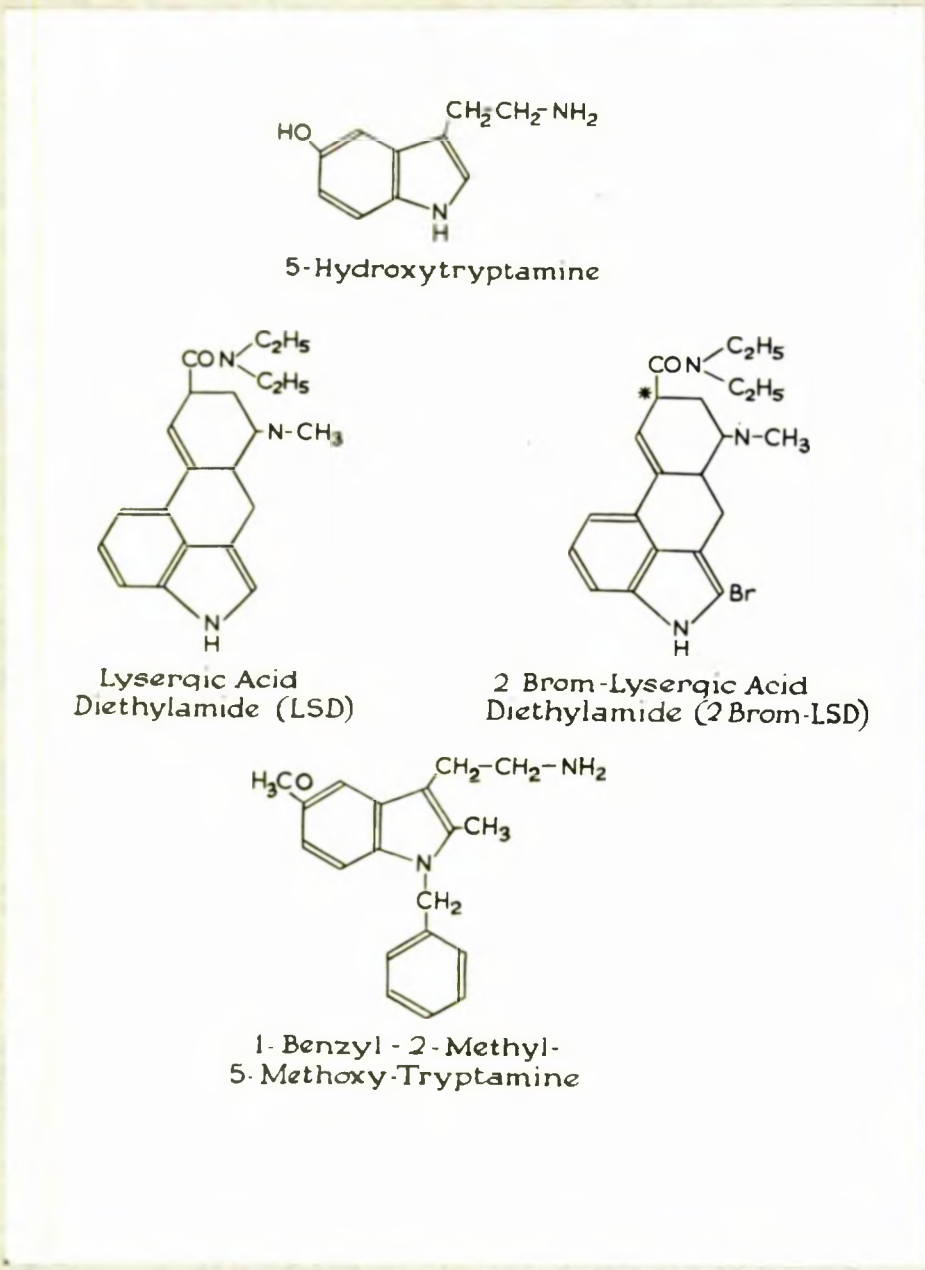

Fig. 1.2

Structural formulae of 5-hydroxytryptamine, lysergic acid diethylamide (LSD), 2 brom lysergic acid diethylamide (2 brom-LSD) and 1:benzyl-2 methyl 5-methoxy tryptamine.

to the evidence that the pressor effect is actually due to 5-hydroxytryptamine (Fig.1.2).

Cerletti and Rothlin[43] have observed that brom-LSD is a powerful antagonist of 5-hydroxytryptamine but does not cause the mental disturbances characteristic of LSD. As the theory of the mode of action of 5-hydroxytryptamine is based, to a great extent, on the fact that LSD is a strong antagonist of 5-hydroxytryptamine, the finding that brom-LSD is as active an antagonist as LSD *in vitro* but that it produces none of the mental disturbances makes it necessary to reconsider this hypothesis. It cannot be argued that brom-LSD lacks actions on the cortex because it does not penetrate into the brain tissue, since the sedative action it produces is a central effect. The mere existence of a pharmacological antagonism between LSD and 5-hydroxytryptamine, however, no longer provides evidence for the hypothesis that the inhibition of the actions of the latter in the brain is the cause of the mental disturbance[44].

If 5-hydroxytryptamine is a mediator of the action of reserpine, then it follows that all substances releasing /

releasing this amine should produce the same actions as reserpine. However, amphetamine - in doses which produce marked central excitation - causes a fall in 5-hydroxytryptamine storage[27], while the histamine liberator 48/80, which also releases 5-hydroxytryptamine from mast cells, does not evoke the sedation in rats or guinea-pigs which is so characteristic of reserpine[29].

The symptoms exhibited by patients with carcinoid tumours which liberate 5-hydroxytryptamine are of considerable interest. These patients characteristically show diarrhoea, bronchoconstriction and flushing of the skin, especially of the face. These symptoms have all been observed following reserpine; on the other hand some of the symptoms - for example, insomnia and tachycardia - are in direct contrast to the effects of reserpine[45].

Thus it can be seen that while certain actions of reserpine may be related to the release of 5-hydroxytryptamine, the evidence that the release of this amine is responsible for all its effects is inconclusive. It may be as Bein suggested[46] that the depletion of endogenous 5-hydroxytryptamine produces a suitable "milieu" /

"milieu" for reserpine or a metabolite of this compound to exert their characteristic pharmacological effects.

Reserpine not only decreases the brain concentration of 5-hydroxytryptamine but also of other mono-amines, notably noradrenaline. Holzbauer and Vogt[47] have shown that the noradrenaline content of the cat's hypothalamus was reduced by reserpine; this reduction was accompanied by central sympathetic stimulation, since the fall in medullary amines was greater in the innervated adrenal gland than in the denervated adrenal gland. Reserpine reduces the noradrenaline content of the cells and fibres of peripheral adrenergic neurones[48]. Organs with an adrenergic innervation no longer respond to the electrical stimulation of preganglionic and postganglionic fibres when the loss of noradrenaline is severe and has persisted enough. Muscholl and Vogt[49] state that a low noradrenaline content of the superior cervical ganglion is compatible with excitability of the sympathetic trunk and suggest that the loss of the amine does not proceed evenly along the whole neurone and that there is a period when the nerve endings have still enough transmitter to enable them /

them to function while the ganglion cell has already reached its lowest noradrenaline concentration. Recently a disappearance of noradrenaline from heart tissue of animals injected with reserpine has been reported by several authors(50 to 52); this supports the above findings.

The rise in circulating adrenaline(53) seen after an injection of reserpine confirms the view that the loss of amines from adrenals and ganglia is caused by a release from the tissue and not by an inhibition of synthesis. In this respect, there is a complete parallelism with the effect of reserpine on 5-hydroxytryptamine the synthesis of which is not impaired in tissue homogenates obtained from reserpinised rabbits(31), and the urinary metabolites of 5-hydroxytryptamine rise to 4 to 5 times the normal value in dogs injected with reserpine(24).

These observations pose the question as to whether the impaired function of the peripheral sympathetic system is a factor which contributes to the clinical picture produced by reserpine in man. The answer is suggested by the experiments(49) in which small doses

were /

were injected over a long period of time, since it is under these conditions that the animal experiments are more closely comparable with the therapeutic procedures. Daily doses of the order of 0.1 mg./kg. are used in psychiatry, and these doses were shown to be as effective as single large doses in depleting the noradrenaline content of the sympathetic ganglia of rabbits. These doses were also found to be more effective than single doses in causing the disappearance of adrenaline from the solar ganglion and from the adrenal medulla. It is, therefore, very likely that an action on the peripheral sympathetic system contributes to the overall effect of reserpine in man. Gaddum et al.(54) have shown that patients treated for weeks with daily doses of reserpine excrete much less noradrenaline than normally; this change might be caused by the reduction in noradrenaline stores in adrenergic nerves. Is the loss of noradrenaline from the sympathetic centres more important than the loss from the peripheral sympathetic system, and is the loss of 5-hydroxytryptamine from the hypothalamus more important than the loss of sympathin from the same region?

Muscholl /

Muscholl and Vogt[48,49] show that noradrenaline is readily lost from the sympathetic neurones of all species; moreover this loss is independent of any connections with the central nervous system. In contrast, there was no evidence that adrenaline was lost from any tissue - such as prevertebral ganglia and postganglionic fibres - in which its initial concentration was very low. In the prevertebral ganglia, which contain high concentrations of adrenaline, the loss was easily demonstrable in the dog and rabbit but not in the cat. The difference between the rabbit and the cat is even more pronounced with regard to the adrenal medullary amines. The adrenal medulla of the rabbit is easily depleted by moderate doses of reserpine and denervation affords only a little protection. In the cat losses of medullary hormones from the innervated gland are very variable, and denervation affords a high degree of protection. This difference in action of reserpine in different tissues is quite obscure, and perhaps points to different modes of binding of the amines at various sites.

Recovery of the normal concentration of amines after a /

a single injection of reserpine takes a long time - usually more than 9 days - which is somewhat longer than the time required for the restoration of 5-hydroxy-tryptamine lost from brain[30].

During the early period of the action of reserpine, some manifestation of the release of medullary amines and adrenergic transmitter into the circulation might be expected. Thus Everett et al.[55] described evanescent pilo-erection in mice or rats half an hour after large doses of reserpine (50 to 100 mg./kg. by mouth); Kuschke and Frantz[56] saw hyperglycaemia in the rabbit; tachycardia has been seen to occur in the heart-lung preparation of the dog[9]; and many workers have observed rises in blood pressure in different species[57,58]. As mentioned earlier, a raised level of blood adrenaline has been found in rabbits[53] during the first hour after intravenous reserpine. It has been recently shown by Burn and Rand[59] that reserpine depletes noradrenaline from the thoracic aorta of the rabbit.

Reserpine releases noradrenaline from different tissues but this release is not only a specific action of /

of reserpine since ether, morphine and insulin also cause a fall in brain sympathin[60]. Carlsson and Hillarp[61] have also shown depletion of adrenal medullary amines by morphine and insulin. The interesting observation was that not only the adrenal medullae were depleted of their catechol amines but there was a parallel fall in the ATP levels of the glands. Another important observation by Weil-Malherbe and Bone[62] which deserves consideration is the fact that reserpine depletes the free extracellular - but not the bound intracellular - amines of the rabbit adrenal glands. Different fractions of the gland extract were obtained by centrifugation but the authors were unable to demonstrate any significant change in the amine contents of the mitochondrial fraction. It is difficult to understand why reserpine should deplete the amines only from the supernatent and not from the mitochondrial fraction. It may be perhaps, as Vogt[49] suggested, due to different modes of binding of the amines at various sites.

Studies on the metabolism of nervous substrates which might shed light on the peculiar mode of action of reserpine /

reserpine have so far been very few. It has been reported that, in rats, S^{35} labelled methionine is concentrated in the region of hippocampus under the influence of reserpine(63). Reserpine *in vitro* depresses the oxygen consumption of slices of cerebral cortex taken from the rat, but only in high concentrations(64). In hypertensive patients it has no significant influence on glucose and oxygen uptake of the brain(8,65). Recently Gillis and Lewis(66) have pointed out that the effects of reserpine upon spontaneous and drug-induced tone are similar in many ways to those of anoxia. It is possible that reserpine in effect renders the intestinal smooth muscle anoxic by virtue of its ability to interfere with the energy producing reactions of carbohydrate metabolism. An effect on oxidative metabolism is supported by their observations that reserpine, after a latent period, decreases oxygen uptake in isolated rabbit intestinal muscle and causes an increased release of potassium from skeletal muscle. Another observation by Abood and Romanchek(67) points out that reserpine inhibits oxidative phosphorylation in brain. Attempts to correlate metabolic disturbances to narcosis have been numerous, although /

although the evidence in favour of such a view is not conclusive. The maintenance of the intramitochondrial geometric pattern, or of some similar structure[68] within it, is apparently essential for carrying on oxidative phosphorylation. Under normal conditions the oxidative metabolism of living cells and of certain isolated systems leads to the synthesis of compounds containing high energy phosphate bonds. These compounds are known to be essential for such functions as growth, muscle contraction and nerve conduction. A number of compounds known as uncoupling agents can alter this relationship by depressing the formation of high energy bonds without depressing the oxygen consumption of the system. Reserpine alters this relationship because it depresses the P/O ratios. The fact that a substance has the ability to uncouple phosphorylation from oxidation does not indicate that it is devoid of other pharmacological actions. The relative importance of uncoupling as a mechanism of action will differ with different drugs. T. M. Brody[69] concluded that "uncoupling is of major importance in the action of certain drugs and is at present the most attractive hypothesis for explaining drug action".

/

Hydrallazine.

A group of phthalazine derivatives, reported by Gross, Druey and Meier[70] and Craver and Yonkman[71], produced a sustained depressor action in experimental animals with a mode of action "not common to other blood pressure depressing substances". Hydrallazine and dihydrallazine are the most potent of the series which have found their way into clinical practice.

Hydrallazine causes the blood pressure to fall after a latent period of 10 to 15 minutes, an effect which is associated with increased cardiac output[72] and increased femoral[70], renal[70,72,73] and coronary blood flow.[70,74,75] The characteristic gradual hypotension seen in several species suggested the possibility of either metabolic alteration of hydrallazine to an active (chemically different) compound, slow penetration to the site of action or a slow response of the central mechanism[76]. Blood pressure reduction also occurs in neurogenic[77,78] and renal hypertensive animals[79], although the drug is less effective in the latter.

Several /

Several hypotheses have been advanced to explain the mode of action, and it has been claimed that hydrallazine acts by central inhibition of vasomotor reflexes. However, the evidence seems inconclusive. The carotid sinus pressor reflex is reported by different workers to be reduced or blocked[78] or not affected[80,81]. The pressor response to anoxia is not inhibited[78,82] but Bein et al.[83] have shown inhibition. Bein et al.[83] and Taylor et al.[84] have shown that the rise in blood pressure following central vagal stimulation is inhibited; but according to other investigators[82,78] it is unaffected. These reflex pressor responses may have the same mechanism of action, i.e. liberation of adrenaline and noradrenaline from the endings of adrenergic nerves, the reflex being mediated through the higher centres of the brain. In view of this fact, it is difficult to understand why a centrally acting drug should fail to antagonise some reflexes and not others. The drug does not lower further the blood pressure of the spinal cat, but when the blood pressure is raised to normal levels by ephedrine or ergotamine, the drug reduces it more markedly than in the anaesthetised animal[70]. This observation supports a peripheral site of action.

Craver and Yonkman[71], Grimson and Chittum[77] and Barret et al.[74] have shown that adrenaline, noradrenaline, pitressin, angiotonin, hypertensin and 5-hydroxytryptamine are antagonised. Page et al.[84] however state that neither renin nor any of the other substances mentioned above - with the exception of 5-hydroxytryptamine - are antagonised. Page et al.[84] claim that their "cerebral vasopressor hormone" and 5-hydroxytryptamine are both antagonised by hydrallazine. Erspamer[85] advances reasons for concluding that hydrallazine is quite non-specific in the antagonism to 5-hydroxytryptamine since other hypotensive drugs have similar effects. The drug, however, possesses only weak anti-adrenalytic action; furthermore, the role of these pressor substances in maintaining the blood pressure level of the normal individual or hypertensive patient is disputed. There is no evidence of peripheral ganglionic blockade in animals, and the failure of the drug to enhance the pressor effects of noradrenaline argues against ganglionic block. The possibility of a direct action on the blood vessels cannot be excluded: intra-arterial injections of hydrallazine in man have produced a rise in temperature of the skin of the corresponding area[86].

Hydrallazine /

Hydrallazine seems to have marked effects on regional circulations. Study of cardiac output and peripheral flow in the human suggests an increase - as in the animals. Hecht and his co-workers[86] confirm that an increase in cardiac output of approximately 100 per cent occurs. It seems possible that such cardiac stimulation exceeds any potential coronary dilatation, since it has been reported that anginal attacks were induced by the drug[87]. The renal effects of the drug are of special interest, since the increase in renal blood flow exceeds that reported with any other drug, except pyrogens[86,88 to 91]. The drug decreases the renal extraction of paraaminohippuric acid in animals[72]. The glomerular filtration rate is not changed despite increases in the renal blood flow. Thus it is not likely that the drug will improve excretion of metabolites in hypertensives with uraemia, or lower the nonprotein nitrogen, since these functions depend particularly on glomerular filtration rate and tubular reabsorption. Cerebral blood flow is maintained (as with the dihydrogenated ergot alkaloids and protoveratrine), despite the fall in pressure[92] but there is no true cerebral hyperaemia /

hyperaemia as might be expected from the development of headache. Splanchnic blood flow appears to be increased to almost twice control levels[83,93]. It would thus appear that this drug is a potent vasodilator which stimulates cardiac output and increases splanchnic and renal blood flow very considerably. Bein, Tripod and Meier[94] reported that hydrallazine, when perfused through the hearts of rabbits and cats, overcame coronary artery constriction induced by posterior pituitary extract and histamine. Tripod and Meier[95] have shown that hydrallazine dilated the blood vessels of the isolated rabbit's hindquarters, and that it antagonised vasoconstriction due to barium chloride or histamine. It is therefore obvious that the magnitude of the reduction in blood pressure will be the result of the summation of the effects of the drug on these different vascular beds or on regional circulations, and on the cardiac output.

Schuler and Meier[96] have shown that hydrallazine and dihydrallazine inhibit diamineoxidase much more effectively than monoamineoxidase. Similarly Werle et al.[97] studied the effects of hydrallazine and related /

related compounds on different enzyme systems such as monoamineoxidase from homogenised livers, diamineoxidase from pig's kidney and red clover, and dopadecarboxylase and histidine decarboxylase from guinea pig kidney. Hydrazine and guanylhydrazone derivatives strongly inhibited all these enzymes, with the exception of monoamineoxidase, but they had no influence upon the formation or inactivation of kallikrenin, kallidin and bradykinin. Certain hydrazino derivatives, with hardly any blood pressure reducing properties, also have an analogous effect on these enzymes. This observation makes it difficult to explain the hypotensive property of this drug on this basis. Another interesting observation was that of Douglass et al.[98] who have shown that hydrallazine inhibits the acetylation of sulphanilamide and glucosamine in pigeon liver extracts. The works of Defranceschi and Zamboni[99] and Makino et al.[100] on the metabolism of isoniazid, and the work of Johnson[101] on several aromatic and heterocyclic hydrazides have demonstrated that acetylation is an important pathway of metabolism of these compounds. They have concluded that this inhibition is of a quantitative nature and appears to be /

be due to the drug being acetylated by acetyl-coenzyme A, thereby indicating a disturbance in the metabolic function of a living cell.

In summary, the pharmacological effects of hydrallazine are not well understood but appear to be related to direct vascular effects, and perhaps also to changes in vasomotor regulation. Reserpine produces definite effects on the central nervous system to produce its characteristic tranquillizing effect, while the hypotensive character of the drug appears to be secondary in origin. The means by which it does so are not known. Reserpine does release 5-hydroxytryptamine and noradrenaline from brain and other tissues, but it remains to be seen whether this is purely coincidental or whether it does in fact give some clue to its mode of action.

The work described in this thesis was undertaken to investigate certain aspects of the modes of action of reserpine and hydrallazine. The earlier part of the thesis is devoted to a study of the pharmacological properties of hydrallazine and some related compounds. It is felt that interpretation of data obtained in such /

such experiments would give some information as to the action of reserpine and hydrallazine at cellular level. It appears that the fundamental action of reserpine or hydrallazine must ultimately involve the biochemistry of the cell, as is probably true of all pharmacologically active agents. With this fact in mind, the possibility of biochemical sites of action has also been investigated.

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CHAPTER II

CHAPTER II

EXPERIMENTAL WORK

Materials

Throughout the thesis the names of certain drugs have been abbreviated. The list of drugs used, together with their shortened names, is as follows:-

(1)	Acetylcholine chloride	is described	as	acetylcholine
(2)	Atropine sulphate	"	"	atropine
(3)	(+)-Tubocurarine chloride	"	"	tubocurarine
(4)	(-)-Adrenaline hydrochloride	"	"	adrenaline
(5)	(-)-Noradrenaline bitartrate	"	"	noradrenaline
(6)	Histamine acid phosphate	"	"	histamine
(7)	5-Hydroxytryptamine creatinine sulphate	"	"	5-hydroxytryptamine
(8)	Decamethonium iodide	"	"	decamethonium
(9)	Nicotine hydrogen tartrate	"	"	nicotine.

The composition and methods of preparation of all physiological saline solutions used in this investigation are to be found in Appendix I.

The /

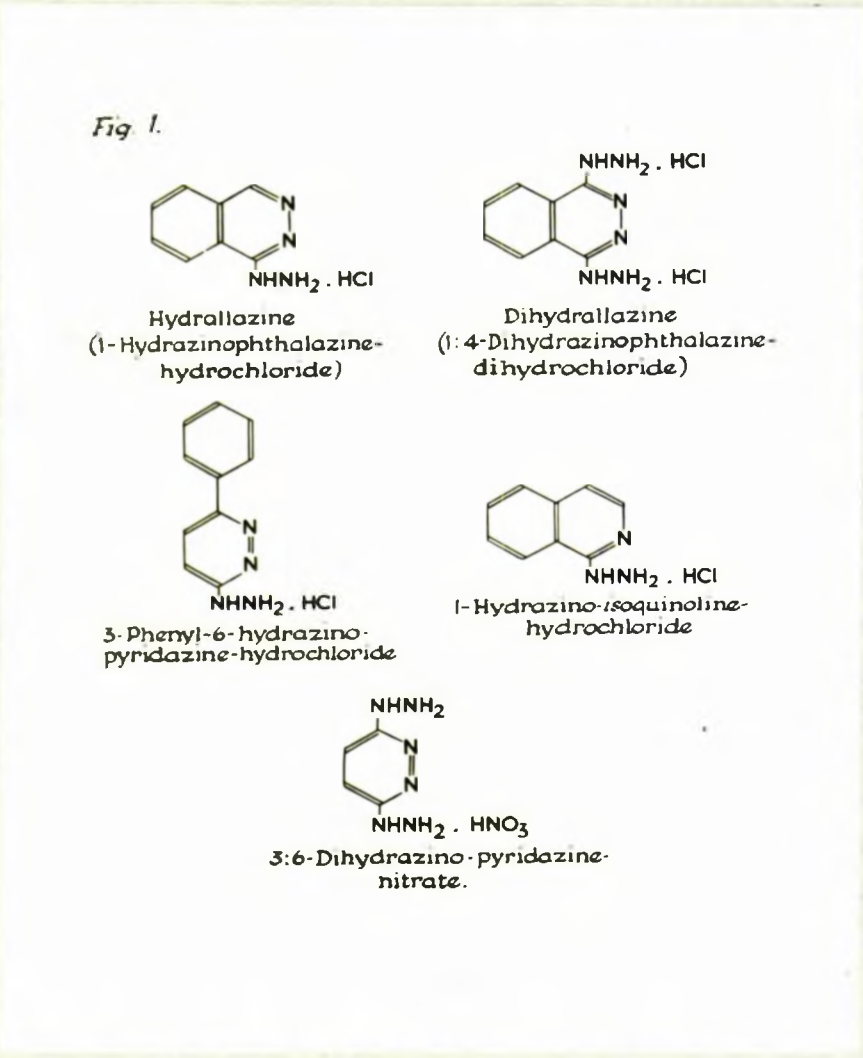

Fig. 2.1

Structural formulae of hydrallazine, dihydrallazine, 3-phenyl-6-hydrazino-pyridazine nitrate, 1-hydrazino-iso-quinoline hydrochloride and 3:6 dihydrazino pyridazine nitrate.

The conventional abbreviations for volumes and weights of the metric system are used throughout this thesis.

In the first part (A) of this chapter, the various experimental procedures employed are described, and the second half (B) is devoted to the results. The pharmacological properties of hydrallazine, dihydrallazine, 3-phenyl-6 hydrazino-pyridazine hydrochloride, 1-hydrazino isoquinoline hydrochloride and 3-6-dihydrazino-pyridazine nitrate have been investigated (Fig. 2.1).

A. EXPERIMENTAL

1. EXPERIMENTS ON THE BLOOD PRESSURE OF THE ANAESTHETISED CAT

Method.

Cats of either sex, weighing between 2.0 and 4.0 kg. were used. Anaesthesia was induced by means of an intraperitoneal injection of chloralose. A one per cent solution of chloralose in distilled water was made. When this solution was used, a dose of 8 ml. per kg. (80 mg. per kg.) was usually sufficient to produce surgical anaesthesia in about 30 minutes.

In some cats pentobarbitone was used as the anaesthetic. 60 mg. per kg. injected intraperitoneally was found to be an adequate anaesthetic dose.

The anaesthetised cat was laid on its back upon an operating table; the legs were secured to the table and the head extended by passing a string through the skin at the apex of the lower jaw. The skin covering the neck was cut away from the sternum up to the apex of the mandible. The fascia covering the trachea was divided at the midline and the blunt point of an aneurysm needle was passed between the muscles of the neck and thence around the trachea. A strong linen thread /

thread was passed around the trachea and a transverse incision made by means of a scalpel. The cut edge of the partly severed trachea was held in a pair of blunt forceps and a tracheal cannula inserted and tied into place. This was done as a precautionary measure in case it became necessary (due to drug-induced depression or failure) to keep the cat alive by means of artificial respiration. The amount of air entering and leaving the cannula could be controlled by means of an adjustable sleeve or side tube.

The external jugular vein, usually of the left side, was then cannulated. To do this the skin of the left anterolateral part of the neck was removed and the left external jugular vein exposed. The fascia covering the vein was carefully removed, a thread was tied around the cephalad end and a bulldog clip was put on the vein on the cardiac aspect. A small transverse cut was made in the dilated vein by means of a pair of sharp pointed iris scissors. A vein cannula, filled with a solution of heparin, was then inserted through the incision with the pointed end towards the heart. The cannula was connected by means of rubber tubing to a 50 ml. burette containing normal /

normal physiological saline. All the air bubbles had been previously removed from the system. The observation that the saline in the burette ran freely into the vein indicated that the cannula had been correctly inserted. Having completed the cannulation of the trachea and of the external jugular vein, an artery cannula was inserted into one of the carotid arteries. The artery was first tied off as near to the head as possible: a bulldog clip was then placed on the artery about three cm. below the ligature and a thread was passed under the vessel midway between the ligature and the bulldog clip. A small transverse cut was made in the artery by means of a pair of sharp pointed iris scissors. An artery cannula, filled with a solution of heparin, was then inserted through the incision with the pointed end towards the heart. The cannula was connected to a mercury manometer, and the space between the mercury and the artery filled with a twenty-five per cent solution of sodium thiosulphate as an anticoagulant. Air having been displaced from the cannula and the pressure in the manometer set at about 120 mm. of mercury, the artery clip was removed. A writing flag

on /

on one arm of the mercury manometer recorded the blood pressure on a smoked surface.

Drug solutions were injected into the rubber connection between the vein cannula and the burette. Each injection was followed by the infusion of 3 ml. of saline from the burette. The blood pressure was usually recorded from the cannulated common carotid artery, but when a reflex pressor response was elicited by means of bilateral occlusion of the common carotid arteries, the blood pressure level was recorded from one of the cannulated femoral arteries.

In a number of anaesthetised cats (pentobarbitone or chloralose), the blood pressure level was raised by the continuous infusion of solutions of adrenaline or noradrenaline, and drugs were injected when the elevated blood pressure level had become steady. The concentration of the adrenaline and noradrenaline solutions which were infused was 0.10 mg. per ml. and the rate of infusion was 1.0 ml. per minute from a Palmer's constant rate slow infusion apparatus.

Vasopressor Reflexes.

The /

The effects of drugs on various pressor reflexes were studied by comparing the nature and magnitude of the reflex responses observed before and after their injection. At least three uniform reflex responses were obtained before drug injection. Pressor responses were obtained following the elicitation of the following reflexes:-

(a) Carotid Sinus Pressor Reflex. Bilateral occlusion of the common carotid arteries for a period of twenty to forty seconds: this reflex was elicited either by placing bulldog clips on to the arteries or by pulling upon threads looped loosely around them.

(b) Occlusion of the Abdominal Aorta. The abdominal cavity was opened by a midline incision. The rectus muscles and the fasciae were retracted, the viscera pushed to the right and the abdominal aorta carefully dissected free from the fascia at a point a little below the diaphragm. A thread was then passed loosely around the aorta by means of an aneurysm needle at a point just above the origin of the coeliac artery. The abdominal aorta was occluded for fifteen to forty seconds by pulling upon the thread.

(c) /

(c) Stimulation of the Cut Central End of the Cervical Vagus. The right vago-sympathetic trunk was carefully dissected free from its fascia and separated from adjacent structures. The vagus was then freed from the cervical sympathetic trunk with which it runs. The vagus was divided by means of scissors at as low a point in the neck as possible. The central end of the cut vagus was placed upon a pair of platinum electrodes which were connected to the output of a stimulator. The nerve was stimulated by means of square wave impulses at a frequency of 800 to 1,000 per minute, at 8 to 12 volts and with a pulse width of 0.5 to 1.0 msec. Stimulation was continued for periods of fifteen to twenty seconds.

(d) Stimulation of the Greater Splanchnic Nerve. This nerve joins the coeliac ganglion which is situated a little below the coeliac artery near to its origin from the aorta, and distributes fibres to the renal and suprarenal plexuses and to the other abdominal nerve plexuses. The coeliac ganglion was carefully dissected free and the greater splanchnic nerve traced upwards and cleared from fascia. The nerve was divided close to the ganglion and the cut central end was /

was placed upon a pair of platinum electrodes which were held in position by means of a clamp, and the abdomen closed. The nerve was stimulated with fifteen to twenty seconds bursts of impulses by means of a Dobbie McInnes square wave stimulator, at a frequency of 800 to 1400 per minute, at 5 to 10 volts and with a pulse width of 1.0 to 1.5 msec.

13/12 to 23/12

(e) Stimulation of the Cut Central End of the Sciatic Nerve. The sciatic nerve can be seen when dissecting the muscles of the hip and thigh. The main sciatic trunk gives off a fairly large branch to the inner surfaces of the biceps, femoris, semitendinosus and semimembranosus. The sciatic nerve passes down median to the biceps and femoris branches supplying the long slender suralis. The main trunk of the sciatic nerve branches in the popliteal space into the lateral peroneal and tibial nerves. The sciatic nerve was stimulated at a point above the emergence of the peroneal and tibial nerves. The nerve was carefully freed from adhering connective tissue etc. and divided by means of scissors. The central end was placed across a pair of platinum electrodes which were connected to a Dobbie, McInnes square wave stimulator. The /

The nerve was stimulated by means of square impulses at a frequency of 800 to 1000 per minute, at 10 to 12 volts and with a pulse width of 0.5 to 1.0 msec. Stimulation was continued for periods varying with different cats but did not exceed 40 seconds. Conditions were maintained constant for the particular experiment.

(f) Hypoxia. The cat was allowed to inhale a mixture of 95 per cent nitrogen and 5 per cent carbon dioxide from a Douglas bag for one to three minute periods.

(g) Local Application of Drugs to the Region of Carotid Sinus. Both carotid arteries of chloralose anaesthetised cats were exposed by dissecting away the muscles and connective tissue. Anterior to the base of the occipital artery, the common carotid divides into two branches - the smaller internal and larger external carotid arteries. The place at which the branching takes place is called the carotid sinus area. This is prominent and easy to dissect because of the bulging of the carotid sinus. The sinuses were exposed carefully. Care was taken to avoid /

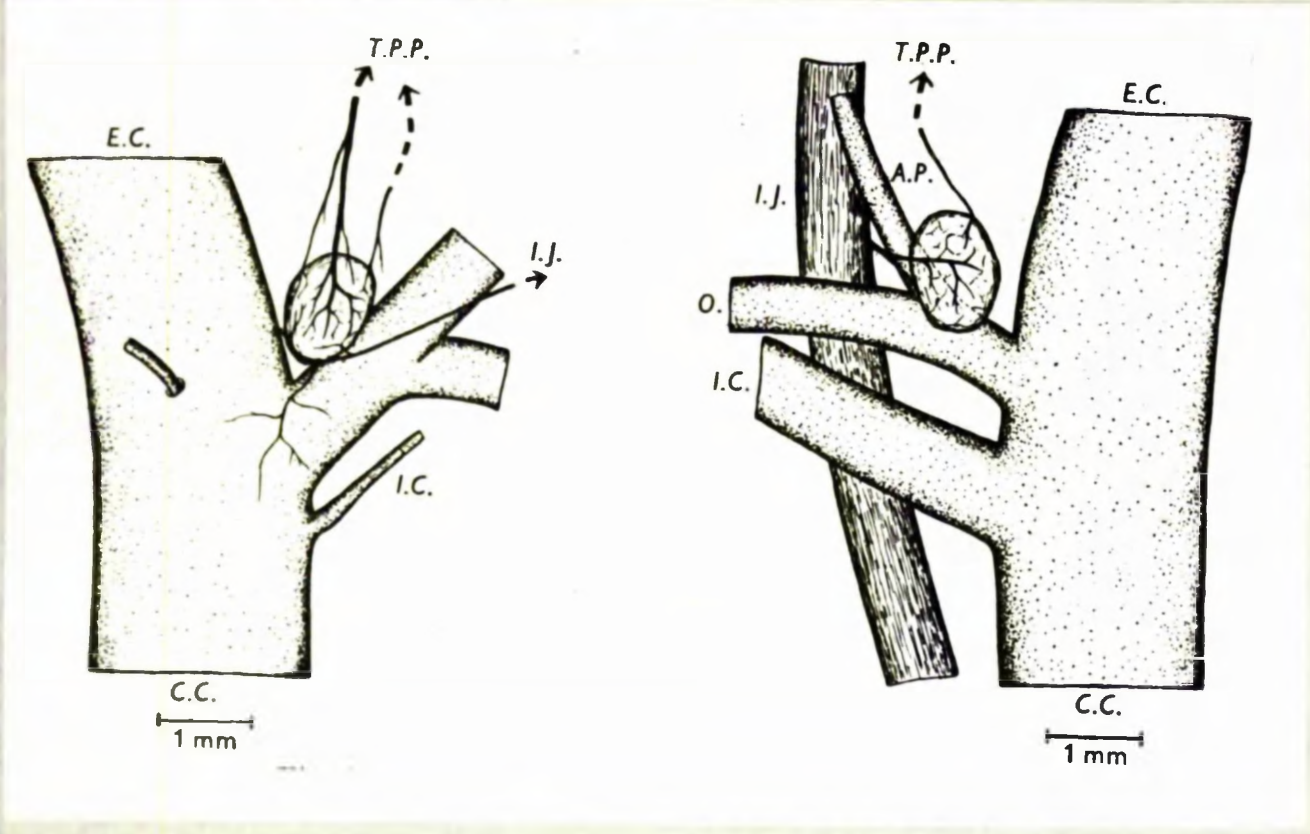

Fig. 2.2

Diagram showing the carotid sinus region and carotid body of the cat injected with indian ink.

Left diagram shows the ventro-medial view of the right carotid. The right one shows the ventro-medial view of the left carotid.

C.C.= common carotid artery, I.C.= internal carotid artery,
E.C.= external carotid artery, O = occipital artery,
A.P.= ascending pharyngeal artery, I.J.= internal jugular vein,
T.P.P.= transverse posterior pharyngeal vein.

(Chungcharoen,D., de Burgh Daly,M., and Schweitzer,A. (1952), The blood supply of the carotid body in cats, dogs and rabbits.

J. Physiol. 117, 347.)

avoid loss of blood by tying off the collateral vessels during the entire procedure. In order to limit vagal reflexes, both the vagi were severed. The systemic blood pressure was recorded from one femoral artery using a mercury manometer. The drugs were applied ? systemically to the walls of the arteries of both the carotid sinuses by gently injecting 1 ml. of solution into the sinus areas (Fig.2.2).

2. EXPERIMENTS ON THE BLOOD PRESSURE OF SPINAL CATS.

Method.

Cats within the weight range of 2.0 to 3.0 kg. were given atropine (1.0 mg. per kg.) by intraperitoneal injection, about 15 minutes before induction of anaesthesia by ether. The common carotid arteries were dissected free from the accompanying vago-sympathetic trunks and tied. The trachea was next freed from adjoining tissues and cannulated. The tracheal cannula was connected by means of rubber tubing to a bottle containing ether. The ether bottle could be joined rapidly and easily by means of rubber tubing to an /

an artificial respiration pump. The cat was then turned over, and the spinal cord exposed in the vicinity of the long spine of the second cervical vertebra. The bony covering of the spinal cord and finally the cord itself were cut by means of bone forceps. As soon as the cord was transected, artificial respiration was commenced. Bleeding was arrested by means of cotton wool swabs soaked in hot normal saline. A probe was inserted through the foramen magnum, pushed up into the brain and the brain destroyed. The cut end of the spinal canal was plugged with plasticine and the area swabbed clear. The skin over the back of the neck was closed with the aid of surgical clips, and the animal again turned on to its back. One of the carotid arteries was cannulated and connected to a conventional pressure recording system filled with a 25 per cent sodium thiosulphate solution. A mercury manometer carrying a writing flag on one arm was incorporated into this system and arranged so as to record the blood pressure on a smoked surface. The external jugular vein was cannulated and connected by rubber tubing to a burette filled with normal saline. Drug solutions were injected into the rubber /

rubber connection between the vein cannula and the burette. Each injection was followed by the infusion of 3 ml. of saline from the burette.

In all experiments, the preparation was left to settle for at least one hour before any experiment was carried out. No drug was given unless the blood pressure level had remained constant for 15 to 30 minutes.

3. EXPERIMENTS ON ISOLATED, PERFUSED RABBIT AND KITTEN HEARTS.

The isolated hearts of both rabbits and kittens were perfused according to the method of Langendorff[1]. This involves perfusion of the coronary vessels through the aorta. Wegria[2], in his review on the pharmacology of the coronary circulation, quotes several published criticisms of this method. It is pointed out that the recorded outflow will give a true picture of the state of tonus of the coronary vessels only if the aortic valves are competent. This is not always so. In the event of aortic incompetence, some perfusion fluid will leak past the valves into the left ventricle and so into the left atrium, and thence to the exterior. The increased /

increased outflow may therefore exceed the true coronary outflow by the amount of fluid which has passed into the left ventricle. The volume of fluid draining into the right atrium *via* the ventricle is not constant and, in addition, cannot be measured satisfactorily. It is also pointed out that the volume of coronary perfusate may be increased by a purely mechanical massaging effect which cardiac muscle - stimulated by a cardiotonic drug - has upon the coronary vessels. Under these circumstances, an increase in outflow might be taken to indicate a coronary dilatation which in fact was not present. For these reasons it was decided that the fluid draining from the heart should be described simply as the "cardiac outflow". In spite of the objections raised to the use of this method, it was felt that the Langendorff preparation would still give some useful information about the effects of drugs on cardiac function *in vitro*. By carefully observing the heart rate, the amplitude of the contractions, and at the same time measuring the outflow, an estimate of alterations of cardiac function as well as of the tonus of the coronary vessels can be obtained.

Method.

/

Method.

Rabbits and kittens used were within the weight ranges of 1.0 to 2.0 kg. and 0.6 to 1.0 kg. respectively. The animal was killed by a blow on the head. The throat was cut and the blood allowed to drain out. The animal was then placed upon its back on a dissecting board and the thoracic cavity exposed, care being taken not to damage the heart with scissors or other instruments. The lungs were removed and a thread was tied loosely around the aortic arch proximal to the origin of the innominate artery. The venae cavae and aorta were then severed and after removing the pericardium, the heart was lifted out of the thorax and placed in a dish of warm Locke's solution (Appendix 1) at a temperature of about 37°C, and containing a little heparin to prevent the blood inside the heart from clotting. A stream of Locke's solution was allowed to run through the superior vena cava from a pipette, and the heart was squeezed gently. After washing, a cannula was tied into the aorta, taking care that its tip was distal to the coronary ostia. The preparation was then set up by connecting the cannula to the perfusion apparatus. Perfusion of oxygenated

Locke's /

Locke's solution, containing double the normal concentration of glucose, was started at a constant rate of flow and at a pressure of 35 mm. of mercury, care being taken that no air bubbles entered the aorta. Any blood remaining in the preparation was rapidly washed away, and as a rule the heart started to beat immediately. After about thirty minutes, when the beat had become regular, a supporting thread was tied by means of a fine needle through the tip of the left ventricle. A bent entomological pin was inserted into the wall of the right ventricle and connected to a Starling's heart lever which recorded the contractions of the heart on the surface of a smoked drum. Doubling the normal concentration of glucose in the perfusion fluid gave a more active preparation, and one which was fatigued less easily. The Locke's solution from the two reservoirs used flowed through heating coils in a water bath maintained thermostatically at 37°C. The two coils were connected by a glass Y-piece which was joined to the aortic cannula by a short length of rubber tubing. The temperature drop between the thermostatically controlled water bath and the cannula was never more than 0.2°C.

Hydrallazine /

Hydrallazine and other compounds were dissolved in Locke's solution in one of the reservoirs to give the concentration of 10.0 µg. per ml. The other bottle contained Locke's solution. Solutions of other drugs in Locke's solution were injected by means of a one ml. tuberculin syringe into the rubber tubing attached to the aortic cannula. The heart rate was counted by inspection of the tracing or by direct observation, and the outflow measured at five minute intervals by collecting the perfusate for a period of one minute. In some experiments the outflow was measured by means of a Gaddum outflow recorder.

4. EXPERIMENTS ON THE ISOLATED AURICLES OF THE GUINEA PIG.

These experiments were carried out in order to study the action of drugs on isolated cardiac muscle.

Method.

Adult guinea pigs of either sex were killed by a blow on the head. The throats were cut and the blood allowed to drain out. The hearts were removed as rapidly as possible and immersed in well-oxygenated Locke's solution. Using a pair of fine scissors, the ventricles /

ventricles were carefully removed and the auricles placed upon a cork mat and moistened frequently with Locke's solution. All extraneous tissue was dissected away until the horseshoe shaped auricles alone remained. These were then suspended in a 50 ml. organ bath by means of two bent entomological pins to which fine cotton threads were tied. One thread was connected to the oxygen delivery tube at the base of the bath, the other to a Starling's heart lever set up so as to record the contractions of the auricles upon a smoked surface. After about twenty to thirty minutes, the beat of the auricles had usually become regular and the experiment was commenced. All drugs were added to the bath as solutions in Locke's solution, and by means of a one ml. tuberculin syringe. The effect of each drug, other than hydrallazine and the other compounds, was observed for a period of sixty seconds, after which the fluid in the bath was replaced. Sufficient time was allowed for the auricles to regain a normal regular rhythm and amplitude of beat before the next addition of the drug. Hydrallazine and the other compounds were added 5 minutes before each of the stimulant or depressant drugs tested.

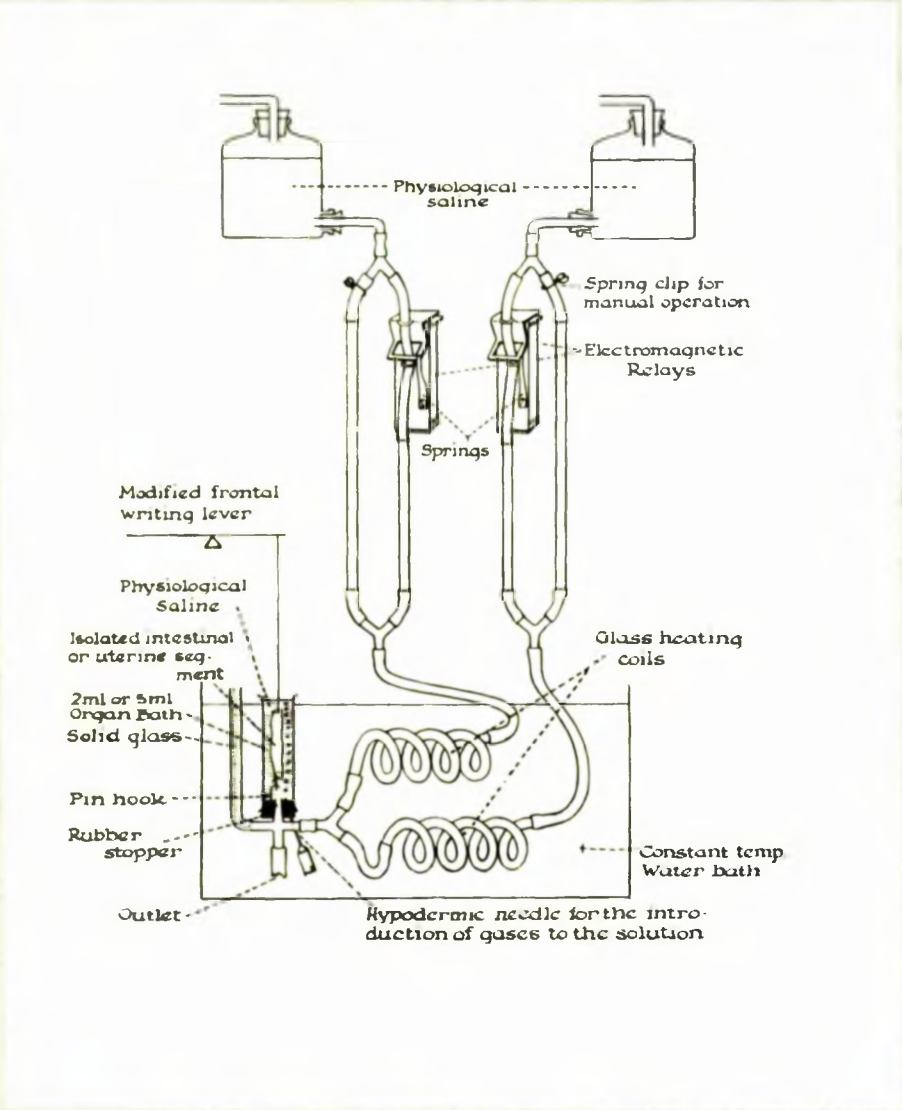

Fig. 2.3

Diagram of the semi-automatic isolated organ bath used for experiments upon isolated strips of guinea pig ileum and rat uterus.

5. EXPERIMENTS UPON THE ISOLATED GUINEA PIG ILEUM.

Method.

Guinea pigs of either sex, weighing between 0.3 and 0.5 kg., were killed by a blow on the head and the throats cut in order to drain out the blood. The abdominal cavity was opened and a piece of ileum about 3 cm. long was removed from the region about 3 cm. proximal to the ileocaecal junction. It was then freed from extraneous tissue and the contents washed out by means of a stream of Tyrode's solution (Appendix 1). Threads were tied to both ends of the segment which was then set up in a 2 ml. organ bath (Fig.2.3) containing oxygenated Tyrode's solution. One thread was attached to a modified frontal point writing lever, and the other to a hook fixed into the base of the bath. The fluid in the bath was oxygenated by passing oxygen through a hypodermic needle fixed into the base of the bath. The temperature was maintained thermostatically at 34 to 35°C. Solutions containing the stimulant drugs were added and washed out automatically using the overflow principle. The inlet tube at the lower end of the bath was /

was connected *via* heating coils to two reservoirs, one of which contained Tyrode's solution, the other a solution of the stimulant drug in the same fluid. The dose of the stimulant drug to be used was determined at the start of each experiment by adding it to the bath by hand. The stimulant drug was then dissolved in Tyrode's solution to give the dilution required, and the automatic apparatus switched on. The electrical controlling equipment replaced the saline solution in the bath by a solution containing the stimulant drug, at three minute intervals. Since the solution containing the stimulant drug flowed into the bath for five seconds, there was complete replacement of the solution in the bath. Reproducible submaximal contractions were obtained to acetylcholine or histamine added at three minute intervals and left in contact with the tissue for thirty seconds. At the end of this period the acetylcholine or histamine solution was washed out by the automatic inflow for a period of five seconds of Tyrode's solution. The drug solution was added to the bath by hand one minute before the next inflow of acetylcholine, 5-hydroxytryptamine, histamine or barium chloride. This point in the cycle

was /

was indicated by a signal light placed in the circuit. The contractions were allowed to return to a constant level before the next addition of the drug.

6. EXPERIMENTS UPON THE ISOLATED RABBIT DUODENUM.

Method.

Rabbits of either sex, weighing between 1.5 and 2.5 kg., were killed by a blow on the head. The throat was cut and the blood allowed to drain out. The abdominal cavity was opened and a piece of duodenum about 4 cm. long removed. This was freed from fatty and other tissues. Threads were tied to both ends of the segment, and a piece of duodenum set up in a 40 ml. organ bath containing oxygenated Locke's solution at 37± 0.5°C. The thread at one end of the duodenum was fixed to the lower end of a glass tube supplying oxygen to the bath, and the thread at the other end was attached to a modified frontal point writing lever giving a magnification of 1 in 8. Adrenaline and acetylcholine in solution in Locke's solution were added to the bath by means of a one ml. tuberculin syringe, and the effect was observed for two minutes /

minutes. At the end of this period the fluid in the bath was replaced several times by running in fresh Locke's solution. The next addition of adrenaline or acetylcholine was not made until the tissue regained its original length. The solution of drugs was added to the bath two minutes before adding acetylcholine, adrenaline, histamine or barium chloride and the effects observed.

7. EXPERIMENTS ON THE ISOLATED RAT UTERUS

Virgin female rats, weighing between 150 to 200 g., were brought into oestrous by the subcutaneous injection of 0.10 mg. stilboesterol per 100 g. body weight. Injections were given 24 hours before use. The animals were killed by a blow on the back of the head, the throats cut and the blood drained out. The uteri were removed and placed in the oxygenated De Jalon's solution (Appendix 1).

Segments of uterus were freed from fat and other tissues. Threads were tied to both ends of the segments which were then set up in a 2 or 5 ml. organ bath at 29°. One thread was attached to a modified frontal

point /

point writing lever, the other to the hook fixed into the base of the bath. Drug solutions were added and the bath washed out automatically using the overflow principle. Some uteri showed spontaneous activity and therefore had to be rejected. The inlet tube at the lower end of the bath was connected *via* heating coils to two reservoirs, one of which contained the physiological solution, the other a solution of the drug. The uterus was stimulated by using acetylcholine, carbachol or 5-hydroxytryptamine. The approximate dose of the drug was determined previously for each experiment. It was then dissolved in De Jalon's solution to obtain the required dilution and the automatic apparatus switched on. When the response to the drug had become constant, hydrallazine or other drugs were added 60 seconds before the next addition of the spasmogen. The contractions were allowed to return to the constant level before the next addition of hydrallazine. Other experimental details are similar to those described for the guinea-pig ileum (p.62).

8. /

8. EXPERIMENTS USING THE ISOLATED RAT PHRENIC-NERVE DIAPHRAGM PREPARATION

The procedure adopted was essentially that described by Bülbring[3]. Adult rats of either sex were killed by a blow on the head, the throats cut and the blood allowed to drain out. The rat was then laid on its back upon a cork-covered dissecting board to which it was pinned. The skin over the chest was removed and the thorax opened along the right side of the sternum. The frontal part of the right thoracic wall was removed. The mediastinum behind the sternum was severed, and a cut was made just above the frontal insertion of the diaphragm. Care was taken not to damage the phrenic nerve which is sometimes attached to the ribs. The frontal part of the left thoracic wall was then removed and the phrenic nerve exposed. Both lobes of the left lung were removed, and the left phrenic nerve carefully freed from fat and other tissues: the utmost care was taken not to injure it. The left abdominal muscles were cut along the costal margin. Holding the last rib with a pair of forceps, a segment of diaphragm was then dissected out. Two converging cuts were made through the ribs towards the tendinous part /

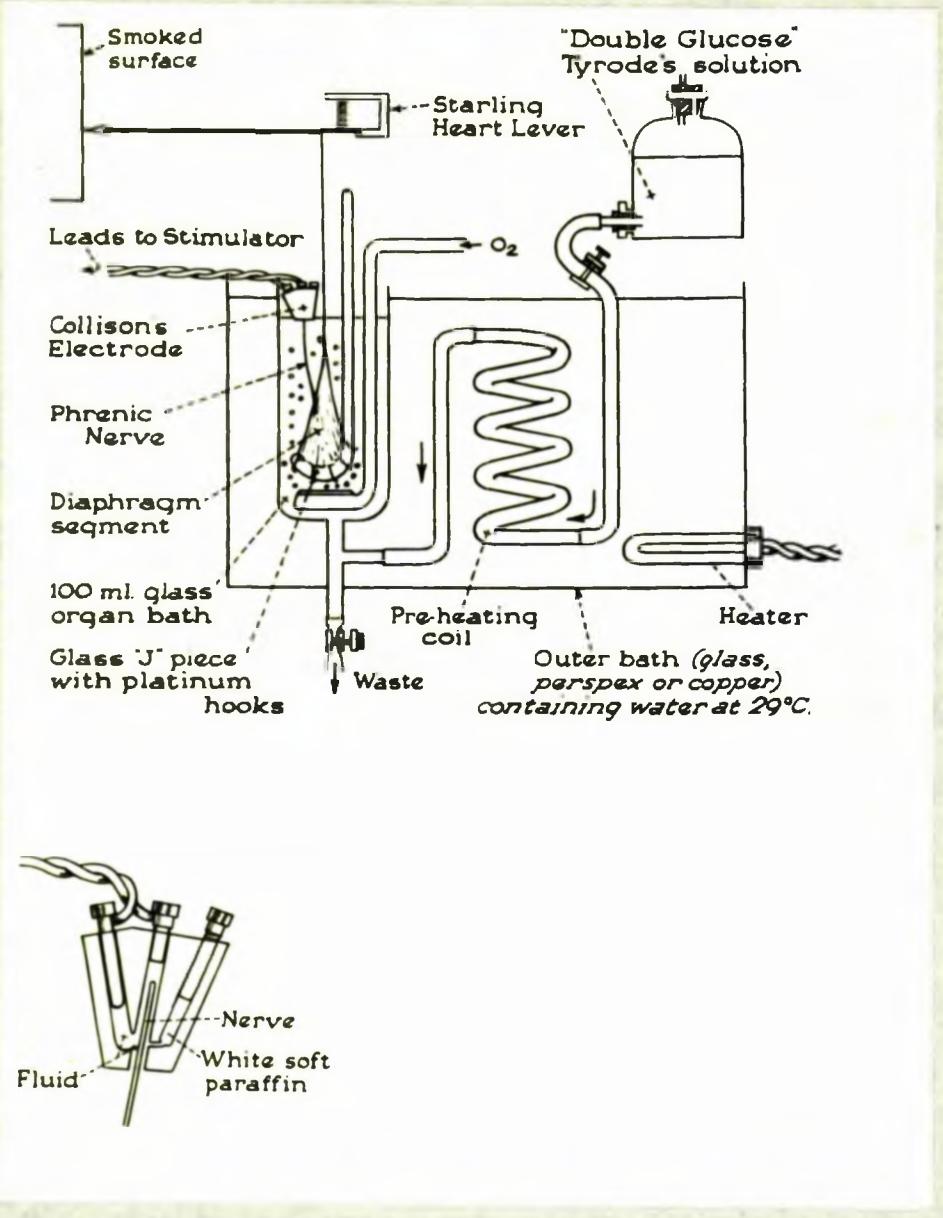

Fig. 2.4

Diagram of the apparatus used for experiments on isolated rat phrenic nerve diaphragm, inset showing Collison's electrodes and the position of the nerve.

(After Lewis, J.J. (1959), Textbook of Pharmacology, Livingstone, Edinburgh.)

part of the diaphragm, and parallel to the direction of its muscle fibres. The cuts were made about 5 mm. to the right and 5 mm. to the left of the point where the phrenic nerve entered the diaphragm. The strip of diaphragm was dissected out beyond its tendinous part with about 5 cm. of phrenic nerve attached to it. The final preparation was fan-shaped, 2 mm. wide at the tendinous end and about 20 mm. wide at the costal margin. The costal margin of the diaphragm segment was fixed to a J-shaped glass rod by means of platinum wires, and a thread was tied around the tendinous end. The preparation was then set up in a 100 ml. organ bath (Fig 2.4) containing double glucose Tyrode's solution. The J-piece held the costal margin of the segment in position at the bottom of the organ bath, while the thread tied at the tendinous end was fixed to a light isotonic heart lever writing upon a smoked drum surface. Tyrode's solution, containing double the usual amount of glucose, was supplied to the bath from a reservoir via the heating coils. The temperature of the bath was maintained thermostatically at 29± 0.5°C. A sintered glass distribution tube was fixed at the bottom of /

of the bath to provide a vigorous supply of oxygen with which the bath fluid was aerated in a form of fine bubbles. A thread was now tied to the cut end of the phrenic nerve and, by means of a fine needle, about one cm. of the phrenic nerve was drawn into a fluid electrode(4). The fluid electrode containing the nerve was filled with double glucose Tyrode's solution and the hole at the bottom of the electrode was sealed off with soft paraffin. By this arrangement, the fluid surrounding the nerve at the point of stimulation was not in electrical contact with the fluid surrounding the muscle in the bath. The nerve was left sufficiently slack to allow for any movement caused by the contraction of the diaphragm. Single square wave impulses were applied to the nerve by means of a Dobbie McInnes square wave generator at a frequency of 6 to 10 per minute, at 8 to 12 volts, the pulse width being 0.5 to 2.0 msecs. In any one experiment frequency, voltage and width were kept constant.

Drugs (in solution in double glucose Tyrode's solution) were added to the bath by means of a 1 ml. tuberculin syringe. The drug was allowed to act for three //

three minutes after which the Tyrode's solution in the bath was changed. Between the addition of one dose of the drug and the addition of the next, there was an interval of about 15 minutes during which the Tyrode's solution was changed several times.

West[5] observed that the preparation gave a constant response of good magnitude for a longer period, if the temperature of the bath was lowered from 37°C. to 20°C. He also found that the rate of recovery of the muscle after the addition of a dose of tubocurarine was greatly accelerated by adding potassium chloride to the bath after the tubocurarine had been washed out. In the present series of experiments, it was observed that lowering the bath temperature from 37°C to 29°C without addition of potassium chloride gave a satisfactory recovery, and that the magnitude of the muscle contraction was maintained in a satisfactory manner.

Chou[6], using the phrenic nerve-diaphragm preparation for the assay of curare-like substances, allowed the solution of tubocurarine to act for 3 minutes. In the experiments described in this section, this /

this feature of Chou's technique was adopted because it was thought that if the drug was allowed to act upon the tissue for long enough to produce its maximal effect, then so much time would be needed to wash the drug off the receptors that few comparisons could be made upon one preparation.

Hydrallazine and dihydrallazine were allowed to act for 3 minutes before the drugs were added.

9. EXPERIMENTS USING THE ISOLATED FROG RECTUS ABDOMINIS MUSCLE.

Method.

The procedure used for preparing the muscle to record the effects of drugs was essentially similar to that described by Burn[7]. An adult frog was stunned by means of a blow on the head, decapitated and pithed. The frog was laid on its back upon a cork covered dissecting board to which it was pinned. The rectus muscle was exposed by cutting away the skin of the abdomen, and then it was dissected from its insertion into the pelvic girdle to its insertion into the cartilage of the pectoral girdle. The rectus muscle

was freed from the underlying connective tissues, removed from the frog and then suspended in an organ bath of 20 ml. capacity by means of two threads tied to either end of the muscle. A loop was made in the thread at one end in order to fix the muscle to the bent wire in the base of the bath, and a long thread left at the other end. The long thread was tied to a modified frontal point writing lever which gave a magnification of 8 to 10 times. The bath contained 20 ml. of oxygenated frog Ringer's solution (Appendix 1) at room temperature. Acetylcholine, decamethonium and nicotine were dissolved in frog Ringer's solution to give the concentration required and added to the bath by means of a one ml. graduated tuberculin syringe.

Solutions of drugs were added to the bath in a similar way. Uniform submaximal contractions to the same dose of acetylcholine were obtained before the effects of drugs were studied. The time interval between each dose of acetylcholine was six minutes; the resulting contractions were recorded for ninety seconds. With nicotine and decamethonium the time interval between /

between doses was about 15 to 20 minutes; the resulting contractions were recorded for 2 minutes. The bath was washed out with fresh frog Ringer's solution between doses of acetylcholine, decamethonium and nicotine. Hydrallazine and other compounds were added one minute before the next addition of acetylcholine, decamethonium or nicotine. Before the next addition of drugs, sufficient time was allowed for the muscle to regain its original length.

The effects of drugs on the antagonism of atropine and tubocurarine to acetylcholine were also observed.

10. EXPERIMENTS ON THE NICTITATING MEMBRANE OF THE ANAESTHETISED CAT.

Method.

In these experiments cats of either sex, weighing between 2.0 and 3.0 kg., were used. The cat was anaesthetised by means of an intraperitoneal injection of chloralose, and tracheal and vein cannulae inserted as described on pages 46 to 50.

The head was rigidly fixed by passing a brass rod between the jaws and then tying the jaws firmly together with /

with string. The ends of the rod were then gripped firmly in clamps, and these were supported on uprights fixed to the side of the operating table. By means of a fine needle, a silk thread was passed through the mid-point of the margin of the nictitating membrane of the right eye, and was tied firmly into place. The thread was then pulled forward and to one side, thus making an angle of about 30^{o} to the long axis of the cat. It was then led around pulleys and attached to a frontal point writing lever. The contractions of the nictitating membrane were recorded on a smoked surface.

The right cervical sympathetic chain was now dissected out and a fine cotton thread tied tightly around it at as low a point as possible in the neck. The chain was severed above the ligature and low in the neck. The cut preganglionic cervical sympathetic chain was then placed upon a pair of platinum electrodes and kept moist with normal saline. Contractions of the nictitating membrane were elicited by stimulation of the cervical sympathetic by means of square impulses at a frequency of 800 to 1,200 per minute, 8 to 15 volts, the pulse width being 0.5 to 1.0 msec. In any one experiment /

experiment frequency of stimulation, voltage and pulse width were constant. The nerve was stimulated, at 3 minute intervals, for 15 seconds. Having obtained standard reproducible responses of the nictitating membrane by stimulating the nerve trunk, solutions of drugs were injected into the external jugular vein one minute before the next period of stimulation. Contractions of the nictitating membrane were also obtained in response to adrenaline or noradrenaline and the effects of drugs studied.

B. RESULTS.

1. EXPERIMENTS ON THE BLOOD PRESSURE OF THE ANAESTHETISED CAT

Hydrallazine, dihydrallazine and 3:6 dihydrazino pyridazine nitrate in doses of from 1 to 2 mg. per kg. produced a delayed and gradual fall in blood pressure with slight stimulation of respiration and heart rate. After intravenous injection of hydrallazine, the blood pressure did not fall suddenly, but relatively slowly within 10 to 20 minutes and then remained for hours at this lower level. With an adequate dose (1 mg. per kg.), the pressure sometimes remained lowered for 6 to 8 hours. Even relatively large doses of the hydrallazines and pyridazines did not usually cause any abrupt or marked fall of pressure, such as is obtained with ganglionic blockers or after histamine release. A correlation between the extent of the hypotension and the dose used was rather difficult to demonstrate since increasing the dose did not increase the extent of the fall in blood pressure but, at most, prolonged the duration of the effect. The extent of the fall in blood pressure was dependent, at least in part, on the initial blood pressure level; the higher the latter, the more marked was the fall. Increases

in /

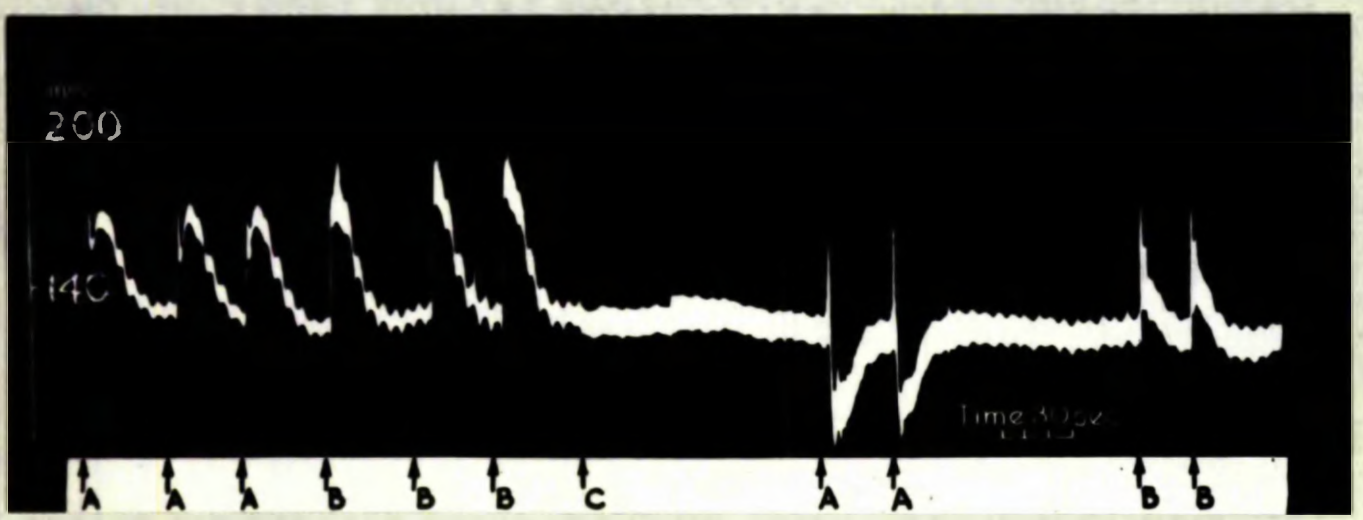

Fig. 2.5

Modification of adrenaline and noradrenaline responses by hydrallazine.

Cat. Chloralose anaesthesia. Blood pressure recorded from the common carotid artery. Drugs administered intravenously.

At A, adrenaline, 2 µg. per kg.
B, noradrenaline, 2 " " "
C, hydrallazine, 1 mg. per kg.

in respiratory and cardiac rates were not prolonged and soon returned to the control level. 1-Hydrazino isoquinoline hydrochloride and 3-phenyl-6 hydrazino-pyridazine hydrochloride in doses of 1 to 2 mg. per kg. occasionally failed to produce a fall in the blood pressure; instead there was a gradual rise. The observations recorded in this thesis are similar to those of Bein, Gross, Tripod and Meier(8).

Hydrallazine, dihydrallazine and 1-hydrazino isoquinoline hydrochloride in the dose of 1 mg. per kg. altered the pressor response to adrenaline (0.15 to 2.0 µg. per kg.) into a biphasic pressor, depressor response indicating an effect upon adrenaline-induced vasoconstriction but no effect upon the stimulant actions of adrenaline on the heart (Fig. 2.5). 1-hydrazino isoquinoline hydrochloride and 3-phenyl-6 hydrazino pyridazine hydrochloride had similar effects against adrenaline but occasionally failed to reverse the response. Hydrallazine, dihydrallazine and 3:6 dihydrazino pyridazine nitrate (1 mg. per kg.) partially antagonised the pressor response to noradrenaline (0.5 to 2 µg. per kg.), but the other compounds had little or no effect. Hydrallazine and other compounds (1 mg. per kg.) /

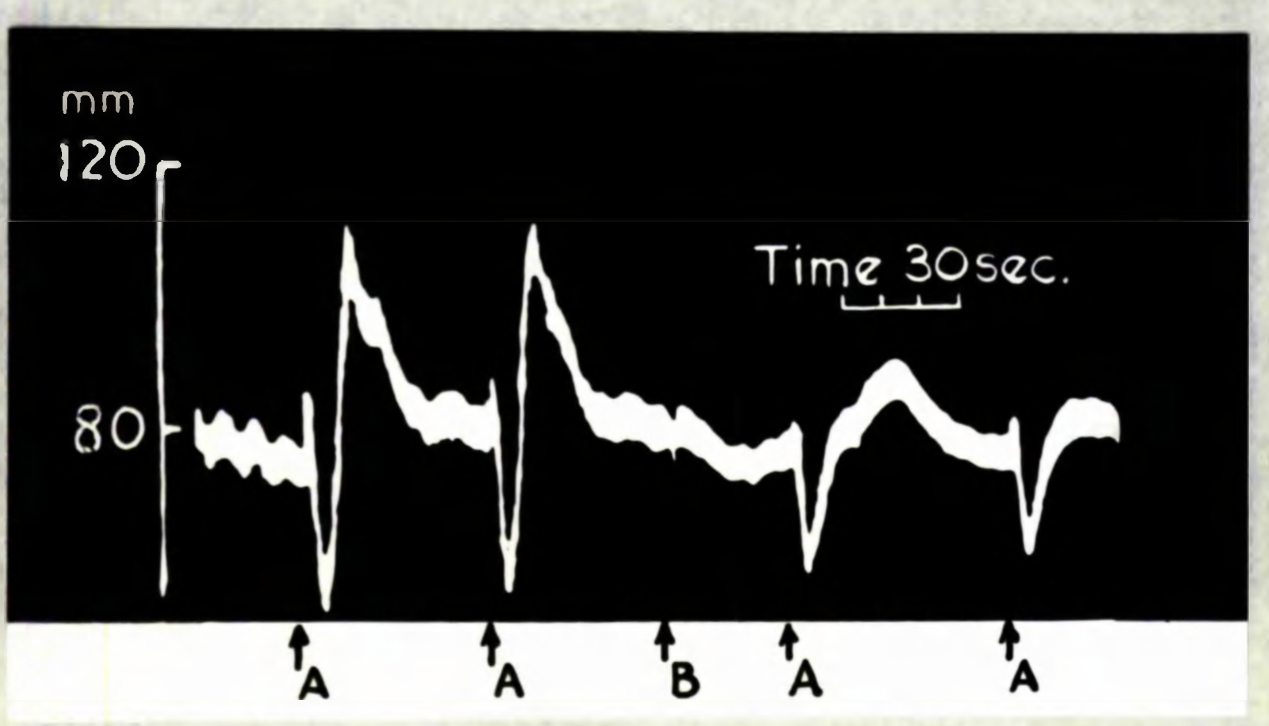

Fig. 2.6

Modification of the histamine response by dihydrallazine. Spinal cat. Blood pressure recorded from the common carotid artery. Drugs administered intravenously.

At A, histamine, 1 µg. per kg.

B, dihydrallazine, 1 mg. per kg.

(1 mg. per kg.) showed slight antagonism to the depressor effects of acetylcholine and histamine (0.5 to 2.0 µg. per kg.). In some cats the depressor effects of acetylcholine and histamine appeared to be slightly prolonged after hydrallazine and dihydrallazine. When the administration of histamine was followed by a biphasic depressor-pressor response, hydrallazine, dihydrallazine and 1-hydrazino isoquinoline hydrochloride in a dose of 1 mg. per kg. strongly antagonised the pressor component (Fig. 2.6).

Hydrallazine and the other chemically related compounds in concentrations of 1 mg. per kg. effectively and rapidly reduced the level of the blood pressure which had been artificially raised by a continuous infusion of adrenaline (10 to 100 µg. per ml. per min.). The blood pressure usually remained at the normotensive level although the adrenaline infusion was continued. Drugs were not so effective against noradrenaline infusions.

Vasopressor Reflexes.

(a) Carotid Sinus Pressor Reflex: The pressor response to bilateral occlusion of the carotid arteries

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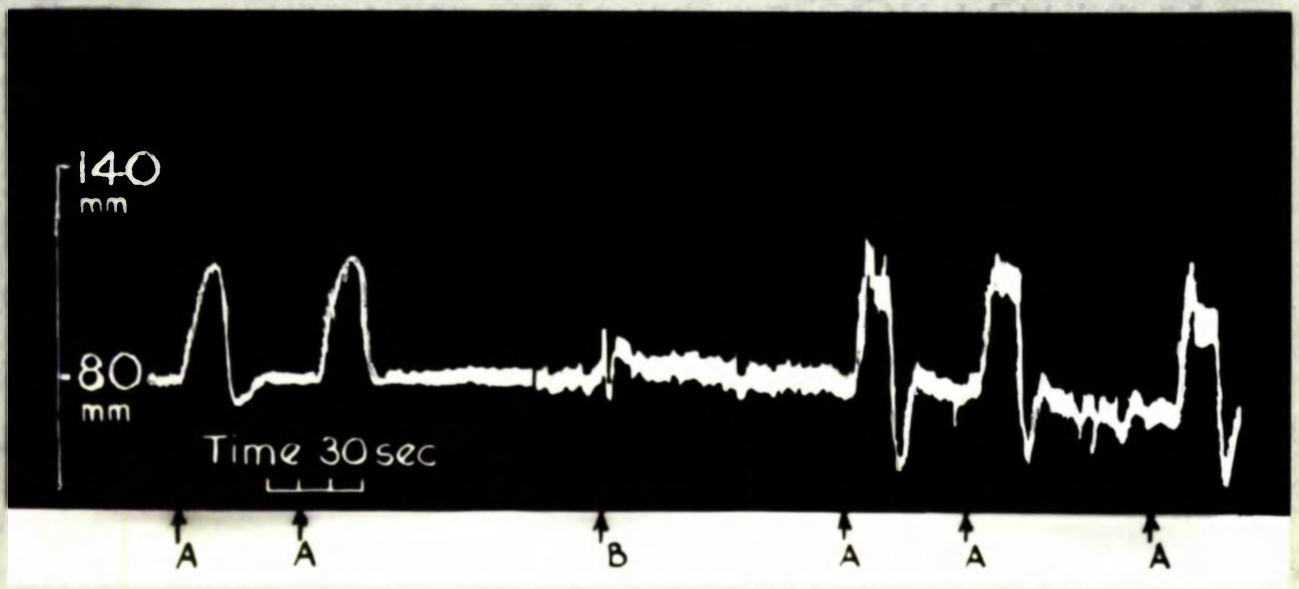

occlusion

Fig. 2.7

Effect of hydrallazine on the pressor response to compression of both common carotid arteries.

Cat. Chloralose anaesthesia. Blood pressure recorded from the femoral artery. Drugs administered intravenously.

At A, bilateral carotid occlusion for 30 seconds

B, hydrallazine, 1 mg. per kg.

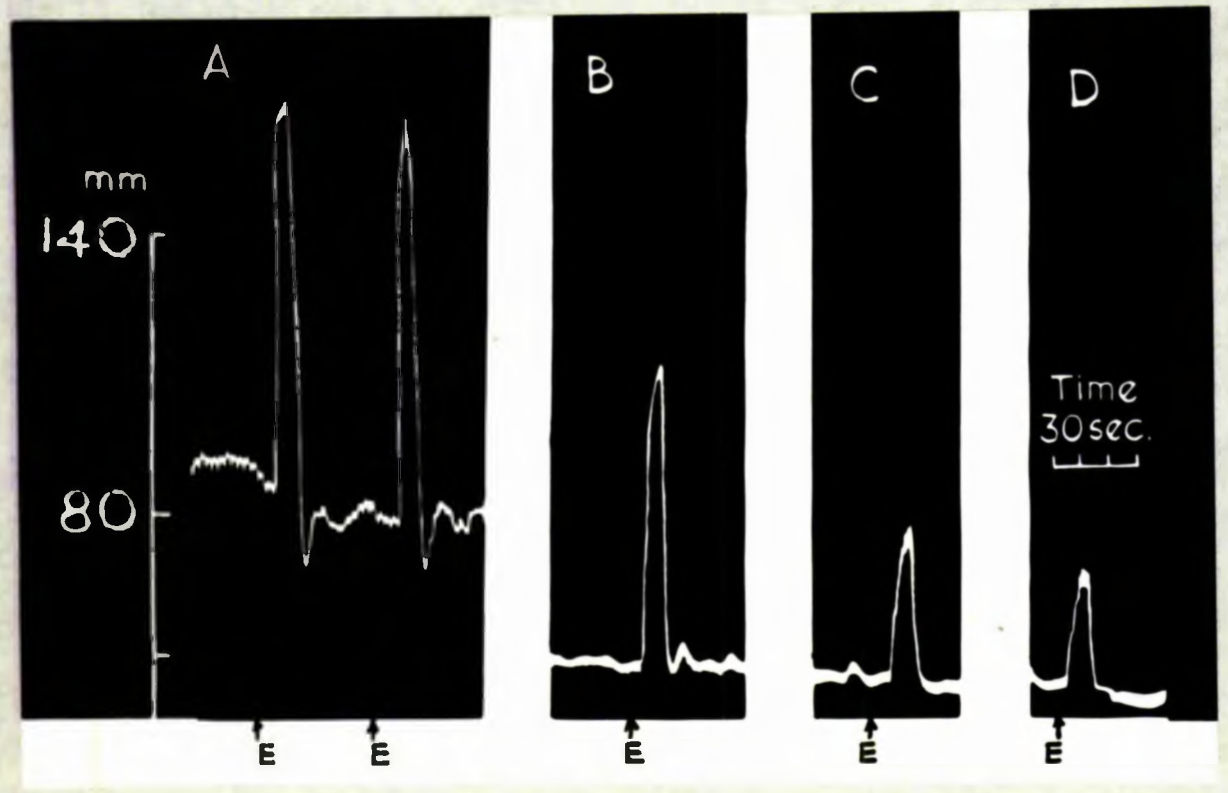

Fig. 2.8

Effect of hydrallazine on the pressor response to compression of the abdominal aorta.

Cat. Chloralose anaesthesia. Blood pressure recorded from the common carotid artery. Drugs administered intravenously.

At E, compression of abdominal aorta for 15 seconds.

A - before hydrallazine

B - 10 minutes after hydrallazine, 1 mg. per kg.

C - 20 " " " 1 " " "

D - 30 " " " 1 " " "

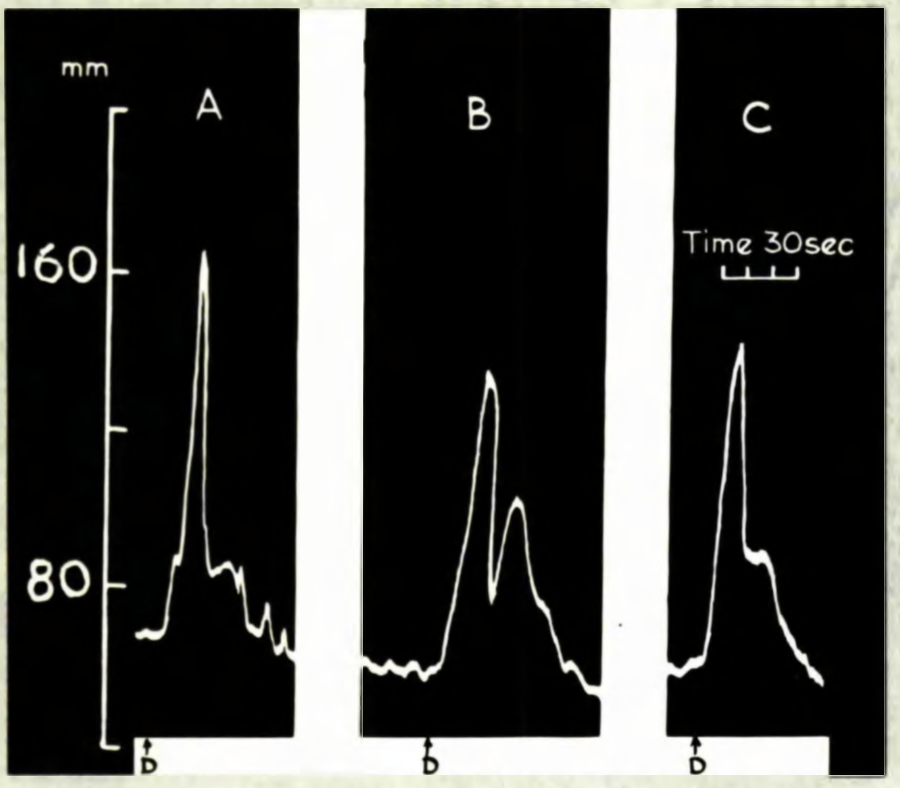

Fig. 2.9

Effect of hydrallazine on the pressor response to stimulation of the central end of the vagus.

Cat. Chloralose anaesthesia. Blood pressure recorded from the common carotid artery. Drugs administered intravenously.

At D, central end of the vagus stimulated at 7.5 volts, pulse width 1.0 m.sec., frequency 1400 per minute for 20 seconds.

A - before hydrallazine

B - 10 minutes after hydrallazine, 1 mg. per kg.

C - 30 " " " 1 " " "

was not altered following 1 mg. per kg. doses of the five drugs tested (Fig. 2.7). Drugs had little effect even after 1 to 2 hours. These observations do not support the findings of Bein et al.[8] who showed that this reflex response was significantly diminished after hydrallazine administration. Walker and his colleagues[9], however, could not show blockade of the carotid sinus pressor reflex in dogs.

(b) Occlusion of the Abdominal Aorta: Hydrallazine and dihydrallazine in doses of 1 mg. per kg. reduced the pressor response to occlusion of the abdominal aorta (Fig. 2.8). Responses were rarely abolished but were diminished by about 60 per cent at the end of one hour. The other three compounds did not reliably block this response and in some preparations no antagonism was shown.

(c) Stimulation of the Cut Central End of the Cervical Vagus: The five compounds, even at large dose levels, had little or no effect upon the pressor response to electrical stimulation of the central end of the cut vagus (Fig. 2.9). These observations are in contrast to those of Bein et al.[8].

(d) /

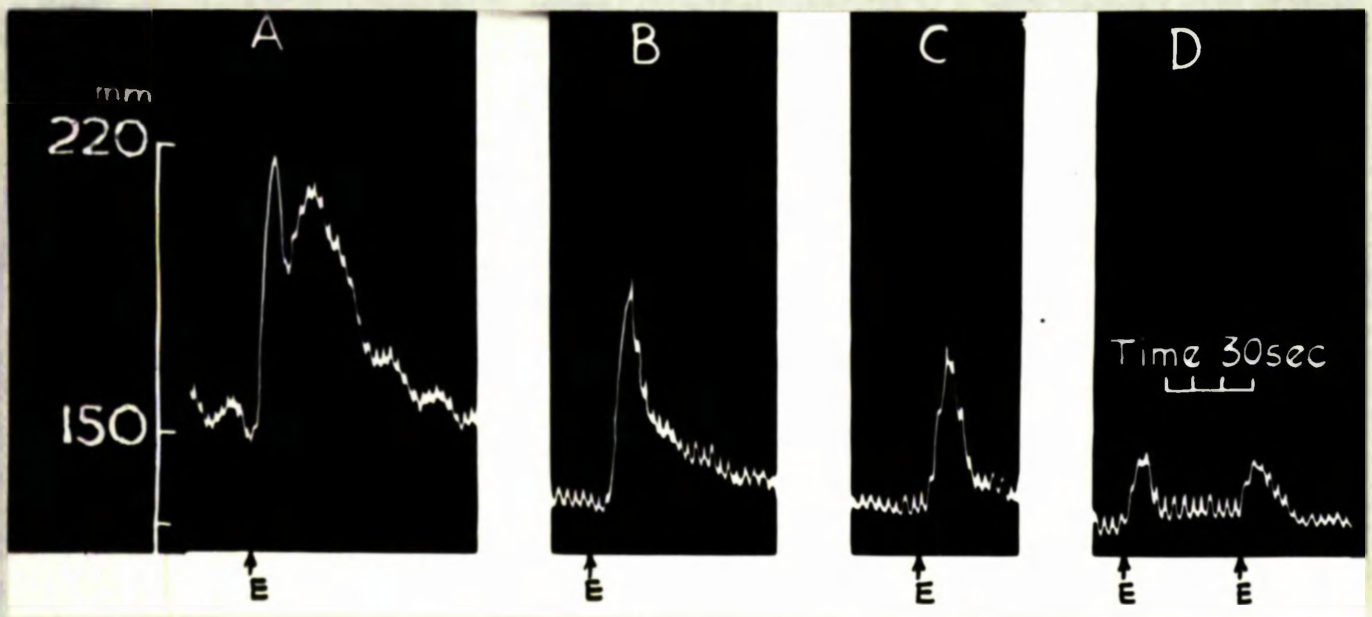

Fig. 2.10

Effect of hydrallazine on the pressor response to stimulation of the splanchnic nerve.

Cat. Chloralose anaesthesia. Blood pressure recorded from the common carotid artery. Drugs administered intravenously.

At E, stimulation of the central end of the greater splanchnic nerve at 7.5 volts, pulse width 3.0 m.sec., frequency 1400 per minute for 20 seconds.

A - before hydrallazine

B - 15 minutes after hydrallazine, 1 mg. per kg.

C - 45 " " " 1 " " "

D - 75 " " " 1 " " "

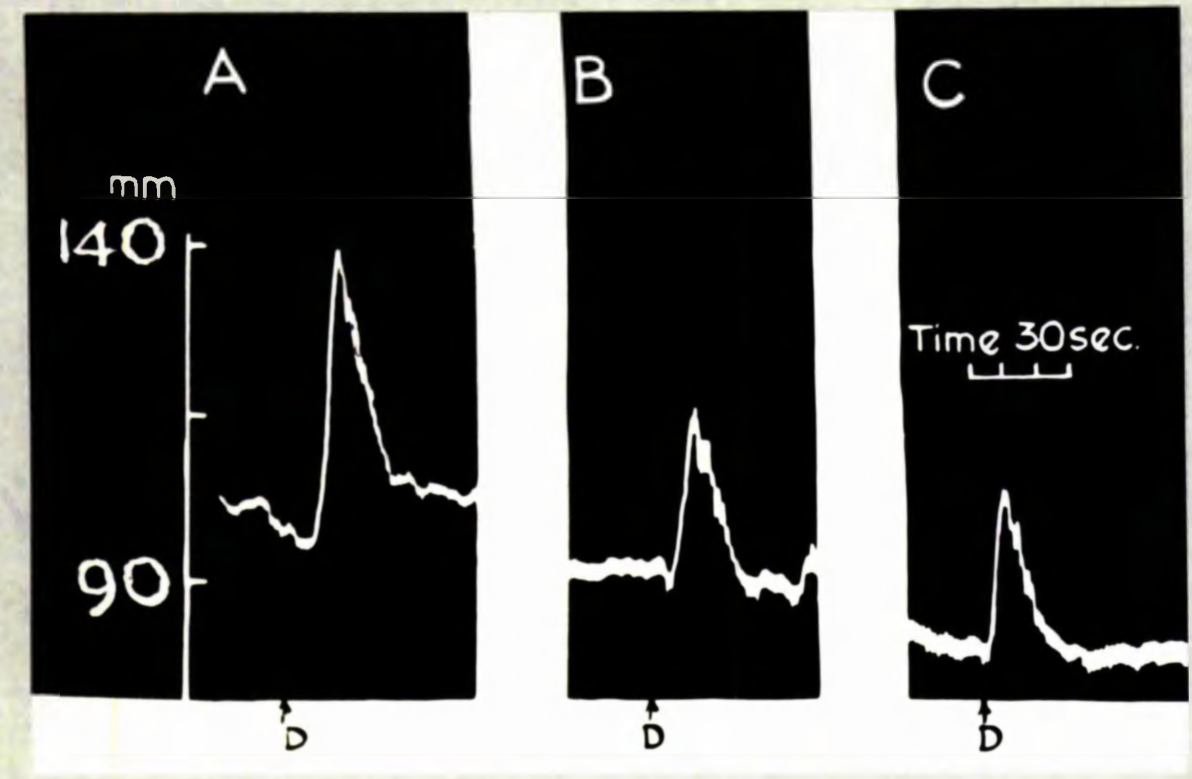

Fig. 2.11

Effect of hydrallazine on the pressor response to stimulation of the central end of the cut sciatic nerve.

Cat. Chloralose anaesthesia. Blood pressure recorded from common carotid artery. Drugs administered intravenously.

At D, central end of the sciatic nerve stimulated at 10 volts, pulse width 1 m.sec., 1400 impulses per minute for 15 seconds.

A - before hydrallazine

B - 10 minutes after hydrallazine, 1 mg. per kg.

C - 20 " " " 1 " " "

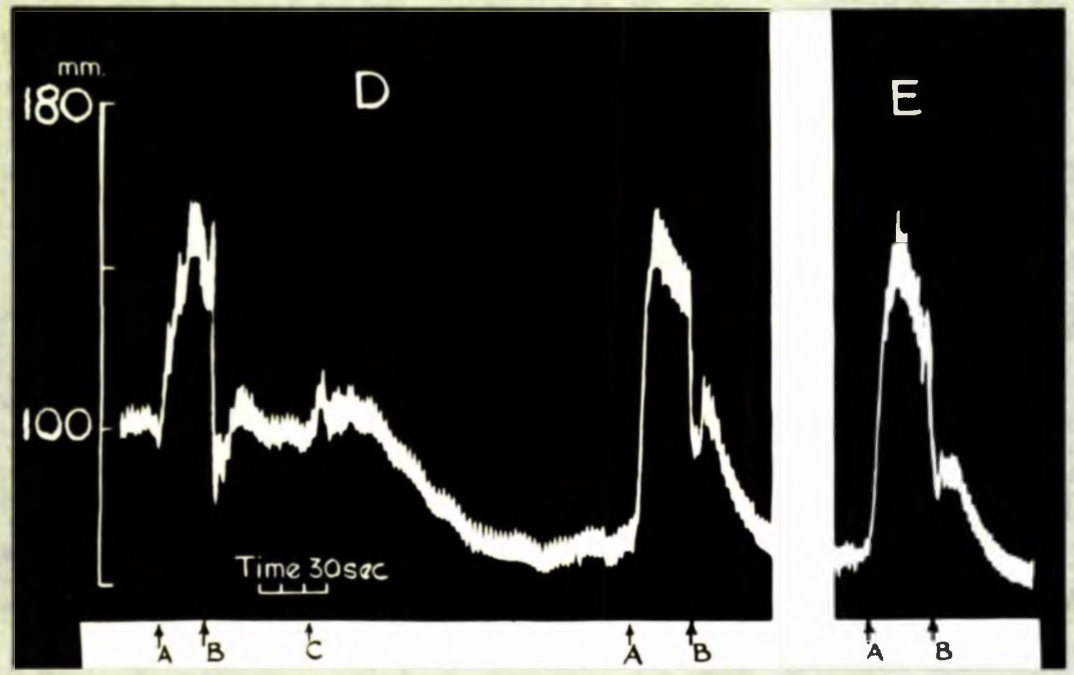

Fig. 2.12

Effect of hydrallazine on the pressor response to hypoxia.

Cat. Chloralose anaesthesia. Blood pressure recorded from common carotid artery. Drugs administered intravenously.

Between A and B, inhalation of a gas mixture containing 95 per cent nitrogen and 5 per cent carbon dioxide.

At C, hydrallazine, 1 mg. per kg.

Record E, 30 minutes after hydrallazine administration.

(d) Stimulation of the Central End of the Greater Splanchnic Nerve: Hydrallazine and dihydrallazine in doses of 1 mg. per kg. reduced considerably the pressor responses to stimulation of the central end of the greater splanchnic nerve (Fig. 2.10). The findings reported here are in agreement with those of Bein et al.[8]. The other three drugs did not consistently block the responses and in some cats failed to antagonise them.

(e) Stimulation of the Cut Central End of the Sciatic Nerve: Hydrallazine and the other compounds tested antagonised the pressor response to stimulation of the central end of the sciatic nerve (Fig. 2.11). Responses were never completely abolished even after one hour.

(f) Hypoxia: None of the compounds tested had any effect upon the pressor response to hypoxia (Fig. 2.12). Even high doses, after a prolonged time, hardly affected the response. The observations support those of Britton and his colleagues[10] but again are in contrast with those of Bein et al.[8].

(g) Local Application of Drugs to the Region of the Carotid Sinus: Experiments were only done with hydrallazine /

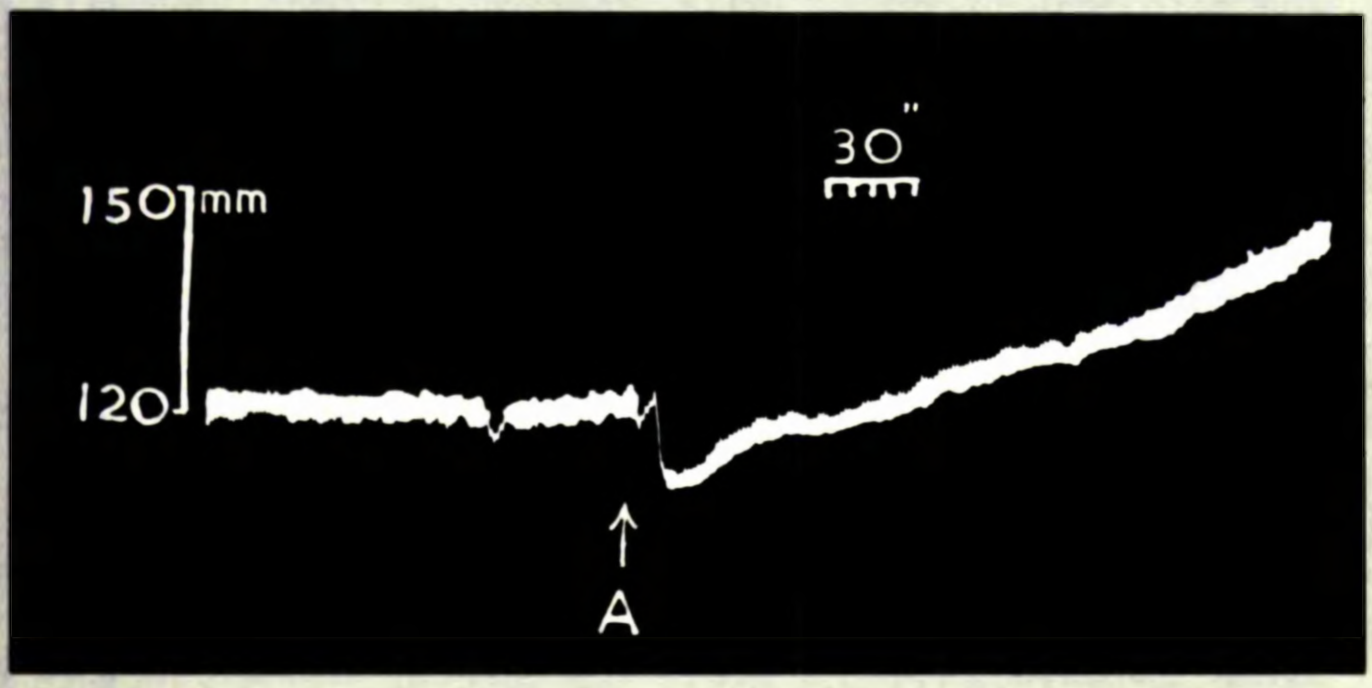

Fig. 2.13

Local application of hydrallazine to the region of carotid sinus.

Cat. Chloralose anaesthesia. Blood pressure recorded from femoral artery.

At A, hydrallazine, 6 mg., was injected around both sinuses.

hydrallazine. Hydrallazine 6 mg. in 1 ml. of saline was infiltrated gently around both the carotid sinuses of the cat. This infiltration caused a rise in the systemic blood pressure (Fig. 2.13), an effect similar to that obtained by Heymans[11] using adrenergic blocking agents including the ergot alkaloids. This points to a direct relaxant effect upon the muscle fibres of the walls of the carotid sinus. Heymans[11] has argued that the drugs relaxing the arterial walls of the carotid sinus and increasing their distensibility, decrease the stimulation of presso-receptors and thus induce reflexly a rise in the blood pressure.

2. EXPERIMENTS ON THE BLOOD PRESSURE OF THE SPINAL CAT.

In spinal cats, in which the blood pressure is usually lowered to about 50 to 60 mm.Hg. by eliminating the higher nervous mechanisms regulating blood pressure, the phthalazine derivatives either cause no diminution of pressure or have only a slight effect. Since the vessels are almost maximally dilated, the drug appears to be unable to cause further dilatation; but if the blood pressure is raised by adrenaline infusion, the /

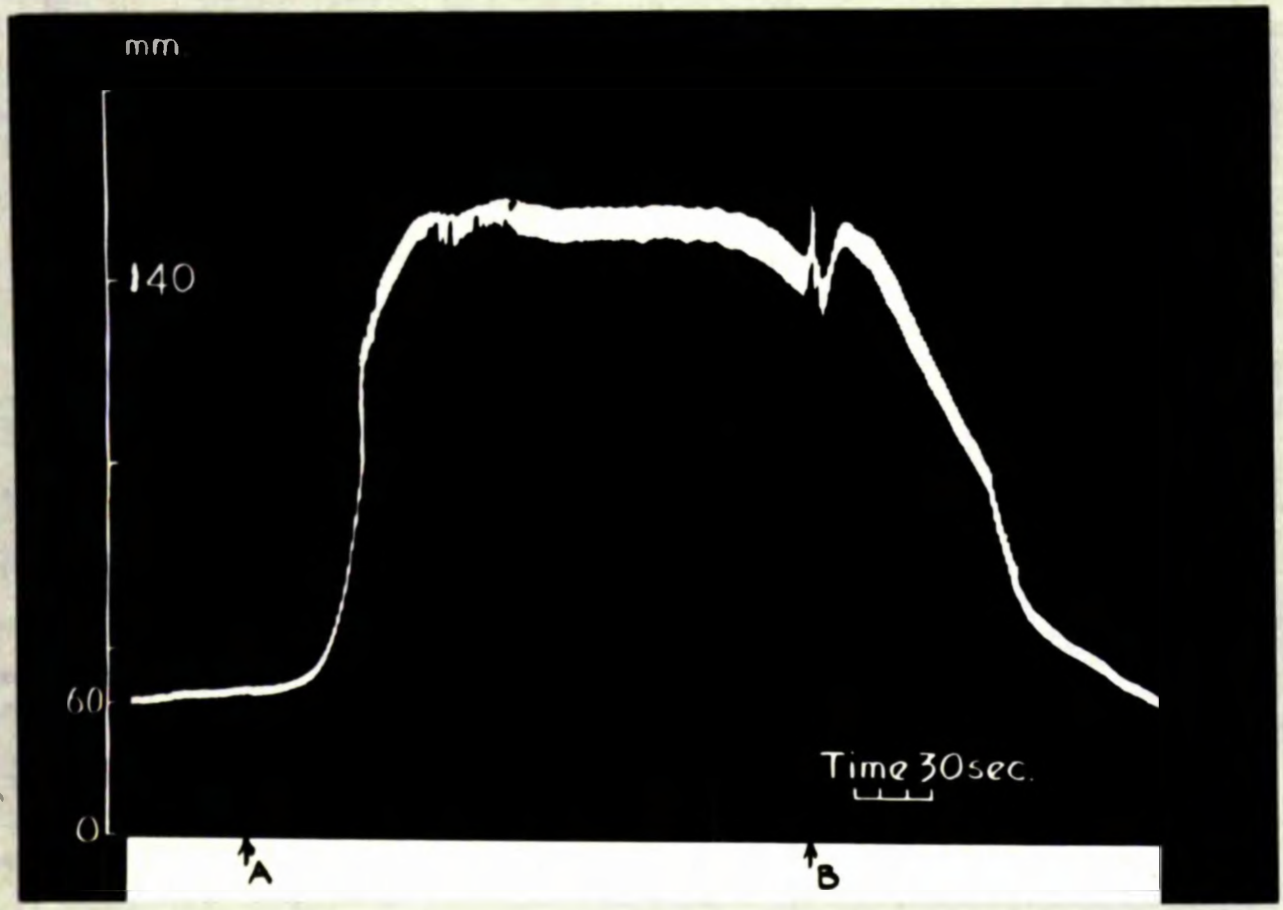

Fig. 2.14

Spinal cat. Blood pressure record from common carotid artery. Drugs administered intravenously.

At A, start continuous infusion of adrenaline 0.10 mg.per ml. at the rate of 1.0 ml. per minute.

B, hydrallazine, 1 mg. per kg.

the phthalazines then cause a fall of pressure which is reduced to approximately the original level (Fig.2.14). Hydrallazine and other compounds altered responses to adrenaline and noradrenaline in the same way as in the anaesthetised cat. The pressor component of the response to histamine was antagonised by all of the five compounds.

The fact that hydrallazine cannot produce hypotension in spinal animals cannot be explained simply on the assumption that these substances are effective only in the presence of an intact central nervous system, but the explanation is probably based on the particular circulatory conditions - especially the marked hypotension which is seen after destruction of the medulla oblongata.

3. EXPERIMENTS ON ISOLATED PERFUSED RABBIT AND KITTEN HEARTS.

No differences were noted in the results obtained when experiments were carried out either with rabbit or kitten hearts. Perfusion of 1.0 to 10 µg. per ml. of hydrallazine or dihydrallazine caused an irreversible decrease in amplitude but an increase in the rate and

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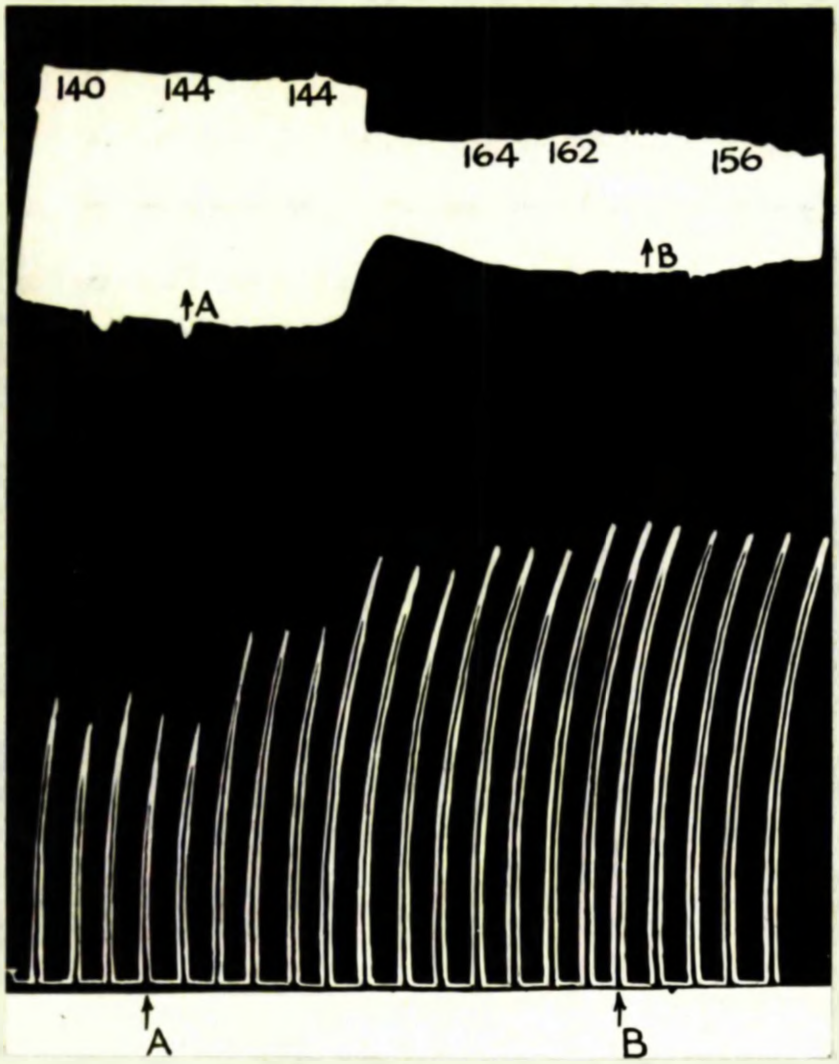

Fig. 2.15

Isolated perfused rabbit heart.

Top record shows amplitude. Figures indicate heart rate. Lower record shows outflow as recorded by Gaddum's outflow recorder.

At A, perfusion with dihydrallazine, 1 µg. per ml.

B, " " Locke's solution.

in the outflow (Fig. 2.15). The decrease in amplitude was gradual and became evident within a few minutes after the start of the perfusion of hydrallazine solution. Decreased amplitude did not return to normal even after the drug was withdrawn. 1-hydrazino isoquinoline hydrochloride (10 µg. per ml.), irreversibly decreased the rate, amplitude and outflow of the heart.

The increased rate and amplitude of contractions produced by adrenaline (0.50 to 2 µg.) and noradrenaline (0.50 to 2 µg.) were slightly potentiated by hydrallazine and dihydrallazine at a dose level of 10 µg. per ml. These compounds had little or no effect on acetylcholine (0.05 µg.) induced depression of the heart.

4. EXPERIMENTS ON THE ISOLATED AURICLES OF THE GUINEA PIG.

When hydrallazine and dihydrallazine in concentrations of 2.5 to 10 µg. per ml. were added to the bath, there was an initial increase in amplitude and rate which was followed by a secondary depression. Other compounds (2.5 to 12.5 µg. per ml.) were ineffective

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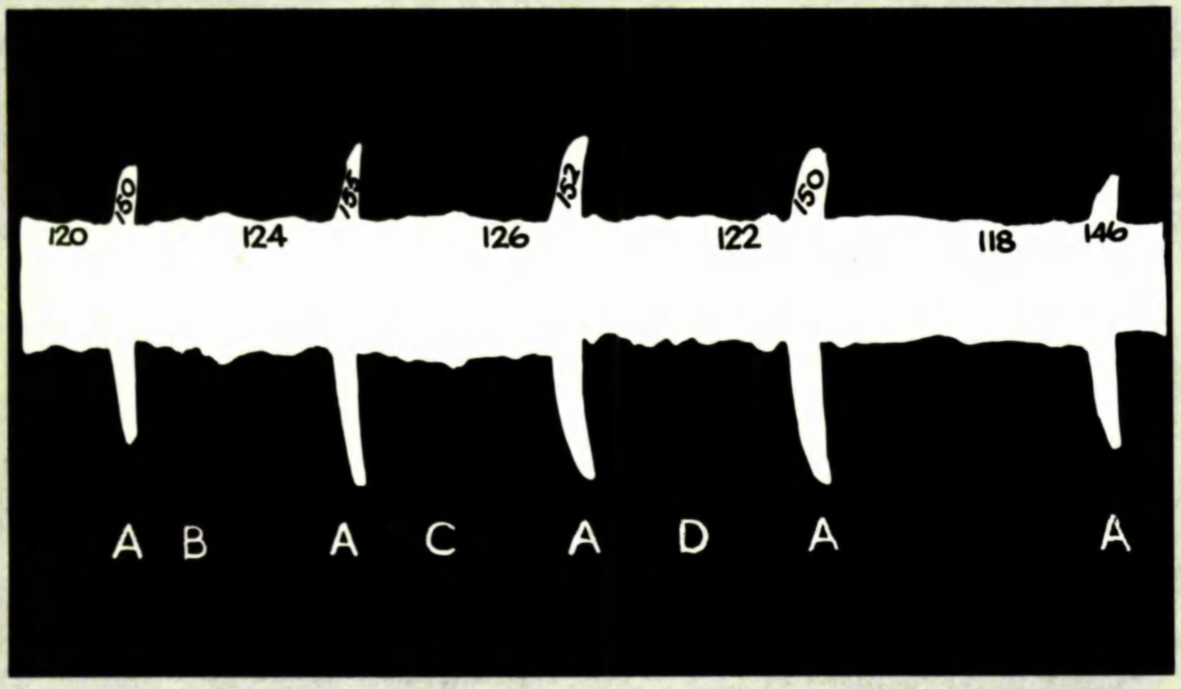

Fig. 2.16

Isolated guinea pig auricles.

At A, adrenaline, 0.0375 µg. per ml.
B, hydrallazine, 2.50 " " "
C, " 7.50 " " "
D, " 25.0 " " "

Figures show the number of beats per minute.

or caused a fall in rate and amplitude. After washing the drugs out the rate and amplitude were restored to the original levels.

Hydrallazine and dihydrallazine (10 µg. per ml.) caused slight potentiation of the stimulant effects of adrenaline (0.05 µg. per ml.) and noradrenaline (0.025 µg. per ml.) (Fig.2.16) but 1-hydrazino isoquinoline hydrochloride (2.5 to 12.5 µg. per ml.) and 3:6 dihydrazino pyridazine nitrate (2.5 to 12.5 µg. per ml.) had no effect or caused slight potentiation, while 3-phenyl-6 hydrazinopyridazine hydrochloride, in similar concentrations, had no effect. Hydrallazine and dihydrallazine (10 to 25 µg. per ml.) had no effect upon histamine (0.013 µg.per ml.) induced acceleration and on acetylcholine (0.01 to 0.10 µg. per ml.) induced depression of the auricles.

5. EXPERIMENTS UPON THE ISOLATED GUINEA PIG ILEUM.

In some preparations which were very sensitive to histamine, hydrallazine and dihydrallazine in doses of 5 to 20 µg.per ml. showed a direct stimulant effect but in others no direct action, even with high doses

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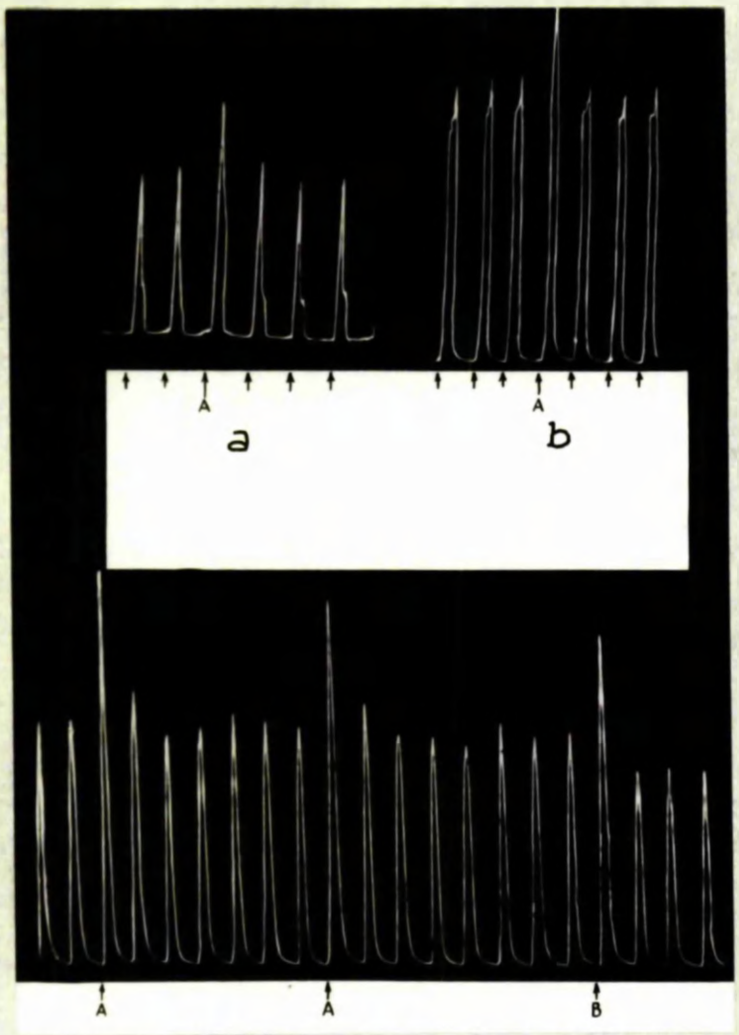

Fig. 2.17

(a) All contractions of the isolated guinea pig ileum produced by 5-hydroxytryptamine, 0.3 µg. per ml.

At A, hydrallazine, 25 µg. per ml.

(b) All contractions of the isolated guinea pig ileum produced by acetylcholine, 0.025 µg. per ml.

At A, hydrallazine, 25 µg. per ml.

(c) All contractions of the isolated rat uterus produced by acetylcholine, 0.25 µg. per ml.

At A, hydrallazine, 75 µg. per ml.
B, dihydrallazine, 25 µg. per ml.

(100 to 200 µg. per ml.) , was observed.

Contractions of the guinea pig ileum following the addition to the bath of acetylcholine (0.10 to 1 µg. per ml.) were potentiated by 12.5 to 125 µg.per ml. of hydrallazine and dihydrallazine (Fig. 2.17). The extent of potentiation of the amplitude of contraction did not appear to be related to the dose of hydrallazine. Recovery to the original level was usually prompt. Hydrallazine and dihydrallazine in smaller doses (12.5 to 25 µg. per ml.) potentiated histamine (0.1 to 1.0µg. per ml.) induced contractions but antagonised them with higher doses (100 to 125µg. per ml.). 3-phenyl-6 hydrazinopyridazine hydrochloride and 1-hydrazino isoquinoline hydrochloride (12 to 125 µg. per ml.) antagonised contractions due to acetylcholine and histamine, but 3:6 dihydrazino pyridazine nitrate at similar dose levels was ineffective.

Hydrallazine and dihydrallazine (10 to 125 µg.per ml.) potentiated the response of the guinea pig ileum to 5-hydroxytryptamine (30 ng. to 3 µg.per ml.)(Fig.2.17). 3-phenyl-6 hydrazinopyridazine hydrochloride and 1-hydrazino /

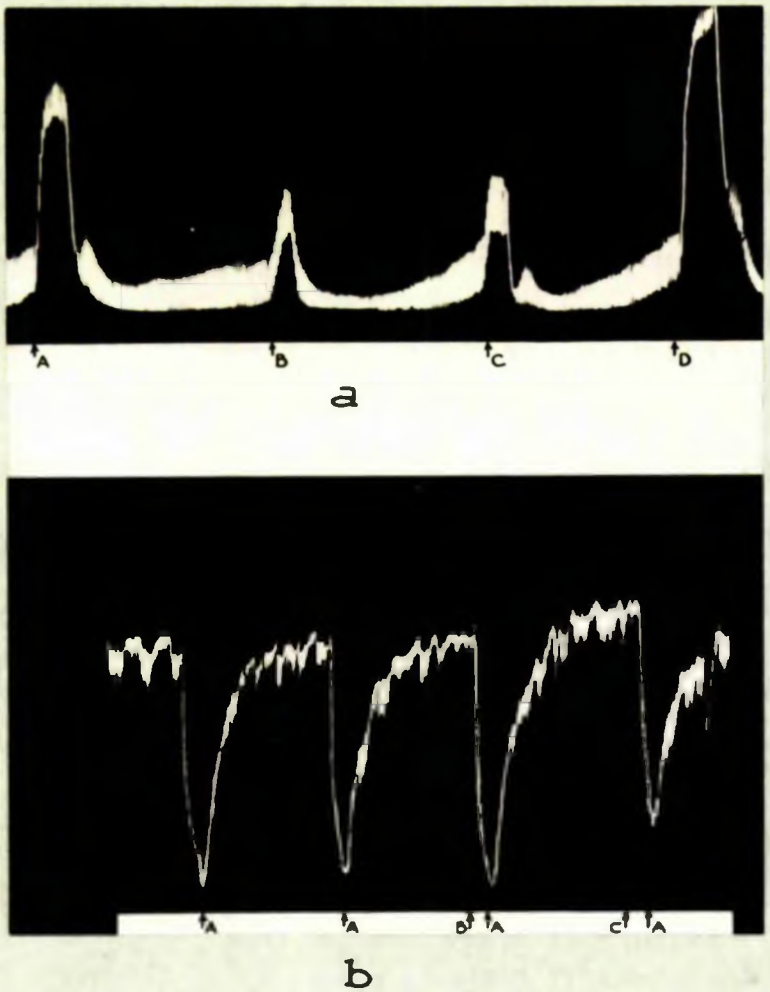

Fig. 2.18

Isolated rabbit duodenum.

(a) At A, dihydrallazine, 30 µg. per ml.
B, hydrallazine, 12.5 " " "
C, " 25.0 " " "
D, " 50.0 " " "

(b) At A, adrenaline, 0.125 " " "
B, hydrallazine, 25.0 " " "
C, " 75.0 " " "

hydrazino isoquinoline hydrochloride (25 to 125 µg.per ml.) antagonised 5-hydroxytryptamine while 3:6 dihydrazino pyridazine nitrate was ineffective. Contractions of the guinea pig ileum induced by barium chloride (0.20 to 0.50 mg. per ml.) were slightly antagonised by 3-phenyl-6 hydrazinopyridazine hydrochloride (12.5 to 125 µg.); the other drugs had no effect.

6. EXPERIMENTS UPON THE ISOLATED RABBIT DUODENUM.

Hydrallazine and dihydrallazine (0.50 to 10 µg.per per ml.) increased the tone and the amplitude of peristaltic movements of isolated strips of rabbit duodenum. Higher doses (25 to 50 µg. per ml.) caused a sharp contraction (Fig. 2.18). The other three compounds caused a slight increase in tone at doses of 2.5 to 12.5 µg. per ml.

Contractions of the duodenum following the addition of acetylcholine (0.02 to 0.10 µg. per ml.) or histamine (2.0 to 20 µg.per ml.) were potentiated by 12.5 to 50 µg. per ml. of hydrallazine and dihydrallazine. Acetylcholine-induced contractions were slightly antagonised /

antagonised by the other three compounds (2.5 to 12.5 µg. per ml.). Contractions due to 5-hydroxytryptamine (1 to 3 µg. per ml.) were slightly potentiated by hydrallazine and dihydrallazine (10 to 50 µg. per ml.) but the other compounds had no demonstrable effect. Hydrallazine or dihydrallazine (5 to 50 µg. per ml.) and the other compounds showed only slight or no antagonism to the reduction in tone induced by 0.013 to 0.1 µg. per ml. of adrenaline or noradrenaline (Fig.2.18).

7. EXPERIMENTS ON THE ISOLATED RAT UTERUS.

None of the compounds had any direct effects on this preparation. Hydrallazine and dihydrallazine (25 to 50 µg. per ml.) showed no, or slight, antagonism to adrenaline (0.01 to 0.10 µg. per ml.) or noradrenaline (0.25 to 1 µg. per ml.) inhibition of acetylcholine induced contractions of the rat uterus. The other three compounds themselves antagonised acetylcholine and were therefore not tested.

Hydrallazine and dihydrallazine (12.5 to 125 µg. per ml.) caused a slight potentiation of the response to /

to acetylcholine (0.1 to 0.25 µg. per ml.)(Fig. 2.17) but the other three compounds (5 to 75 µg. per ml.) antagonised it. 1-hydrazino isoquinoline hydrochloride was the most potent.

Hydrallazine and dihydrallazine (10 to 100 µg. per ml.) showed antagonism to contractions induced by 5-hydroxytryptamine (0.1 to 2 µg. per ml.). Similar effects were shown by other three compounds but 1-hydrazino isoquinoline hydrochloride appeared to be a potent antagonist (2.5 to 25 µg. per ml.)

8. EXPERIMENTS USING THE ISOLATED RAT PHRENIC NERVE DIAPHRAGM PREPARATION

Hydrallazine and dihydrallazine had practically no direct effects unless used at a concentration of 1 mg. per ml. when they reduced the twitch height of indirectly stimulated muscle. Hydrallazine and dihydrallazine (200 to 500 µg. per ml.) almost completely antagonised the block induced by decamethonium (0.10 to 0.15 mg. per ml.) and nicotine (0.20 to 0.40 mg. per ml.) but had no effect on the block caused by tubocurarine (0.01 to 0.03 mg. per ml.). The other compounds were not tested on this preparation.

9. EXPERIMENTS USING THE ISOLATED FROG RECTUS ABDOMINIS MUSCLE.

Hydrallazine, dihydrallazine and 1-hydrazino isoquinoline hydrochloride (5.0 to 50 µg. per ml.) potentiated the response of the frog rectus abdominis muscle to acetylcholine (0.10 to 0.50 µg. per ml.) while the other two compounds (5.0 to 25 µg. per ml.) caused neither potentiation nor antagonism. Hydrallazine and dihydrallazine in similar doses potentiated the response of the frog rectus muscle to 0.25 to 0.75 mg. per ml. of potassium chloride, while the other three compounds had no effect or caused slight antagonism. Decamethonium induced contractions (2 to 3 µg. per ml.) were antagonised by all the five compounds but the doses required of hydrallazine and dihydrallazine were higher (50 to 100 µg. per ml.); on the other hand 50 µg. per ml. of hydrallazine or dihydrallazine potentiated the response of nicotine (1 to 3 µg. per ml.) while the other three compounds at doses of 2.5 to 12.5 µg. per ml. showed slight antagonism. After decamethonium was washed out, 50 µg. per ml. of hydrallazine, dihydrallazine or 25 to 50 µg. per ml. of 1-hydrazino isoquinoline hydrochloride /

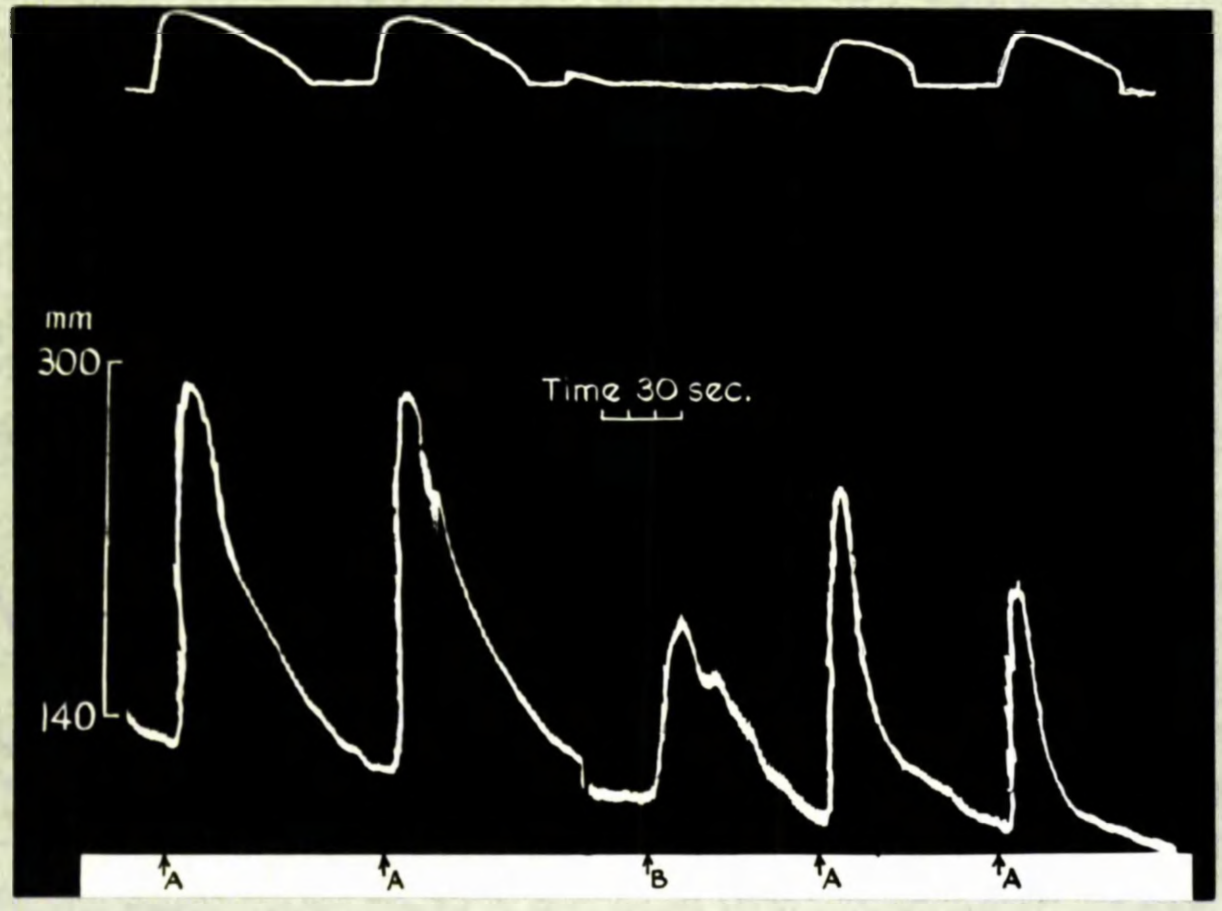

Fig. 2.19

Effects of hydrallazine on the response of the nictitating membrane. Cat. Chloralose anaesthesia. Upper record showing contractions of the nictitating membrane. Lower record showing responses of blood pressure to adrenaline.

At A, adrenaline, 5.0 µg. per kg. intravenously

B, hydrallazine, 1.0 mg. per kg. "

hydrochloride caused the rectus to contract even though they had no direct effect on the muscle prior to decamethonium. After 5 to 25 µg. per ml. of hydrallazine or dihydrallazine, atropine (0.10 to 2 µg. per ml.) or tubocurarine (0.40 to 0.50 µg. per ml.) did not antagonise acetylcholine induced (0.10 to 0.50 µg. per ml.) contractions. Eserine (2 to 4 µg. per ml.) potentiation of acetylcholine induced contractions was further enhanced by hydrallazine and dihydrallazine (5.0 to 25.0 µg. per ml.).

10. EXPERIMENTS ON THE NICTITATING MEMBRANE OF THE ANAESTHETISED CAT.

Hydrallazine and other related compounds in doses of 1 to 2 mg. per kg. showed no direct effects on this preparation. Contractions of the nictitating membrane induced by adrenaline (5 to 8 µg. per kg.) were slightly antagonised by all the compounds (Fig.2.19), excepting 3-phenyl-6 hydrazinopyridazine hydrochloride at doses of 1 mg. per kg. Contractions due to nor-adrenaline (10 µg. per kg.) or preganglionic electrical stimulation of the cervical sympathetic were not affected by any of the drugs tested.

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CHAPTER III

CHAPTER III

This chapter has been devoted to the study of the effects of hydrallazine, dihydrallazine, 3-phenyl-6-hydrazinopyridazine hydrochloride, 1-hydrazino *iso*-quinoline hydrochloride, 3:6 dihydrazino pyridazine nitrate and reserpine on arterial smooth muscle. The following preparations have been used:

1. (a) The isolated rat hindquarters.
   (b) The isolated rabbit ear.

2. Isolated strips of the thoracic aorta of the rabbit and cat.

3. Isolated strips of horse carotid artery.

In this section hydrallazine and other compounds were tested on the isolated rat hindquarters and rabbit ear only. Hydrallazine and reserpine were tested on isolated strips of rabbit and cat aorta, and on horse carotid artery.

A. EXPERIMENTAL

1(a). EXPERIMENTS ON THE ISOLATED PERFUSED RAT HINDQUARTERS.

Method.

In these experiments the pressure at which the physiological fluid passed through the blood vessels was kept constant, and alterations in the rate of outflow of the perfusion fluid which were produced by the drug were recorded by means of Gaddum's drop recorder. The vessels were perfused with oxygenated Locke's solution at room temperature.

Rats of either sex, weighing between 200 and 300 g., were killed by a blow on the head. The throats were cut and the blood allowed to drain out. The abdominal cavity was opened by means of a longitudinal incision extending from the sternum to the anus. The rectum, the oesophagus and the inferior and superior mesenteric arteries were divided between ligatures. The abdominal viscera were then removed. This brought into view the abdominal aorta which was cannulated. The body wall and vertebral column were transected above the point of cannulation and the cannula attached to the perfusion system by means of fine rubber tubing. The perfused hindquarters /

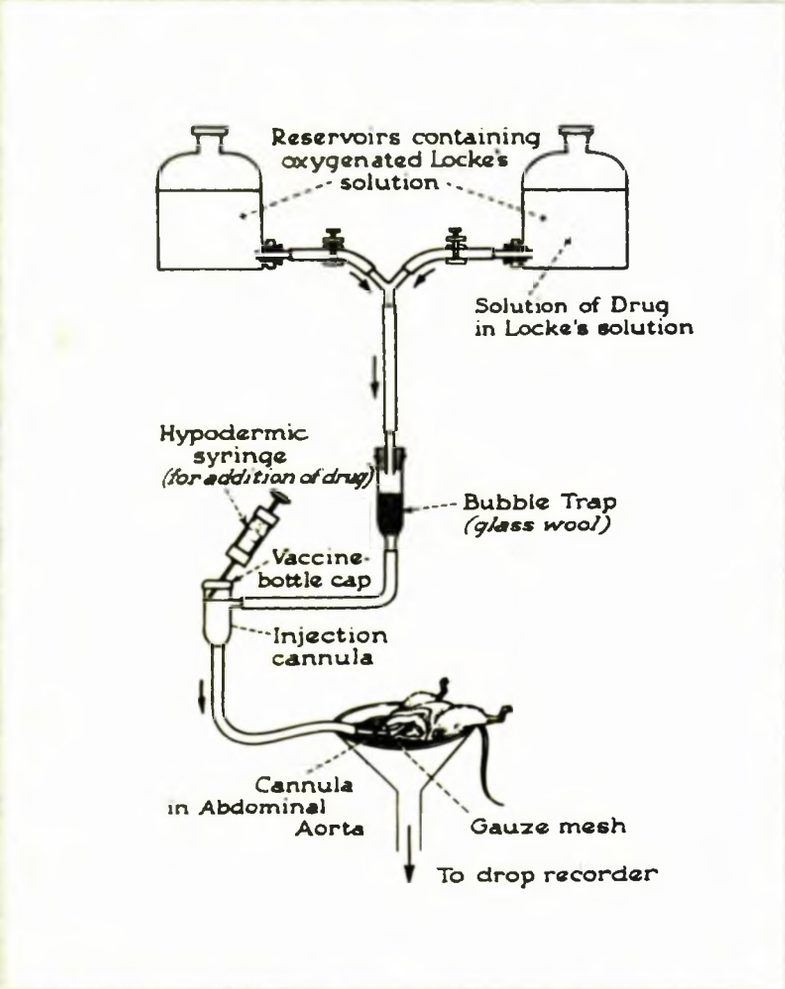

Fig. 3.1

Diagram of the apparatus used for perfusing rat hindquarters or rabbit ear. Electronic arrangement for recording the drop is not shown in this figure. (After Lewis, J.J. (1959), Textbook of Pharmacology, Livingstone, Edinburgh.)

hindquarters were set up in the apparatus shown in Fig.3.1.

Two reservoirs are included in this apparatus. The rate of flow of fluid from the bubble trap to the injection cannula is controlled by an adjustable screw clip, and can be adjusted to a suitable rate at the beginning of each experiment. The injection cannula, (the design of which was based upon that suggested by Gaddum and Kwiatkowski[1]) (Fig. 3.1), allowed the drug solutions to be injected at a constant rate. This was achieved by injecting the solution with a tuberculin syringe, fitted with a fine needle, through the rubber cap at a rate such that the level of fluid in the cannula was unaltered during the process. The hindquarters preparation was placed on a muslin rest lying in a filter funnel. The outflow was led *via* the filter funnel to the contacts of a Gaddum drop recording assembly[1].

After setting up the preparation, a uniform outflow record was obtained for at least 15 minutes before drugs were injected. Drugs were dissolved in the Locke's solution in one of the reservoirs to give the desired concentration (10 µg. per ml.). The drug solution was allowed to perfuse through the preparation for 10 minutes /

minutes and the same concentration of the agonist injected again. After about 30 minutes, the drug perfusion was stopped, the hindquarters perfused with Locke's solution and the doses of the agonists repeated.

1(b). EXPERIMENTS ON THE ISOLATED RABBIT EAR.

Rabbits were killed by a sharp blow on the back of the neck. It was found that subsequent cannulation was more easily carried out if the fur at the base of the ear was shaved and the path of the dorsal auricular artery traced in ink before killing the animal. If the animal was not bled, the vessels of the ear did not tend to collapse. The animal was placed on a dissecting board and a small strip of skin at the base of the ear removed. The dorsal auricular artery (the central vessel of the ear) was freed from adherent tissue using the blunt dissection technique. A fine polythene cannula, filled with perfusion fluid containing a little heparin, was then tied into the vessel. The ear was severed from the head and connected to the perfusion apparatus by means of fine rubber tubing (Fig. 3.1). Other details are similar to those applied to the preparation /

preparation of the rat hindquarters.

2. EXPERIMENTS ON STRIPS OF RABBIT AND CAT THORACIC AORTA.

For each experiment a rabbit or a cat, weighing from 2 to 3 kg., was rapidly decapitated. The descending thoracic aorta was removed and placed in a Petri dish containing Tyrode's solution at room temperature. Excess fat and connective tissue were trimmed off. The whole length of aorta was then cut along a closed spiral. During the cutting - which was done with a pair of small, sharp pointed scissors - the uncut portion of the aorta , which was held between the thumb and fingers of the operator's free hand, was gradually rotated and moved forwards towards the scissors in such a manner as to permit a continuous spiral incision. The resulting strip was usually about 2 mm. wide and 20 cm. long and contained smooth muscle fibres (the circular fibres of the intact aorta). From this strip shorter strips of 2 to 4 cm. in length were cut for use in the experiments. During the whole procedure of preparing strips of artery, the tissue was kept moistened with Tyrode's solution. (Appendix 1)

Strips /

Strips of artery were set up in organ baths containing 20 ml. of oxygenated Tyrode's solution at 37± 0.5°C. Drug solutions were washed out from the bath by allowing fresh fluid to flow into the bath from the reservoir so as to displace the contents. Contractions were recorded by means of a modified frontal point lever adjusted to give a 9-fold magnification. The strip was kept under a tension of 5 g. for one hour before any drugs were added. The gradual elongation under tension is the result of a physical process in which certain undefined structural elements of the aortic strips slowly lengthen, and does not appear to be due to gradual loss of spontaneous tone.

Contractions of the strips were elicited by the addition of a stimulant drug to the bath. Generally a gradual contraction starts and reaches a maximum in about 5 minutes. While the artery strip was still in a state of contraction, hydrallazine or dihydrallazine was added to the bath. After the maximum relaxation, the Tyrode's solution was changed by running in a fresh solution.

Certain observations were made on the nature of these /

Histology?

these strips. The thoracic part of the aorta of both the rabbit and the cat was more sensitive to stimulant drugs than the abdominal one. The aortae of cats were more sensitive than those of rabbits.

Preparations from the aortae of cats and rabbits were not very satisfactory because the magnitude of contraction or relaxation was usually small. Furthermore, the response was slow and recovery prolonged, so that the time interval between doses was often as much as two hours. Many of the strips were refractory to stimulant drugs including acetylcholine, 5-hydroxytryptamine and histamine.

3. EXPERIMENTS UPON STRIPS OF HORSE CAROTID ARTERY.

Histology?

An isolated arterial smooth muscle preparation which responded readily to stimulant drugs was necessary for the study of drug antagonisms. In the previous section the preparations described were the aortae of cats and rabbits, but they suffered from certain disadvantages. The author, therefore, made a search for a suitable artery preparation and found one in the horse /

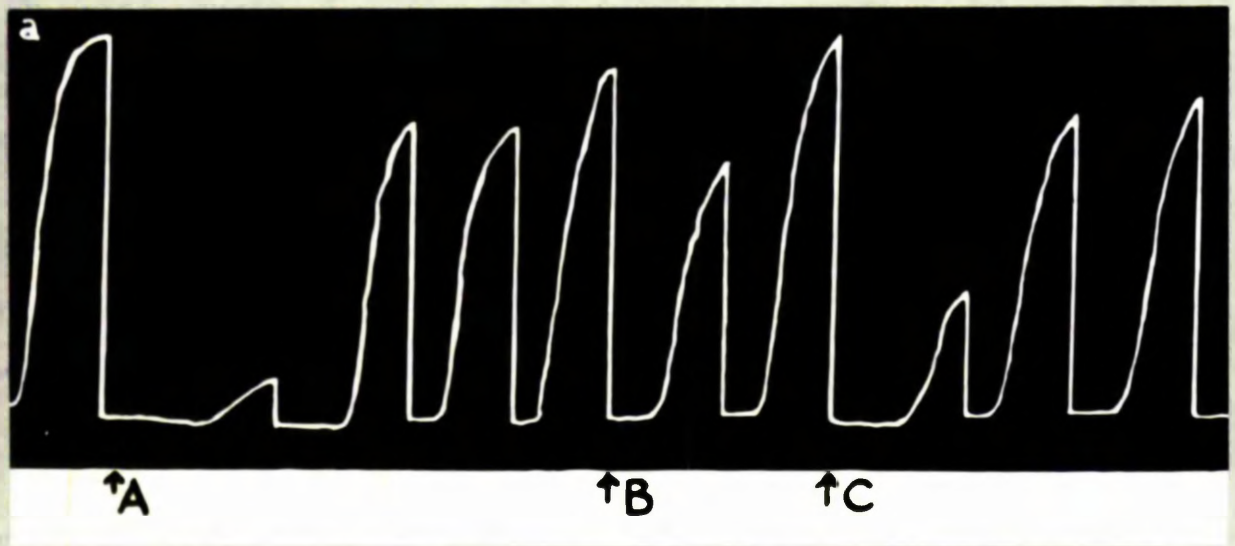

Fig. 3.2(a)

Noradrenaline-phentolamine antagonism in horse carotid artery strips.

All contractions induced by noradrenaline, 0.5 µg.per ml.

At A, phentolamine, 0.03 µg. per ml.

B, " 0.01 " " "

C, " 0.02 " " "

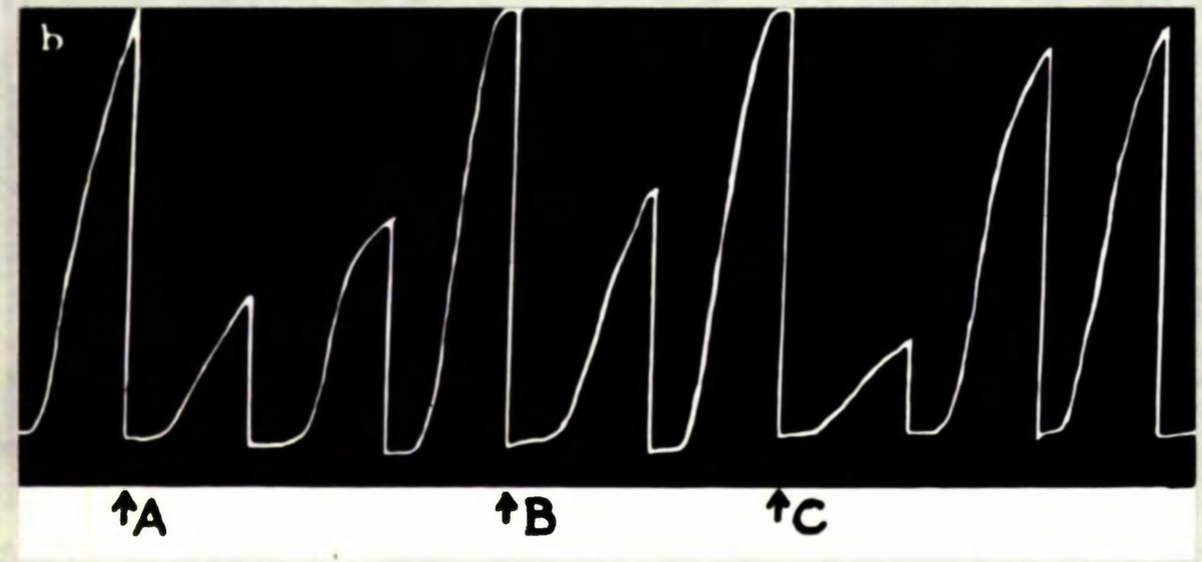

Fig. 3.2(b)

Acetylcholine-atropine antagonism in horse carotid artery strips.

All contractions are due to acetylcholine, 0.2 µg.per ml.

At A, atropine, 0.02 µg. per ml.

B, " 0.01 " " "

C, " 0.04 " " "

horse carotid artery. The advantages of this tissue were sensitivity to most of the stimulant drugs, rapid recovery after washing, specificity and an ability to retain sensitivity for 3 to 4 days if stored at 0° in Tyrode's solution. All these features are desirable in studying drug antagonisms. There were disadvantages, however, in that the material had to be obtained from a slaughter-house, and that the arteries in themselves were thick.

Drug antagonisms were specific since very low doses of atropine and mepyramine blocked the response to acetylcholine and histamine; and atropine did not antagonise histamine induced contractions and mepyramine did not block responses to acetylcholine. A few typical illustrative antagonisms are shown in Fig. 3.2.

Method.

Lengths of carotid artery were removed at the slaughter-house from freshly killed horses. A portion of the artery was freed from fascia and a strip about four cm. in length and two mm. wide was made by cutting the artery into a spiral by means of a pair of iris scissors. Threads were tied to both ends of the segment and /

and the strip of artery set up in a forty ml. organ bath containing oxygenated Tyrode's solution (Appendix 1) at 37± 0.5°C. The thread at one end of the artery strip was fixed to the lower end of a glass tube supplying oxygen to the bath; the thread at the other end was attached to a modified frontal point writing lever giving a magnification of about 1 in 10. The strip was stretched for about one hour by means of a 10 g. weight. Before the experiment was started the additional weight was removed and the lever readjusted. Contractions of the strips of artery were induced by addition to the bath of adrenaline, noradrenaline, 5-hydroxytryptamine, acetylcholine, histamine, barium chloride or potassium chloride. These were added to the bath as solutions in Tyrode's solution by means of a tuberculin syringe. Two types of experiments were performed.

(1) Stimulant drugs were added to the bath and the effects observed for five minutes. In some experiments the contraction was complete in four minutes and, in others, in six minutes. When this happened the period of contact between the drug and the tissue was decreased or increased. Some strips showed rhythmic /

Fig. 3.3

Spontaneous rhythmic activity in a strip of horse carotid artery.

At A, histamine, 0.66 µg. per ml.

W, wash out.

rhythmic activity in the presence of adrenaline or histamine and these were rejected. An example is shown in Fig. 3.3. After the drug was washed out, the lever returned slowly to the base line. With acetylcholine contractions, relaxation on washing took place in 5 to 20 minutes but with other drugs, the relaxation took 30 minutes or more. Standard, reproducible submaximal responses were obtained to acetylcholine, adrenaline, noradrenaline, histamine, 5-hydroxytryptamine, barium chloride and potassium chloride. Once these had been obtained, reserpine or hydrallazine was added 20 minutes before the next addition. Total time of contact of hydrallazine and reserpine with the tissue was 25 minutes.

Artery strips always contracted after the addition of acetylcholine but were less sensitive to other drugs, and sometimes did not respond unless high doses were used.

(2) Furchgott[2], in experiments with strips of rabbit aorta, has shown that the presence of a high concentration of a stimulating drug during exposure to a specific antagonist can protect against the effects of the antagonist. Such "self-protection" implies that /

that the antagonist is blocking the same receptors with which the stimulant drug combines, since these have been saturated by the high concentration of the stimulant drug and are, therefore, not available to the antagonist.

A few experiments were carried out on this assumption. Artery strips were stimulated using small concentrations of acetylcholine, histamine, adrenaline and noradrenaline (1 to 5 µg. per ml.). The low concentration of the stimulant drug was allowed to act for 5 minutes and then a second higher dose of 0.5 mg. to 1 mg. of the same drug was added. The second dose was left in the bath for 15 minutes; reserpine (5.0 to 25 µg. per ml.) or hydrallazine (25.0 to 500 µg.) was added and left in contact with the tissue for a further 20 minutes. After washing out, the addition of the smaller dose was repeated.

Experiments of this type are rather difficult since the second response to the smaller dose, after its own high dose, is generally reduced even without the presence of the antagonist. Hence only the strips in which the second contraction after the high doses was not appreciably reduced, were used.

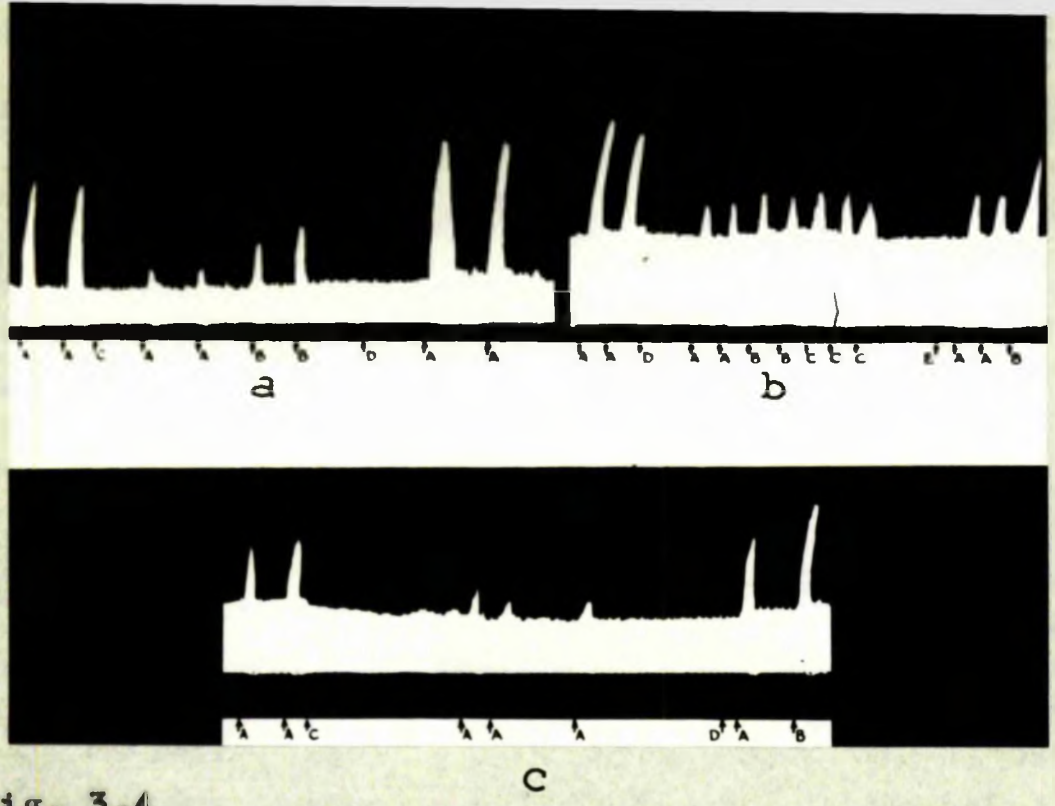

Fig. 3.4

Isolated perfused rat hindquarters.

(a) At A, 5-hydroxytryptamine, 0.2 µg.
B, " 0.3 µg.
C, perfusion with 10 µg. per ml. of hydrallazine in Locke's solution.
D, perfusion with Locke's solution.

(b) At A, adrenaline, 0.1 µg.
B, " 0.2 µg.
C, " 0.3 µg.
D, perfusion with 10 µg. per ml. of hydrallazine in Locke's solution.
E, perfusion with Locke's solution.

(c) At A, barium chloride 1 mg.
B, " " 1.6 mg.
C, perfusion with 10 µg. per ml. of hydrallazine in Locke's solution.
D, perfusion with Locke's solution.

B. RESULTS

1(a). ISOLATED PERFUSED RAT HINDQUARTERS

(b). ISOLATED PERFUSED RABBIT EAR.

Hydrallazine, dihydrallazine, 3-phenyl-6-hydrazino-pyridazine hydrochloride, 1-hydrazino isoquinoline hydrochloride and 3:6 dihydrazino pyridazine nitrate (1 to 100 µg. per ml.) increased the outflow from rat hindquarters and rabbit ear preparations. Both hydrallazine and dihydrallazine (1 to 10 µg. per ml.) reduced the vasoconstriction produced by adrenaline or noradrenaline (10 ng. to 1 µg.), 5-hydroxytryptamine (10 ng. to 1 µg.), histamine (1 to 10 µg.) or barium chloride (0.1 to 5.0 mg.) (Fig. 3.4). This effect was shared by 10 µg. per ml. of 1-hydrazino isoquinoline hydrochloride and 3:6 dihydrazino pyridazine nitrate but not invariably by 10 µg. per ml. of 3-phenyl-6 hydrazino pyridazine hydrochloride. The recovery of the response to the vasoconstrictor agents was generally complete over the period of the observations, although in some preparations recovery was never complete even after three hours. Hydrallazine and dihydrallazine showed a marked antagonism to 5-hydroxytryptamine and barium chloride; antagonism to adrenaline and nor-adrenaline /

noradrenaline was less marked. In general the rat hindquarters preparation was found to be less sensitive to vasoconstrictor agents than the rabbit's ear: compounds tested had qualitatively similar effects on both preparations.

Tripod and Meier[3], using the perfused hindquarters of the rabbit, demonstrated that hydrallazine and dihydrallazine only slightly antagonised the vasoconstriction produced by adrenaline and noradrenaline on this preparation. They were, however, able to show a marked antagonism to histamine and barium chloride . They found that these drugs had no direct effects on this preparation at concentrations of 100 µg. per ml. They concluded that hydrallazine and dihydrallazine had peripheral sites of action.

In this preparation the vessels are probably no longer under any neuro-humoral control; hence, without tone, they are almost maximally dilated. Observations upon the direct dilator effects of drugs on this preparation are, therefore, somewhat difficult to explain. Tripod and Meier[3] failed to show any antagonism between adrenaline and noradrenaline and hydrallazine /

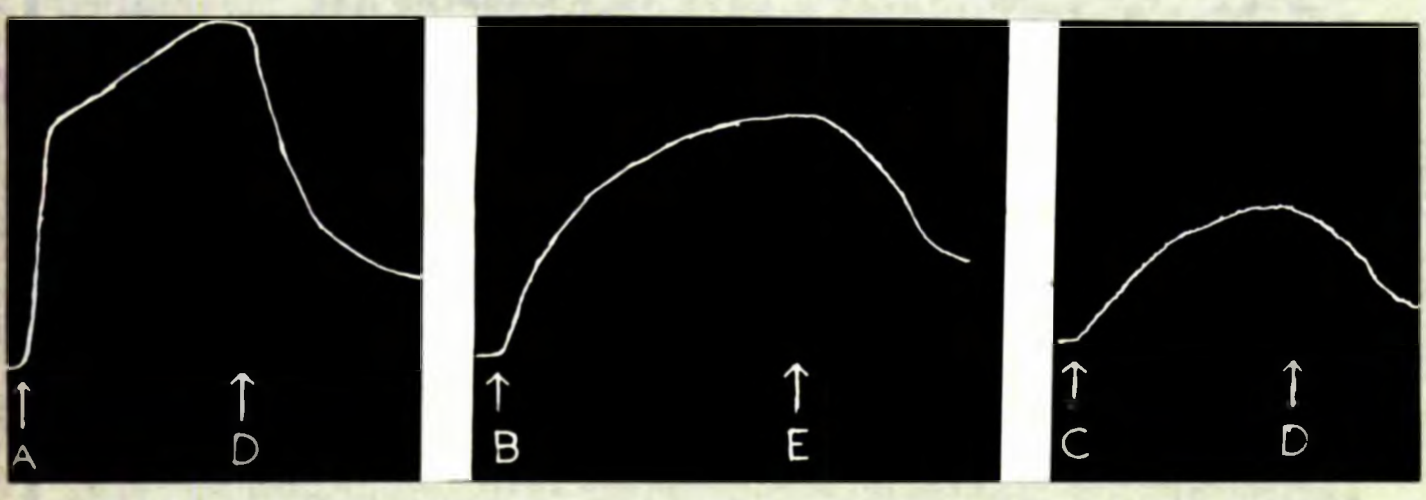

Fig. 3.5

Influence of hydrallazine on contractions of rabbit aortic strips induced by adrenaline, noradrenaline and 5-hydroxytryptamine.

At A, adrenaline 2 µg. per ml.

At D, hydrallazine, 0.4 mg. per ml.

At B, noradrenaline, 5 µg. per ml.

At E, hydrallazine, 1 mg. per ml.

At C, 5-hydroxytryptamine, 2 µg. per ml.

hydrallazine but the author has been able to show antagonism in all cases. The species difference between the hindquarters of the rabbit and of the rat might also have played a part in producing the variation in the observed degree of inhibition of vasoconstriction.

2. ISOLATED STRIPS OF RABBIT AND CAT AORTA.

Hydrallazine (400 µg. per ml.) and dihydrallazine (500 µg. per ml.) caused a slight relaxation of aortic strips obtained from cats and rabbits. Both compounds in similar concentrations relaxed sustained contractions of artery strips which were induced by adrenaline (1 to 10 µg. per ml.), noradrenaline (1 to 10 µg. per ml.), 5-hydroxytryptamine (2 to 5 µg. per ml.), histamine and acetylcholine (1 to 10 µg. per ml.), but not those induced by barium chloride (0.1 to 5 mg. per ml.) (Fig. 3.5). Hydrallazine was slightly more effective than dihydrallazine. Prior addition of the antagonist was not possible since the contraction or the relaxation was slow and recovery prolonged. In some preparations, the time interval between doses was often two hours or more. A few experiments were carried out in this way, however, and showed /

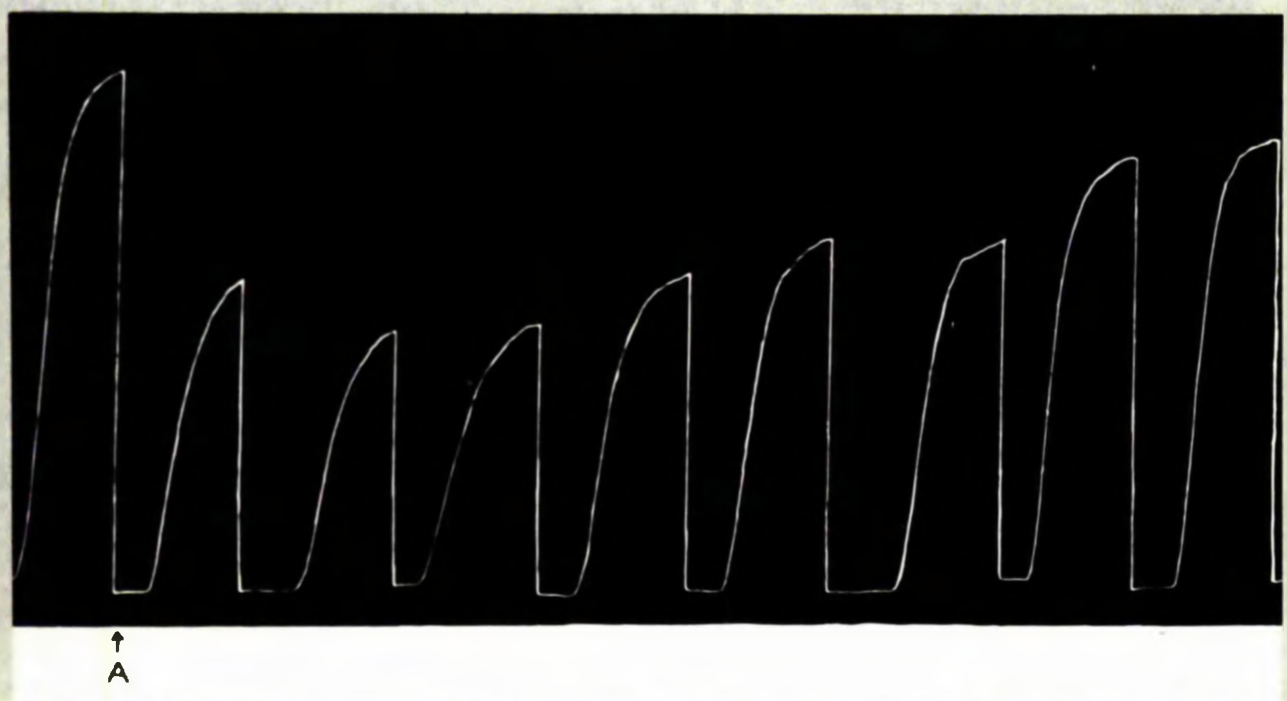

Fig. 3.6

Effect of reserpine upon acetylcholine-induced contractions of horse carotid artery strips.

All contractions due to acetylcholine, 0.05 µg. per ml. for 5 minutes.

At A, reserpine, 12.5 µg. per ml. 20 minutes before acetylcholine.

showed a reversible antagonism between hydrallazine and adrenaline and noradrenaline.

The effects seen on the isolated artery strips were similar to those on isolated vascular beds in the rat hindquarters and the rabbit ear. Hydrallazine caused a direct relaxation of the artery strips. A solitary human artery preparation was also found to relax under the influence of hydrallazine. It, therefore, appears that the drug may be disturbing some fundamental mechanism which maintains tone in arterial smooth muscle.

3. EXPERIMENTS USING STRIPS OF HORSE CAROTID ARTERY.

Reserpine (1.0 to 10.0 µg. per ml.) or hydrallazine (25 to 100 µg. per ml.) had a direct relaxant effect. Relaxation due to hydrallazine was reversible after a few washings but the effect of reserpine was much more persistent.

(1) Reserpine (1.0 to 25.0 µg. per ml.) and hydrallazine (25 to 500 µg. per ml.) antagonised the stimulant effects of acetylcholine (Fig. 3.6) (0.1 ng. to 2.0 µg. per ml.), adrenaline (10.0 ng. to 5.0 µg. per /

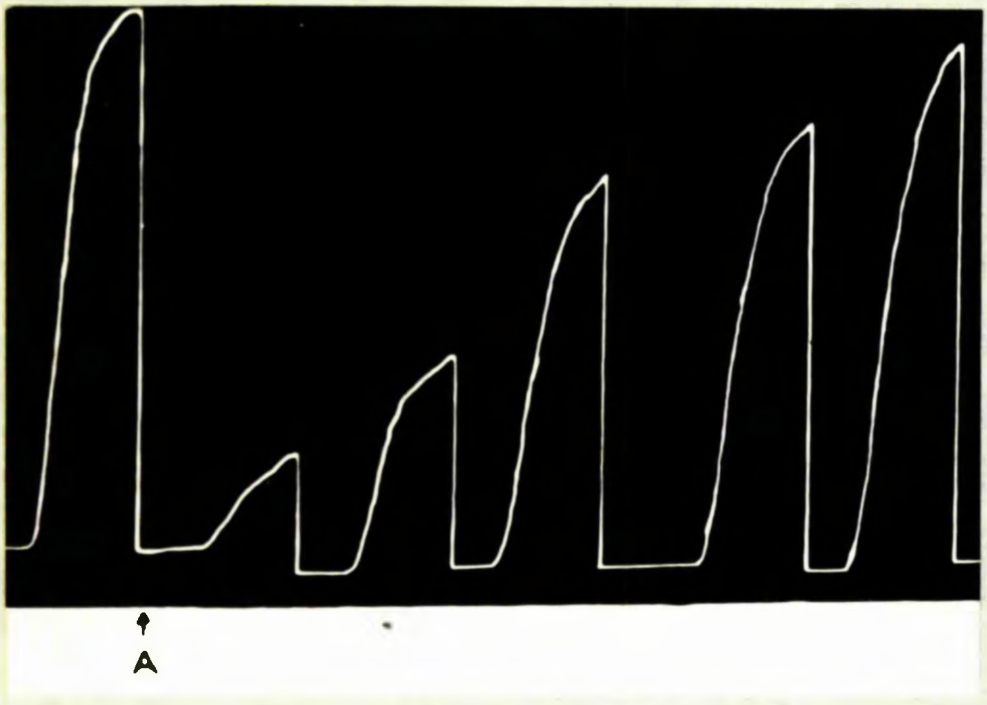

Fig. 3.7

(a) Effect of hydrallazine upon noradrenaline-induced contractions of horse carotid artery strips.

All contractions due to noradrenaline, 0.66 µg.per ml. for 5 minutes.

At A, hydrallazine, 26.5 µg.per ml. 20 minutes before noradrenaline.

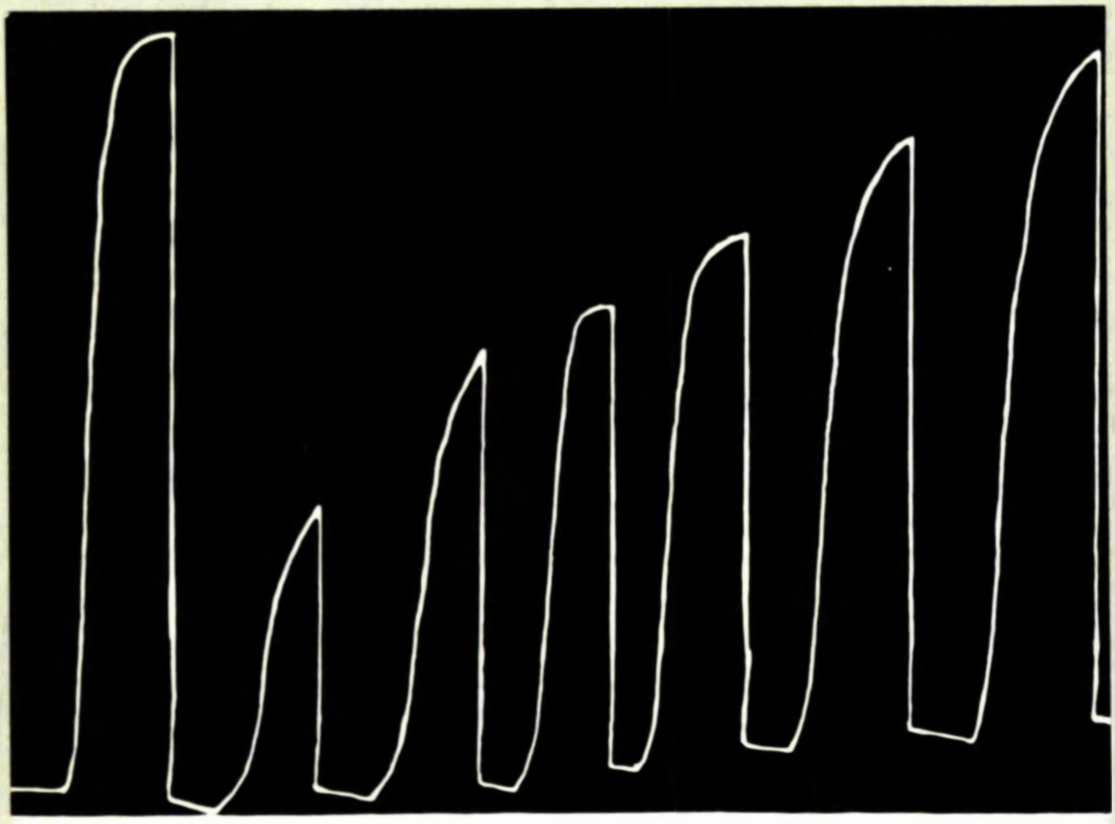

Fig. 3.7

(b) Effect of hydrallazine upon acetylcholine-induced contractions of horse carotid artery strips.

All contractions due to acetylcholine, 0.13 µg. per ml. for 5 minutes.

At A, hydrallazine 0.10 mg. per ml., 20 minutes before acetylcholine.

per ml.), noradrenaline (10.0 ng. to 5.0 µg. per ml.) (Fig. 3.7(a) and 7(b)), 5-hydroxytryptamine (40 ng. to 3.0 µg. per ml.), histamine (0.1 to 5.0 µg. per ml.), barium chloride (0.1 to 0.5 mg. per ml.) and potassium chloride (3.0 to 5.0 mg. per ml.). No evidence of specificity or selectivity was obtained. Recovery after hydrallazine was usually rapid and complete but in a few experiments it was incomplete. After reserpine it was rare for the tissue to recover, although occasionally recovery was seen after 6 to 12 hours. In some experiments using smaller doses of hydrallazine (5.0 to 10.0 µg. per ml.), there was slight potentiation of contractions due to acetylcholine and histamine.

It could be seen from the concentrations of the agonists used, that the preparation responded to much lower doses. It was especially sensitive to acetylcholine, since some preparations responded even to 1×10^{-12} g. of acetylcholine.

(2) These experiments were carried out to test whether reserpine and hydrallazine had a selective action on a specific drug receptor. High concentrations of adrenaline, /

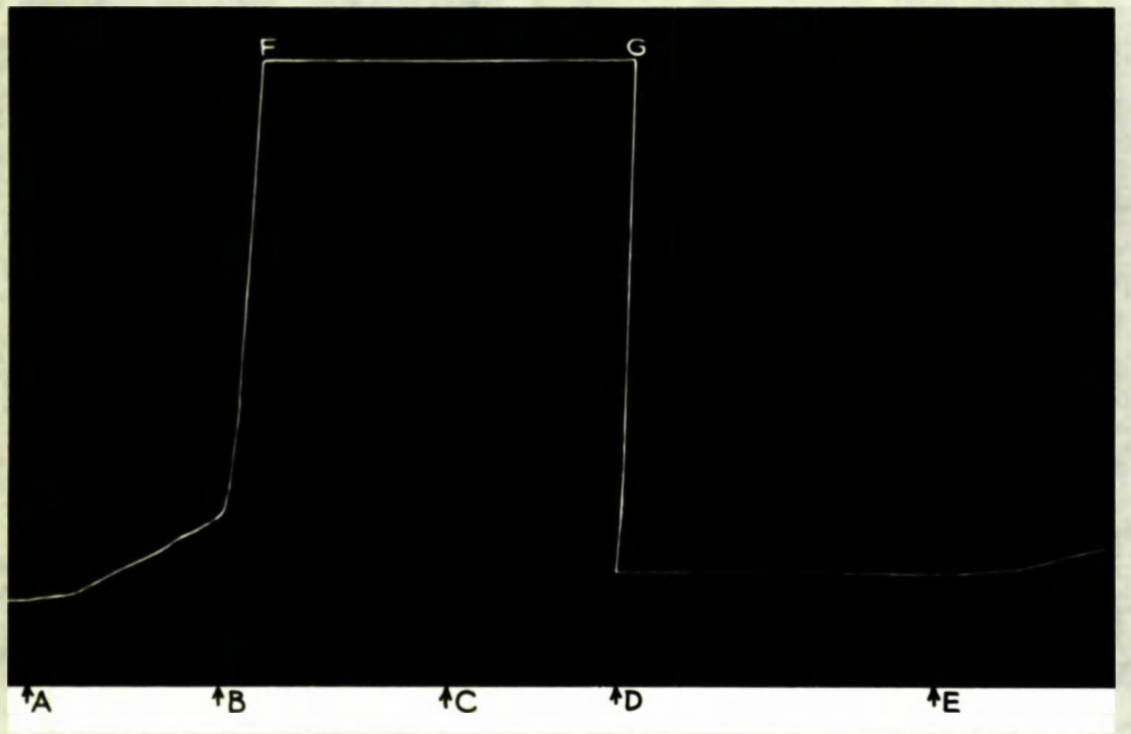

Fig. 3.8

Failure of a high dose of histamine to protect against hydrallazine inhibition.

At A, histamine,	1	µg.	per	ml.	for	5	minutes
B, "	10	"	"	"	"	15	"
C, hydrallazine,	25	"	"	"	"	20	"
D, wash out.							
E, histamine,	1.2	"	"	"	"	5	"

(Plateau FG accounts for a stop placed on the lever to prevent it from leaving the drum.)

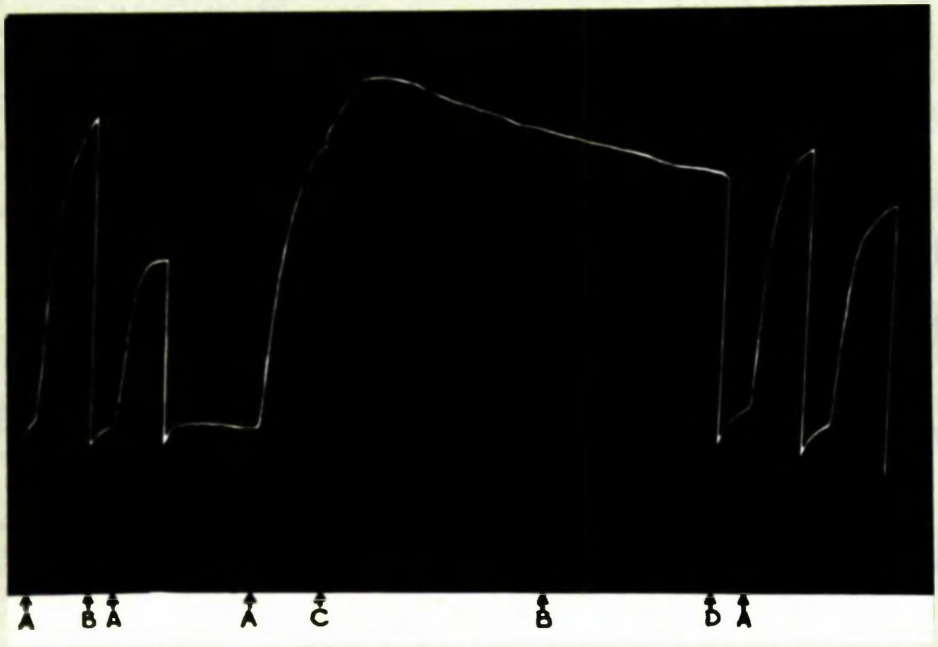

Fig. 3.9

Self-protection against hydrallazine inhibition by a high dose of adrenaline.

At A, adrenaline, 1 µg. per ml. for 5 minutes.

At B, hydrallazine, 20 µg. per ml. for 20 minutes before A.

At C, adrenaline, 10 µg. per ml. for 15 minutes.

At D, wash out.

adrenaline, noradrenaline, histamine or acetylcholine did not give protection against reserpine antagonism, thereby showing non-specificity of drug action. Hydrallazine behaved in a manner similar to that of reserpine with respect to its antagonism to acetylcholine and histamine (Fig. 3.8) but the effect of the second dose of adrenaline and noradrenaline was not reduced to the same extent, thereby showing that hydrallazine had some affinity for adrenergic receptors (Fig. 3.9). If it could be assumed that when there was no "self-protection" there was no specificity, then this method could be used to differentiate between drugs which acted on specific receptors and those which did not. Both reserpine and hydrallazine seemed to have non-specific drug effects rather than an action on specific receptors.

Andersson[4], using isolated rabbit aorta, showed that reserpine antagonised the vasoconstrictor effect of adrenaline. Tripod and Meier[5], using the perfused hindquarters of the rabbit, demonstrated that a sustained vasoconstriction produced by the continuous perfusion of adrenaline or noradrenaline was only slightly antagonised by 1 µg. per ml. of reserpine.

The /

The tone induced by histamine and 5-hydroxytryptamine was somewhat more effectively antagonised. They were unable to show any direct action of reserpine on this preparation. MacQueen and his colleagues[6], however, using the innervated but otherwise isolated rabbit's hindquarters found that the injection of 0.125 mg. reserpine caused an immediate diminution of vasomotor tone. Both groups of workers concluded that reserpine had a peripheral site of action. MacQueen et al.[7] added that the hypotensive effect of the drug could not be ascribed purely to a central action. Gillis[8], however, showed that reserpine antagonised drug induced tone in the isolated rat hindquarters and rabbit ear preparations; but he was unable to demonstrate any specificity of drug action.

Experiments carried out and reported above by the author support these findings. Hydrallazine behaved in a similar way to reserpine when its effects were tested against different vasoconstrictor agents on all of these preparations.

Adrenaline, noradrenaline, histamine, 5-hydroxy-tryptamine or acetylcholine act on specific receptors, while barium chloride or potassium chloride are believed to /

to act directly on the muscle. It is rather interesting to find that reserpine and hydrallazine - which belong to two entirely different classes of drugs - have similar actions on arterial smooth muscle. Experiments described on "self-protection" indicate that reserpine and hydrallazine do not act on specific receptors. Gillis and Lewis(9) have shown that reserpine may act by virtue of an interference with the metabolic processes which underly the contraction of intestinal smooth muscle. It seems not unlikely that reserpine and hydrallazine are acting in a similar manner on arterial smooth muscle.

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CHAPTER IV

CHAPTER IV

DISCUSSION OF DATA PRESENTED IN CHAPTERS II AND III

It is perhaps appropriate at this point to summarise the results presented in chapters II and III, and examine them in some detail. The discussion will be mainly confined to hydrallazine and dihydrallazine.

Hydrallazine and dihydrallazine cause a delayed fall in the blood pressure level of the anaesthetised cat, with slight stimulation of respiration. So far, knowledge is lacking as to whether these compounds themselves have a hypotensive effect, whether they are changed chemically *in vivo* before acting, or whether they act on intermediary processes. Werle *et al.*[1] and Schuler and Meier[2] have shown that certain phthallazines have a pronounced inhibitory effect on diamine-oxidase, but there are no indications that this effect is responsible for the typical hypotension induced by these drugs since other hydrazine derivatives which can inhibit diamine-oxidase have no hypotensive properties.

Hydrallazine and other compounds possess certain anti-adrenergic /

anti-adrenergic properties. Hydrallazine and dihydrallazine abolish the hypertension induced by infusion of adrenaline much more effectively than that induced by noradrenaline, while ganglion blocking agents and reserpine increase the hypertension caused by adrenaline and noradrenaline. Hydrallazine alters the pressor response to adrenaline into a biphasic pressor-depressor response, indicating thereby an effect upon vasoconstriction but no effect upon the stimulant actions of adrenaline on the heart (page 77). Both compounds only partially antagonise the pressor response to noradrenaline, and they also antagonise the pressor component of the biphasic pressor-depressor response to histamine which is supposed to be due to liberation of adrenaline. Antagonism to adrenaline is also shown in the isolated perfused rat hindquarters and rabbit ear (page 104), on the nictitating membrane and on isolated aortic strips. There are wide variations in potency which is greatest on the isolated perfused rat hindquarters and rabbit ear, moderate on aortic strips and the cat blood pressure, and low on the heart, auricles and nictitating membrane (pages 104-106, 76,83,90). Hydrallazine and dihydrallazine seem to be adrenergic blocking /

blocking agents but appear to be neither specific, selective nor potent.

Taylor et al.[3] consider hydrallazine to be a specific antagonist of 5-hydroxytryptamine. The observations presented in this thesis do not support this view. Marked antagonism to 5-hydroxytryptamine is shown only on the isolated rat hindquarters or rabbit ear. Contractions of the rat uterus in response to 5-hydroxytryptamine are partially blocked by high doses, and on the isolated guinea pig ileum they are potentiated. Erspamer[4] does not consider hydrallazine to be a specific antagonist of 5-hydroxytryptamine. Hydrallazine does not have any marked effects on acetylcholine and histamine responses on isolated tissue or organ preparations, or on the blood pressure of the anaesthetised cat.

Vasopressor reflexes.

Hydrallazine and dihydrallazine do not completely eliminate any of the pressor reflexes, but responses to stimulation of the cut central end of the sciatic nerve, to compression of the abdominal aorta and to stimulation of the splanchnic nerve are reduced by both /

both compounds. The responses to anoxia, hypoxia, carotid sinus occlusion and stimulation of the cut central end of the vagus are almost unaffected.

These reflex pressor responses may have the same mechanism of action, i.e. liberation of adrenaline and noradrenaline from the endings of the adrenergic nerves[5], the reflex being mediated through higher centres in the brain. The fact that adrenergic and ganglion blocking agents depress these reflexes supports this view. Although the peripheral sympathetic nervous system is considered to represent the final pathway responsible for the rise of blood pressure which is brought about by these reflexes[6], and noradrenaline is the transmitter set free from the adrenergic nerve endings[5,7], the differences in the composition of the adrenal medullary secretion after stimulation of afferent nerves and of pressor and chemoreceptors, are also of importance in causing the blood pressure to rise[6,8]. In cats it has been shown that, when the pressor reflexes are brought into play, about two-thirds of the medullary secretion consists of noradrenaline. The author's observations do not support the finding of Bein et al.[9], Grimson et /

et al.[10] and Taylor et al.[3] and indicate that hydrallazine does not antagonise all the pressor reflexes. Bein, Gross, Tripod and Meier[9] have shown that both drugs antagonise all the pressor reflexes; but Walker and his colleagues[11] could not show blockade of the carotid sinus reflex, and Britton and his colleagues[12] could not inhibit the pressor response to hypoxia. Failure to antagonise these pressor reflexes indicates that hydrallazine may not act by interfering with sympathetic activity in the central nervous system, because it antagonises the peripheral effects of adrenaline more effectively than those of noradrenaline. Hydrallazine, even in high doses, has no depressant action on sympathetic ganglia. An effect upon the pressor and chemoreceptors of the carotid body and carotid sinus seems unlikely, since responses to hypoxia, anoxia or carotid sinus occlusion are not altered. Neither hydrallazine nor dihydrallazine antagonises the pressor response to electrical stimulation of the central end of the cut vagus. As already pointed out, hydrallazine antagonises the pressor response due to splanchnic nerve stimulation, abdominal aortic compression and sciatic /

sciatic nerve stimulation. Although two-thirds of the medullary secretion usually consists of noradrenaline, it is possible that when different reflexes are evoked the ratio of noradrenaline to adrenaline may change. Thus, after electrical stimulation of the sciatic nerve, the ratio shifts in favour of adrenaline[8] while after stimulation of the central end of the vagus, it consists mostly of noradrenaline[3]. Hydrallazine antagonises adrenaline more effectively than noradrenaline, and this may partly explain why hydrallazine antagonises the pressor response to sciatic nerve stimulation in which the secretion is mostly adrenaline and not that due to central vagal stimulation in which the secretion consists mostly of noradrenaline. In any case, antagonism to these pressor reflexes cannot be explained by assuming the drug to interfere with the sympathetic activity in the central nervous system; if this were so it would be rather difficult to explain why it should antagonise some responses and not others.

Depression of the rhythmic activity of isolated cardiac muscle preparations is observed following hydrallazine. The amplitude of the contractions of the isolated kitten and rabbit hearts is decreased.

This /

This is accompanied by an increased outflow from the hearts; this may not have been due entirely to coronary dilatation. It seems clear, however, that it reflects either a general reduction in the vascular support of the coronary bed, or an increased aortic incompetence. In either case, the mechanism should involve muscular relaxation.

In perfused blood vessel and isolated artery preparations, drug induced vasoconstriction was reduced both by hydrallazine and reserpine, which were also capable of causing direct relaxation of the artery strips. The fact that both hydrallazine and reserpine antagonise vasoconstriction produced by such different agents as adrenaline, histamine and 5-hydroxytryptamine (which act on specific receptors) and barium chloride and potassium chloride (which act directly on the muscle) suggests a fundamental disturbance by these drugs in the smooth muscle cell. The pharmacological nature of the agents used to cause contraction does not appear to be decisive in determining the type of antagonism. The suggested non-specificity of action may be supported by the very wide variation in the degree of inhibition produced by the same dose of hydrallazine

or //

or reserpine. Finally, these characteristics develop only slowly; the reserpine effect on arterial muscle was practically irreversible, while that of hydrallazine was reversible.

Many of these observations may be explained, however, by assuming that hydrallazine and reserpine act by depressing the ability of the smooth muscle to contract. This implies that a process common to muscular contraction - whether myogenic or drug induced - is influenced by the drugs. Coronary dilatation can also be explained on this hypothesis. Infiltration of the carotid sinus area with hydrallazine causes the systemic blood pressure to rise. This also points to a direct relaxant effect on the muscle fibres of the carotid sinus walls. Drugs relaxing the arterial walls of the carotid sinus, and increasing their distensibility, decrease the stimulation of pressor receptors and thus induce a reflex rise of the systemic arterial pressure[14]. It is therefore felt that these non-specific effects of reserpine and hydrallazine are due to interference with normal cell metabolism.

The experimental work to be described in the next chapters /

chapters deals with attempts to investigate in more detail the possibility of a metabolic site of action of hydrallazine and reserpine.

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CHAPTER V

(Tables will be found at the end of this chapter)

CHAPTER V

AN INVESTIGATION OF POTENTIAL INHIBITORS OF THE ACTION OF HYDRALLAZINE AND RESERPINE ON ARTERIAL SMOOTH MUSCLE

Reserpine and hydrallazine have been shown to antagonise contractions of arterial smooth muscle irrespective of the nature of the stimulant drug used. Both drugs are capable of causing direct relaxation of strips of arteries. These antagonisms are considered to be non-specific drug effects, rather than actions upon specific receptors. Gillis and Lewis (1 to 3) have shown that reserpine may act by virtue of its ability to interfere with the metabolic processes which underlie the contraction of intestinal smooth muscle. It therefore appears likely that reserpine and hydrallazine may be acting in a similar manner on arterial smooth muscle.

Mammalian smooth muscle, isolated and maintained under good conditions, maintains its normal spontaneous tone and activity for many hours. When stimulated electrically or by histamine or acetylcholine, the muscle contracts and remains contracted for as long as the stimulus lasts. It has been known for many years that the/

the contractile properties of isolated smooth muscle alter reversibly when the supply of oxygen or of substrate is stopped. Thus, Gross and Clark[4] and Garry[5] have shown that anoxia rapidly diminishes the tone of isolated uterus and intestine, and it abolishes the spontaneous movements of intestinal muscle. Effects similar to anoxia are seen when the isolated smooth muscle is deprived of glucose or of other substrates[6]. The effects of anoxia, of substrate deficiency and of metabolic inhibitors have been attributed to an interruption of the normal supply of energy to the muscle. Such an interruption might be expected to show itself in a fall in the concentrations of the labile high energy phosphate compounds, adenosine triphosphate (ATP) and creatine phosphate, which are generally thought to be the immediate - or almost immediate - sources of energy for muscular activity.

Methods.

The tissue chosen for most of the work was horse carotid artery. As has been indicated in the previous chapter, this preparation was found to be suitable for the study of drug antagonisms. The method of preparation /

preparation has already been described (Chapter III).

A series of known intermediates of carbohydrate, fat and protein metabolism was tested for possible antagonism to the actions of reserpine and hydrallazine in depressing drug-induced contraction of strips of isolated carotid arteries. The effects of the intermediary metabolites on reserpine or hydrallazine inhibition were compared with those of anoxia and of known enzyme inhibitors such as cyanide, iodoacetate and azide. Attempts were also made to compare these drugs with p-chloromercuribenzoate, a known sulphydryl enzyme inhibitor, and to antagonise their actions with glutathione which protects these enzymes. Solutions of the metabolites in Tyrode's solution were used as their sodium salts to avoid any pH effects, with the exception of maleic acid (pH 1.8) and (±) leucine (pH 1.0). Each metabolite was tested in concentrations of 500 µg. and 1 mg. The dose most frequently used was 1 mg.

In testing for possible antagonism to the actions of reserpine and hydrallazine, the antagonists were added to the bath 10 minutes before the addition of the drugs and remained in contact with the tissue for 30 minutes. /

minutes. Potassium cyanide, sodium azide, sodium iodoacetate or p-chloromercuribenzoate were added to the bath in place of reserpine or hydrallazine using the same time cycle. To render the tissue anoxic, the bicarbonate free Tyrode's solution was first of all boiled to drive off dissolved gases. It was cooled under a mixture of 95 per cent nitrogen and 5 per cent carbon dioxide, and the bicarbonate was added to the cooled solution. The final pH was adjusted to 7.6 to 7.8. During the experiment the same gas mixture replaced oxygen in the bath fluid. The tissue was kept under these conditions for 30 minutes before any drugs were added. Intermediary metabolites were added in the same way as before.

Contractions of the artery strips were obtained in response to acetylcholine (0.1 ng. to 2.0 µg.), adrenaline (10.0 ng. to 5.0 µg.), noradrenaline (10.0 ng. to 5.0 µg.), 5-hydroxytryptamine (40.0 ng. to 3.0 µg.), and histamine (0.1 to 5.0 µg.). The drugs remained in contact with the tissue between 4 and 6 minutes depending upon the nature of the response. The ability of the added compound to antagonise hydrallazine or reserpine depression of contractile responses is expressed /

expressed as the average inhibition per cent of the contractions induced by the stimulant drug ± standard error of the mean. This approximate figure was calculated as follows:

The height of the control contraction (A) is measured and also that of the contraction after addition of the antagonist (B). When there is complete recovery to the control level - which may be slightly greater or less than the original control - the height of the contraction after the addition of antagonist with intermediary metabolite is measured (C). The protection per cent was then calculated:

$$\frac{C - B}{A - B} \times 100 = \text{protection per cent.}$$

Values from all experiments using the same combination of spasmogen, antagonist and the intermediary metabolite, have been averaged to obtain these figures.

Summary of Results.

Certain intermediates of carbohydrate, fat and protein metabolism in concentrations of 1 mg. were found to give protection against hydrallazine depression of /

of acetylcholine, (-) adrenaline, noradrenaline, histamine and 5-hydroxytryptamine-induced contractions of spirally cut strips of arteries. Strips made anoxic behave in a manner similar to that of hydrallazine-treated strips. Both caused loss of tone, relaxation and reduction in the response to the same dose of the stimulant. Recovery of the tissue after anoxia was similar to recovery after hydrallazine, but was different from that seen after potassium cyanide, sodium azide, sodium iodoacetate or reserpine. Intermediary metabolites which gave protection against anoxia were also found to protect against hydrallazine inhibition, although complete parallelism was not to be found.

Inhibition of drug-induced contractions by cyanide, iodoacetate and azide was of a different character from that caused by hydrallazine and anoxia. Few intermediates gave significant protection. Hydrallazine probably exerts its effect by a non-specific depression of metabolism rather than by an action upon specific receptors. Reserpine depression of drug-induced contractions in artery strips was so persistent that experiments using intermediary metabolites could not be made.

RESULTS

Since the contents of the bath were a complex mixture of salts and drugs, and the possibility of chemical inactivation was present, some experiments were carried out in which hydrallazine did not come in contact with the added substrate. The substrate was allowed to remain in the bath for 10 minutes and was then washed out. Hydrallazine was then added and the experiment continued. Qualitatively similar results were obtained, and it was concluded that the manner in which the drug and its antagonists were added made very little difference, if any. None of the compounds tested caused a direct contraction of the artery strip when added alone to the bath, nor did they appreciably potentiate the contractile responses to acetylcholine and adrenaline. It therefore appears that the possibility of chemical inactivation does not exist.

It is well known that certain di- and tricarboxylic acids form undissociated complexes with calcium ions and therefore effectively remove these from the solution. It seemed possible that some of the observed effects may be due to this phenomenon. It has been shown by Gillis[7] that removal of calcium ions by these compounds is /

is not significant, and that citrate - which is known to remove ionic calcium - was inactive as an antagonist of hydrallazine. To exclude possible effects due to liberation of histamine, the experiments were repeated using Tyrode's solution containing 200 µg. per lit. of mepyramine. Under these conditions there was no alteration in the inhibition of hydrallazine effect produced by the active substances. It was concluded, therefore, that there was no evidence for histamine release.

It is difficult to explain why certain metabolites did not antagonise inhibitions due to hydrallazine. If the active substances are effective because they are intermediates of cell metabolism, then it is reasonable to expect that such metabolic intermediates should show antagonism. However, it may be possible that the inactive compounds do not penetrate the cells or that they are not needed.

The results shown in Table 5.1 indicate that most of the compounds can antagonise hydrallazine depression of acetylcholine and adrenaline-induced contractions. Many of the active compounds were intermediates of the tricarboxylic acid cycle, though this has not been so far /

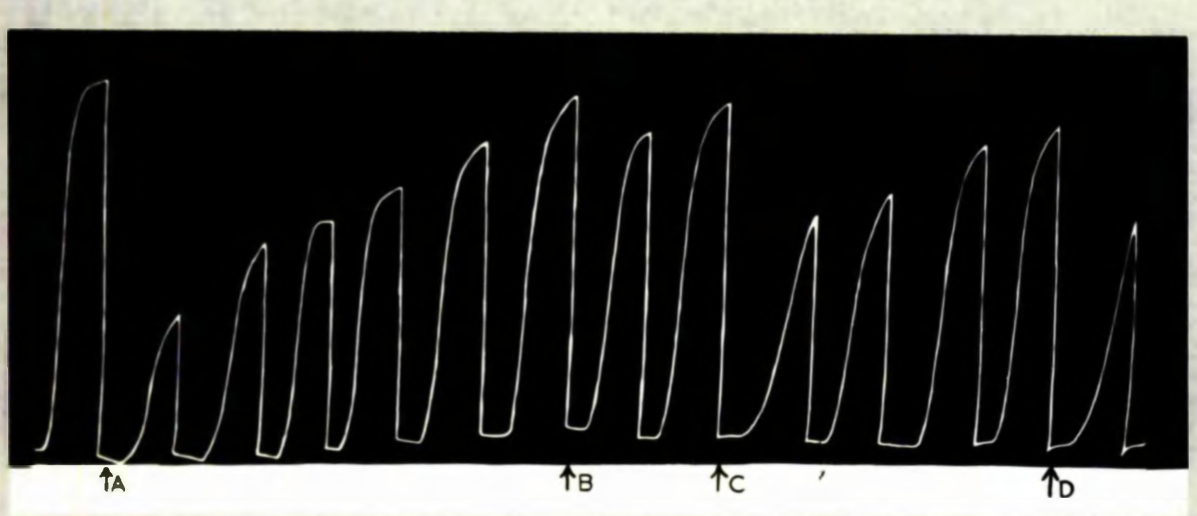

Fig. 5.1

Effects of pyruvate, succinate and fumarate on hydrallazine inhibition of acetylcholine-induced contractions of artery strips.

All contractions due to acetylcholine, 0.13 µg. per ml.

At A, hydrallazine, 0.13 mg. per ml.

At B, pyruvate, 1 mg. per ml. + " " " " "

At C, succinate, 1 mg. per ml. + " " " " "

At D, fumarate, 1 mg. per ml. + " " " " "

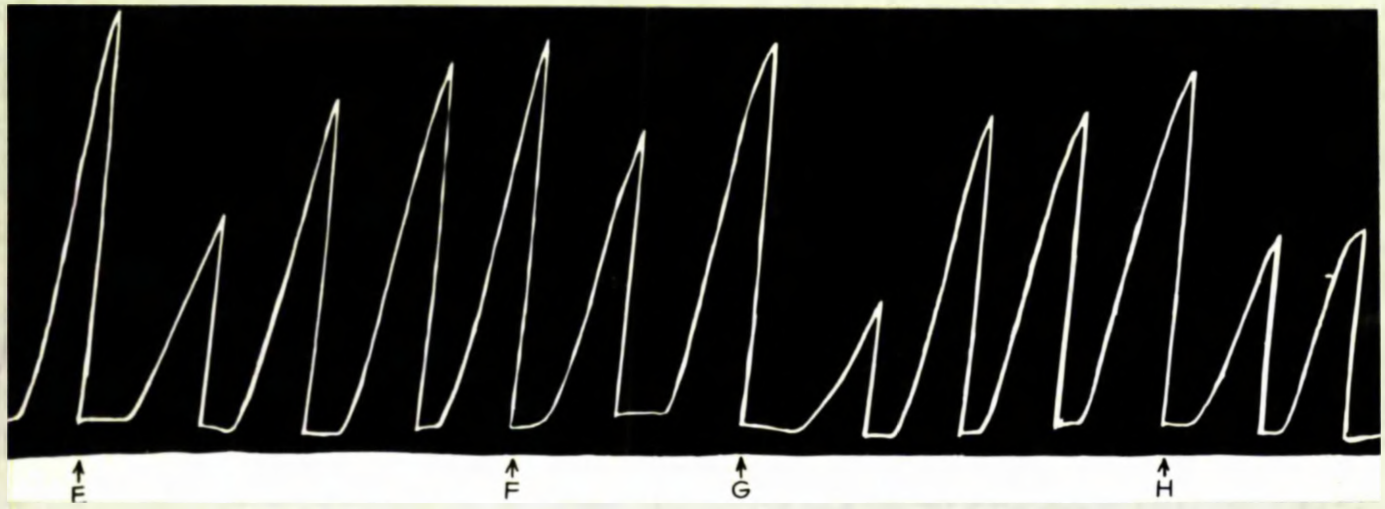

Fig. 5.2

Effects of oxaloacetate, cisaconitate and citrate on hydrallazine inhibition of acetylcholine-induced contractions of artery strips.

All contractions due to acetylcholine, 0.02 µg. per ml.

At E, hydrallazine, 0.066 mg. per ml.

At F, oxaloacetate, 1 mg. per ml.+ " " " " "

At G, cisaconitate, 1 mg. per ml.+ " " " " "

At H, citrate, 1 mg. per ml.+ " " " " "

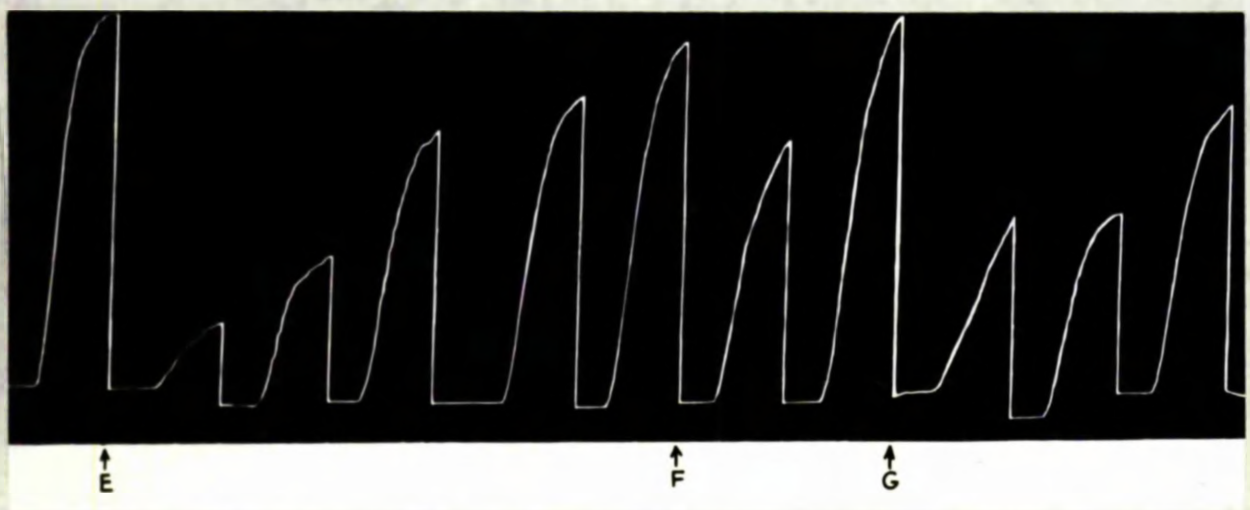

Fig. 5.3

Effects of pyruvate and succinate on hydrallazine inhibition of noradrenaline-induced contractions of artery strips.

All contractions due to noradrenaline, 0.13 µg.per ml.

At E, hydrallazine, 26 µg.per ml.

At F, pyruvate, 1 mg. per ml. + " " " " "

At G, succinate, 1 mg. per ml. + " " " " "

far shown to exist in arterial smooth muscle. Pyruvate, succinate, fumarate, α-ketoglutarate and oxaloacetate (Fig. 5.1) were found to be potent antagonists of hydrallazine-induced inhibition, though oxalosuccinate also gave a moderate protection. Citrate, isocitrate, cisaconitate and maleic acid were inactive or less potent (Fig. 5.2). In general, intermediates of glycolysis were less potent though 3-phosphoglycerate and fructose-6-phosphate are moderately potent. Intermediary metabolites in general were more active when adrenaline was used as a stimulant. Pyruvate, succinate, fumarate, cisaconitate, α-ketoglutarate, and oxaloacetate were found to give marked protection. Among the members of the glycolysis cycle, 3-phosphoglycerate, glucose-6-phosphate, and fructose-6-phosphate afforded maximum protection, while glucose-1-phosphate was intermediate in action. Glucose-1-6-diphosphate, citrate, and maleic acid were found to be ineffective against hydrallazine. The most active compounds, when selected for use with noradrenaline (Fig. 5.3), histamine and 5-hydroxytryptamine, showed a similar parellelism. Thus pyruvate, succinate, α-ketoglutarate, 3-phosphoglycerate and fructose-6-phosphate were shown

to /

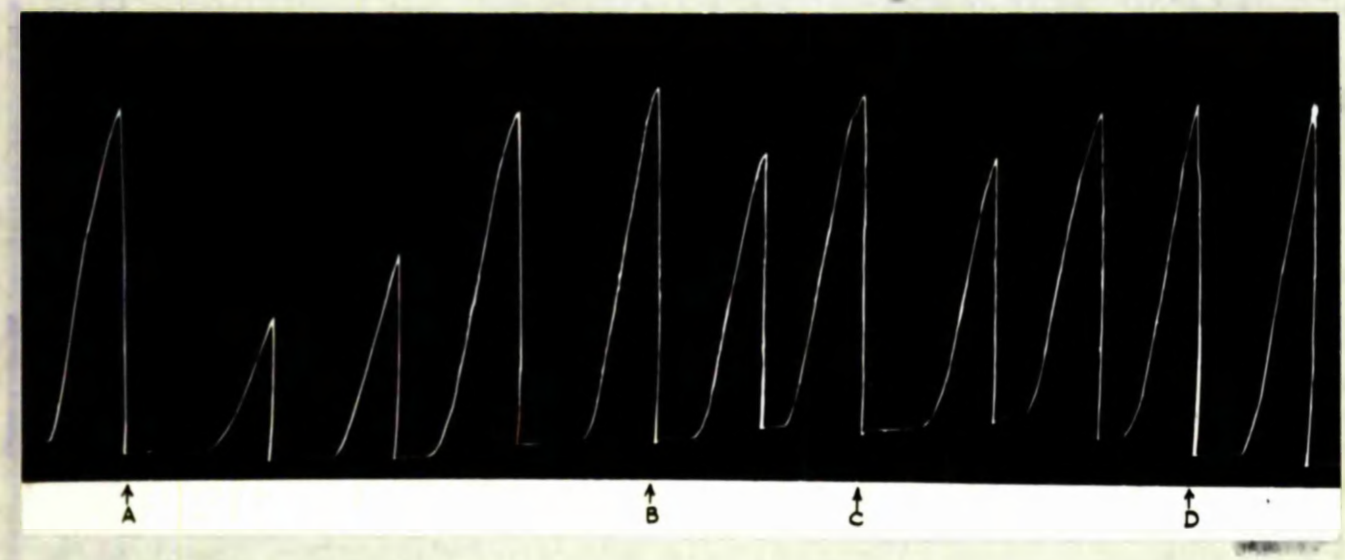

Fig. 5.4

Effects of 3-phosphoglycerate, (±) alanine and butyrate on hydrallazine inhibition of adrenaline-induced contractions of artery strips.

All contractions due to adrenaline, 0.2 µg. per ml.

At A,		hydrallazine, 0.26 mg. per ml.
At B,	3-phosphoglycerate, 1 mg. per ml.+	" " " "
At C,	(±) alanine, 1 mg. per ml.+	" " " "
At D,	butyrate, 1 mg. per ml.+	" " " "

to behave in more or less the same manner (Table 5.2).

Among the intermediates of fat and protein metabolism, 3-hydroxybutyrate, propionate, glutamate and alanine gave considerable protection against hydrallazine inhibition of acetylcholine or adrenaline-induced contractions, while (±)-leucine was ineffective (Fig. 5.4). Glutathione - which protects the thiol groups - did not give protection against hydrallazine, and the thiol group inhibitors, such as p-chloromercuribenzoate and arsphenamine, did not antagonise the drug-induced contractions unless used in very high doses.

Reserpine-induced inhibition was antagonised to a slight extent by pyruvate and succinate. Since only two experiments were possible, these effects might be doubtful. Reserpine caused what was virtually an irreversible effect showing a remarkable affinity for the tissue.

Results in Table 5.3 show the antagonism of intermediary metabolites against the inhibitory effects of cyanide and anoxia on this preparation. Both potassium cyanide and anoxia were used to antagonise acetylcholine/

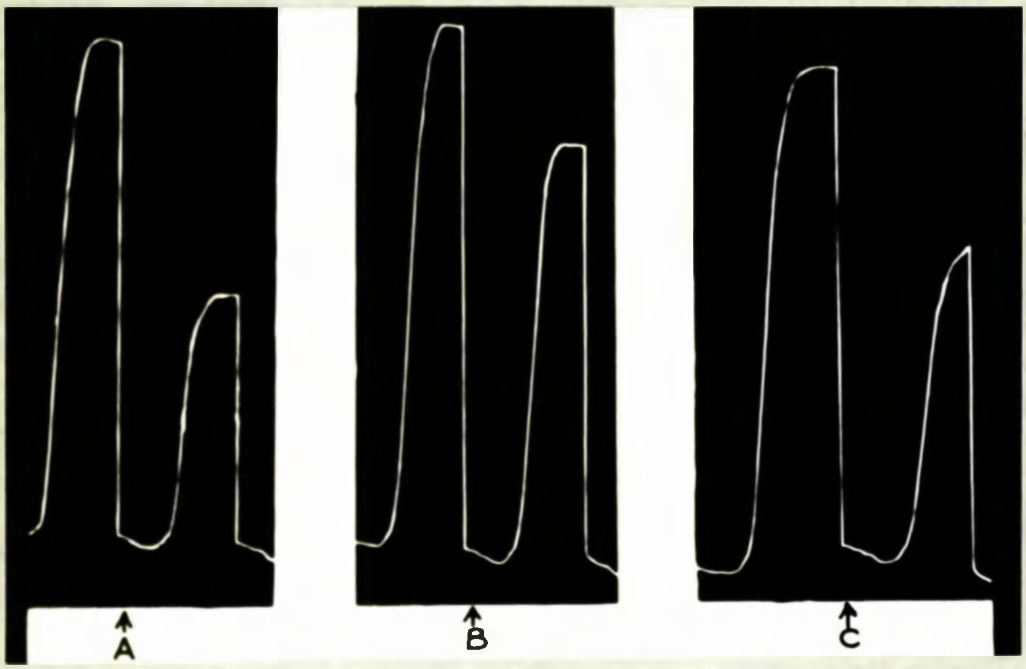

Fig. 5.5

Effects of pyruvate and succinate upon cyanide inhibition of acetylcholine-induced contractions of artery strips.

All contractions due to acetylcholine, 0.2 µg. per ml.

At A, cyanide, 0.1 mg. per ml.

B, pyruvate, 1 mg. per ml. + " " " " "

C, succinate, 1 " " " + " " " " "

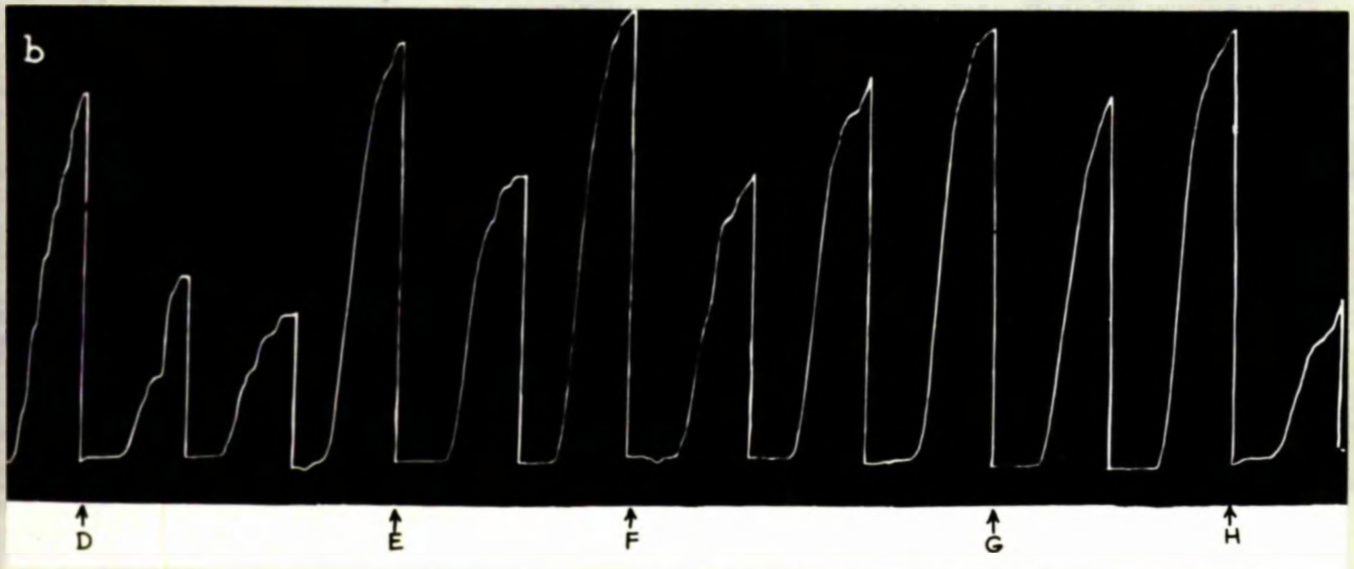

Fig. 5.6

Effects of (±) alanine, glutamate, α-ketoglutarate and fumarate upon cyanide inhibition of acetylcholine-induced contractions of artery strips.

At D, cyanide, 0.15 mg. per ml.

E, (±) alanine, 1 mg. per ml. + " " " " "

F, glutamate, 1 " " " + " " " " "

G, α-ketoglutarate, 1 " " " + " " " " "

H, fumarate, 1 " " " + " " " " "

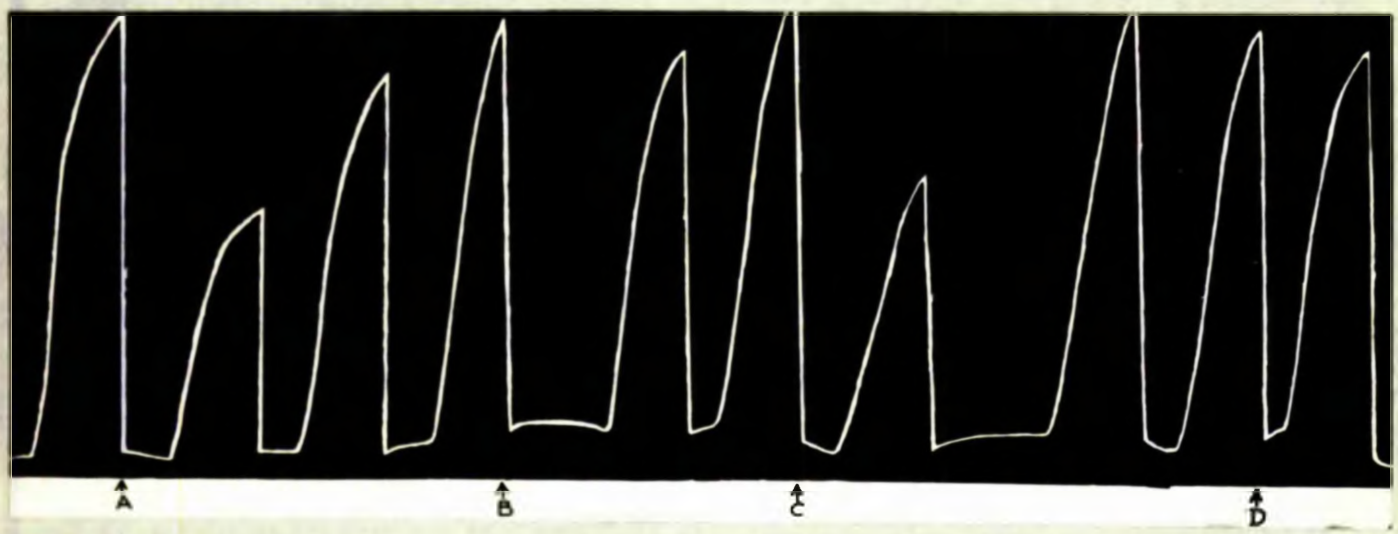

Fig. 5.7

Effect of succinate, fumarate and pyruvate upon anoxic inhibition of acetylcholine-induced contractions of artery strips.

All contractions due to acetylcholine, 0.04 µg. per ml.

At A, effect of anoxia

At B, succinate, 1 mg. per ml. and anoxia

At C, fumarate, 1 mg. per ml. and anoxia

At D, pyruvate, 1 mg. per ml. and anoxia

acetylcholine-induced contractions. Potassium cyanide (0.04 to 0.2 mg.) antagonised drug-induced contractions, causing prolonged inhibition, and at times the tissue failed to recover. Tissues made anoxic lost their tone and the response to the same dose of the stimulant was considerably reduced. However, the tissue recovered quite promptly on resuming the oxygen supply, and the response to the stimulant drug also returned. The recovery of the anoxic and hydrallazine-treated tissues was strikingly similar, and could be contrasted with that of cyanide-treated tissue.

It seems quite obvious from the Table that very few intermediates gave protection against potassium cyanide. Only pyruvate and α-ketoglutarate (Fig. 5.5) gave considerable protection, but glutamate and (±) alanine also showed significant effects (Fig. 5.6). In general, all other compounds tested were ineffective. The results obtained with anoxic tissues were quite different, - since pyruvate, succinate, fumarate, α-ketoglutarate and oxalosuccinate gave considerable protection against anoxia (Fig. 5.7). Few intermediates of glycolysis such as glucose-6-phosphate, fructose--6-phosphate and 3-phosphoglycerate antagonised the effects /

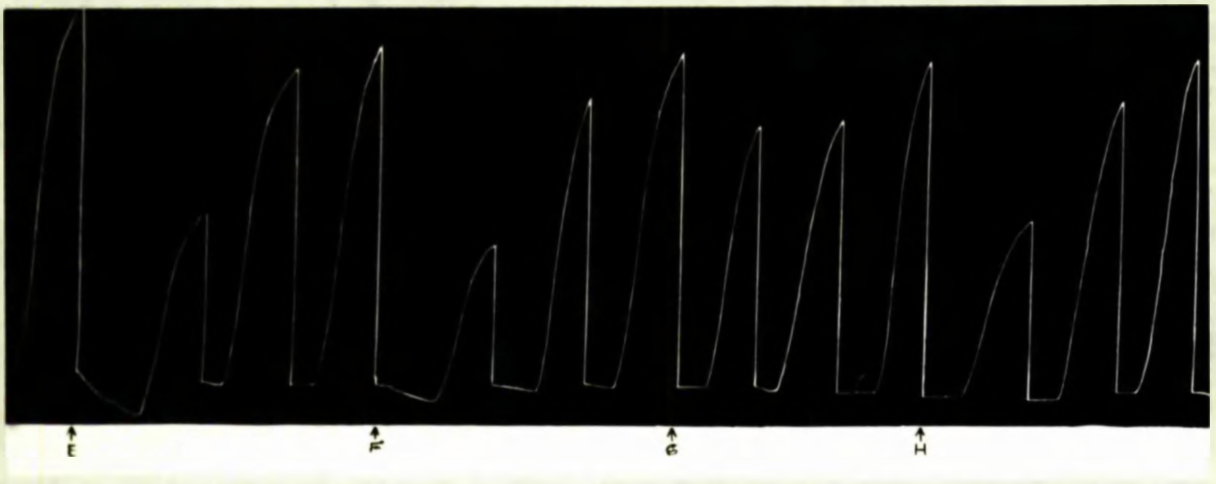

Fig. 5.8

Effect of butyrate, oxalosuccinate, citrate upon anoxic inhibition of acetylcholine-induced contractions of artery strips.

All contractions due to acetylcholine, 0.2 µg. per ml.

At E, effect of anoxia

At F, butyrate, 1 mg. per ml. and anoxia

At G, oxalosuccinate, 1 mg. per ml. and anoxia

At H, citrate, 1 mg. per ml. and anoxia.

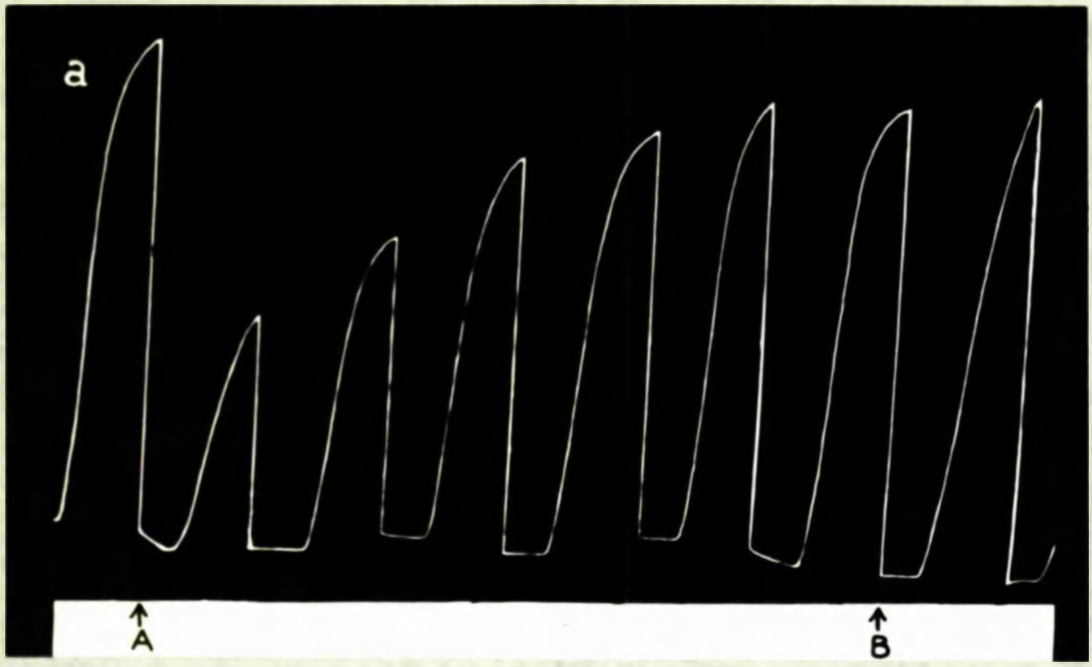

Fig. 5.9(a)

Inconsistent effects of pyruvate in antagonising azide inhibition of acetylcholine-induced contractions of horse carotid artery strips.

(a) All contractions due to acetylcholine 0.1 µg. per ml.

At A, inhibition due to azide, 0.4 mg. per ml.

At B, protection due to pyruvate, 1 mg. per ml. before azide.

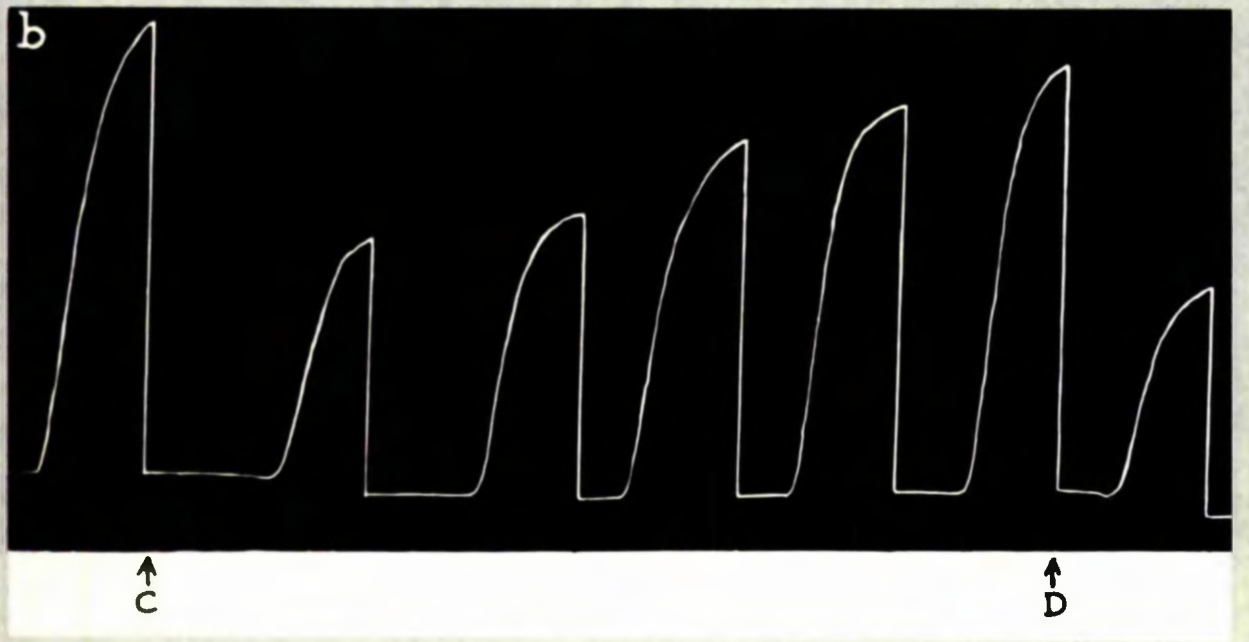

Fig. 5.9(b)

(b) All contractions due to acetylcholine 0.4 µg. per ml.

At C, inhibition due to azide, 0.8 mg.per ml.

At D, lack of protection by pyruvate, 1 mg. per ml. before azide.

effects of anoxia. Members of the fat series were ineffective (Fig. 5.8) while glutamate, and especially (±) alanine, gave a significant protection against anoxia. Results obtained with anoxia and hydrallazine treatment bear a close - though not complete - resemblance.

Similar attempts were made to antagonise the inhibitory effects of sodium azide on drug-induced contractions by supplying the tissue with metabolites. Sodium azide (0.1 to 1.0 mg. per ml.) caused a pronounced and prolonged inhibition of acetylcholine-induced contractions, but the compounds added had irregular effects and reproducible results could not be obtained (Fig. 5.9). Sodium iodoacetate markedly, but irreversibly, inhibited the drug-induced contractions.

DISCUSSION.

It seems unlikely that hydrallazine and reserpine act on specific receptors in arterial smooth muscle. The experiments described were, therefore, carried out to see whether the effects of hydrallazine and reserpine could be antagonised by supplying the tissue with a series of known intermediates of carbohydrate, and /

and of fat and protein metabolism; and also to see whether the effects of hydrallazine or reserpine resembled those of anoxia or of known enzyme inhibitors, such as azide, cyanide and iodoacetate.

Substances which restore contractility may act either as substrates or as pharmacological agents. All the evidence so far accumulated, although indirect, appears to favour the substrate concept. Rona and Neukirch[8] observed a reduction in glucose and mannose content during a two and a half-hour period of active contractions. More specific evidence against a pharmacological action was provided by Feldberg and Solandt[6]. The addition of acetylcholine during a period of diminished activity in a glucose-free Tyrode's solution produced a relatively small increase in tonus of the intestinal smooth muscle. However, following the restoration of amplitude by glucose or pyruvate, acetylcholine produced a marked and well-sustained increase in tonus. It was, therefore, inferred that glucose and pyruvate did not increase smooth muscle activity by increasing the production of acetylcholine, and that this agent could exert its pharmacological effects only when energy for contraction

was provided by appropriate substrates. The discussion of the results has, therefore, been based on the concept that the capacity of a substance to augment and sustain the amplitude of contraction is derived from the ability to provide energy for smooth muscle contraction.

Results shown in Table 5.1 indicate that most of the compounds can antagonise hydrallazine depression of drug-induced contractions and, on the basis of the above hypothesis, provide energy for contraction. Most of the intermediates are members of the tricarboxylic acid cycle. Intermediates of carbohydrate metabolism - 3-phosphoglycerate, pyruvate, succinate, fumarate, α-ketoglutarate and oxaloacetate - are potent antagonists and in general the intermediates of glycolysis are less potent. Propionate, glutamate, (±) alanine and 3-hydroxybutyrate are moderately active. The ability of an amino acid like (±) alanine to protect against hydrallazine inhibition may be explained by assuming that the arterial smooth muscle can deaminate (±) alanine to its corresponding keto acid - pyruvic acid. In the same way the behaviour of oxaloacetic acid suggests that it may have exerted its effect after decarboxylation

to /

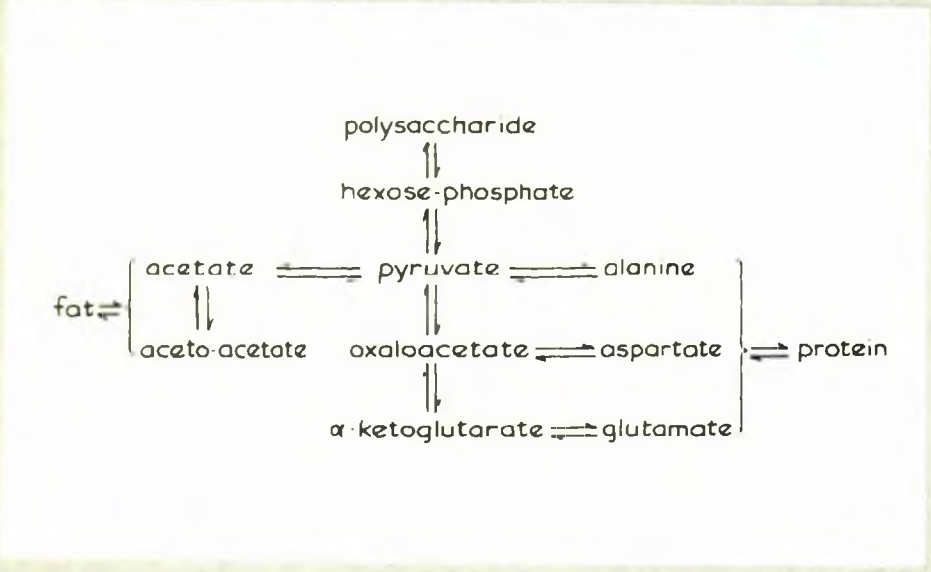

Fig. 5.10

The relationship between carbohydrate, protein and fat metabolism.

(after Work, T.S. and Work, E. (1948), The Basis of Chemotherapy, Oliver and Boyd Ltd., London.)

to the readily oxidisable pyruvic acid. Similarly, protection afforded by glutamate may be due to its deamination to α-ketoglutaric acid. The intermediates of fat and protein metabolism are so interdependent[9] that the protection afforded by intermediates of fat and protein metabolism may be due to interconvertibility. This relationship between carbohydrate, fat and protein metabolism is shown in Figure 5.10. It is, therefore, possible to speculate that hydrallazine is interfering with cellular metabolism - possibly in the tricarboxylic acid cycle - and that the more potent compounds are supplying energy requirements or replacing a missing metabolite.

The results obtained with anoxic tissue bear close, but not complete, resemblance to those obtained from experiments in which hydrallazine is the antagonist. Anoxic tissues lose their tone and are less sensitive to acetylcholine, but sensitivity is never completely lost. Moreover, the recoveries of the anoxic and hydrallazine-treated tissues are strikingly similar. Pyruvate, succinate, fumarate, and ketoglutarate and oxalosuccinate are as active with hydrallazine, and glucose-6-phosphate, fructose-6-phosphate, and 3-phosphoglycerate /

phosphoglycerate are also active. (±) alanine is very potent. The similarity between the two effects is obvious, and it is rather tempting to suggest that hydrallazine may alter or interfere with cell metabolism in a manner similar to oxygen lack.

Effects of cyanide, iodoacetate, azide and reserpine appear to be different. In the case of cyanide, very few intermediates gave any protection, while with the rest the recoveries were very prolonged and many times it was impossible to get recovery at all. The protection afforded by glutamate and (±) alanine against cyanide inhibition may be due to their deamination to α-ketoglutarate and pyruvate respectively. The effects of added metabolites to antagonise azide inhibition are inconsistent. In short, it appears that hydrallazine and reserpine may have different mechanisms of action. It would appear to be fairly reasonable to assume that hydrallazine may exert its effects by making the tissue anoxic. The fact that reserpine and the enzyme inhibitors bear some relationship during the recovery of the tissue, does not necessarily mean that reserpine behaves in a manner similar to these agents. It may be merely fortuitous.

Born /

Born and Bülbring[10] found that as the concentration of 2:4-dinitrophenol is increased the spontaneous tension of taenia coli decreases and the concentration of ATP in the muscle decreases in proportion. Moreover, when the muscle is stimulated with histamine, the tension increases but the increase is not maintained, although histamine is still present. Hydrallazine and reserpine appear to disturb some fundamental mechanism underlying muscle contraction. The effects of anoxia, of metabolic inhibitors and of substrate deficiency are supposed to be due to an interruption of the supply of energy to the muscle. Such an interference may mean a fall in the levels of labile high energy phosphate compounds - ATP and creatine phosphate - which are generally regarded as the immediate sources of energy for muscle contraction. Hydrallazine and reserpine (assuming that they interfere in cell metabolism) may be expected to lower the labile high energy phosphate compounds of different tissues. Any interference at the cellular level may also be reflected in the oxygen uptake of various tissues in the presence of these compounds. They may inhibit tissue respiration, as with cyanide, or they may stimulate it, as with 2:4 dinitrophenol.

In /

In the next chapter the effects of reserpine and hydrallazine are studied on respiration and on adenosine nucleotide levels of different tissues of the rat.

TABLE 5.3

Activity of compounds tested for antagonism to the actions of KCN and anoxia on horse carotid artery strips.

(Protection per cent ± S.E.)

Stimulant Drug	Cyanide		Anoxia	
	Acetylcholine		Acetylcholine	
		Percentage Protection		Percentage Protection
(a) Carbohydrate Metabolism				
Glucose - 1-phosphate	0	+10(±10.0)	0	0
Fructose- 1:6-diphosphate	0	0(± 4.1)	0	0
Glucose - 6-phosphate	0	0	++	+40(± 6.4)
Fructose- 6-phosphate	+	+30(±10.0)	++	+45(± 5.0)
3-phosphoglycerate	0	0	+	+25(±13.4)
Pyruvate	++	+68(± 9.69)	++	+74(± 8.6)
Succinate	+	+16(± 2.50)	++	+68(±13.7)
Fumarate	0	0	++	+54(±13.1)
Citrate	0	-70(±30.0)	0	-50(±40.0)
Cisaconitate	0	-50(± 5.0)	0	-80(±10.0)
Isocitrate	0	-50(± 4.0)	0	0
α-ketoglutarate	++	+70(±15.2)	+	+25(± 4.0)
Oxalo-acetate	0	-55(± 5.0)	0	0(±13.1)
Oxalo-succinate	+	+22(± 5.8)	++	+44(± 6.0)
(b) Fat Metabolism				
3-hydroxybutyrate	0	0	0	0
Propionate	+	+20(±11.2)	0	0(± 5.0)
(c) Protein Metabolism				
Glutamate	++	+50(±21.7)	+	+11(± 9.5)
(±)-alanine	++	+85(±17.4)	++	+76(±18.0)
(±)-leucine	0	-35(± 3.0)	0	+6(± 6.0)
(d) Other compounds				
Malate	0	-50(± 5.0)	0	+10(±10.0)

0 = no activity or antagonism
+ = some activity
++ = marked activity

TABLE 5. 1

Activity of compounds tested for antagonism to the actions of hydrallazine and reserpine on horse carotid artery strips.

(Protection per cent ± S.E.)

Stimulant Drug	Hydrallazine				Reserpine	
	Acetylcholine		Adrenaline		Acetylcholine	
		Percentage protection		Percentage protection		Percentage protection
Intermediates of:						
(a) Carbohydrate Metabolism						
Glucose - 1-phosphate	0	0	+	+28(±12.9)	)	
Fructose- 1:6-diphosphate	0	0(±10.0)	0	-15(±15.0)	)	
Glucose - 6-phosphate	0	0	++	+75(± 2.8)	) *	
Fructose- 6-phosphate	+	+24(± 7.1)	++	+43(± 9.5)	)	
3-Phosphoglycerate	+	+29(± 9.6)	++	+54(±11.3)	)	
Pyruvate	++	+78(± 6.7)	++	+72(±12.3)	+	+12
Succinate	++	+58(±11.1)	++	+42(± 6.0)	+	+35
Fumarate	++	+60(±10.1)	0	+10(± 6.5)	)	
Citrate	0	+5(± 5.8)	0	0	)	
Cisaconitate	0	-40(± 4.0)	++	+50(± 6.9)	)	
Isocitrate	0	0(± 4.1)	+	+18(± 9.2)	)	
α-ketoglutarate	++	+51(± 6.8)	++	+55(±13.2)	)	
Maleic acid	0	-50(± 5.0)	0	-100(±10.0)	) *	
Oxalo-acetate	+	+26(± 8.1)	++	+60(±11.5)	)	
Oxalo-succinate	+	+27(±15.0)	+	+19(±10.8)	)	
(b) Fat Metabolism					)	
3-hydroxybutyrate	+	+17(±10.0)	++	+57(±17.1)	)	
Propionate	++	+37(±12.0)	++	+58(±22.0)	)	
(c) Protein Metabolism					)	
Glutamate	++	+35(± 8.9)	++	+58(±17.2)	)	
(±)-alanine	++	+38(± 7.5)	++	+53(±10.4)	)	
(±)-leucine	0	0(±10.0)	0	0	)	
(d) Thiol compounds					)	
Glutathione	0	0	0	0	)	
(e) Other compounds					)	
Malate	+	+23(±12.7)	0	0	)	

0 - no activity or antagonism ++ - marked activity

+ - some activity * - experiments could not be done.

TABLE 5.2

ACTIVITY OF SOME INTERMEDIATES OF CARBOHYDRATE AND PROTEIN METABOLISM IN ANTAGONISING THE ACTIONS OF HYDRALLAZINE ON HORSE CAROTID ARTERY STRIPS.

Stimulant Drug	Noradrenaline	Hydrallazine	
		Histamine	5-hydroxytryptamine
Intermediates of :			
(a) Carbohydrate metabolism			
Pyruvate	++	++	++
Succinate	++	++	++
α-ketoglutarate	++	++	+
3-phosphoglycerate	+	+	+
Fructose-6-phosphate	++	++	++
(b) Protein metabolism			
Glutamate	+	+	+
(±)-alanine	0	0	0

0 = no activity or antagonism; + = some activity; ++ = marked activity.

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CHAPTER VI

(Tables will be found at the end of this chapter)

In Chapter VI the concentrations of drugs used in experiments on tissue respiration refer to the weight of the drug per ml. of physiological saline used. Q_{O_2} values are expressed as µl. of oxygen uptake per mg. of wet weight of the tissue.

The concentrations of drugs used in <u>in vivo</u> experiments refer to the weight of the drug per 100 g. body weight. Adenosine nucleotides are expressed in µ moles per g. of wet weight of the tissue.

CHAPTER VI

EFFECTS OF RESERPINE AND HYDRALLAZINE UPON TISSUE RESPIRATION AND ADENOSINE NUCLEOTIDE LEVELS

As discussed previously (Chapter 5, p. 137) reserpine and hydrallazine appear to have an effect upon the metabolic processes which supply energy for the contraction of muscle, an effect which may be reflected either in a decrease or increase in the oxygen uptake of different tissues. It is apparent that any interference in cell metabolism may alter the synthesis of energy rich phosphate compounds.

In this chapter are described attempts made to investigate the effects of hydrallazine and reserpine on the respiration of rat brain, kidney and liver. Hydrallazine alone is tested on rabbit aorta. The studies on tissue respiration have been supplemented by estimations of the adenosine nucleotide content of brain, heart, skeletal muscle and liver of the rat following *in vivo* treatment with reserpine and hydrallazine.

Methods.

For the determination of tissue respiration a

2.5 mg. per ml. solution of reserpine was used. This solution was diluted as required. The pH was 3.6. A control solution without reserpine was supplied by the manufacturers. A 20 mg. per ml. aqueous solution of hydrallazine hydrochloride was prepared and diluted as required before use with Krebs Ringer phosphate solution or Krebs Medium IIA[2] (Appendix 1).

Respiration in Isolated Rat Tissue.

Rats of either sex weighing from 150 to 200 g. were used. To obtain preparations of brain, the animals were killed, bled out and the brain rapidly removed and weighed on a torsion balance. A 10 per cent suspension was prepared by grinding the brain in a glass pestle and mortar with ice-cold Krebs Ringer phosphate solution (Appendix 1). The suspension was filtered through muslin; 1 ml. of this extract - which is equivalent to 100 mg. of brain tissue - was used in each flask. Liver and kidney were removed from animals, washed with Krebs Ringer Phosphate to remove excess blood, and sliced on a McIlwain tissue slicer to give sections of 0.364 mm. thickness. The fluid from the suspension containing the /

the slices was drained off and the slices were gently rolled on a filter paper with a pair of blunt forceps. A sufficient number of liver or kidney slices to weigh 100 mg. wet wt. were used in each flask.

Respiration in Isolated Rabbit Thoracic Aorta.

Rabbits weighing between 2.0 to 2.5 kg. were killed by a blow on the head and bled out. About 5 cm. of thoracic aorta was rapidly removed and washed with ice-cold Krebs Ringer phosphate solution or Krebs Medium IIA[2] (Appendix 1). The excised aorta was opened by a longitudinal incision and sliced on the tissue slicer to give pieces of the same thickness as liver and kidney. A sufficient number of slices to weigh 100 mg. wet wt. were used in each flask.

Experiments on oxygen consumption were carried out by the Warburg "Direct" method at 37 ± 0.05°C. using an atmosphere of air. It was found that initial oxygenation of the flasks was not necessary since the Q_{O_2} values of the oxygenated flasks did not appreciably differ from the ones in air. Carbon dioxide produced during /

during the aerobic metabolism was absorbed by 0.2 ml. of a 20 per cent potassium hydroxide solution in the centre well of the Warburg flask. A small piece of fluted, alkali resistant Whatman No.50 filter paper was seated in the centre well after the addition of the potassium hydroxide solution. This served to increase the surface area of the solution, thus ensuring an efficient removal of carbon dioxide formed within the flask. The main chamber of each Warburg flask contained 2.4 ml. of Krebs Ringer phosphate or Krebs Medium IIA, together with the appropriate tissue preparation. The side-arm of the flask contained 0.4 ml. of drug solution or the control. After equilibration for 10 minutes, the contents of the side-arm were tipped into the main chamber and readings taken at 10 minute intervals for 1 hour. In some experiments the contents of the side-arm were tipped in after one hour of initial control period, and the readings continued for one more hour. This made it possible to use the same flask as its own control, thus dispensing with many variables.

The total volume of the reaction mixture and approximately 100 mg. of tissue was taken to be 3 ml. The /

The flask constants were, therefore, calculated using this value. The manometer fluid used was that described by Krebs[3] which had excellent flowing properties.

Two thermobarometers, each containing 3 ml. of water, were incorporated, one on either side of the apparatus.

Estimations of Adenosine Nucleotides.

Attempts were made to estimate adenosine nucleotides by two methods:

(a) Chromatographic methods

(b) Enzymatic methods.

(a) Chromatographic methods.

In view of the applicability of both paper and column chromatography to the resolution of the components of biochemical systems, it is not surprising that these techniques have been applied to the separation of nucleotides. Cohn and Carter[4] have made possible the separation of dibasic acids such as adenosine triphosphate (ATP), adenosine diphosphate (ADP) and phosphoglyceric acid on ion exchange resin columns /

columns by varying the pH or by varying the ionic strength of the eluant. Monophosphate derivatives can be separated by the addition of borate. Volkyn, Khym and Cohn[5], on the other hand, have used ammonium chloride solutions as eluants.

The ion exchange resin used by Cohn and Carter was Dowex I, on which the nucleotides were absorbed at a pH of 8, while the anion concentration was kept lower than 0.01N and eluted thus -

(i) adenosine - 0.01 M NH_4Cl in 0.1 M NH_4OH.

(ii) adenine - 0.01 M NH_4Cl in water.

(iii) adenosine monophosphate (AMP) - 0.003 M HCl.

(iv) adenosine diphosphate (ADP) + inorganic orthophosphate - 0.02 M NaCl in 0.01 M HCl.

(v) adenosine triphosphate (ATP) - 0.2 M NaCl in 0.01 M HCl.

It can be seen that the ADP fraction is contaminated with orthophosphate and that there is a contaminating phosphate compound - in concentrations of 2 to 4 per cent and having labile phosphate groups - in the ATP fraction. Koshland, Budenstein and Kowalsky[6] have modified this method in so far as they elute ADP with 0.01 M HCl /

0.01 M HCl, and ATP with 0.1 M HCl.

Deutsch and Nilsson[7] have used Dowex 2, in the chloride form, as an exchange resin in attempts to separate the adenosine nucleotides from the inosine ones. AMP is eluted with 0.003 M HCl which is followed closely by inorganic orthophosphate. The inosine monophosphate (IMP), which trails ADP, is eluted with 0.02 M NaCl in 0.01 M HCl; the pyrophosphate and inosine diphosphate (IDP) are eluted with 0.05 M NaCl in 0.01 M HCl and the ATP with 0.2 M NaCl in 0.01 M HCl. Obviously these fractions are not homogeneous and a certain amount of cross-contamination occurs. Between AMP and orthophosphate it is of the order of 2 to 3 per cent, between IMP and ADP about 4 per cent, and between IDP and ATP 2.9 per cent. Bergkvist and Deutsch[8], in an attempt to separate the mono, di- and triphosphates of adenosine, guanosine, inosine and uridine, used a Dowex 1 formate exchange column. The nucleotides were eluted with formic acid-formate solutions containing increasing amounts of formate. Once more the separations were incomplete; ATP was contaminated with inosine triphosphate (ITP), and uridine monophosphate (UMP) with orthophosphate.

A /

A similar method has been reported by Schmitz[9], the main difference being the eluant, which contains increasing concentrations of formic acid (0 to 4N) followed by 4N formic acid + 4N ammonium formate.

All the methods which have been previously discussed suffe from various shortcomings. The most common one is that large volumes of eluant are required and therefore either the nucleotides have to be present initially in relatively large concentrations, or some means of concentrating them has to be employed. Another disadvantage is the fact that ion exchange chromatography takes too long to run to be of much use as a routine analytical tool where large batches of samples have to be analysed. Moreover, it can be seen from the previous discussion that often the fractions are contaminated to a slight extent by other compounds which may prove a source of serious error. In addition, hydrolysis of compounds such as ATP and ADP may occur on the column. For example, if hexose phosphates, inorganic phosphate, ATP and ADP are separated by the method of Volkin et al.[5] then, in the time taken for hexose phosphates to be eluted from the column, the ATP and ADP still remaining suffer a partial hydrolysis.

(b) /

(b) Enzymatic methods.

While adequate separations of nucleotides from associated metabolites, and even from other nucleotides, can be achieved by the previously described chromatographic method, a reliable estimation of the individual nucleotides can only be made by the enzymatic method introduced some ten years ago.

The finding of Schmidt and Engel[10], that the purine content of tissues could be estimated by using specific deaminases and measuring the amount of ammonia released from the purines, was developed by Parnes et al.[11] who estimated ATP by using phosphatase to convert ATP to AMP which was then deaminated. These observations were forgotten until Kalckar[12] described the determination of AMP by the highly sensitive and specific adenylic acid deaminase and measuring the drop in extinction at 265 mμ spectrophotometrically. This was further elaborated by Kalckar[13] in 1945 to measure adenyl pyrophosphates by coupling the deaminase with a potato apyrase.

In 1947 Kalckar[14] published a series of papers describing procedures for estimating hydroxy purines and /

and adenine compounds. Nucleotides are characterised by intense ultraviolet absorption and because many different purine derivatives have the same absorption spectra, e.g. adenine, adenosine, adenylic acid and adenyl pyrophosphates, direct measurement of the absorption completely lacks specificity; but if changes in the absorption as a result of specific enzymes are studied then the method - differential spectrophotometry - is a highly sensitive and specific analytical tool. The importance of the method can be appreciated when its application to the estimation of various adenosine compounds is considered. When AMP is deaminated IMP is formed and the absorption maximum shifts to 248 mμ: at 265 mμ, the extinction of IMP is only 40 per cent of that of AMP. Therefore if we follow the deamination of AMP as represented by the drop in extinction of 265 mμ (Δ 265), we have a measure of AMP concentration since the drop is directly proportional to the AMP concentration. Similarly, if it is wished to estimate ADP, then by using myokinase which dismutates ADP thus -

$$2\ \text{ADP} \rightleftharpoons \text{ATP} + \text{AMP}$$

and /

and adding deaminase, we get a further drop in extinction which is proportional to half the concentration of ADP. ATP can also be estimated by adding apyrase which splits ATP to AMP. By adding these enzymes in the order - deaminase, myokinase and apyrase - to a mixture of nucleotides or to a tissue extract, we can estimate each separately by the successive drops in extinction at 265 mμ. Some of the ATP estimated will have its origin in the dismutated ADP but this will be equivalent to half the ADP concentration and so a correction can be made.

This elegant method has been criticised by Munch-Peterson[15] as being too cumbersome to be of use as routine analytical procedure because of the time taken by the individual reactions to go to completion. However, the method has been employed either *per se* or in a modified form. Albaum and Lipshitz[16] have extended Kalckar's method by measuring the rate of change of extinction rather than the magnitude of the drop, and by using hexokinase instead of apyrase to measure the ATP concentration. This method, although quicker, necessitates measuring the rate of change in standard solutions of known purity, and suffers from the /

the disadvantage that some means must be devised for measuring the purity of these solutions.

Column chromatography was found to be too lengthy a technique in view of the number of estimations which had to be carried out, and the recovery of the added nucleotides was generally 10 per cent. lower. No attempts were, therefore, made to use tissue extracts with this technique and the preliminary exploratory work was done with commercial preparations of ATP, ADP and AMP.

The method that has been used by the author is the enzymatic method of Kalckar as a routine technique to assay the tissue nucleotide levels. It was felt that a much more reliable approach lay in enzymatic methods.

Preparation of Enzymes.

(1) Deaminase:

The method of preparation was that described by Kalckar[14]. Rabbit muscle was minced and extracted with ice-cold distilled water. On standing overnight, the lactic acid formed from glycogen acidified the mixture to pH 6.0, and thus precipitated the deaminase /

deaminase. The precipitate was spun off and extracted twice with 1.0 M ammonium acetate at pH 8.0 with shaking. The coarse precipitate was spun down and the turbid supernatant subjected to ammoniacal ammonium sulphate fractionation. The fraction which was precipitated between 0.3 and 0.5 saturation, was centrifuged off, dialysed and stored at 0°C.

(2) Myokinase:

The method of preparation was that described by Colowick and Kalckar[17]. Rabbit muscle was ground with sand and extracted with ice-cold distilled water. The extract was heated to 90°C, cooled and the pH adjusted to 6.5. After removal of the precipitate, the filtrate was half-saturated with ammonium sulphate and the enzyme precipitated by full saturation with this salt. It was purified by dialysing against dilute ammonium sulphate, and its activity assayed against a stock solution of ADP.

(3) Apyrase:

This enzyme was prepared from potatoes according to the procedure adopted by Krishnan[18]. The method is /

Scheme for the isolation of Apyrase from Potatoes

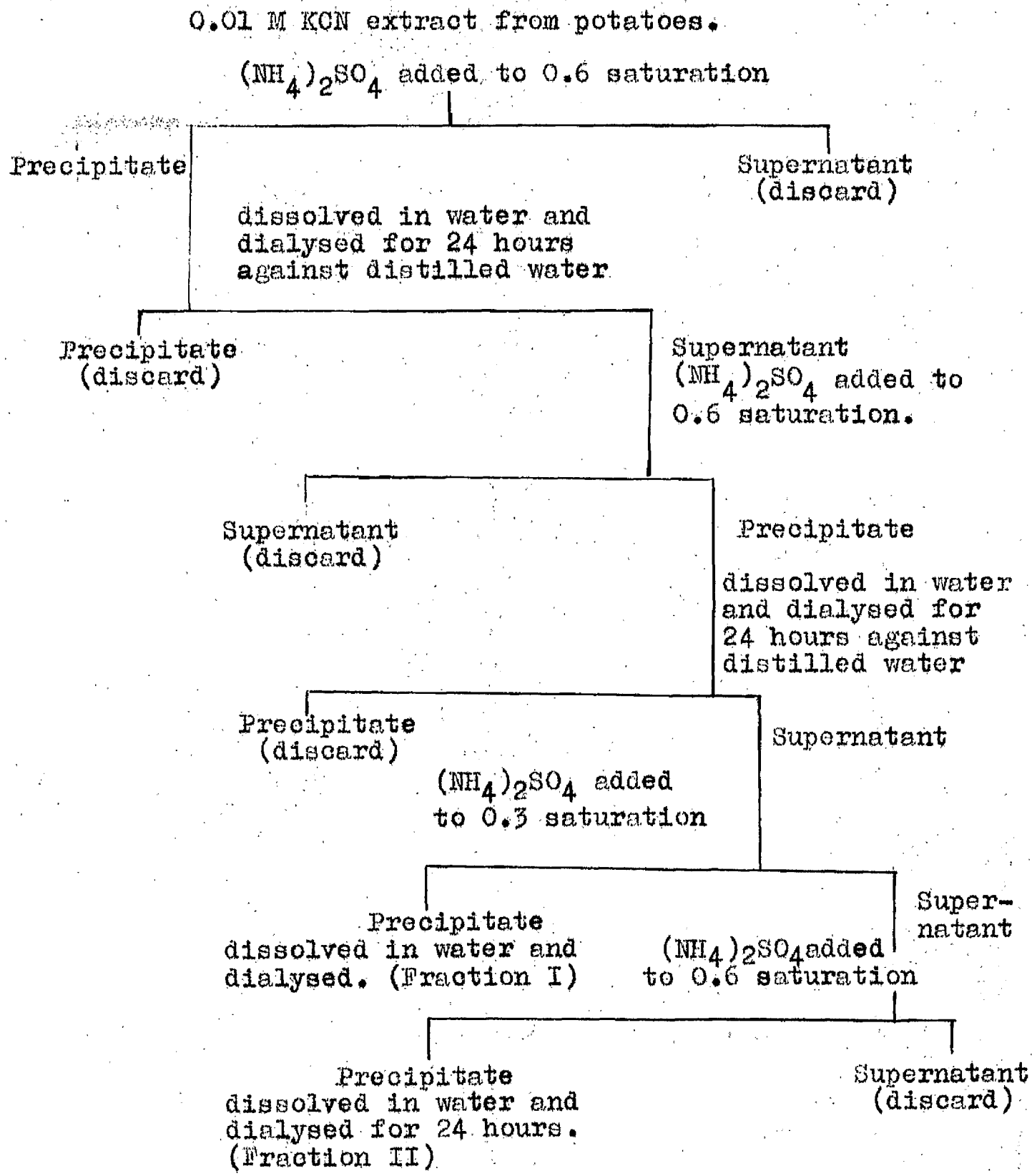

Fig. 6.1

is shown in Figure 6.1. Most of the activity was found in Fraction II which was used in subsequent assays. It was found that a 1 :25 dilution of the enzyme was adequate.

Animals.

Female albino rats weighing between 180 and 200 g. were used for these estimations. Rats were given 10 g. of powdered diet No.86 (Laboratory Animals Bureau) every day for at least 5 days before killing. The powder was made into a smooth paste with water to avoid spilling. Water was given ad lib. This regimen was strictly adhered to since it was shown by Munroe and Goodlad[19] that the ATP level of the liver was very sensitive to variations in dietary energy intake. Immediately after the last feed, 0.5 mg. (per 100 g. body weight) of reserpine was given by intraperitoneal injection or 5.0 mg. (per 100 g. bodyweight) of hydrallazine by subcutaneous injection. The controls were given equivalent volumes of control solution or physiological saline respectively. Only those rats which had completely consumed /

consumed their food were used for the experiments. Reserpine treated animals and their controls were killed between 11 and 12 hours after injection; hydrallazine treated animals and their controls were killed 3 to 4 hours after injection.

To minimise struggling, since such movements cause the breakdown of energy rich phosphate compounds, the rats were injected with 200 mg. per kg. of mephenesin 5 to 10 minutes before killing. This usually gave complete muscular relaxation. Liver, heart and gastrocnemius muscle were rapidly removed from the same animals but brains were obtained from separate animals. This was necessary because of the length of time required for dissection. Since the doses of the drugs used were sublethal, estimations were always made after killing the animals. It was found, however, that 5 mg. of hydrallazine per 100 g. was quite toxic and some of the animals were in poor condition, while a few had convulsions and died. Animals which died due to drug administration were not used for the experiments.

The animals were killed by cutting the throat.

The /

The tissues were removed, washed with ice-cold water and the excess water removed by lightly touching with tissue paper. The tissues were then weighed on a torsion balance and homogenised in 1.5 per cent perchloric acid solution. Extraction procedures for individual tissues are described below:

(a) Skeletal Muscle:

Gastrocnemius muscle was homogenised by using a glass pestle and mortar at 0°C. For each g. of muscle 5 ml. of 1.5 per cent perchloric acid solution was used. The contents of the mortar were transferred to a glass centrifuge tube and the mortar and pestle washed with 2 x 5 ml. portions of 1.5 per cent perchloric acid solution. The washings were added to the tubes and the contents centrifuged for 10 minutes at 2000 r.p.m. at 0°C. The supernatant was decanted and the residue re-extracted with 5 ml. of 1.5 per cent perchloric acid solution, centrifuged, the supernatant added to the previous extract and the total volume noted. 10 ml. extract was pipetted into a beaker containing 10 ml. of succinate buffer at pH 6.1. The contents of the beaker were adjusted to pH 6.1 by the addition of 5N NaOH using the glass electrode. The volume /

volume was adjusted to 25 ml. with water giving an approximately 1 in 50 tissue extract.

(b) Liver:

About 2 g. of liver was excised and washed with ice-cold distilled water to remove excess of blood, transferred to 20 ml. of ice-cold 1.5 per cent perchloric acid solution in a "Nalco" breaker vessel and homogenised. The homogenate was transferred to a cooled centrifuge tube and the breaker vessel and the homogeniser blades rinsed with 2 x 5 ml. aliquots of ice-cold 1.5 per cent perchloric acid; the washings were then added to the homogenate. The contents of the tube were centrifuged at 2000 r.p.m. at 0°C. for 10 minutes, and the supernatant transferred. The residue was re-extracted with 5 ml. of 1.5 per cent ice-cold perchloric acid, centrifuged, and the supernatant added to the previous extract. The total volume was noted and a 10 ml. aliquot transferred to a beaker containing 10 ml. of succinate buffer at pH 6.1. The pH was adjusted to 6.1 with 5N NaOH, and the final volume adjusted to 50 ml. to give an approximately 1 in 75 extract.

(c) /

(c) Heart:

Hearts were removed, washed with ice-cold water and extracted in a manner similar to liver to give an approximately 1 in 75 tissue extract.

(d) Brain:

Brain was extracted in a manner similar to that adopted for liver and heart to give an approximately 1 in 50 extract.

All tissue extracts were stored in the frozen state (- 15°C.) until assayed.

Summary of Results.

Reserpine (10 and 50 µg. per ml.) had nc significant effects on the respiration of rat brain homogenates, and liver and kidney slices. Reserpine (100 µg. per ml.) caused significant depression of respiration of brain homogenates but had no significant depressant effects on liver and kidney respiration. But higher doses (330 µg. per ml.) significantly depressed respiration of all the tissues. Hydrallazine (50 or 100 µg. per ml.) depressed respiration of the brain, liver /

liver, kidney and arterial smooth muscle.

After sublethal doses of reserpine, depletion of energy rich phosphate compounds occurred in brain and liver but not in skeletal muscle and heart. Hydrallazine also depleted brain of energy rich phosphate compounds but did not affect liver, heart or skeletal muscle.

RESULTS.

Tissue Respiration:

The results are shown in tables 6.1 and 6.2.

Reserpine (10 or 50 µg. per ml.) had no significant effects on the respiration of brain, liver and kidney. Only 100 µg. per ml. showed significant depression ($p < 0.01$) of brain homogenates but in similar doses did not affect liver and kidney respiration significantly. The control solution had very little effect on brain and kidney respiration, but significantly stimulated ($p < 0.01$) liver respiration. Hydrallazine (50 or 100 µg. per ml.) depressed the oxygen uptake of brain homogenates ($p < 0.01$) and liver and kidney slices ($p < 0.01$) and arterial smooth muscle ($p < 0.01$).

Reserpine /

Reserpine (330 µg. per ml.) depressed the oxygen uptake of brain homogenates ($p < 0.01$) and of liver and kidney slices ($p < 0.01$).

Experiments were carried out using Krebs IIA Medium which contained pyruvate, glutamate, fumarate and dextrose to confirm the observations discussed in the previous chapter. It was shown that the inhibiting effects of hydrallazine on arterial smooth muscle could be overcome to a certain extent by some of the intermediates of the tricarboxylic acid cycle. Hydrallazine inhibited respiration of arterial smooth muscle using Krebs Ringer phosphate solution but it failed or slightly depressed the respiration in the presence of Krebs IIA Medium.

Adenosine Nucleotides:

Tables 6.3 and 6.4 summarise the effects of reserpine and hydrallazine upon the levels of adenosine nucleotides in rat skeletal muscle, heart, brain and liver. Mephenesin administration does not affect tissue ATP levels[20]. Mephenesin induced relaxation was partially antagonised by hydrallazine, and rats treated with hydrallazine required one and a half to twice the /

the usual relaxant dose of mephenesin, while the time of onset of relaxation was 20 to 25 min. as against the more usual 5 to 10 min. The corresponding controls were treated similarly.

Neither reserpine nor hydrallazine had a significant effect upon the adenosine nucleotide content of gastrocnemius muscle or heart. In the brain , reserpine caused a significant decrease in ATP ($p < 0.01$) and a corresponding increase in ADP ($p < 0.05$). The ATP/ADP ratio which may be taken as a measure of the synthetic potential of the cell is also significantly reduced ($p < 0.01$). In liver, reserpine caused a significant lowering in ATP content ($p < 0.01$) and there was a corresponding increase in ADP ($p < 0.05$); the ATP/ADP ratio and total nucleotides were also significantly reduced ($p < 0.05$). Hydrallazine had no significant effect upon liver ATP: on the other hand, ADP was significantly raised ($p < 0.05$), and ATP/ADP ratio was significantly lowered ($p < 0.05$). In brain, hydrallazine reduced the ATP level significantly ($p < 0.01$), ADP was significantly increased ($p < 0.01$) and the ATP/ADP ratio was also significantly reduced ($p < 0.05$).

Discussion.

Discussion

Under normal conditions the oxidative metabolism of living cells and of certain isolated systems leads to the synthesis of compounds containing high energy phosphate bonds. These compounds are essential for growth, muscle contraction and nerve conduction etc. Uncoupling agents can disturb metabolism by depressing the formation of high energy phosphate bonds without depressing or even slightly stimulating the oxygen consumption of the system.

Reserpine depresses the P/O ratio[21] in rat brain mitochondria, a property it shares with other uncoupling agents. Hydrallazine depresses tissue respiration at all concentrations used, thereby indicating a general depressant effect upon the oxidative metabolism of living cells. Hydrallazine, therefore, appears to act differently from reserpine.

Douglass et al.[22] have shown that hydrallazine inhibits the acetylation of sulphanilamide and glucosamine in pigeon liver extracts, and have concluded that this inhibition is of a competitive nature and appears to be the result of the drug being preferentially

acetylated /

acetylated by acetyl-coenzyme A. The acetylation of hydrallazine by acetyl-coenzyme A may ultimately mean interference in the functioning of the tricarboxylic acid cycle. The author's findings (Chapter V), that some intermediates of the tricarboxylic acid cycle antagonise the effects of hydrallazine, support this view. The observation in experiments on the respiration of arterial smooth muscle, that the inhibition of oxidation by hydrallazine can be overcome to a certain extent by supplying the tissue with pyruvate, glutamate, fumarate and dextrose, also points in the same direction.

The effects of reserpine on the brain and liver adenosine nuclectides indicate interference with oxidative phosphorylation. No effect could be demonstrated in skeletal muscle or heart. It is possible that in these tissues phosphocreatine levels are affected. The accepted role of phosphocreatine is to maintain the level of ATP via the Lohmann reaction. Therefore, in the absence of the major synthetic source of energy-rich phosphate bonds, the loss of phosphocreatine will be the first indication of a disturbed phosphate metabolism. Hydrallazine inhibits /

inhibits oxidative phosphorylation in brain but not in skeletal muscle, heart or liver.

Many substances which uncouple phosphorylation from oxidation have been employed in experimental pharmacology and therapeutics. A number of anti-psychotic drugs and central nervous system depressants inhibit oxidative phosphorylation e.g. chlorpromazine and barbiturates. Whether the effectiveness of these substances or drugs is dependent upon their effectiveness as uncoupling agents is not yet clear. The fact that a substance uncouples phosphorylation from oxidation does not mean that it is devoid of other actions. The relative importance of uncoupling as a mechanism of drug action differs with different drugs. Brody(23) concludes that uncoupling is of major importance in the action of certain drugs and is at present the most attractive hypothesis which explains, at least in part, the mechanism of action of barbiturates, antibiotics such as gramicidin and tetracyclines, salicylates and thyroid hormones.

Reserpine has been shown to cause depletion of noradrenaline and 5-hydroxytryptamine from brain and other /

other tissues[24,25]. Carlsson and Hillarp[26] have shown that the depletion of the adrenal medulla of catechol amines by morphine or insulin is accompanied by a parallel depletion of ATP. Depletion of rat brain ATP may be linked with depletion of brain catechol amines. In the case of hydrallazine, in vitro studies have indicated a mainly peripheral site of action and have led the author to conclude that this drug is a general depressant of cell metabolism. The effect of this compound on brain adenosine nucleotide level was therefore not unexpected but the lack of effect on skeletal muscle, heart and liver cannot yet be explained.

TABLE 6.1

EFFECTS OF RESERPINE AND HYDRALLAZINE ON THE OXYGEN UPTAKE OF BRAIN HOMOGENATES, LIVER AND KIDNEY SLICES

(oxygen uptake in µl ± S.E./mg. wet tissue)

BRAIN	No. of observations	Incubation Time (Min.) 20'	40'	60'
Reserpine (10 µg/ml)	(12)	0.158(±0.0089)	0.320(±0.0099)	0.457(±0.01)
Control	(12)	0.163(±0.005)	0.316(±0.005)	0.46(±0.0075)
Reserpine (50 µg/ml)	(27)	0.188(±0.006)	0.328(±0.0095)	0.476(±0.0107)
Control	(24)	0.185(±0.0063)	0.348(±0.0102)	0.498(±0.013)
Reserpine (100 µg/ml)	(13)	0.162(±0.0119)	0.292(±0.012)	0.397(±0.07)
Control	(13)	0.166(±0.008)	0.318(±0.0097)	0.477(±0.007)
Reserpine (330 µg/ml)	(9)	0.156(±0.0085)	0.188(±0.0106)	0.232(±0.010)
Control	(9)	0.151(±0.005)	0.282(±0.011)	0.386(±0.012)
Krebs Ringer Phosphate	(30)	0.179(±0.008)	0.362(±0.013)	0.514(±0.016)
Hydrallazine (50 µg/ml)	(11)	0.122(±0.011)	0.209(±0.015)	0.268(±0.018)
" (100 µg/ml)	(8)	0.154(±0.025)	0.278(±0.017)	0.323(±0.010)

/continued...

TABLE 6.1 (continued)

LIVER	No. of observations	Incubation Time (Min.)		
		20'	40'	60'
Reserpine (10 µg/ml)	(8)	0.347(±0.050)	0.550(±0.032)	0.764(±0.033)
Control	(7)	0.294(±0.0407)	0.516(±0.029)	0.710(±0.035)
Reserpine (50 µg/ml)	(18)	0.434(±0.0105)	0.7947(±0.0148)	1.10(±0.0146)
Control	(12)	0.404(±0.0081)	0.7609(±0.0151)	1.06(±0.019)
Reserpine (100 µg/ml)	(10)	0.4938(±0.0116)	0.898(±0.025)	1.222(±0.0124)
Control	(10)	0.5200(±0.0119)	0.935(±0.020)	1.316(±0.031)
Reserpine (330 µg/ml)	(8)	0.3935(±0.0031)	0.768(±0.0106)	1.152(±0.001)
Control	(9)	0.4659(±0.011)	0.934(±0.009)	1.378(±0.0086)
Krebs Ringer Phosphate	(41)	0.201(±0.007)	0.388(±0.012)	0.658(±0.013)
Hydrallazine (50 µg/ml)	(14)	0.151(±0.014)	0.263(±0.022)	0.478(±0.02)
" (100 µg/ml)	(11)	0.111(±0.003)	0.216(±0.014)	0.311(±0.016)

/continued...

TABLE 6.1 (continued)

KIDNEY	No. of observations	Incubation Time (Min.) 20'	40'	60'
Reserpine (10 µg/ml)	(12)	0.657(±0.016)	1.276(±0.021)	1.913(±0.0245)
Control	(12)	0.6212(±0.0181)	1.219(±0.027)	1.817(±0.036)
Reserpine (50 µg/ml)	(9)	0.6627(±0.036)	1.271(±0.053)	1.90(±0.089)
Control	(10)	0.608(±0.026)	1.23(±0.0359)	1.861(±0.0616)
Reserpine (100 µg/ml)	(12)	0.639(±0.0275)	1.24(±0.036)	1.837(±0.047)
Control	(11)	0.648(±0.0176)	1.272(±0.0297)	1.883(±0.0334)
Reserpine (330 µg/ml)	(9)	0.484(±0.0087)	0.821(±0.041)	1.389(±0.0356)
Control	(9)	0.536(±0.013)	1.05(±0.021)	1.589(±0.0303)
Krebs Ringer Phosphate	(12)	0.63(±0.016)	1.18(±0.022)	1.75(±0.04)
Hydrallazine (50 µg/ml)	(6)	0.389(±0.035)	0.823(±0.040)	1.20(±0.04)
" (100 µg/ml)	(6)	0.541(±0.045)	1.02(±0.045)	1.46(±0.05)

TABLE 6.2

EFFECTS OF HYDRALLAZINE ON THE OXYGEN UPTAKE OF ARTERIAL SMOOTH MUSCLE

(Oxygen uptake in µl ± S.E./mg. wet tissue)

	No.	20'	40'	60'
Krebs Ringer Phosphate	(11)	0.12(0.013)	0.243(0.026)	0.373(0.019)
Hydrallazine (50 µg/ml)	(6)	0.059(0.008)	0.125(0.013)	0.217(0.014)
Hydrallazine (100 µg/ml)	(8)	0.078(0.0027)	0.117(0.020)	0.170(0.032)
Krebs Medium 11	(11)	0.173(0.019)	0.318(0.020)	0.414(0.028)
Hydrallazine (50 µg/ml)	(6)	0.185(0.018)	0.335(0.028)	0.451(0.041)
Hydrallazine (100 µg/ml)	(5)	0.164(0.015)	0.230(0.015)	0.292(0.016)

TABLE 6.3

EFFECTS OF RESERPINE AND HYDRALLAZINE ON ADENOSINE NUCLEOTIDES OF RAT SKELETAL MUSCLE AND HEART

Figures in parentheses refer to the number of rats used.

All values are expressed in μ moles/g. wet wt. ± S.E.

Muscle

Group No.	Dosage mg./100g.	ATP	ADP	AMP	ATP + ADP + AMP
I(4)	Reserpine, 0.5	7.21(±0.17)	0.35(±0.19)	0.14(±0.015)	7.52(±0.36)
II(4)	Hydrallazine, 5.0	6.88(±0.33)	0.17(±0.06)	0.05(±0.02)	7.09(±0.36)
III(8)	Controls	7.57(±0.31)	0.31(±0.08)	0.12(±0.05)	7.97(±0.35)

Heart

I(4)	Reserpine, 0.5	3.27(±0.35)	0.78(±0.15)	0.60(±0.12)	4.65(±0.32)
II(4)	Hydrallazine, 5.0	4.21(±0.35)	0.79(±0.15)	0.70(±0.17)	5.71(±0.17)
III(8)	Controls	3.67(±0.20)	1.18(±0.17)	0.94(±0.11)	5.79(±0.23)

TABLE 6.4

EFFECTS OF RESERPINE AND HYDRALLAZINE ON ADENOSINE NUCLEOTIDES OF RAT BRAIN AND LIVER

Figures in parentheses refer to the number of rats used.
All values are expressed in μ moles/g.wet wt. ± S.E.

Liver

Group No.	Dosage mg./100g.	ATP	ADP	AMP	ATP + ADP + AMP	ATP/ADP
1(4)	Reserpine, 0.5	0.49(±0.05)	1.16(±0.07)	1.18(±0.08)	2.82(±0.19)	0.42(±0.03)
11(4)	Hydrallazine, 5.0	1.15(±0.15)	1.47(±0.19)	1.33(±0.15)	3.95(±0.18)	0.77(±0.07)
111(8)	Controls	0.91(±0.03)	0.94(±0.06)	1.48(±0.09)	3.33(±0.07)	1.00(±0.061)
			Brain			
1(6)	Reserpine, 0.5	0.68(±0.06)	0.50(±0.08)	0.87(±0.05)	2.02(±0.10)	1.40(±0.30)
11(5)	Hydrallazine, 5.0	0.52(±0.09)	0.55(±0.09)	0.89(±0.09)	1.96(±0.07)	1.08(±0.28)
111(9)	Controls	1.10(±0.06)	0.26(±0.03)	0.90(±0.07)	2.30(±0.08)	4.74(±0.88)

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CHAPTER VII

(Table will be found at the end of this chapter)

CHAPTER VII

EFFECTS OF RESERPINE ON ADENOSINE NUCLEOTIDE LEVELS OF THE RAT SUPRARENAL MEDULLA

In the previous chapter it has been shown that reserpine interferes with oxidative phosphorylation in rat brain and liver *in vivo* but has little effect on oxidative phosphorylation in skeletal muscle and heart.

A number of reports[1,2] have shown that reserpine depletes the brain and adrenal medulla of catechol amines. Since adrenal medulla contains a high concentration of ATP[3,4], it seemed of interest to investigate whether reserpine had an effect on the adenosine nucleotides of this gland.

Methods:

Experiments were carried out using groups of 9 female rats, each rat weighing about 200 g. The control groups were injected with an appropriate volume of the vehicle solution, while the treated groups were given 0.5 mg. per 100 g. body weight of reserpine by intraperitoneal injection. Food was withheld and the animals were killed 16 hours after the injection. The adrenal /

adrenal glands were dissected out, chilled and freed from adhering tissue. The adrenal medullae from all of the 18 glands in both groups were dissected out, freed as much as possible from cortical tissue, weighed and pooled. Care was taken to keep the glands ice-cold throughout the preparation. The pooled medullae were transferred to a centrifuge tube containing 1 ml. of 1.5 per cent perchloric acid. A sandpaper-roughened glass rod and a little silver sand facilitated crushing. The crushed samples were centrifuged at 2000 r.p.m. for 10 minutes at 0° and the supernatant transferred. The residue was extracted twice with 1 ml. of 1.5 per cent perchloric acid. An equal amount of succinate buffer (pH 6.1) was added to the extract, and the pH of the extract was adjusted to 6.1 using a glass electrode. The final volume was adjusted to 10 ml. with succinate buffer.

Total catechol amines were estimated on the blood-pressure of the spinal cat and calculated in nor-adrenaline equivalents. Adenosine nucleotides were estimated by the specific enzymatic method of Kalckar(5).

Summary. /

Summary:

Reserpine (0.5 mg. per 100 g. body weight) depletes the adrenal medullae of catechol amines (80 per cent) and ATP (66 per cent). Since very little ADP or AMP is found in the medullae of the treated group, it is concluded that reserpine acts differently in the brain and in the medulla.

Results:

The results are presented in Table 7.1. Only four groups of rats were used; two served as controls and two were treated with reserpine. All results are expressed in terms of g. wet tissue.

It can be seen from the column showing weight of pooled medullae that reserpine-treated glands weighed about 33 per cent more than the control ones. It seems possible that the long-lasting stimulation caused an increase in the weight of the adrenal medulla.

The reserpine treatment caused a marked drop in catechol amine content of the adrenal medullae. Reserpine caused, on an average, as much as an 80 per cent /

cent depletion of the catechol amines. The results corroborate the findings of a number of workers[1,2]. Similarly, total nucleotide and ATP levels in treated animals fell by about 66 per cent from the controls. There was not much change, however, in the ADP and AMP levels of the control and treated groups. Hence, the differences in the total nucleotide levels between the two groups were merely due to differences in ATP levels.

Discussion:

It was shown in the previous chapter that reserpine inhibits oxidative phosphorylation in brain *in vivo*. It was also suggested that this ATP depletion of the brain could be linked with depletion of brain catechol amines.

Rat adrenal medullae contain a very large amount of ATP - 10.1 and 9.25 μ moles per g. of the wet tissue. Hillarp et al.[4] have shown that cow adrenal medullae contain ATP in even larger amounts than striated muscle. The author's observations support these findings. This surprisingly high ATP content suggests that ATP does not participate only /

only in the synthetic functions of the medullary cell, but perhaps is involved in the storage and release mechanism of the sympathomimetic amines which are stored in large amounts in specific cytoplasmic granules[6,7].

Since reserpine depletes the medullae of catechol amines and ATP in roughly the same proportions, ATP must have a role either in the storage of catechol amines, or in the mechanism of their release. Similar observations were made by Hillarp and Carlsson[8] who showed that the catechol amines and ATP disappeared in the same proportions during stimulation of the adrenal medullae of cats and rats either by insulin or morphine. They also found that the granules had a high adenosine triphosphatase activity from which the ATP of the unimpaired granules was protected in an unknown way, although ATP added to the suspension medium was readily attacked by the enzyme.

It is interesting to note that as a result of reserpine treatment, ATP levels are lowered but there is no parallel increase in ADP or AMP as might be expected./

expected. It is therefore obvious that the ATP molecule disappears as a whole along with the catechol amines. There appears to be a fundamental difference between the effect of reserpine on the adrenal medulla and on the brain, since in the former depletion of ATP is not accompanied by a corresponding rise in ADP and AMP but in the brain the fall in ATP concentration is accompanied by a corresponding and expected rise in ADP. It may be possible that the functions of ATP may differ in different tissues since in the striated muscle the chief function of ATP is in contraction, while in the adrenals it may be involved in either synthesis, storage or release of catechol amines. The effect of reserpine on the adrenal medulla is not suggestive of interference with oxidative phosphorylation, but it may be connected with storage or release of these amines. It has very recently been shown by Schumann[9] that reserpine and insulin deplete hen adrenal medulla of catechol amines and ATP. The results reported in this chapter show that a similar phenomenon occurs in rat adrenal medulla but that there is no accumulation of breakdown products of ATP, i.e. ADP and AMP. In Schumann's investigations, adenosine nucleotides were /

were separated by paper chromatography, and ATP was estimated by the fire-fly method. ADP or AMP levels were not estimated.

TABLE 7.1

EFFECTS OF RESERPINE ON THE CONTENT OF CATECHOL AMINES AND ATP OF THE ADRENAL MEDULLA

Group No.*	Treatment	Wt. of pooled medullae in mg. wet wt.	Total catechol amines in noradrenaline equiv.mg/g. wet wt.	(Expressed in μ moles/g. wet wt.) Total Nucleotides	ATP	ADP	AMP
1.	Control	29.5	11.7	10.1	10.0	nil	0.10
2.	Control	29.7	12.15	9.25	9.0	nil	0.25
3.	Reserpine (1 mg.)	39.1	2.9	3.32	3.0	nil	0.32
4.	Reserpine (1 mg.)	39.9	2.1	3.30	2.95	nil	0.35

* Each group contains 18 medullae taken from 9 rats.

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CHAPTER VIII

(Tables will be found at the end of this chapter)

CHAPTER VIII

EFFECTS OF RESERPINE AND HYDRALLAZINE UPON THE MOVEMENTS OF POTASSIUM (K+) AND SODIUM (Na^+) IN RESTING FROG SKELETAL MUSCLE

Transfers of K+ and Na^+ take place in both directions across the membranes of living cells. These movements can be demonstrated to occur under a wide variety of physiological and pathological conditions, but the mechanisms by which they are brought about are not completely understood. Experimental evidence has been put forward[1] to support the view that in addition to a permeability system which allows ions to move down concentration gradients during electrical activity (passive transport), there is a secretory mechanism which depends upon energy derived from underlying metabolic processes (active transport) and which ejects Na^+ and absorbs K+ against the concentration gradients. Conduction of nerve impulses, but not recovery, can take place if the secretory mechanism is put out of action with inhibitors. Na^+ efflux and K+ influx are coupled but do not seem to be linked rigidly. Probably the same considerations apply to skeletal muscle.

Experimental evidence indicates that K+ plays an important role in carbohydrate metabolism . Thus, in adrenalectomised /

adrenalectomised animals, it has been shown[2,3] that K+ accumulates within the cells. K+-accumulation is accompanied by an increase in the concentration of glucose-1-phosphate and a decrease in those of glucose-6-phosphate and fructose-6-phosphate. It has been demonstrated that the fall in blood sugar following insulin administration is accompanied by a simultaneous fall of K+ and phosphate[4 to 7]. This suggests a formation of hexose-phosphate in the muscle and actually the number of equivalents of glucose, K+ and phosphate which disappear from the blood are of the same order of magnitude. In many cases, both K+ and sugar appear to move into or out of blood plasma together. One possible reason for this behaviour may be that deposition of glycogen in the liver has necessarily been accompanied by the deposition of water and K+[8]. Similar evidence that K+ favours glycogenesis is seen in the observation of McQuarrie et al.[9,10] that in diabetes a high K+ diet increases glycosuria while Na^+ has the opposite effect. These facts are in agreement with the observation that K+ stimulates the liberation of adrenaline which then mobilises both glucose and K+ from the liver. It has also been shown[11,12] in the isolated rat diaphragm that both glucose utilisation /

utilisation and K+ uptake are increased by the addition of insulin to the incubating medium, and in intact rats K+ depletion retards glycogenesis[13]. Just as plasma levels of glucose and K+ rise and fall together with the movement of glucose into or out of the liver, so also, under certain conditions related chiefly to muscular activity, K+ and lactic acid appear to move together[14]. It has, therefore, been suggested[7] that K+ leaves liver or enters muscle in company with glucose while it leaves muscle or enters liver along with lactic acid. The existence has therefore been suggested of a K+ cycle from muscle to liver and other viscera and back again comparable to the carbohydrate cycle.

Data already presented (Chapters V and VI) indicates that reserpine and hydrallazine interfere with oxidative phosphorylation and cellular oxidation. Gillis and Lewis[15] have put forward evidence that reserpine may interfere with carbohydrate metabolism. Evidence has been presented (Chapter V) by the author that hydrallazine has a similar effect. Since a close relationship exists between carbohydrate metabolism and potassium accumulation, it is possible that /

that reserpine and hydrallazine influence the distribution of K+ and Na^+ ions in the cells and extracellular fluids. It has in fact been shown by Gillis and Lewis[16] that reserpine releases K+ from frog skeletal muscle.

Certain drugs and poisons inhibit the transport of Na^+ and K+ ions in tissues. Poisons such as 2:4 -dinitrophenol (DNP) and cyanide block the active transport of these ions in the gastric mucose[17], kidney slices[18], frog skin[19], giant sepia or squid axons[1], and chicken erythrocytes[20]. Mammalian erythrocytes[21] and amphibian muscle[22] do not appear to be affected by these agents. Under the influence of these inhibitors of metabolism, tissues lose K+ and gain Na^+. In view of the fact that reserpine and hydrallazine interfere with the metabolism of living cells, and thus act as "poisons", the effects of these agents on the movements of Na^+ and K+ ions were studied.

For the reasons outlined above an attempt has been made to demonstrate qualitatively the effects of reserpine and hydrallazine on movements of K+ and Na^+ ions in frog skeletal muscle and to compare these effects with those of known enzyme inhibitors and anoxia.

Experiments /

Experiments were carried out using radioactive sodium ($^{24}Na^+$) and potassium ($^{42}K+$) and in certain experiments K+ concentration was estimated directly by flame photometry.

Methods.

Frog sartorius muscle was used in most of the work. Some experiments were also carried out using the frog rectus abdominis muscle. Experiments were designed to study the release and uptake of $^{42}K+$ by resting frog sartorius muscle under the influence of drugs or compounds known to be enzyme inhibitors. Since tissue sodium is mostly extracellular, studies were made on the uptake of $^{24}Na^+$ only. The experiments may be grouped as follows:-

(a) Efflux of $^{42}K+$ from resting frog sartorius or rectus abdominis muscle.

(b) Uptake of $^{42}K+$ by resting frog sartorius or rectus muscle.

(c) Uptake of $^{24}Na^+$ by resting frog sartorius muscle

(d) Measurements of tissue levels of K+ by the flame /

(d)... /flame photometer.

(a) EFFLUX OF $^{42}K^+$ FROM RESTING FROG SARTORIUS OR RECTUS MUSCLE:

$^{42}K^+$ was used for this study. The radioactive material was obtained as a 1.15 per cent w/v solution of ^{42}KCl from the Atomic Energy Research Establishment, Amersham, and had an activity of approximately 1 mc.per ml. at the time of despatch. Frogs of either sex and weighing between 25 to 50 g. were injected with 1.0 ml. of this solution into the dorsal lymph sac. After a 2-hour equilibration period, the frogs were decapitated and pithed. The sartorius and rectus muscles were removed from both sides. One muscle served as a test object and the other was used as a control.

The experimental procedure adopted for this study was a slight modification of the procedure used by Gillis[23]. The dissected muscles were weighed on a torsion balance. In general the weights of the paired sartorius muscles were similar - differing by not more than 1 to 2 mg. - but the paired rectus muscles differed in weight by about 10 mg. The muscles were suspended in test tubes containing /

containing 10 ml. of frog Ringer's solution (Appendix I), through which oxygen was bubbled.

The dissected muscles were suspended from a support made from a bent entomological needle and fitted to the top of the test tube. The muscle was stretched by suspending a bent hypodermic needle at the lower end. After a 10 minute immersion in the first tube, the muscle was removed to the next tube in the series. After transferring the muscle, the $^{42}K^{+}$ content of the bathing fluid was measured by means of a Geiger-Muller liquid counter (Type M6). Corrections for decay and lost counts were not made as a control muscle was compared under similar conditions.

Each series contained ten tubes. In the control series each tube contained 10 ml. of frog Ringer's solution. The control series gave the normal rate of release of $^{42}K^{+}$ from the resting muscle. In the test series, the first 3 and the last 4 tubes each contained 10 ml. of normal frog Ringer's solution while the 4th, 5th and 6th contained frog Ringer's solution together with the test substance. To render the tissue anoxic, oxygen was replaced by a mixture of 95 per cent nitrogen and /

and 5 per cent carbon dioxide.

The advantage of this method is that any change in the release of $^{42}K^{+}$ taking place when the muscle is in contact with the drug will be shown by the change in slope of the curve obtained by plotting the number of counts released against time. Hence the muscle can serve as its own control. This dispenses with the need for running a simultaneous control i.e. if it is not desired to compare the $^{42}K^{+}$ remaining in the test and control muscles after treatment. In these experiments, however, both the control and test muscles were dissolved in concentrated nitric acid and the solution counted to give the total number of counts remaining after treatment. All values are expressed in counts per minute per mg. of wet tissue. The rate and total exchange of $^{42}K^{+}$ in the treated muscle was compared with that of the control muscle by plotting the output of $^{42}K^{+}$ against time.

(b) UPTAKE OF $^{42}K^{+}$ BY RESTING FROG SARTORIUS MUSCLE:

SARTORIUS MUSCLE:

The method employed was essentially the same as that /

that used by Lister[24]. The paired sartorius or rectus muscles of a pithed frog were carefully exposed, dissected free and weighed on a torsion balance.

Stainless steel hypodermic needles bent into identically shaped hooks were attached to the tendon at each end of the muscle and suspended from the barrel of a 1 ml. tuberculin syringe into a tube containing 10 ml. of frog Ringer's solution, in which some of the $^{39}K^{+}$ had been replaced by $^{42}K^{+}$. The control muscle was placed into the radioactive frog Ringer's solution and the other was placed in radioactive frog Ringer's solution to which drugs had been added.

Before exposing the muscle to the radioactive frog Ringer's solution, both the test and control solutions were tested for radioactivity in an M6 liquid counter. A count of about 10,000 per ml. per minute was adequate for the muscle to attain measurable radioactivity in 30 minutes.

The bathing solution was continuously agitated and oxygenated by a stream of oxygen and maintained at room temperature at pH 7.4 to 7.6. The muscles were left immers-

ed for . /

for 30 minute periods; they were then removed from the solution and each side of the muscle was washed for 5 seconds by a stream of non-radioactive Ringer's solution and lightly blotted with absorbent tissue to remove adhering moisture.

The muscle was then attached by means of the bent needle to a syringe barrel which was so arranged that the muscle was suspended 1.5 cm. from the window of a GM4 end-window Geiger counter tube, with the centre of the muscle approximating to the centre of the counter window. The muscle was placed in front of the counter for two and a half minutes. It was then rotated through 180° and counted for a further two and a half minutes. This procedure ensured that both surfaces of the muscle were adequately counted. After counting, the muscle was returned to the bathing (radioactive) solution.

The Geiger tube was connected to a recording ratemeter, the controls of which were adjusted to give optimal recording conditions, the degree of radioactivity being recorded by a pen writing upon a moving paper surface.

The /

The muscles were exposed at 30 minute intervals for 2 to 3 hours. Equilibration was reached in about 1 to 1½ hours and further exposure of the control muscle to the radioactive solution did not appreciably increase the number of counts. The control and the test muscles were exposed alternately to the counter for a total of five minutes. The total activity in the muscle at the end of this period (usually 2 to 3 hours) was measured by dissolving it in concentrated nitric acid and counting the solution in an M6 counter. The method described gives qualitative measurements only and suffers from the disadvantage that the muscle must be exposed for a considerable time under unphysiological conditions. Corrections for decay, lost counts etc. were not applied since in every experiment a control muscle treated under identical conditions was compared with the test muscle.

(c) UPTAKE OF $^{24}Na^{+}$ BY RESTING FROG SARTORIUS MUSCLE:

Paired frog sartorius muscles were used in these experiments. The method was the same as that described in section (b) and used for measuring the uptake /

uptake of $^{42}K^+$, but part of the $^{22}Na^+$ of the frog Ringer's solution was replaced by $^{24}Na^+$. $^{24}Na^+$ was obtained in the form of an isotonic saline solution from the Atomic Energy Research Establishment, Amersham and had an activity of about 1 mc. at the time of despatch. A count of about 10,000 per ml. of Ringer's solution per minute was adequate for the muscle to attain measurable radioactivity in 30 minutes. Other experimental details were the same as described under section (b).

(d) (d) MEASUREMENTS OF TISSUE LEVELS OF K^+ BY FLAME PHOTOMETER.

Frogs of either sex and weighing from 25 to 50 g. were used in these experiments. They were decapitated, pithed and both sartorius muscles removed and weighed on a torsion balance. One muscle served as a test object while the other was used as a control.

The test muscle was exposed to the frog Ringer's solution containing the drug for 2 to 3 hours, after which it was removed and blotted carefully to remove excess moisture. It was then dissolved in 0.4 ml. of concentrated nitric acid with gentle heating and the

final /

final volume was made up to 10 ml. with glass distilled water. 2.5 ml. of this solution was diluted to 50 ml. with glass distilled water and the diluted solution used in the flame photometer. The control muscle was similarly treated.

All potassium determinations were made using the flame photometer manufactured by Evans Electroselenium Limited. All standard solutions were made up with "Analar" standard chemicals and in freshly glass distilled water.

For determination of K^+, "stock" potassium chloride solution was prepared by dissolving 1.910 g. of KCl in a litre of distilled water. This was diluted 100 times with distilled water to give a working standard containing 10 parts per million (p.p.m.) of K^+ ion. Standards containing 2, 4, 6 and 8 p.p.m. of K^+ ion were also prepared. A calibration curve was obtained by running each of the standard solutions through the instrument and noting the scale reading. A curve was drawn by plotting the scale readings against the concentration of K^+ in the standard. The concentration of K^+ in the sample was determined by reading off the concentration equivalent to the reading from the calibration /

calibration curve. All values are expressed as mEq. of K^+ per kg. of wet tissue.

Summary of Results.

Reserpine (0.16 to 1.6 x 10^{-4}M) and hydrallazine (0.5 x 10^{-3}M) did not cause release of $^{42}K^+$ from resting frog sartorius and rectus abdominis muscles. The effects were not markedly different from those obtained when K^+ free Ringer's solution was used as a bathing fluid. Hydrallazine (10^{-2}M), however, caused a marked release of $^{42}K^+$. Cyanide (1.5 x 10^{-2}M) and azide (10^{-2}M) caused a similar efflux of $^{42}K^+$. DNP (10^{-4}M) and anoxia had no apparent effects.

Reserpine (0.16 to 1.6 x 10^{-4}M) had no effect on the uptake of $^{42}K^+$ but hydrallazine (0.5 to 1.0 x 10^{-2}M) decreased it. Cyanide (10^{-2}M), azide (10^{-2}M), DNP (10^{-4}M) and anoxia markedly reduced the $^{42}K^+$ uptake.

Results obtained from studies on $^{24}Na^+$ uptake showed changes in the opposite direction. Thus cyanide, azide, DNP (all in similar concentrations) increased the $^{24}Na^+$ uptake of the sartorius muscle. Hydrallazine (0.5 to 1.0 x 10^{-2}M) increased it while reserpine in any of /

of the concentrations used had no effect or slightly decreased it.

Direct measurements using the flame photometer of K^+ content of the treated muscles corroborated the above findings.

Results.

As far as possible frogs of similar gross weight were used but no correlation between the body weight and weight of the sartorius muscle could be found, and the weights of individual muscles differed quite widely from experiment to experiment. Although the muscles of a pair were usually comparable with respect to both uptake and release of $^{42}K^+$ and $^{24}Na^+$, these factors were widely different in muscles from different frogs. Due to this lack of correlation between experiments, it was found impossible to compare the results directly, using standard statistical procedures. In experiments where K^+ was estimated by the flame photometer, the results were calculated statistically.

(a) EFFLUX OF $^{42}K^+$ BY FROG SARTORIUS OR RECTUS ABDOMINIS MUSCLE.

The /

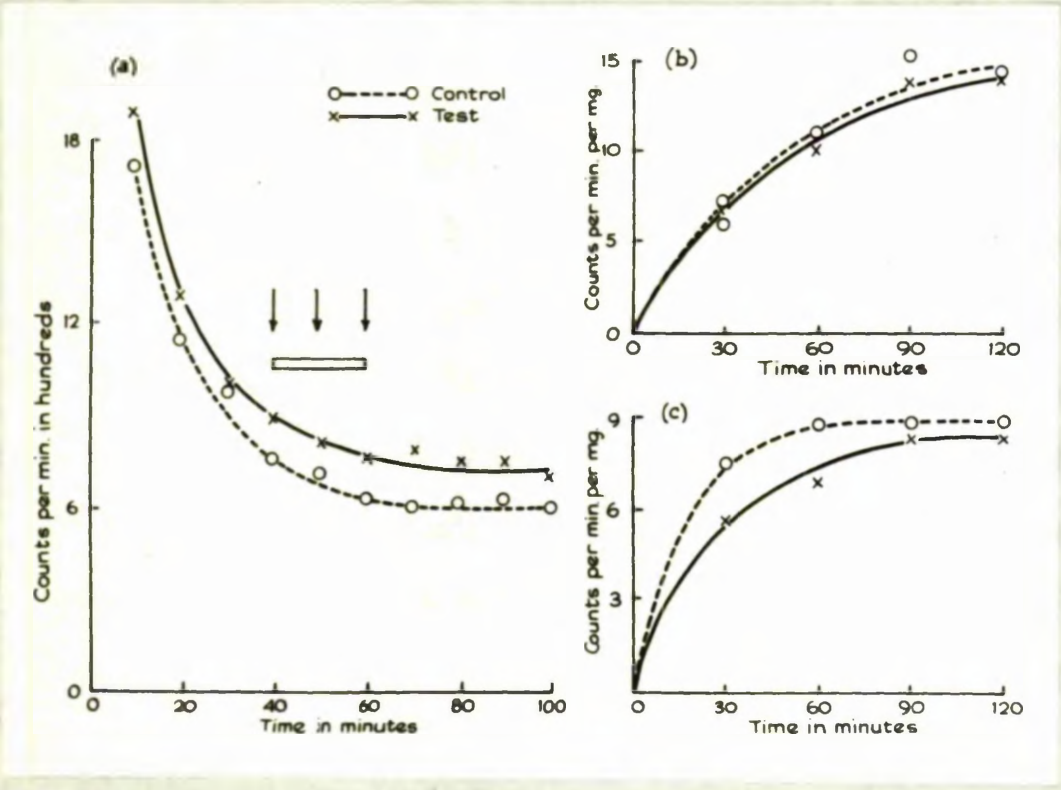

Fig. 8.1

(a) Effect of reserpine (1.6×10^{-4}M) on the release of $^{42}K^{+}$ by frog sartorius muscle.

Arrows indicate exposure of the muscle to the test substance.

(b) Effect of reserpine (1.6×10^{-4}M) on the uptake of $^{42}K^{+}$ by frog sartorius muscle.

(c) Effect of reserpine (1.6×10^{-4}M) on the uptake of $^{24}Na^{+}$ by frog sartorius muscle.

Ordinate - number of counts

Abscissa - time in minutes.

The results obtained from typical experiments for the efflux of $^{42}K^{+}$ by frog skeletal muscles which were exposed to drugs and certain inhibitors of metabolism are presented in tables 8.1 to 8.6, and the curves therefrom in figures 8.1a to 8.6a. In general, the counts released after the first 10 minute exposure of the same pair of muscles differed considerably. This may have been due to inadequate washing. From the second tube onwards, however, the counts were uniform and comparable.

Reserpine (0.8 to 1.6 x 10^{-4}M) had practically no effect on the release of $^{42}K^{+}$ from frog muscle. The control solution, when used in equivalent amounts, also had no effect on $^{42}K^{+}$ release (Table 8.1, fig. 8.1a). The total number of counts released by the test muscles during the incubation period was more than that released from the control, and there was practically no difference in the final counts remaining in the test and control muscles (Table 8.1). The total number of counts released by the test muscle was not always greater than that released by the control. This may have been due to variations in the individual muscle. A few experiments were carried out using K^{+} free Ringer's solution /

solution. Both control and the test muscles were exposed to the K^+ free Ringer's solution instead of normal Ringer's solution, and the number of counts released during 10 minute incubation periods were counted as before. Drugs were added to the K^+ free Ringer's solution in the 4th, 5th and 6th tubes. Under these conditions reserpine did not show appreciable differences in the rate of release of $^{42}K^+$ from experiments in which normal frog Ringer's solution was used as a bathing fluid. Gillis and Lewis[16] have, however, shown that reserpine causes the release of K^+ from frog skeletal muscle. The method used by the author for this study is different from the one used by them. Gillis and Lewis studied the release of potassium by a method in which the bathing fluid was continuously circulated through a small organ bath (which held the muscle) by means of a pump to a flow counter and thence back to the bath. Thus a continuous, integrated record of the $^{42}K^+$ in the bathing fluid was obtained with a recording ratemeter. This method has the decided advantage over the author's method in that the tissue was maintained in a constant environment throughout the experiment. In addition, frogs differ in their sensitivity to drugs in different seasons. It

is /

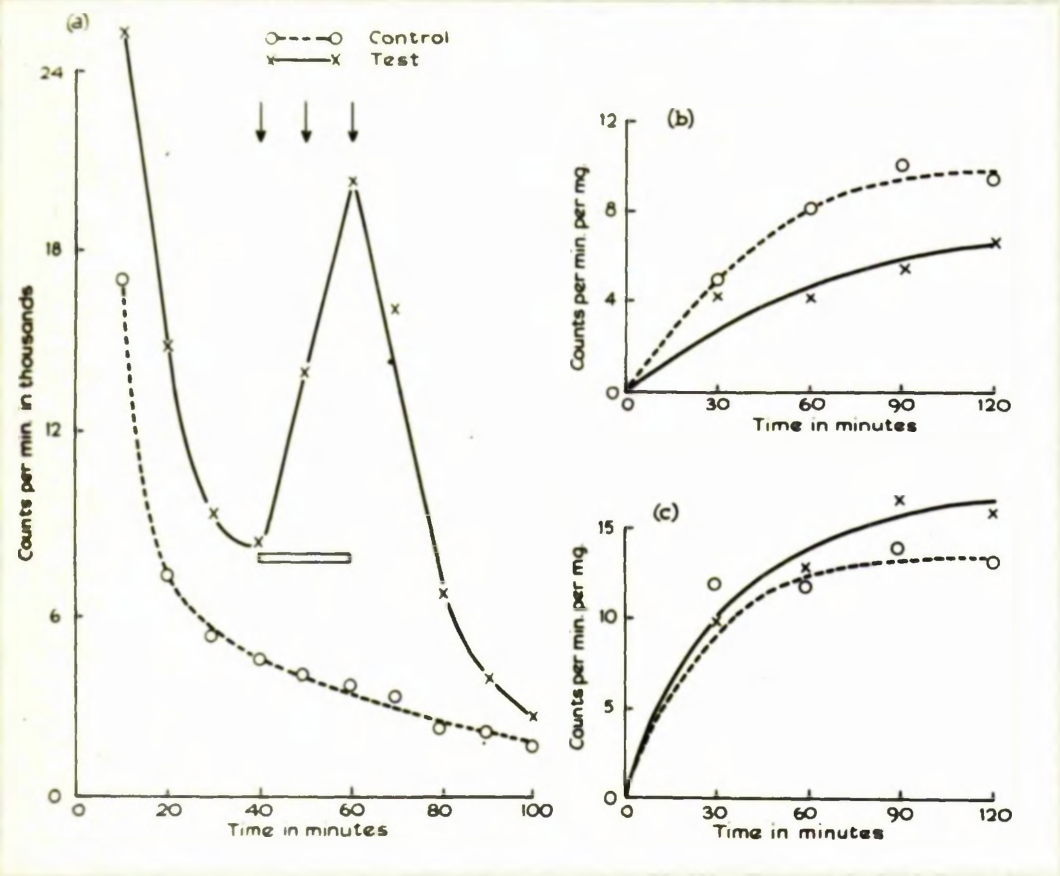

Fig. 8.2

(a) Effect of hydrallazine (10^{-2}M) on the release of $^{42}K^{+}$ by frog rectus abdominis muscle in K^{+}-free frog Ringer's solution.

Arrows indicate exposure of the muscle to the test substance.

(b) Effect of hydrallazine (0.5×10^{-2}M) on the uptake of $^{42}K^{+}$ by frog sartorius muscle.

(c) Effect of hydrallazine (0.5×10^{-2}M) on the uptake of $^{24}Na^{+}$ by frog sartorius muscle.

Ordinate - number of counts.

Abscissa - time in minutes.

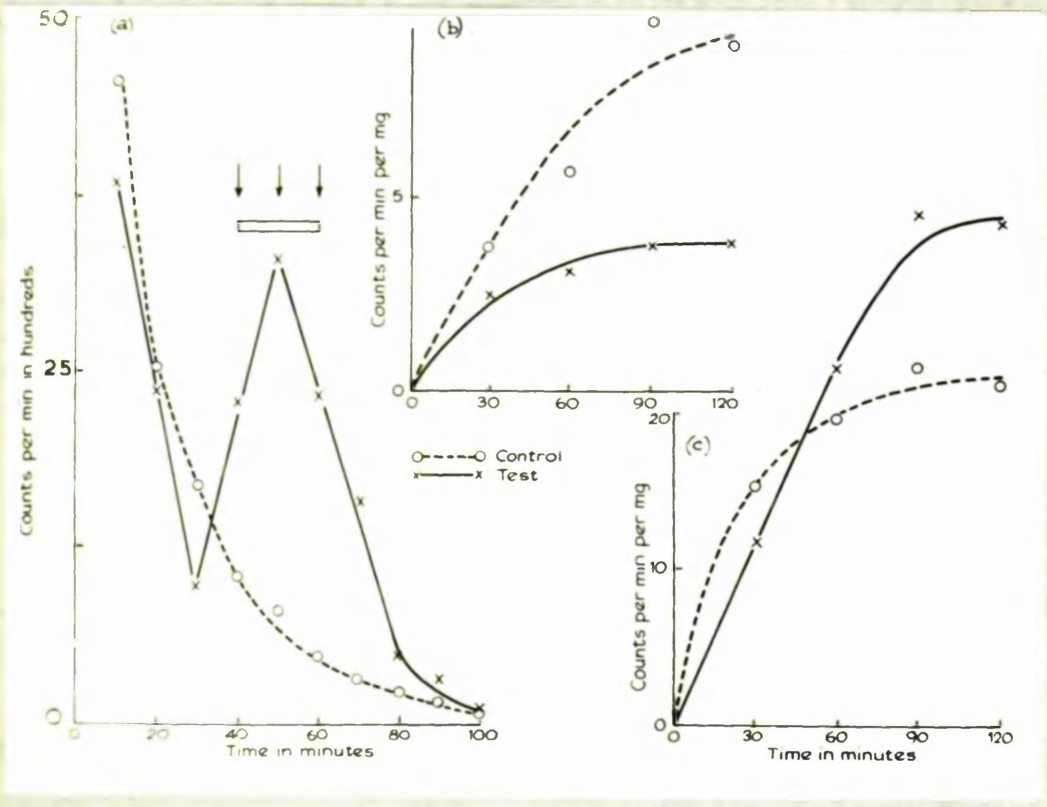

Fig. 8.3

(a) Effect of azide (10^{-2}M) on the release of $^{42}K^{+}$ by frog rectus abdominis muscle.

Arrows indicate exposure of the muscle to the test substance.

(b) Effect of azide (10^{-2}M) on the uptake of $^{42}K^{+}$ by frog sartorius muscle.

(c) Effect of azide (10^{-2}M) on the uptake of $^{24}Na^{+}$ by frog sartorius muscle.

Ordinate - number of counts
Abscissa - time in minutes.

possible that this second factor may also account for a part of the discrepancy.

Hydrallazine (0.5×10^{-3}M or 0.5×10^{-2}M) had practically no effect on the release of potassium but at times appeared to change the slope of the curve. In doses of 10^{-2}M hydrallazine caused a release of $^{42}K^{+}$ from the muscle. A typical experiment is shown in Table 8.2, Fig. 8.2a, in which hydrallazine caused a very marked increase in the release of $^{42}K^{+}$. This effect was even more pronounced when K^{+} free Ringer's solution was used. The number of counts remaining in the test muscle per mg. per minute was found to be approximately 2.5 times less than in the control muscle.

Sodium azide (10^{-2}M) caused a marked increase in the release of $^{42}K^{+}$ (Tables 8.3, Fig. 8.3a). When the muscle was returned to the normal Ringer's solution, the number of counts released fell quite rapidly and approximated to the control values. Final counts remaining in the test muscle per mg. per minute were about half those remaining in the control. A similar effect was shown when potassium cyanide at 1.5×10^{-2}M (the pH was adjusted to 7.5) was used and the curves obtained /

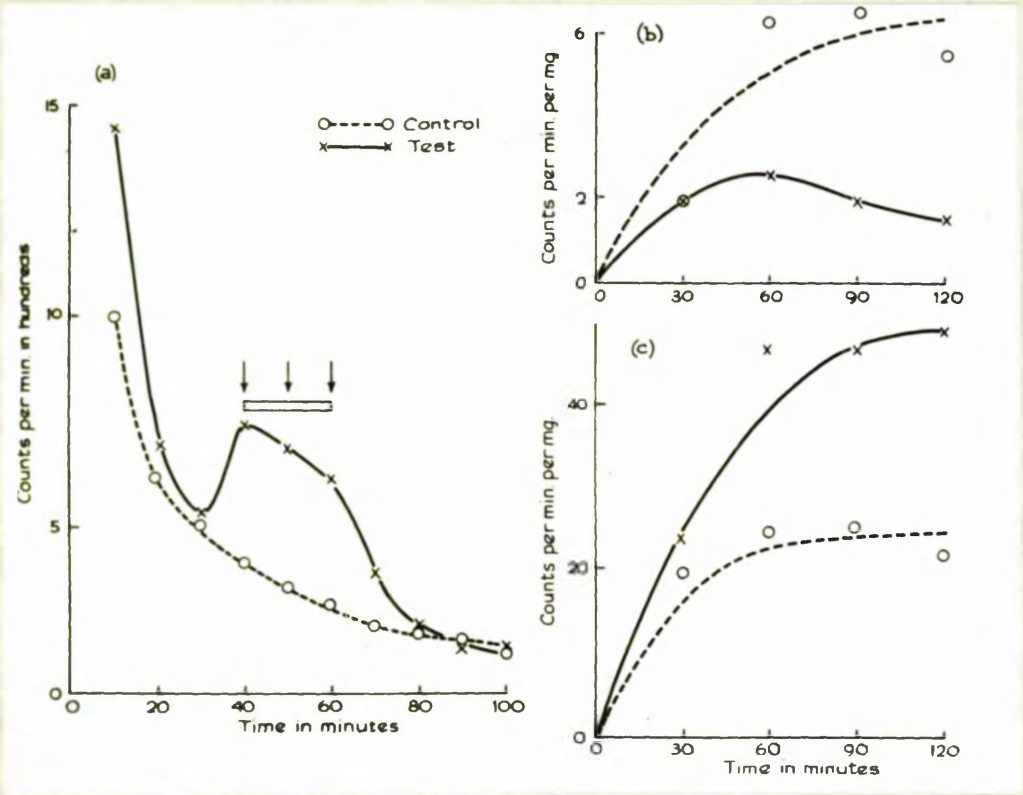

Fig. 8.4

(a) Effect of cyanide (1.5×10^{-2}M) on the release of $^{42}K^{+}$ by frog sartorius muscle.

Arrows indicate exposure of the muscle to the test substance.

(b) Effect of cyanide (1.5×10^{-2}M) on the uptake of $^{42}K^{+}$ by frog sartorius muscle.

(c) Effect of cyanide (1.5×10^{-2}M) on the uptake of $^{24}Na^{+}$ by frog sartorius muscle.

Ordinate - number of counts.
Abscissa - time in minutes.

obtained (Fig. 8.4a) were similar to those obtained from azide and hydrallazine. On removing the cyanide the rate of release of $^{42}K^+$ tended to come back to normal (Table 8.4).

However, DNP (0.5 to 1 x 10^{-4}M) and anoxia had only a slight effect on the release of potassium from the muscle. Typical curves and experiments are shown in Fig. 8.5a and 8.6a, and in Tables 8.5 and 8.6. At times DNP changed the slope of the curve and gave an impression that more $^{42}K^+$ was leaking out. With anoxia, the slope of the curve changed but the very distinct release of $^{42}K^+$ following azide and cyanide was not seen. When the muscle was supplied with oxygen, a considerable number of counts were lost. This occurred either immediately or 10 to 20 minutes after supplying oxygen. In the experiment cited (Table 8.6), this can be seen 20 minutes after supplying oxygen to the tissue. No correlation could be found between the final number of counts remaining in the muscles treated with DNP or anoxia and the controls. Thus, DNP and anoxia had no marked effects on the release of $^{42}K^+$ from the skeletal muscle.

(b) /

(b) and (c) UPTAKE OF $^{42}K^+$ AND $^{24}Na^+$ BY FROG SARTORIUS OR RECTUS ABDOMINIS MUSCLE:

Reserpine (0.8 to 1.6 x 10^{-4}M) had little or no effect either on the uptake of $^{42}K^+$ or of $^{24}Na^+$ (Fig. 8.1, b and c, and Table 8.7). Since the method used for these studies kept the muscle in contact with the drug solution for a considerable length of time, it could be assumed that considerable penetration of the drug into the cells had taken place, and that the drug was probably exerting its maximum effects. It might be argued from the results obtained in the previous section that the period of contact of reserpine with the muscle was rather short, and that the drug probably did not penetrate sufficiently into the muscle. In addition, reserpine is only very slightly soluble at pH 7.5 and hence it was decided to increase the time of contact between the muscle and the drug solution to 2 to 3 hours. Anoxia exerts its effects rather slowly and it is possible that reserpine may behave similarly. It has been shown previously (Chapter VI) that reserpine may inhibit oxidative phosphorylation and uncoupling agents, e.g. DNP, have very characteristic effects on the uptake of both K^+ and Na^+ ions. The details of one experiment /

experiment using reserpine are shown in Table 8.7. Counts for $^{42}K^+$ remaining in the test muscle did not appreciably differ from those remaining in the control muscle. Similar results were obtained when $^{24}Na^+$ was used. Thus reserpine in any of the doses used had no appreciable effects on the uptake of $^{42}K^+$ or $^{24}Na^+$.

Hydrallazine (0.5 to 1.0 x 10^{-2}M) reduced the $^{42}K^+$ and slightly increased $^{24}Na^+$ uptake of the muscle but had little effect at 0.5 x 10^{-3}M (Table 8.8 and Fig. 8.2, b and c). Final counts remaining in the test muscle were lower than in the control for $^{42}K^+$ and higher for $^{24}Na^+$. Since it has been shown in chapters V and VI that hydrallazine may inhibit cellular oxidation, these results were expected. The hydrallazine effect is compared with anoxia and in anoxia the muscle loses K^+ and gains Na^+. Similarly, tissues treated with azide and cyanide (which are known to inhibit oxidation) lose K^+ and gain Na^+. Thus hydrallazine at higher dose levels influences the movements of these ions in ways similar to anoxia, azide and cyanide.

Azide (10^{-2}M) and cyanide (1.5 x 10^{-2}M) markedly inhibited $^{42}K^+$ uptake and augmented $^{24}Na^+$ accumulation. Typical /

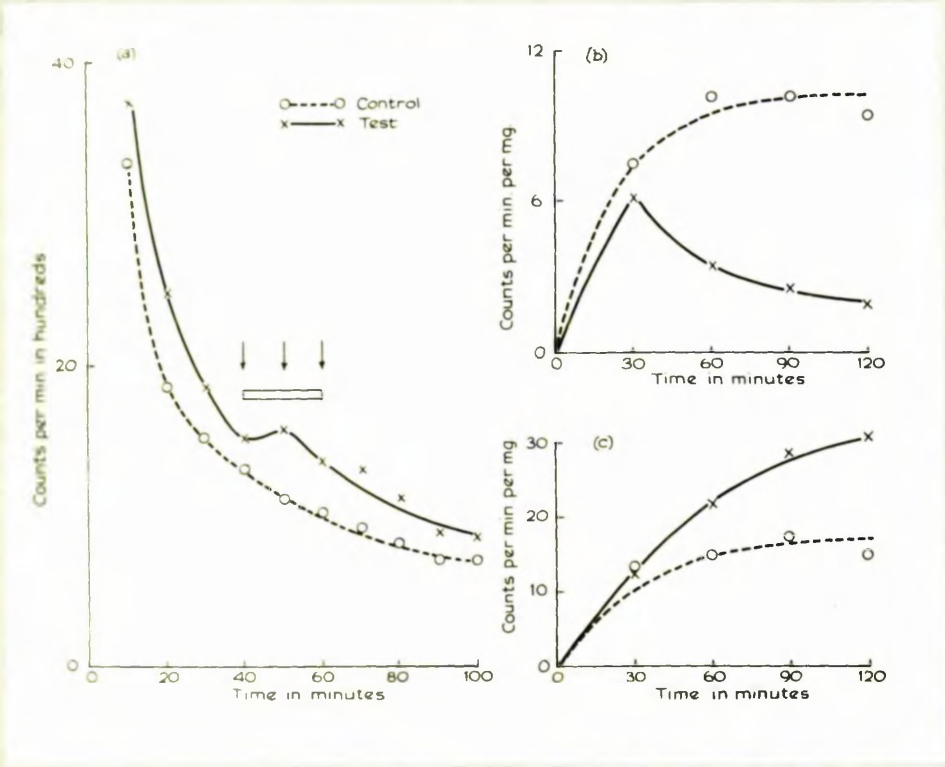

Fig. 8.5

(a) Effect of DNP (10^{-4}M) on the release of $^{42}K^{+}$ by frog rectus abdominis muscle.

Arrows indicate exposure of the muscle to the test substance.

(b) Effect of DNP (10^{-4}M) on the uptake of $^{42}K^{+}$ by frog sartorius muscle.

(c) Effect of DNP (0.5×10^{-4}M) on the uptake of $^{24}Na^{+}$ by frog sartorius muscle.

Ordinate - number of counts.
Abscissa - time in minutes.

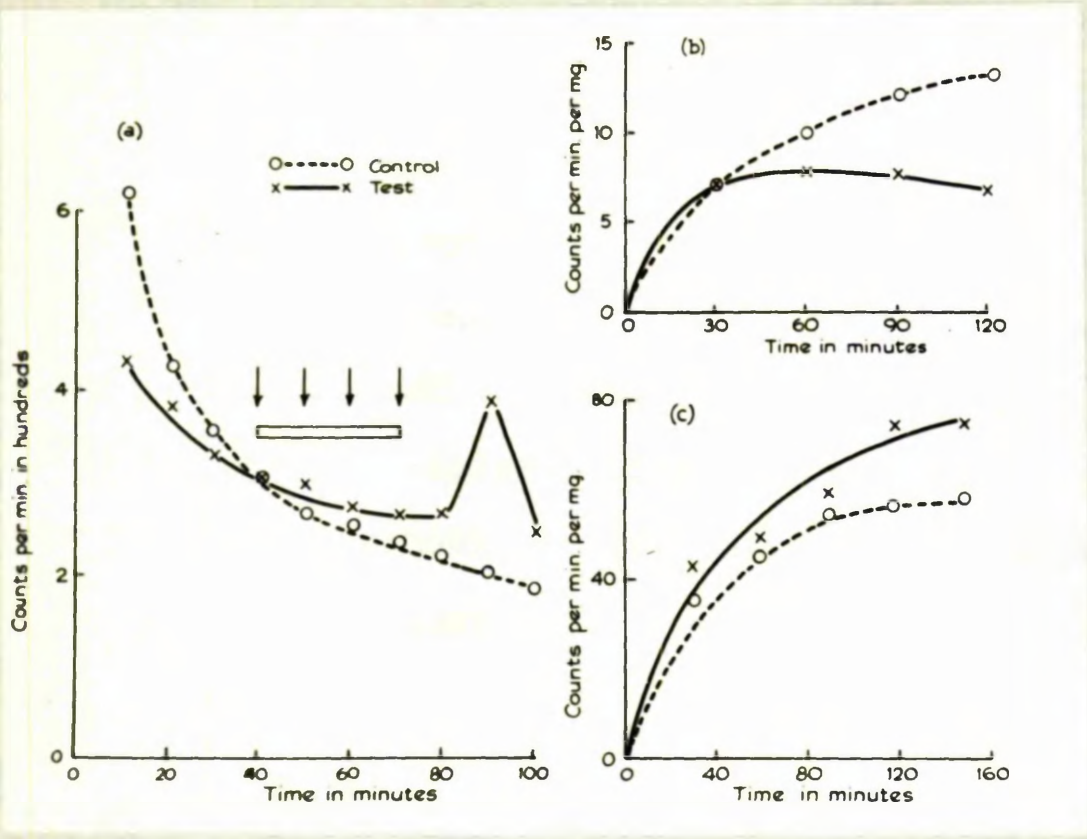

Fig. 8.6

(a) Effect of anoxia on the release of $^{42}K^{+}$ by frog sartorius muscle.

Arrows indicate exposure of the muscle to the test substance.

(b) Effect of anoxia on the uptake of $^{42}K^{+}$ by frog sartorius muscle.

(c) Effect of anoxia on the uptake of $^{24}Na^{+}$ by frog sartorius muscle.

Ordinate - number of counts.

Abscissa - time in minutes.

Typical experiments are shown in Tables 8.9 and 8.10 and curves obtained from the data in Figs. 8.3, b and c, and 8.4, b and c. After azide treatment, the $^{42}K^+$ counts remaining in the muscle were about one third of those remaining in the control. The $^{24}Na^+$ counts for the test muscle were almost doubled. Muscles treated with cyanide contained only one fourth the number of $^{42}K^+$ counts as the control, and twice the control value for $^{24}Na^+$ (Table 8.10).

DNP (0.5 to 1 x 10^{-4}M), anoxia, cyanide and azide had qualitatively similar effects on Na^+ and K^+ movements. DNP (0.5 x 10^{-4}M) appeared to inhibit $^{42}K^+$ uptake and to stimulate $^{24}Na^+$ uptake quite markedly. In general, the effects of DNP and anoxia developed slowly and began to show themselves after about 1 hour, while azide and cyanide exerted their effects earlier(30 minutes). Anoxia also retarded $^{42}K^+$ accumulation and increased $^{24}Na^+$ retention. Typical experiments are shown in Tables 8.11 and 8.12, and the curves obtained from the data are shown in Figs.8.5, b and c, and 8.6, b and c. With DNP, the test muscle had one fourth the number of $^{42}K^+$ counts as the control and double the control value for $^{24}Na^+$. With anoxia, $^{42}K^+$ /

$^{42}K^+$ counts were half and $^{24}Na^+$ counts double the control values.

(d) MEASUREMENTS OF K^+ BY FLAME PHOTOMETER:

The results are summarised in Tables 8.13 and 8.14. Thus, reserpine (0.16 to 1.6 x 10^{-4}M) in any of the concentrations used had no significant effect on the K^+ content of the muscle, while hydrallazine (10^{-2}M) significantly lowered it ($P < 0.01$). Hydrallazine (0.5 x 10^{-2}M) did not significantly lower muscle K^+, although in every experiment the value for the control muscle was higher than the test. This may be due to the large scatter of individual estimations e.g. the lowest and the highest values of 5 such estimations for the control muscle were 53.3 and 101.97 mEq. per kg. and for the test muscle 40.00 and 89.00 mEq. per kg. By using the "t" test, P was found to be nearly equal to 0.1. Hydrallazine (0.5 x 10^{-3}M) had no significant effects.

Cyanide (1.5 x 10^{-2}M), DNP (10^{-4}M) and anoxia significantly lowered the muscle K^+ ($P < 0.02$). Azide (10^{-2}M) had also significant effects ($P < 0.05$).

The /

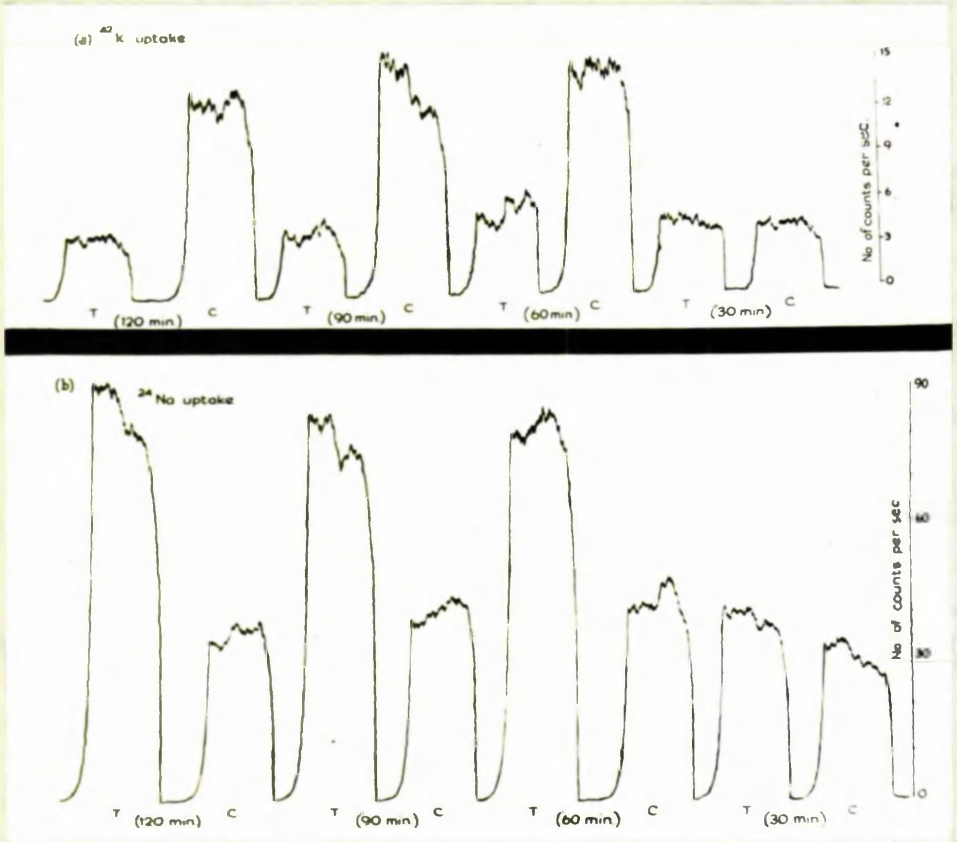

Fig. 8.7

Record of the effects of cyanide on $^{42}K^{+}$ (a) and $^{24}Na^{+}$(b) uptake by frog sartorius muscle. This is a photograph taken from an actual record and shows the uptake at four intervals during the experiment.

C - Activity of control muscle

T - Activity of test muscle exposed to cyanide. In (a) cyanide concentration was 1.5×10^{-2}M and in (b) it was 10^{-2}M.

The results obtained by using tracer techniques are therefore substantiated by direct measurements using the flame photometer.

A typical record for the muscle uptake of $^{42}K^+$ and $^{24}Na^+$ and the effects of cyanide on this uptake are shown in Fig. 8.7.

Discussion.

The uptake of K^+ from low external concentrations is related to cellular metabolism as measured by the rate of oxygen consumption(25). K^+ accumulation in the cells is an example of active transport and can be modified by such variables as oxygen tension, temperature and metabolic inhibitors(17 to 22). The uptake of K^+ is associated with a reciprocal change in Na^+ efflux.

As indicated in the results, anoxia depressed K^+ and increased Na^+ uptake. It has similarly been shown in kidney slices(25) that anoxia depressed respiration and K^+ uptake in a parallel manner. This appears to indicate that anoxia affects the transport system by decreasing metabolic activity and that other cellular components, either cellular or functional, are not altered. /

altered. This is supported by the fact that the effects of anoxia are reversible. Cyanide and azide both depress K^+ uptake and stimulate Na^+ uptake. It is well known that cyanide inhibits cytochrome-oxidase, and hence it is possible that respiration and K^+ accumulation are depressed approximately to the same extent. Azide behaves similarly to cyanide but Mudge[18] has made a distinction between the effects of cyanide and those of azide. Thus a significant inhibition of K^+ accumulation occurred in kidney slices at a concentration of azide which did not depress respiration. Thus it is possible that cyanide affects the mechanisms responsible for K^+ accumulation by acting on cellular functions involved in aerobic oxidation, while azide may affect the transport mechanism by an action on cellular functions not involved in the maintenance of oxidative metabolism. As the inhibitor concentration was increased, the anticipated depression of respiration was observed, but this effect occurred at concentrations significantly greater than those required to produce large changes in electrolyte composition[18,26].

DNP has been shown to inhibit K^+ accumulation and to stimulate Na^+ uptake. It is well known that DNP inhibits /

inhibits the synthesis of energy-rich phosphate bonds which normally accompanies certain aerobic oxidative reactions. The results obtained with DNP may indicate that the energy liberated by aerobic oxidative reactions becomes available to the cell for K^+ and Na^+ transport by the synthesis of energy-rich phosphate bonds. In intact cells, DNP stimulates respiration[27]. Respiratory stimulation with DNP is associated with a depression of potassium accumulation[18].

Reserpine has very little effect on the uptake of K^+ or Na^+, while hydrallazine depresses K^+ accumulation and slightly increases Na^+ uptake. Reserpine has no significant effect on respiration unless used in high doses; hydrallazine depresses respiration (Chapter VII). Since transport of K^+ appears to depend on oxidative processes, inhibition of K^+ accumulation by hydrallazine is likely. On this basis reserpine should have no effect on K^+ transport. Since reserpine inhibits oxidative phosphorylation <u>in vitro</u>[28] and depresses ATP levels in brain and liver, it is suggested (Chapter VI) that reserpine may act as an uncoupling agent. On this assumption, considerable K^+ loss and Na^+ accumulation would be expected, but no such change has been /

observed by the author. Gillis and Lewis[16] have however shown a considerable release of K^+ from reserpine-treated frog muscle. It appears that their method of estimation was more sensitive than the one used by the author and reserpine may in fact be releasing K^+. In this case reserpine has one more of the properties of an uncoupling agent.

It is possible to divide the active inhibitors into two general groups; those which depress oxidation and K^+ accumulation in a parallel manner and those which inhibit K^+ uptake without affecting respiration. It may be that these compounds like DNP block enzymic reactions not primarily or directly involved in oxygen consumption. It is also possible that their action is upon a nonenzymic function, such as an alteration in membrane permeability due to chemical changes induced in an otherwise stable cellular component. Net accumulation of K^+ seems to be dependent upon the utilisation of phosphate bond energy which may be essential either for the formation of the hypothetical ion carrier complex, its dissociation, or the regeneration of new carrier molecules.

It has been shown by a number of workers that certain poisons, e.g. cyanide and azide, block the active transport /

transport of ions in different tissues[1,17 to 22]. Two preparations which are not affected by these agents are mammalian erythrocytes[12] and amphibian muscle[13]. In the former case the lack of effect is not surprising, since it may be that cyanide and DNP have little blocking action on the glycolytic system which is the principal metabolic pathway in mammalian blood. This explanation is less satisfactory for frog muscle, since muscles doubly poisoned with cyanide and iodoacetate - which should block both respiration and glycolysis - show no striking reduction in Na^+ efflux after several hours treatment at room temperature[22] and no K^+ leakage at 0°[29]. Schatzmann and Witt[30] have, however, reported that the net loss of K^+ from a resting frog muscle (in K^+-free medium) is increased by DNP and K-strophanthin. It is possible that this muscle is insensitive to these metabolic inhibitors. The apparent lack of effect of low doses of hydrallazine and reserpine on the release of K^+ from a loaded muscle may be a reflection on the peculiarity of this muscle. It may, however, be fruitful to repeat this work with a mammalian muscle which is more readily affected by these poisons.

TABLE 8.1

EFFECT OF RESERPINE (1.6×10^{-4}M) ON THE RELEASE OF $^{42}K^{+}$ FROM FROG SARTORIUS MUSCLE

Weight of control muscle = 134 mg.

" " test " = 130 mg.

No. of tube in series	1	2	3	4	5	6	7	8	9	10	Total	counts remaining in muscle per min.per mg.
Control	1732	1154	986	765	712	645	599	606	628	587	8414	139.7
Test	1899	1295	1018	(T)898	(T)824	(T)782	796	757	763	694	9726	144.8

(T) denotes exposure of the muscle to the test solution and each column shows the number of counts released per minute during a 10 minute incubation period.

TABLE 8.2

EFFECT OF HYDRALLAZINE (10^{-2}M) ON THE RELEASE OF $^{42}K^{+}$ BY FROG RECTUS ABDOMINIS MUSCLE (K^{+} FREE MEDIUM)

Weight of control muscle = 179 mg.

" " test " = 186 mg.

). of be in ries	1	2	3	4	5	6	7	8	9	10	Total	Counts remaining in muscle per min. per mg.
ntrol	16996	7368	5306	4762	4145	3769	3296	2460	2039	1763	51904	284.4
st	25322	14822	9095	(T)8450	(T)13844	(T)20515	16258	6856	3958	2719	121839	104.2

(T) denotes exposure of the muscle to the test solution and each column shows the number of counts released per minute during a 10 minute incubation period.

TABLE 8.3

EFFECT OF AZIDE (10^{-2}M) ON THE RELEASE OF $^{42}K^{+}$ BY FROG RECTUS ABDOMINIS MUSCLE

Weight of control muscle = 104 mg.

" " test " = 104 mg.

No. of tube in series	1	2	3	4	5	6	7	8	9	10	Total	Counts remaining in muscle per min. per mg.
Control	4628	2760	2004	1423	1209	921	779	705	622	532	15583	82.2
Test	3979	2616	1328	(T)2541	(T)3461	(T)2593	1907	919	785	556	20685	46.6

(T) denotes exposure of the muscle to the test solution and each column represents the number of counts released per minute during a 10 minute incubation period.

TABLE 8.4

EFFECT OF CYANIDE (1.5×10^{-2}M) ON THE RELEASE OF $^{42}K^{+}$ FROM FROG SARTORIUS MUSCLE

Weight of control muscle = 86 mg.

" " test " = 88 mg.

o. of ube in eries	1	2	3	4	5	6	7	8	9	10	Total	Counts remaining in muscle per min. per mg.
ontrol	995	622	500	415	355	304	263	248	234	195	4131	65.9
est	1443	695	524	(T)746	(T)685	(T)621	383	271	204	206	5778	52.00

(T) denotes exposure of the muscle to the test solution and each column shows the number of counts released per minute during a 10 minute incubation period.

TABLE 8.5

EFFECT OF DNP (10^{-4}M) ON THE RELEASE OF

$^{42}K^{+}$ FROM FROG RECTUS MUSCLE

Weight of control muscle = 196 mg.
" " test " = 195 mg.

No. of tube in series	1	2	3	4	5	6	7	8	9	10	Total	Counts remaining in muscle per min. per mg.
Control	3341	1865	1526	1327	1126	1022	922	816	711	715	13371	90.4
Test	3742	2483	1853	(T)1512	(T)1579	(T)1362	1301	1131	904	861	16728	58.8

(T) denotes exposure of the muscle to the test solution and each column shows the number of counts released per minute during a 10 minute incubation period.

TABLE 8.6

EFFECT OF ANOXIA ON THE RELEASE OF $^{42}K^{+}$

FROM FROG SARTORIUS MUSCLE

Weight of control muscle = 88 mg.

" " test " = 88 mg.

No. of tube in series	1	2	3	4	5	6	7	8	9	10	Total	Counts remaining in muscle per min. per mg.
ontrol	621	627	362	308	271	260	238	223	204	186	3100	82.20
st	438	387	331	(T)305	(T)300	(T)276	(T)270	274	392	258	3231	96.00

(T) denotes exposure of the muscle to the test solution and each column shows the number of counts per minute released during a 10 minute incubation period.

TABLE 8.7

EFFECT OF RESERPINE ON THE UPTAKE OF $^{42}K^{+}$ AND $^{24}Na^{+}$ BY FROG SARTORIUS MUSCLE

Isotope used	Drug concentration	Wt. of muscle in mg.	Time of exposure in min.	Total counts per min. in muscle		Counts per min. per mg. of muscle		Final counts remaining in muscle per min. per m	
				Control	Test	Control	Test	Control	Test
$^{42}K^{+}$	1.6×10^{-4}M	control = 94 test = 96	30	660	600	7.02	6.25	-	-
			60	1020	960	10.85	10.00	-	-
			90	1440	1320	15.31	13.75	-	-
			120	1380	1380	14.68	14.37	48.19	47.8
$^{24}Na^{+}$	1.6×10^{-4}M	control = 88 test = 86	30	660	480	7.5	5.38	-	-
			60	780	600	8.86	6.97	-	-
			90	780	720	8.86	8.37	-	-
			120	780	720	8.86	8.37	20.26	15.09

TABLE 8.8

EFFECT OF HYDRALLAZINE ON THE UPTAKE OF $^{42}K^{+}$ AND $^{24}Na^{+}$ BY FROG SARTORIUS MUSCLE

Isotope used	Drug concentration	Wt. of muscle in mg.	Time of exposure in min.	Total counts per min. in muscle		Counts per min. per mg. of muscle		Final counts remaining in muscle per min. per mg.	
				Control	Test	Control	Test	Control	Test
$^{42}K^{+}$	0.5×10^{-2}M	control = 96 test = 98	30	480	420	5.0	4.28	-	-
			60	780	420	8.12	4.28	-	-
			90	960	540	10.0	5.51	-	-
			120	900	660	9.37	6.73	28.43	22.27
$^{24}Na^{+}$	0.5×10^{-2}M	control = 82 test = 80	30	960	780	11.70	9.75	-	-
			60	960	1020	11.70	12.75	-	-
			90	1140	1320	13.90	16.50	-	-
			120	1020	1260	12.43	15.75	23.63	28.49

TABLE 8.9

EFFECT OF AZIDE ON THE UPTAKE OF $^{42}K^+$ AND $^{24}NA^+$ BY FROG SARTORIUS MUSCLE

Isotope used	Drug concentration	Wt. of muscle in mg.	Time of Exposure in min.	Total counts per min. in muscle		Counts per min. per mg. pf muscle		Final counts remaining in muscle per min. per mg.	
				Control	Test	Control	Test	Control	Test
$^{42}K^+$	$10^{-2}M$	control = 95 test = 96	30	360	240	3.78	2.50	-	-
			60	540	300	5.68	3.12	-	-
			90	900	360	9.47	3.75	-	-
			120	840	360	8.84	3.75	31.81	10.86
$^{24}Na^+$	$10^{-2}M$	control = 92 test = 92	30	1380	1080	15.00	11.73	-	-
			60	1800	2100	19.56	22.82	-	-
			90	2100	3480	22.82	37.82	-	-
			120	1980	3420	21.52	37.17	36.29	63.39

TABLE 8.10

EFFECT OF CYANIDE ON THE UPTAKE OF $^{42}K^{+}$ AND $^{24}NA^{+}$

BY FROG RECTUS ABDOMINIS AND SARTORIUS MUSCLES

Isotope used	Drug concentration	Wt. of muscle in mg.	Time of exposure in min.	Total counts per min. in muscle		Counts per min. per mg. of muscle		Final counts remaining in muscle per min. per mg.	
				Control	Test	Control	Test	Control	Test
$^{42}K^{+}$	1.5×10^{-2}M	Rectus abdominis Control=152 test =152	30	300	300	1.97	1.97	-	-
			60	960	390	6.31	2.56	-	-
			90	996	300	6.55	1.97	-	-
			120	840	240	5.52	1.57	19.1	4.85
$^{24}Na^{+}$	1.5×10^{-2}M	Sartorius Control=112 test =113	30	2160	2580	19.28	22.83	-	-
			60	2760	5160	24.64	45.66	-	-
			90	2760	5160	24.64	45.66	-	-
			120	2400	5580	21.42	49.38	32.81	67.72

TABLE 8.11

EFFECT OF DNP ON THE UPTAKE OF $^{42}K^{+}$ AND $^{24}NA^{+}$ BY FROG SARTORIUS MUSCLE

Isotope used	Drug concentration	Wt. of muscle in mg.	Time of exposure in min.	Total counts per min. in muscle		Counts per min. per mg. of muscle		Final counts remaining in muscle per min. per mg.	
				Control	Test	Control	Test	Control	Test
$^{42}K^{+}$	$10^{-4}M$	control = 70 test = 68	30	540	420	7.71	6.17	-	-
			60	720	240	10.28	3.52	-	-
			90	720	180	10.28	2.64	-	-
			120	660	132	9.42	1.94	28.34	6.28
$^{24}Na^{+}$	$0.5 \times 10^{-4}M$	control = 74 test = 70	30	960	840	12.97	12.00	-	-
			60	1080	1500	14.59	21.42	-	-
			90	1260	1980	17.02	28.28	-	-
			120	1080	2160	14.59	30.85	23.71	54.13

TABLE 8.12

EFFECT OF ANOXIA ON THE UPTAKE OF $^{42}K^{+}$ AND $^{24}NA^{+}$

BY FROG SARTORIUS MUSCLE

Isotope used	Wt. of muscle in mg.	Time of exposure in min.	Total counts per min. in muscle		Counts per min. per mg. of muscle		Final counts remaining in muscle per min. per mg.	
			Control	Test	Control	Test	Control	Test
$^{42}K^{+}$	control = 144	30	1020	1020	7.08	7.23	-	-
	test = 141	60	1440	1080	10.00	7.65	-	-
		90	1740	1080	12.08	7.65	-	-
		120	1920	1020	13.33	7.23	45.40	25.22
$^{24}Na^{+}$	control = 77	30	2700	3180	35.06	41.29	-	-
	test = 77	60	3420	3720	44.41	48.31	-	-
		90	4200	4560	54.55	59.22	-	-
		120	4300	5700	55.84	74.02	-	-
		150	4500	5700	58.44	74.02	49.67	57.99

TABLE 8.13

EFFECTS OF RESERPINE AND HYDRALLAZINE ON THE K^+ CONTENT OF FROG SARTORIUS MUSCLE

No.	Drug.	Dose	No. of estimations	K^+ in mEq. per kg. wet tissue	Level of significance
(1)	Reserpine	1.6×10^{-4}M	2	68.92	
	control	-	2	63.81	
	Reserpine	0.8×10^{-4}M	3	78.33	Not significant
	control	-	3	82.59	
	Reserpine	0.16×10^{-4}M	3	82.71	
	control	-	3	87.55	
(2)	Hydrallazine	10^{-2}M	3	34.40	$P < 0.01$
	control	-	3	63.33	Not significant
	Hydrallazine	0.5×10^{-2}M	5	59.76	(P nearly equals 0.1)
	control	-	5	77.33	
	Hydrallazine	0.5×10^{-3}M	3	70.49	Not significant
	control	-	3	77.78	

TABLE 8.14

EFFECTS OF DRUGS ON THE K^+ CONTENT OF RESTING FROG SARTORIUS MUSCLE

No.	Drug.	Dose.	No. of estim-ations	K^+ in mEq. per kg. wet tissue	Level of significance
(1)	Azide	10^{-2}M	4	43.20	$P < 0.05$
	control	-	4	70.70	
(2)	Cyanide	1.5×10^{-2}M	1	31.48	
	control	-	1	62.97	
	Cyanide	0.75×10^{-2}M	1	50.15	
	control	-	1	66.86	
	Cyanide	0.15×10^{-2}M	1	43.33	
	control	-	1	58.76	
(3)	DNP	10^{-4}M	2	38.15	$P < 0.02$
	control	-	2	66.64	
(4)	Anoxia	$N_2 + CO_2$	3	45.76	$P < 0.02$
	control	-	3	61.05	

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CHAPTER IX

(Table will be found at the end of this chapter)

CHAPTER IX

EFFECT OF HYDRALLAZINE ON IRON, RED BLOOD CORPUSCLES AND IRON ENZYME SYSTEMS

Introduction.

Drugs formerly used as antihypertensive agents in man and animals and which do not block ganglion transmission, cause adrenergic block or have a direct action on the central nervous system - for example thiocyanate[1], nitroprusside[2], azide[3], dimercaprol, and other thiol compounds and thiohydrazines[4] - have a chemical affinity for certain trace metals. Using normal and renal-hypertensive rats, Schroeder and Perry[5] tested compounds known to be capable of forming chelation complexes with metals but which were not previously tested for effects on the blood-pressure. They found that some lowered the blood-pressure in anaesthetised renal-hypertensive rats but not in the normotensive animals. They put forward the hypothesis that chelating agents and weakly bound metal complexes combined or formed association complexes with one or more trace metal ions in hypertensive rats and induced a fall in blood-pressure.

Schroeder and Perry[5] also suggested that hydrallazine /

hydrallazine inactivates pherentasin - the pressor principle which they found in blood. The hydrazine group in the hydrallazine molecule is the most active part. It has a marked affinity for thiol and carbonyl groups which are present in the molecules of several biologically important substances, including cysteine, glutathione and pyruvic acid. It also combines with heavy metals.

Treatment with hydrallazine for long periods causes, in certain patients, symptoms resembling the collagen disease, and disseminated lupus erythematosus (D.L.E.). This is now known as the "hydrallazine syndrome"(7,8), and is generally accompanied by arthritis, fever, weakness, loss of weight, anaemia and in some cases the presence of "D.L.E." cells in the peripheral blood. Recent studies carried out by Comens(9) and Gardner(10) have shown that prolonged administration of hydrallazine to dogs causes a syndrome which resembles that found in humans suffering from overdosage with this drug but which bears no clear relationship to naturally-occurring D.L.E.

A solution of hydrallazine when withdrawn in a syringe /

syringe with a metal nozzle, went pink after a time. This was considered to be due to a reaction between the metal and the drug in solution, and possibly a reflection of its ability to chelate. An attempt has therefore been made to prepare chelates of hydrallazine with different metals, including iron, and to characterise them. The subsequent isolation and characterisation of a stable iron chelate, and the knowledge that hydrallazine was known to cause anaemia, led to an investigation of the effects of this drug on iron-protein enzyme systems. It was also tested for the inhibition of cysteine oxidation by iron.

Certain hydrazines, for example phenylhydrazine, are known for their haemolytic action. Since hydrallazine has a reactive hydrazine group, it was felt that it might be capable of producing a haemolytic effect. An attempt has therefore been made to study the effects of this drug on red blood corpuscles both in vivo and in vitro.

Methods and Results.

Since it is difficult to treat separately the experimental methods used and the results obtained, these are /

are presented together.

1. THE HYDRALLAZINE-IRON COMPLEX

(a) Chemistry.

When a 2 per cent w/v aqueous solution of hydrallazine hydrochloride was added to a 10 per cent w/v solution of ferric chloride ($FeCl_3$,6 H_2O), the colour changed from red to grey, to green and then to blue, and finally a grey precipitate formed slowly. This was sparingly soluble in water and was recrystallised from methanol to give a product with m.p. 210°C. Hydrallazine has one absorption peak below 210 mμ and a second peak between 255 and 269 mμ. When the UV spectrum of the hydrallazine-iron complex was obtained, the second peak disappeared - possibly due to the formation of the chelate ring.

Certain diamines, for example ethylene diamine, are capable of forming chelates with metals; in these the bond is formed by electron donation and the number of groups associated with the metal is known as the coordination number (Fig. 9.1a).

Iron is know to form cyclic chelation complexes known as chelates and it seems possible that it is forming /

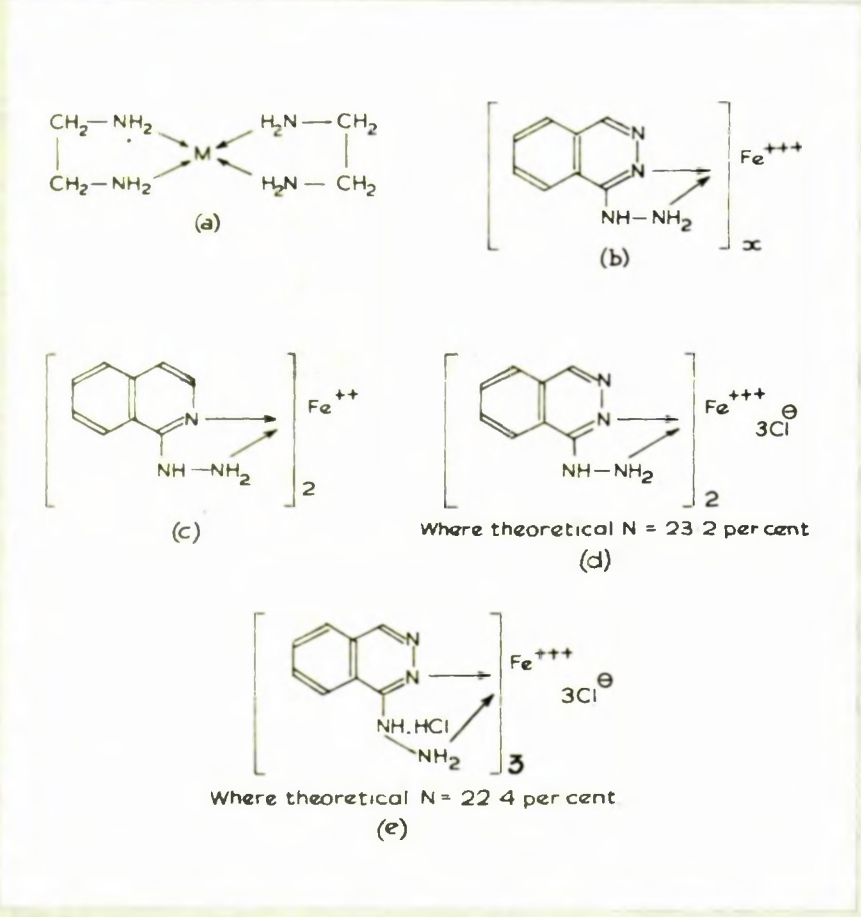

Fig. 9.1

Structural formulae of:

(a) Ethylenediamine-metal complex

(b) postulated hydrallazine-iron complex

(c) β-pyridylhydrazine-iron complex

(d) hydrallazine-iron complex with coordination number 4

(e) hydrallazine-iron complex with coordination number 6.

forming one with hydrallazine (Fig. 9.1(b)).

The five membered ring shown in Fig. 9.1(b) is theoretically the most stable structure. A similar structure is known in the case of the β-pyridylhydrazine complex with ferrous iron (Fig. 9.1c).

An analysis was obtained for nitrogen, and the figures obtained were N= 22.1 and N= 23.0 per cent. On the basis of this, two structures are possible: one in which the coordination number of iron is 4 (Fig. 9.1d) in which the theoretical N= 23.2 per cent; and the other in which the coordination number is 6, and the compound precipitated as the hydrochloride of the secondary amino group (Fig. 9.1e) in which the theoretical N= 22.4 per cent.

As the coordination number of ferric iron (Fe^{+++}) is usually 6, the structure shown in Fig. 9.1e is favoured and the analysis supports this. Similar analytical data also suggests a fixed composition and not a random association of molecules. It may, however, be possible that a mixture has been precipitated in which the form where the coordination number is 6 (Fig. /

(Fig. 9.1e) predominates with a lesser amount of a form with coordination number 4.

Chelates of hydrallazine were obtained from copper acetate, magnesium chloride, and manganous chloride. The copper and magnesium but not the manganese chelates precipitated out readily. Stability indices for iron and copper complexes are much higher than those for maganese and magnesium. The magnesium complex is the least stable. Hydrallazine chelates formed with these metals were not chemically characterised.

(b) Pharmacology of the hydrallazine-iron complex.

It was of obvious interest to see whether the chelate had different pharmacological properties from the chelating agent.

Hydrallazine causes a marked fall in the blood-pressure of the anaesthetised rat, and it antagonises the pressor effects of adrenaline in the same preparation. Its effects on this preparation were compared with those of the chelate.

Rats of either sex, weighing between 300 and 400 g., were anaesthetised by the intraperitoneal injection of 0.7 /

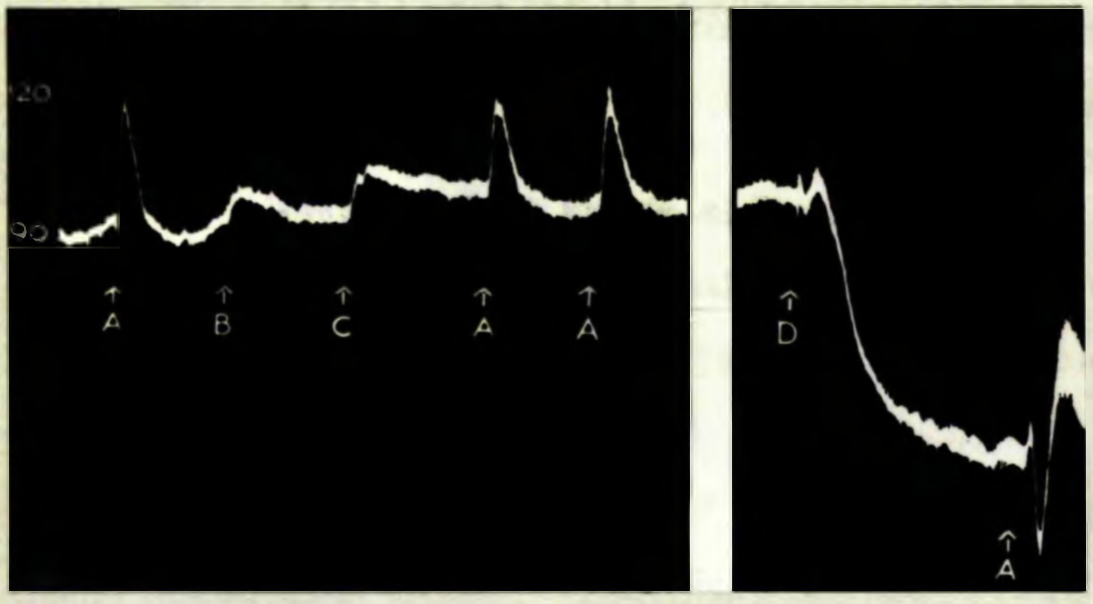

Fig. 9.2

Rat. Urethane anaesthesia. Blood pressure recorded from common carotid artery and drugs injected <u>via</u> the jugular vein.

At A,	adrenaline,	0.5 µg. per kg.
	B, propylene glycol,	0.1 ml. per kg.
	C, hydrallazine-iron complex,	0.50 mg. per kg.
	D, hydrallazine,	0.25 mg. per kg.

0.7 ml. per 100 g. body weight of a 25 per cent w/v urethane solution. Drugs were injected *via* the jugular vein, and the blood-pressure was recorded from the common carotid artery.

A typical record is shown in Fig. 9.2. The chelate which was insoluble in water was dissolved in propylene glycol and injected at dose levels of 0.5 to 2.0 mg. per kg. in a volume of 0.1 to 0.2 ml. followed by 0.4 ml. of saline. The chelate caused virtually no fall in blood-pressure but induced a slight rise. Propylene glycol itself is known to cause a rise in blood-pressure. Ferric chloride was inert in this respect. Small doses of hydrallazine of the order of 0.25 to 0.50 mg. per kg. had a marked hypotensive effect and these effects could be contrasted with those of the chelate. Hydrallazine changed the pressor response of adrenaline into a biphasic pressor-depressor response, but the chelate had no effect on this response.

2. EFFECTS ON CATALASE ACTIVITY.

A crystalline catalase preparation was obtained from the Sigma Chemical Company for this work, and estimations of catalase activity were made by the method of /

of Euler and Josephson[11].

The method used is briefly as follows. About 0.5 ml. of a suspension of the pure enzyme was diluted with 1.0 ml. water. 0.1 ml. of this dilute solution was made up to 100 ml. and 1 ml. of this solution was used for 50 ml. of chilled (0 to 4°) 0.01N hydrogen peroxide in M/150 phosphate buffer, at pH 6.8.

Catalase decomposes hydrogen peroxide according to the following reaction:

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

5 ml. samples were removed by means of a pipette after 4, 8, 12 and 16 minutes. 5 ml. of 2N sulphuric acid was immediately added to stop the reaction, followed by 10 ml. of a 10 per cent w/v solution of sodium iodide and one drop of 1 per cent w/v solution of ammonium molybdate. After 3 minutes the liberated iodine was titrated with 0.005N thiosulphate solution using soluble starch as an indicator. In experiments in which hydrallazine was used, the drug (0.1 ; 1.0 or 10.0 mg. per ml.) was incubated with the dilute solution of catalase. 1 ml. of this was added to the hydrogen /

hydrogen peroxide solution and the same procedure followed.

The velocity constant (K) of the enzyme-catalysed reaction was calculated from the expression

$$K = \frac{1}{t} \log_{10} \frac{a}{a - x}$$

where, t = reaction time

a = initial substrate concentration

x = substrate destroyed in time t

Values for K were calculated for 4, 8, 12 and 16 minutes and plotted against time (t) taking for (a) the titration value for 5 ml. of hydrogen peroxide at zero time in terms of 0.005N thiosulphate and (a-x) the titration value at time (t). Graphs were obtained by plotting K against t.

In <u>in vivo</u> experiments, rats of either sex weighing between 150 and 200 g. were injected with 5 mg. per 100 g. body weight of hydrallazine, and killed after 3 hours. Livers were rapidly dissected out, weighed and homogenised in ice-cold glass-distilled water using a Potter Elvehjem homogeniser. The homogenate was made up to a volume corresponding to 10 ml. per g. of liver and /

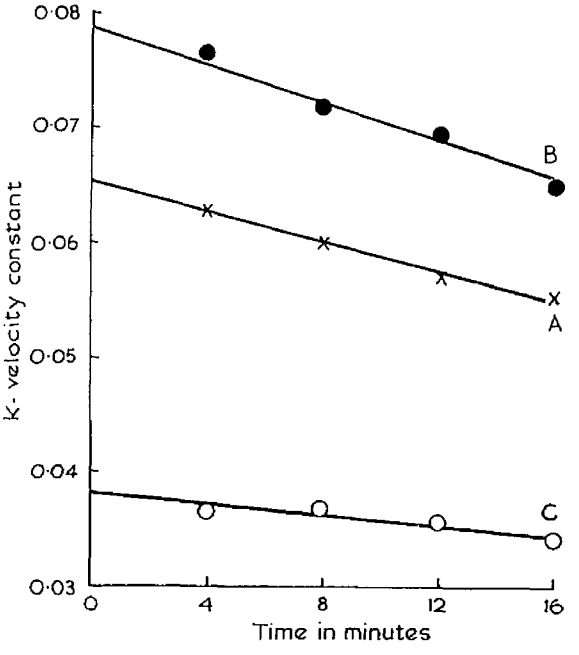

Fig. 9.3

Effect of hydrallazine on in vitro catalase activity.

A - control

B - hydrallazine, 0.10 mg. per ml.

C - " 10.0 " " "

Ordinate - velocity constant
Abscissa - time in minutes.

and an aliquot of this diluted 10 times with ice-cold distilled water. For the determination of catalase activity, 0.2 to 0.4 ml. of this dilute suspension was added to 50 ml. of 0.01N hydrogen peroxide solution. Control rats were injected with saline. This procedure was the same as adopted by Adams[12]. Other details are similar to those already described. Velocity constants were calculated and plotted against time.

Results.

Hydrallazine (0.1 and 1.0 mg.per ml.) had practically no effect on catalase activity in vitro, but 10 mg. per ml. caused about 50 per cent inhibition (Fig. 9.3). A single dose of hydrallazine (5 mg. per 100 g. body weight) 3 hours after administration did not reduce the catalase activity of rat liver.

3. CYSTEINE-CYSTINE OXIDATION.

The oxidation of cysteine to cystine is catalysed by trace metals, and iron and copper are especially effective. Since it has been established that hydrallazine forms chelation complexes with iron and copper, it was of obvious interest to see whether it could /

could inhibit this reaction.

Method.

Oxidation of cysteine to cystine was studied using the Warburg "Direct" method. The main chamber contained 1.9 ml. borate buffer at pH 7.0, together with 0.4 ml. of a 15 mg. per ml. cysteine solution. The side arm contained 0.1 ml. of 3.3×10^{-3}M ferric chloride solution and 0.6 ml. of water. In experiments with hydrallazine, 0.6 ml. water was replaced by the same volume of hydrallazine solution to give final flask concentrations after tipping of 0.01, 0.05, 0.1, 0.5 and 1.0 mg. per ml. The temperature was $24.0^{\circ} \pm 0.10$. After an equilibration period of 10 minutes, the contents of the side arm were tipped into the main chamber and readings taken at 5 minute intervals for 30 minutes.

Results.

Results are shown in Table 9.1. Cysteine itself was slightly oxidised at pH 7.0. Hydrallazine had a very slight effect on cysteine oxidation itself. The iron catalysed oxidation of cysteine was significantly inhibited ($P < 0.001$) by hydrallazine at 0.5 and 1.0 mg. per /

per ml. dose levels but had no effect at 0.1 mg. per ml. or lower doses.

4. EFFECT OF HYDRALLAZINE ON RED BLOOD CORPUSCLES.

BLOOD CORPUSCLES.

In view of the reports in literature regarding the toxic effects of hydrallazine, a few *in vivo* studies have been made using rabbits.

Method

For *in vivo* studies, rabbits weighing 2.5 to 3.0 kg. were injected intravenously with a solution of neutral $^{59}FeCl_3$ containing 2 µc. of radio-activity. Radioactive iron was obtained from Harwell as a solution of $^{59}FeCl_3$ containing 20 µc. per ml. Blood samples were collected from the ear vein into heparinised bottles after 24 hours and then on subsequent days. Blood samples were not collected from the injected ear, since contamination of the samples with spilt over ^{59}Fe was likely.

1 ml. whole blood samples made up to 10 ml. with water were counted on an Ekco scintillation counter. No attempts were made to separate plasma from corpuscles, since /

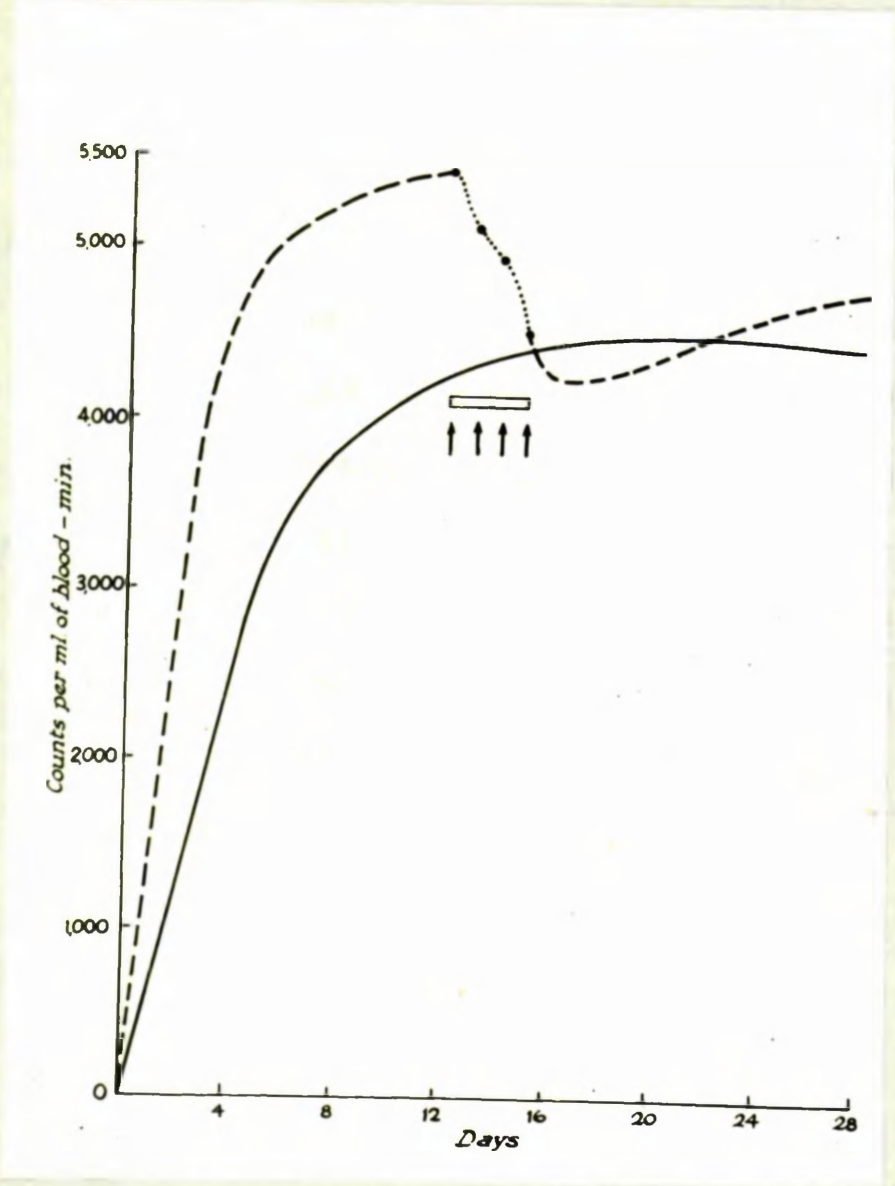

Fig. 9.4

Effect of hydrallazine on red blood corpuscles. Thick and dotted lines indicate control and test animals respectively. Arrows indicate administration of hydrallazine (40 mg. per kg.) to the test animal.

Ordinate - number of counts

Abscissa - time in days.

since most of the activity was found in the cells. Plasma activity was slightly high on the 1st and 2nd day after the injection of ^{59}Fe but it became negligible from the 4th day onwards. When the activity concentrations had become relatively stable, hydrallazine (40 mg. per kg.) was injected subcutaneously, and blood samples collected 24 hours after the injection and counted for radio-activity. The same procedure was repeated on subsequent days. The control animal was given saline. In later experiments, haemoglobin and haematocrit values were also determined. Blood samples were collected for about 3 weeks after the last hydrallazine injection. Curves were obtained by plotting radio-activity (corrected for decay) of the blood sample against number of days.

Results.

For these studies a total of 7 animals of either sex was used, and the results obtained from one rabbit are shown in Fig. 9.4. Iron gets slowly incorporated into red blood cells, and the maximum blood activity was reached in about 7 to 9 days. This level remained constant over the period of observation. The radio-activity /

radio-activity of the blood remained unchanged for 24 to 48 hours after the first or second injection of hydrallazine; but from the 3rd day onwards the activity began to fall. This fall continued on the 4th day and after, even though the injections of hydrallazine were stopped. Activity, however, returned to normal in about 15 days. A similar picture was obtained with 3 more rabbits but the effects were less marked, and one rabbit did not show any change. It had convulsions, so further treatment was stopped. One control animal showed a large variation in activity though the animal looked normal. After the 4th day of injection, 3 rabbits had convulsions and all the treated animals appeared to be extremely sensitive to external stimuli. The animals appeared more conscious and apprehensive. One animal was found to have died during the night. In one animal, in which both haemoglobin and haematocrit estimations were made, both showed a decline after hydrallazine treatment. Thus the haemoglobin value fell from 13.16 per 100 ml. to 10.86, and the haematocrit from 40 to 33, on the fourth day of hydrallazine administration. 16 days after stopping hydrallazine treatment, the haemoglobin value returned to 13.24 and the haemoterit to 42.

5. EFFECT OF HYDRALLAZINE ON IRON EXCRETION.

A few experiments were carried out to see whether the excretion of iron is in any way affected by hydrallazine.

Method.

Rats of either sex and weighing about 150 g. were injected intraperitoneally with 2 μc. of ^{59}Fe in the form of neutral $^{59}FeCl_3$ solution, and the urine and faeces were collected after 24 hours from a metabolism cage. The combined samples were partly dried in an oven at 100° and then transferred to a muffle furnace and ashed at 700° for 18 hours. The ash was taken up in dilute hydrochloric acid and the final volume made up to 5 ml. The ash was not completely soluble. The samples were counted in a well-type scintillation counter. This counter is quite efficient as long as the volume of the sample does not exceed 5 ml. Two groups of 4 animals were used. Two animals in each group were used as controls, and two as tests. Excretion of ^{59}Fe was maximum on the 3rd or 4th day, and thereafter it fell down and reached a plateau. Hydrallazine (2.5 mg. per 100 g. body weight) was then injected intraperitoneally

on /

on four successive days, and the urine and faeces collected, ashed and counted as above.

Results.

The counts were plotted against the number of days. On hydrallazine administration, the number of counts excreted in the urine and faeces increased only in two animals, and two animals showed no effect. It is rather difficult to draw any conclusions from these observations since the number of counts excreted by a control animal varied to a certain extent.

The treated animals looked normal, and there was not much difference between them and the control animals. But when the dose of hydrallazine was increased to 10 mg. per rat, in about 2 hours the animals became breathless and found difficulty in moving. Both animals showed intermittent convulsions and died.

Discussion.

Hydrallazine combines with different metals and forms chelates. Such an iron-hydrallazine chelate is pharmacologically inert. Thus, it has no hypotensive action and it does not antagonise the pressor effects of /

of adrenaline on the blood pressure of the rat.

It has been reported[13,14] that hydrallazine when incubated with sera, proteins or polypeptides for 24 hours at 38°C, loses much of its hypotensive effect. This inhibition is less marked with an ultrafiltrate of serum, indicating the important role of the colloidal part. The reaction is still present when serum first heated at 56° is used, showing that an enzymatic process is unlikely. At the same time as the hypotensive effect of hydrallazine disappears, the ability to antagonise vasoconstriction due to barium chloride and histamine is also lost. Hydrallazine also loses its effect if incubated with ground arteries. It does not appear from this work [14] that there is any gross modification of serum proteins such as the formation of proteinic molecular complexes, since hydrallazine can be chemically detected in the serum, and the electrophoretic pattern of the serum proteins or sedimentation constant after ultracentrifugation remains unaltered. With metals, hydrallazine forms a complex by donating a pair of electrons, and the hydrallazine molecule loses its activity presumably due to the closure of the ring. Hydrallazine may not behave similarly towards proteins /

proteins since Rometsch[15] could not obtain evidence of such a complex.

Hydrallazine inhibits catalase only at very high concentrations; lower concentrations have no effect. Catalase activity *in vivo* is not inhibited. Hydrallazine inhibits the iron catalysed oxidation of cysteine to cystine. This effect is probably due to chelation of hydrallazine to iron.

The effect of hydrallazine on red blood cells appears to be haemolytic in nature, since with high doses of hydrallazine the activity falls rather sharply but is regained in a matter of days. This means that the iron liberated from haemolysed corpuscles is re-utilised for the formation of new cells. The effect is not an indication of inhibition in haemoglobin synthesis, since an average life cycle of red blood cell runs up to 120 days, and in that case one might not expect an early recovery. When a blood sample was incubated at 37° with hydrallazine, haemolysis was observed. The body tries to conserve as much iron as possible and hydrallazine may not be having any effect on iron excretion. Since most of the body iron is stored in haem in a very tightly bound form, it is rather /

rather difficult to visualise that hydrallazine (which chelates with metals) can sequester iron from haem and cause it to be excreted.

A few of the properties of hydrallazine are described in this chapter. Hydrallazine chelates with metals but how far chelation is responsible for its hypotensive effect is not known. Schroeder and Perry(5) have suggested that certain antihypertensive drugs (cyanate, azide and hydrallazine) chelate with metals with a resultant hypotensive effect. In the first instance, we do not know whether any particular metal is involved in hypertensive disease - except, perhaps, sodium. But on chemical grounds the hydrallazine-Na chelate will be very unstable, and hence an effect on sodium may, perhaps, be ruled out. In addition, hydrallazine lowers the blood pressure of a normotensive animal. The effect of hydrallazine on red blood corpuscles might explain the anaemias encountered during a long period of treatment with this drug. This may be due partly to haemolysis while loss of weight, general weakness etc. may be due to inhibition of cellular oxidation. Inhibition of the synthesis of haem is also possible since hydrallazine interferes in the tricarboxylic /

tricarboxylic acid cycle (Chapter V) and haem synthesis also takes place through this.

TABLE 9.1

EFFECT OF HYDRALLAZINE ON IRON-CATALYSED CYSTEINE OXIDATION

Oxygen uptake in µl. ± S.E.

Drug	Concentration µg.per ml.	No. of observations	Incubation Time (min.)		
			10	20	30
Hydrallazine	10	6	136(± 6.08)	156(± 6.24)	152(± 5.29)
"	50	6	128(± 10.44)	154(± 8.06)	153(± 7.21)
"	100	6	128(± 8.18)	159(± 6.16)	154(± 5.65)
"	500	6	10(± 0.85)	9(± 0.87)	7(± 1.1)
"	1000	6	12(± 1.73)	12(± 2.44)	14(± 3.31)
Control	-	29	132(± 2.6)	152(± 2.75)	152(± 2.91)

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CHAPTER X

CHAPTER X

DISCUSSION.

Reserpine and hydrallazine have been shown to interfere with the normal functioning of the living cell. The ways in which these drugs disturb cellular function are, however, different. In a preliminary discussion (Chapter IV) it was suggested that hydrallazine and reserpine may interfere with the energy production of the smooth muscle cell, and some of the observed effects can be explained on this basis.

Szent-Gyorgi[1] described the contraction of skeletal muscle as a reaction between actomyosin, adenosine triphosphate (ATP) and certain inorganic ions. This process utilised endogenously produced energy. The source of the energy is the terminal high energy phosphate bond of ATP which is synthesised by the oxidative reactions of carbohydrate metabolism. While the studies on these relationships have been carried out mainly with skeletal muscle, the same considerations probably apply to the biochemistry of smooth muscle[2]. The enzymes which control tissue oxidation form an integral part of the Krebs tricarboxylic acid cycle[3].

The /

The cell uses nutrient substances for the maintenance of the steady state, growth and reproduction, and it is generally agreed that, of these, carbohydrate is the most efficiently oxidised. This high efficiency is achieved by the introduction of phosphate into the molecule and by interpolation of a series of anaerobic reactions through which carbohydrates pass before the oxidative pathway starts. This chain of reactions starts with the carbohydrate molecule and ends in the production of carbon dioxide and water. The efficiency is increased when a portion of the energy yielded by cellular oxidation-reduction reactions is used for the formation of high energy phosphate bonds; and in normal cell metabolism oxidation leads to the synthesis of energy-rich phosphate bonds.

A considerable amount of interest has recently been shown in substances which depress high-energy phosphate bond formation without affecting oxygen consumption. This dissociation of oxidation from phosphorylation is termed "uncoupling", and the agents which do this are known as "uncoupling agents". It has often been suggested[4] that the fundamental action of many drugs is due to an ability to interact with,

or /

or to influence in some other way, the normal functioning of an enzyme system. The fact that many drugs act in minute amounts makes it attractive to speculate that they act on enzymes which are essential for normal cellular function, and which are themselves present in very small amounts[5]. Growth, reproduction, muscle contraction and nerve conduction involve the integration of chemical reactions which are catalysed by enzymes. Any drug or agent which influences an enzyme system directly or indirectly may have profound effects upon the behaviour of the cell, tissue or organ.

In some cases the action of a drug *in vivo* has been linked with its action on an enzyme system *in vitro*. A classic example of this type was demonstrated by Englehart and Loewi[6] who showed that eserine exerts its pharmacological action by inhibiting cholinesterase. Similarly cyanide, azide and related compounds exert their actions by inhibiting certain essential components of the cytochrome system including cytochrome-oxidase.

It is probably true to say that most drugs act at more than one site. Brody[7], for example, considers it improbable that any compound is sufficiently highly selective for it to act on one enzyme only, but the possibility /

possibility still remains that one receptor site may be so much more sensitive than the others so as to account for the major demonstrable pharmacological actions of an agent *in vivo*.

The idea that certain drugs may act by interfering with phosphorylation is not new[8,9]. It is obvious that any interference with phosphorylation and the production of energy rich compounds will alter the normal function of the cell. A considerable number of drugs including barbiturates, salicylates and antibiotics, apparently have in common the ability to depress energy yielding reactions without significantly depressing the normal oxygen uptake of the cell. Such compounds disrupt the link between oxidation and phosphorylation, while other compounds - for example cyanide and azide - exert their effects primarily upon oxidation, and inhibition of phosphorylation parallels the reduction in oxidation. Uncoupling always implies that the phosphorylative mechanisms are more sensitive to the action of a chemical agent than are the oxidative steps.

It is well known that the oxidation of a Krebs-cycle intermediate (*e.g.* succinate) involves a chain of /

of reactions. By means of flavoproteins, cytochromes etc., hydrogen and electrons are transferred from the substrate to atmospheric oxygen. High energy phosphate bonds are formed not only at the initial step but also at several other points in the chain of reactions. Attempts have been made to show uptake of inorganic phosphate during the later steps of the electron transport chain[10,11]. Thus Slater[12] has shown that phosphorylation, which takes place when α-ketoglutarate is oxidised by oxygen, occurs in those steps of the hydrogen transfer reactions between the substrate and cytochrome C. The number of energy producing steps in this chain seems to vary with the substrate[13,14]. The observed $P/_O$ ratios differ for different substrates, for example, the oxidation of succinate gives ratios of the order of 2.0 whereas with pyruvate the ratio is about 3.0. It has been generally accepted that the P/o ratio or net uptake of inorganic phosphate is an accurate measure of the ability of the tissue to synthesise high energy bond-containing compounds <u>in vitro</u>. It has been argued in Chapter IV on the basis of the results obtained in Chapters II and III, that hydrallazine has mainly a peripheral site of action. Further evidence presented in Chapter V points /

points to some relationship between the pharmacological action of hydrallazine and an effect upon carbohydrate metabolism in arterial smooth muscle. Gillis and Lewis[15] working with intestinal smooth muscle found a similar relationship between reserpine and carbohydrate metabolism. Under suitable conditions of pH and temperature, most intermediates of carbohydrate metabolism antagonised the depression by reserpine of drug-induced contractions of intestinal smooth muscle. In addition, a few intermediates of protein and fat metabolism were found to be effective. The author was, however, unable to study this antagonism between reserpine and intermediates of carbohydrate metabolism on the arterial smooth muscle because of the persistent action of reserpine on this tissue. In one experiment however, where recovery occurred, pyruvate and succinate were found to antagonise the inhibitory effects of reserpine to a certain extent. The results must be accepted with reserve because repeat experiments were not possible. The discussion of the results obtained from experiments on arterial smooth muscle, which is presented in Chapter V, is based on the concept that the ability of the substance to augment and sustain the /

the amplitude of contraction is derived from its ability to provide energy for smooth muscle contraction; and the effectiveness of a metabolite to antagonise the effects of hydrallazine and reserpine is considered to be a measure of its ability to serve as a source of energy for smooth muscle contraction. On this assumption it was shown that hydrallazine-treated tissues behaved in a manner similar to anoxic tissues, and that the intermediary metabolites which were effective against anoxia were also effective against hydrallazine. Thus pyruvate, succinate, fumarate, α-ketoglutarate and oxalsuccinate were active both against hydrallazine and anoxia. Intermediates of glycolysis were less potent, and intermediates of protein and fat metabolism also gave similar protection. Another parallel was seen in the type of recovery. The enzyme inhibitors - cyanide, azide and iodoacetate - behaved differently since none (excepting pyruvate) of the intermediates of carbohydrate metabolism had much effect. Anoxia has been shown to cause either a reduction in, or complete inhibition of, spontaneous tone and activity in isolated rabbit intestinal muscle[16] and the taenia coli muscle of the guinea pig[17]. At the same time the ability of the tissues to maintain electrically[17] or /

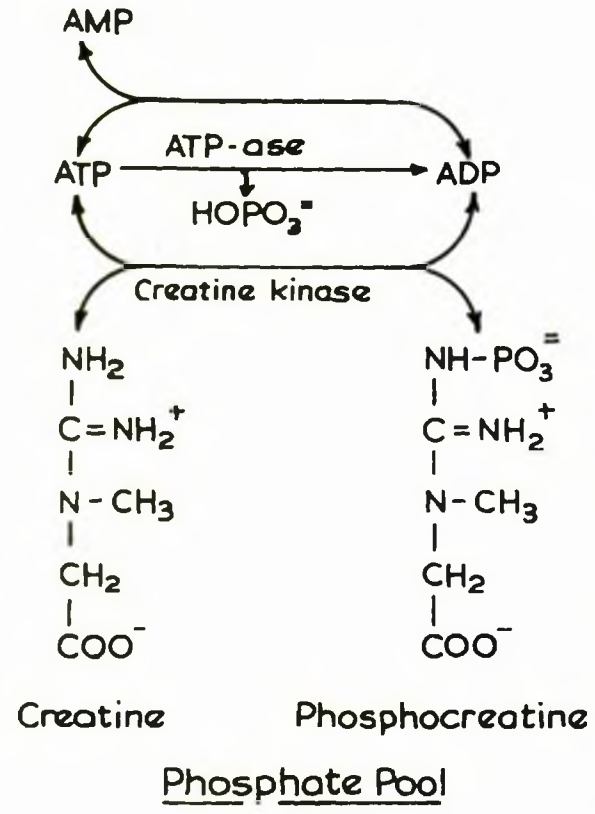

Fig. 10.1

The Phosphate Pool.

(from Intermediary Metabolism, published by GME - 714 Market Place, Madison, Wisconsin.)

or drug-induced tone[16] was completely abolished. These effects were accompanied by a corresponding decrease in creatine phosphate, but not in ATP content[17]. Anoxia has also been shown to cause a reduction in the ATP content of intestinal muscle[18]. The effects of reserpine and hydrallazine upon drug-induced tone were similar in many respects to those of experimentally produced anoxia. The direct measurements of the content of adenosine nucleotides of different rat tissues *in vivo* (Chapter VI) under the influence of hydrallazine and reserpine, supports to a certain extent this assumption. It was shown that reserpine significantly depressed ATP levels in the brain and liver with a corresponding rise in adenosine diphosphate (ADP). Hydrallazine was found to depress brain ATP level and to cause a rise in brain ADP. Hydrallazine and reserpine had virtually no effect on the ATP contents of heart and skeletal muscle. Hydrallazine significantly reduced the ATP/ADP ratio in liver, though it had no effect on ATP content of the liver. It was also suggested that creatine phosphate levels might be affected. The accepted role of creatine phosphate (Fig. 10.1) is to maintain the level of ATP *via* the Lohmann reaction. In brain, heart, liver, and /

and muscle, loss of phosphocreatine might be the first indication of disturbed phosphate metabolism and ATP levels might be affected only after all the available stores of creatine phosphate are exhausted. It was also argued that hydrallazine and reserpine affect tissue metabolism in different ways. It is possible that hydrallazine renders the tissue anoxic probably by virtue of its ability to interfere with the energy-producing reactions of carbohydrate metabolism. Reserpine appears to act one step later than hydrallazine (since oxidation precedes phosphorylation) because it has no effect on tissue respiration but at the same time depresses the process of phosphorylation. It seems, therefore, that hydrallazine interferes with oxidation itself, while reserpine interferes with phosphorylation which is in fact a result of oxidation. Both the processes ultimately lead to inhibition of the synthesis of energy-rich phosphate compounds. Evidence has been presented in Chapter VI which shows that, at least in brain and liver, both drugs depress the formation of energy-rich phosphate compounds *in vivo*. It has been shown in *in vitro* studies[19] that in oxidative phosphorylation reserpine acts as an uncoupling agent.

The /

The normal spontaneous activity of smooth muscle requires the steady production of energy from some readily available source. Under aerobic conditions, the metabolism can cope with the extra requirement, but anoxia eliminates[20] the more efficient sources of energy, namely oxidative metabolism. West and his colleagues[16] considered anaerobic energy to be only a small fraction of that available during aerobic activity. As a result of the loss of its most efficient energy-producing reaction, the total available energy is depressed during anoxia or after treatment with hydrallazine. It is suggested that reserpine may be acting in oxidative phosphorylation. It has been shown by Born and Bülbring[21] that 2-4 dinitrophenol (DNP) stimulates oxygen uptake at lower doses, and depresses the production of ATP at higher dose levels (greater than 3×10^{-5}M). DNP increases tension and spontaneous activity of the taenia coli at lower concentrations but higher concentrations at first increase and, when left in contact with the tissue, decrease tension and spontaneous movements. Moreover, when the muscle is stimulated by histamine, the tension increases but the increase is not maintained although histamine is still present. Similarly, when the /

the muscle is deprived of oxygen and glucose, it loses its spontaneous tension and activity, and the ability to maintain increased tension during electrical stimulation[17]. The immediate component of the contraction (spike phase) is unaffected by DNP. DNP is known to interfere in oxidative phosphorylation more than oxidation. It is, therefore, possible that the inability of DNP-treated muscle to maintain tension may be due to the inhibition of high energy phosphate bond formation. Born[17] suggested that two mechanisms provide energy for contraction:

(a) that responsible for the immediate response to drugs or electrical stimuli which was unaffected by DNP

and

(b) that responsible for both the sustained contraction and spontaneous activity which was presumably the oxidative mechanism, since it was abolished by anoxia or DNP.

Thus the energy for muscular contraction is probably derived from both aerobic and anaerobic processes; the former contributing the larger amount of energy. Prasad[22,23] obtained very active anaerobic spontaneous contractions of the isolated rabbit intestine. /

intestine. West and his colleagues[16] confirmed that anaerobic contractions do occur in rabbit intestinal smooth muscle. It has been observed that the ability of the arterial smooth muscle to maintain drug-induced tone was markedly affected both by reserpine and hydrallazine. The muscle lost tone under the influence of these drugs. Similar observations with reserpine were made by Gillis and Lewis[15] using guinea pig ileum. According to Born[17] both types of activity are supported largely by oxidative metabolism. It was observed that by rendering the tissue anoxic, the drug-induced contractions were reduced in magnitude but never completely eliminated. Since oxidation proceeds both aerobically and anaerobically, it is possible that the small response obtained after rendering the tissue anoxic may be due to anaerobic glycolysis. In the same way it may be true that hydrallazine and reserpine inhibit aerobic oxidative processes more effectively than anaerobic ones. Just as the adverse effects of anoxia develop slowly, reserpine and hydrallazine also exert their effects slowly. The effects of hydrallazine and anoxia on arterial smooth muscle were readily reversible. The fact that phosphorylated glucose and fructose were less effective

as hydrallazine antagonists may be a reflection either of the inability of these compounds to penetrate the cell membrane as such, or of the ineffectiveness of hydrallazine on anaerobic glycolysis.

Reserpine does not affect tissue respiration unless used in very high concentrations, while it has been shown by *in vivo* studies that it does affect the ATP/ADP ratio in brain and liver. It is therefore suggested that reserpine has an effect in oxidative phosphorylation rather than oxidation. The function of ATP may differ in different tissues, *e.g.* ATP is involved in muscular contraction. ATP may be playing a role in the storage and release of catechol-amines from the adrenal medulla, and perhaps it may have a specialised function in maintaining the normal brain processes. ATP may have a similar function in maintaining the normal beat of the heart muscle and it can be very difficult for any drug to affect ATP levels in the heart unless it acts as a poison. It might be useful to study *in vitro* the effect of reserpine on high energy phosphate compounds in mammalian heart and skeletal muscle, as it is possible that reserpine may behave differently towards these tissues when injected into /

into the intact animals. It has been shown by the author (Chapter VII) that reserpine depletes the rat suprarenal medulla of catechol amines and ATP in roughly the same proportions. This may be due to an effect on the release of catechol amines which, according to one view[24], is the primary action of reserpine. Since ATP has been implicated[25,26] in the storage and release of catechol amines from the adrenal medulla, reserpine - which releases catechol amines - should proportionately release ATP. It has also been shown[27] that reserpine depletes brain of noradrenaline, and since reserpine also depletes brain of ATP one wonders whether a similar link exists between catechol amines and ATP contents of brain. However, there is one fundamental difference between the effect of reserpine on brain and suprarenal medulla in that, in the former, depletion of ATP is accompanied by a corresponding rise in ADP while in the latter, depletion of ATP is not followed by the corresponding rise in ADP. This fact, however, lends support to the hypothesis that ATP may have specialised functions in different tissues and that in different tissues it may not necessarily behave in the same way towards drugs. It will be of great interest to study the effects on brain ATP

levels /

levels of compounds such as amphetamine (which stimulates the central nervous system and depletes the brain of catechol amines) and morphine (which depresses the central nervous system and releases catechol amines). On the basis of the above finding, the role of noradrenaline in maintaining normal mental processes may seem doubtful. It is by no means possible to explain the actions of drugs which depress the central nervous system on the basis of one single mechanism of action. According to different schools of thought 5-hydroxytryptamine[28], noradrenaline[24] and oxidative phosphorylation[7] are implicated in the action of certain antipsychotic drugs. Each theory has certain arguments for and against it, and one has to be careful before arriving at definite conclusions.

Studies on the release of potassium, or uptake of both potassium and sodium ions, have added support to some of the above findings (Chapter VIII). It has been shown both by tracer studies and direct potassium measurements that hydrallazine at higher concentrations (10^{-2}M) significantly releases potassium from a resting frog muscle, while reserpine is without any effect. Hydrallazine in similar concentrations reduces $^{42}K^{+}$ /

$^{42}K^+$ and increases $^{24}Na^+$ uptake. Cyanide and azide in high concentrations ($10^{-2}M$) release potassium from resting muscle but DNP and anoxia have no effect; but all four (cyanide, azide, DNP and anoxia) markedly inhibit $^{42}K^+$ and increase $^{24}Na^+$ retention. It is well known that under the influence of these poisons tissues lose potassium and gain sodium.(29) In many cases inhibition of potassium accumulation runs parallel with inhibition of oxygen consumption. It has been argued that hydrallazine exerts its effects by rendering the tissue anoxic, and that this view is reinforced by the observation that hydrallazine inhibits potassium and stimulates sodium retention. Its effects on the release of $^{42}K^+$ are comparable with those of azide and cyanide which act by inhibition of oxidation. Reserpine is believed to act in oxidative phosphorylation. DNP has no effect on the release of $^{42}K^+$ from resting frog muscle, neither has reserpine. DNP, however, markedly influences the uptake of $^{42}K^+$ and $^{24}Na^+$ (which are generally in opposite directions), but reserpine has no such effect. Gillis and Lewis(30) have shown potassium release by reserpine. From their observations, it appears that either their method of estimation was superior /

superior, or the factor of seasonal variation in the sensitivity of frog muscles may have played a part. (Most of their work was done in the late spring and early summer, while the author did his experiments in winter.)

On the basis of their effects on potassium accumulation, the enzyme inhibitors may be grouped in two groups. One group consists of compounds which depress respiration and potassium accumulation simultaneously (e.g. cyanide, azide, anoxia and hydrallazine), and the second group consists of compounds which depress potassium accumulation without affecting respiration (e.g. DNP and reserpine). The second group of compounds stresses the importance of high energy phosphate bonds (creatine phosphate and ATP) in potassium accumulation. Krebs and co-workers[31] have shown that guinea pig brain and kidney cortex retained potassium only in the presence of glutamic acid and α-ketoglutaric acid, to which the former could be easily converted. It is possible, therefore, that in the presence of substances which interfere with oxidative and high bond energy producing reactions, potassium may be released because it cannot be utilised. It has also been /

been argued by Fenn[32] that a close link exists between carbohydrate metabolism and potassium accumulation, and that a potassium cycle from liver to muscle and back - comparable to the carbohydrate cycle - might exist. It has been shown that the effects of hydrallazine on arterial smooth muscle (Chapter V) and of reserpine on guinea pig ileum[15] can be modified by certain intermediates of carbohydrate metabolism. In view of these observations, it is reasonable to suppose that reserpine and hydrallazine may have an effect on tissue potassium.

Hydrallazine and reserpine have a variety of different actions on different tissues, and it is probably unwise to attempt to interprete all the actions on the basis of interference with cellular metabolism. Both drugs lower the blood pressure, but the blood pressure-reducing property of reserpine appears to be secondary to sedation. Hydrallazine antagonises the peripheral effects of adrenaline more effectively than those of noradrenaline while reserpine enhances them. As has been pointed out earlier, hydrallazine may have some antiadrenaline properties besides being largely non-specific in its actions. Vasodilatation by /

by both these drugs is seen only if some vascular tone exists. Both drugs only slightly affect the blood pressure of spinal cats in which the basal tone is minimum. It must be remembered, however, that when the blood pressure is raised by infusion of adrenaline, hydrallazine promptly brings it back to normal, thus reinforcing the view that hydrallazine has a peripheral site of action. The mechanism responsible for maintaining basal tone in blood vessels is not fully understood. It has been suggested that basal vascular tone is a result of specific vasoconstrictor agents circulating in the blood(33). Granaat(34) has suggested that such agents may be released from the spleen. In addition, it is widely assumed that the suprarenal medulla makes a considerable contribution to the maintenance of basal vascular tone. Lofving and Mellander(35) could find no evidence for a circulating vasoconstrictor agent in cats which had been sympathectomised. They suggested that basal tone was the result of "smooth muscle automaticity", which they thought was probably to be regarded as a characteristic property both of heart muscle and many types of smooth muscle cells. Whatever the cause of basal tone, it must involve to a greater or lesser degree /

degree contraction of vascular smooth muscle, and this in turn involves an increased energy turnover. Thus if reserpine and hydrallazine affect the energy-producing mechanism of the smooth muscle cell, then a reduction of inherent tone would be expected. One manifestation of this phenomenon would be the fall in blood pressure. The extent of the observed fall in blood pressure after hydrallazine was dependent on the initial blood pressure level (Chapter II). The higher the latter, the more marked was the fall. Bein[36] stated that, as a rule, in man and experimental animals the higher the level prior to the administration of reserpine, the greater was the subsequent reduction in blood pressure. It must be pointed out that many other factors are likely to be involved in the action of reserpine and hydrallazine in experimental animals and in man. Nevertheless, a direct peripheral component in the depression of blood pressure following hydrallazine - and to some extent reserpine - cannot by any means be ruled out.

It has been shown that hydrallazine causes vaso-dilatation in the denervated hindquarters of the rat and in the rabbit's ear. Both reserpine and hydrallazine cause /

cause vasodilatation of isolated strips of horse carotid arteries. The vasoconstrictor actions of adrenaline, noradrenaline, histamine, 5-hydroxytryptamine or barium chloride are depressed by reserpine and hydrallazine, presumably because of the decreased ability of cellular metabolism to produce energy to support a drug-induced contraction of the muscle. A few experiments were done with the isolated rat hindquarters, and it was found that infusion of sodium pyruvate (1 mg. per ml.) together with hydrallazine could overcome, to a certain extent, the antagonism between adrenaline and hydrallazine. Sodium succinate was, however, found to be ineffective. Tripod and Meier[37] have also shown that increased vascular tone in the isolated hindquarters of the rabbit, produced by the infusion of noradrenaline, adrenaline, histamine and 5-hydroxytryptamine is antagonised by reserpine. Similar considerations probably apply to rescinnamine[38] - a drug closely related to reserpine. In completely isolated perfused blood vessels, the possibility of any action, other than a direct one, upon the vascular smooth muscle seems remote although the presence of nervous elements cannot be ignored.

Reserpine /

Reserpine and hydrallazine have different actions on different muscles. Thus, the action of reserpine upon drug-induced contractions of intestinal muscle is generally reversible. This is not the case in the isolated heart, auricles and arterial smooth muscle. Hydrallazine depresses heart and arterial smooth muscle but it has stimulant effects on rabbit intestine. In the consideration of mechanisms of drug action, it seems probable that all drugs act at more than one site. By pure chance, a compound may act specifically at only one site or on one enzyme and produce an effect. It may, however, be true that one receptor site may be sufficiently more sensitive than others and so account for its major *in vivo* actions. In the same way, drugs may have different affinities towards tissues, and hence may not produce the same effect.

Few studies have been carried out on the actions of reserpine and hydrallazine on metabolism. Reserpine has been found to inhibit the effect of the thyroid hormone on oxygen consumption in rats[40]. An *in vitro* antithyroid action of reserpine has been reported by Mayer and his colleagues[41]. Other workers[42] have been unable to show any direct effect on the thyroid /

thyroid gland. Kuschke and Frantz[43] reported that reserpine caused a hyperglycaemia in rabbits and dogs which was blocked by the hydrogenated ergot alkaloids. They suggested that this was due to stimulation of "hypothalamic substrates" and subsequent suprarenal stimulation. Hyperglycaemia was still seen when the splanchnic nerves were severed, but they assumed that the section of the splanchnic nerves did not guarantee complete suprarenal denervation.

Reserpine inhibits adenosine triphosphatase[44] but only at concentrations of 100 µg. per ml. or above. Oxidative phosphorylation in rat brain is also depressed[19,45]. Reserpine *in vitro* depresses the oxygen consumption of slices of cerebral cortex taken from the rat but only in high concentrations[46]. Recently, Gillis and Lewis[30] have put forward evidence for a reserpine-effect upon metabolism. According to these authors, the effects of reserpine on spontaneous and drug-induced tone resemble those of anoxia. Reserpine in fact renders the tissue anoxic by virtue of its ability to interfere with the energy-producing reactions of carbohydrate metabolism. An effect on oxidative metabolism is substantiated by their /

their observations that reserpine depresses respiration and causes an increased release of potassium from skeletal muscle. It has been shown by the author that reserpine has no effect on tissue respiration at low concentrations but depresses at higher dose levels. Hydrallazine has been shown to inhibit diamine-oxidase more effectively than monoamine-oxidase(47), but this does not help us since other hydrazine derivatives which have an effect on these enzyme systems are not hypotensive agents. Another important observation was that of Douglass and his colleagues(48) who have shown that hydrallazine inhibits the acetylation of sulphanilamide and glucosamine in pigeon liver extracts.

The observations presented in this thesis may explain some of the above data. In Chapter V, it has been shown that most of the intermediates of carbohydrate metabolism can protect arterial smooth muscle against hydrallazine-inhibition, and that these effects can be compared with those of anoxia. Several aromatic and heterocyclic hydrazides are acetylated <u>in vivo</u>(49). Chou and Lipmann(50) have established that the process of biological acetylation occurs in two stages:

(1) /

(1) Activation of acetate:

$$\text{acetate} + \text{CoA} + \text{ATP} \rightleftharpoons \text{acetyl CoA} + \text{AMP} + \text{PP}.$$

(2) Transfer of acetyl group:

$$\text{acetyl CoA} + \text{HX} \rightleftharpoons \text{acetyl} - \text{X} + \text{CoA}$$

where HX represents the substrate being acetylated.

It has been shown[48] that hydrallazine did not affect the rate of formation of acethydroxamic acid from hydroxylamine (which shows that the first reaction is not involved), but that incubation of hydrallazine in the reaction mixture in the absence of sulphanilamide results in its disappearance. In short, the drug has no effect on the formation of acetyl~CoA but reacts with acetyl CoA and is acetylated. Acetyl~CoA plays an important part in the tricarboxylic acid cycle, and the reaction pyruvate $\rightleftharpoons$ citrate is mediated through acetyl~CoA. Since hydrallazine interacts with acetyl~CoA, one might expect interference in the functioning of the tricarboxylic acid cycle. The protection afforded by some of the intermediates of the tricarboxylic acid cycle towards the inhibitory effects of hydrallazine (Chapter V) appears, therefore, to support this view. The observation that the depression /

depression of respiration of arterial smooth muscle can be antagonised to a certain extent by glucose, pyruvate, fumarate and glutamate points in the same direction.

Hydrallazine chelates with metals, inhibits oxidation of cysteine and inhibits catalase at high concentrations. Hydrallazine in high doses has a haemolytic effect, but its influence on iron excretion appears doubtful. It is an interesting finding that hydrallazine can chelate with heavy metals but how far this property is useful in explaining its pharmacological properties is not certain.

What light is shed upon the mode of action of these drugs in hypertensive human subjects by experiments upon isolated tissues and organs, and intact animals? This is really a very difficult question. In general, animal experiments give some indication of the effects produced in human subjects, but this may not be necessarily always true, and data obtained with animal experiments should be treated with some reserve. It may be felt that the doses of hydrallazine and reserpine used in some experiments were far in excess of those which /

which produce a clinically useful effect. While this criticism is undoubtedly valid for some preparations, it is not true of isolated heart and perfused blood vessels in which perfusion with 1 µg. per ml. of reserpine or 1 to 10 µg. per ml. of hydrallazine produced demonstrable effects. The doses used in experiments with cats are similar to those used in man, and the effects are comparable. Thus reserpine induces bradycardia and peripheral vasodilatation, while hydrallazine produces tachycardia and peripheral vasodilatation. The onset of the hypotensive effect of hydrallazine does not start immediately after the administration of the drug but takes some time (usually 15 to 20 minutes) in both man and animals. The peripheral vasodilatation caused by this drug is, in the opinion of the author, its main action, and most of the other pharmacological properties of this drug can be explained on this basis. It has been demonstrated quite conclusively by Stunkard and his colleagues[51] that (a) hydrallazine lowered the blood pressure of four subjects with spinal cord transection as high as T 1-5, and (b) hydrallazine increased bloodflow in extremities denervated by spinal cord transection and sympathectomy. Similarly Wilkinson /

Wilkinson and his colleagues[52] have shown that intra-arterial injection of hydrallazine results in a marked increase in skin temperature. All these observations support the peripheral mode of action of hydrallazine in man. Similarly the doses used and the effects observed after reserpine are comparable in man and animals. It may be true, however, as Tripod and Meier[53] point out that the isolated tissue is generally less sensitive than the intact experimental animal or man, and that the doses used do in fact correspond.

Studies of the action of reserpine upon metabolic function in man are not conclusive. Hafkenschiel and his colleagues[54] were unable to show any alteration in glucose or oxygen uptake in man with reserpine in doses which reduced the blood pressure by 20 per cent when compared with control levels. Cerebral vascular resistance was decreased, but was not followed by any metabolic disturbance in the brain. Hafkenschiel and others[55] in a subsequent publication reported that cerebral glucose utilisation, but not cerebral oxygen uptake, was depressed in 5 hypertensive patients. Clinical investigations are influenced by many considerations /

considerations, and it is often very difficult to get strictly quantitative results.

Burrel[56,57] found that the mental depression following reserpine was abolished by oral administration of sodium succinate, which he considered may have been active by virtue of its ability to restore the disturbed metabolism of brain cells. He pointed out that disorders of carbohydrate metabolism were known to occur in functional psychoses. This observation lends a strong support for a metabolic site of action for reserpine. Reports concerning the metabolic effects of hydrallazine in man are very few, and McCall[58] reports that hydrallazine increases the cerebral bloodflow and oxygen utilisation of the brain. It may be possible that this increase in oxygen consumption may be proportional to the vasodilatation and may simply be a reflection of a physical process.

In conclusion, it may be said that the evidence so far presented supports the possibility of a peripheral site of action for hydrallazine and reserpine. Evidence is more convincing for hydrallazine. Both drugs have marked effects on isolated tissues and, in the author's opinion, /

opinion, they exert their physiological effects by interfering in tissue metabolism, probably that supplying energy for muscular contraction.

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SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Reserpine and hydrallazine are used in the treatment of certain types of hypertension. Reserpine is also used in certain forms of mental illness.

In the introductory chapter of this thesis current hypotheses on the mode of action of reserpine and hydrallazine, as antihypertensive agents, are described. Reserpine is thought to depress central sympathetic tone, although the means by which this is achieved is not clear. Hydrallazine is believed to act upon the vasomotor centre but its actions seem to have a large peripheral component. The work described in this thesis was undertaken to clarify some aspects of the mode of action of these drugs at cellular levels.

In Chapters 2 and 3, experimental studies using preparations of isolated cardiac, smooth and skeletal muscle have been described. Hydrallazine, dihydrallazine, 3-phenyl-6 hydrazinopyridazine nitrate, 1-hydrazino-isoquinoline hydrochloride and 3:6 dihydrazino pyridazine nitrate have been used in preliminary studies. Reserpine was also used on preparations of arterial smooth muscle. Hydrallazine and related compounds were tested for antagonism to various pressor reflexes which are mediated

via /

via the central nervous system. These experiments showed that hydrallazine and related compounds reduced smooth muscle tone, and after a latent period depressed drug-induced contractions of arterial smooth muscle. Reserpine was found to reduce the inherent muscular tone and to depress drug-induced contractions of smooth muscle. In Chapter 3, it was shown that reserpine and hydrallazine non-specifically antagonised drug-induced contractions of arterial smooth muscle, and that they did not act upon specific drug-receptors.

Chapter 4 has been devoted to the discussion of the results obtained in Chapters 2 and 3. It was pointed out that hydrallazine and dihydrallazine failed to antagonise all the pressor reflexes which were elicited, and this evidence speaks against their having a central site and mechanism of action. If this was the case, all pressor reflexes which are mediated via the central nervous system should have been antagonised or depressed. This does not, however, rule out the possibility that hydrallazine acts specifically upon certain cell groups in the central nervous system. If this is true, some reflexes would be antagonised but not others. Hydrallazine was shown to reverse the pressor effects of adrenaline /

adrenaline but not those of noradrenaline. Antagonism by hydrallazine to some pressor reflexes only was explained on the basis of quantitative variations in the amounts or proportions of these humoral agents which are secreted during these reflexes. The fact that hypertension caused by a constant infusion of a solution of adrenaline was promptly brought back to normal levels by hydrallazine, also speaks in favour of a peripheral site of action.

Many of these effects can be explained by assuming that hydrallazine interferes with the normal energy-providing mechanisms of the smooth muscle cell. Chapter 5 describes the results of experiments which indicate that hydrallazine has an effect upon carbohydrate metabolism which underlies its action upon isolated arterial smooth muscle. Intermediates of carbohydrate metabolism antagonised hydrallazine-depression of drug-induced contractions of arterial smooth muscle. Some of the intermediates of protein and fat metabolism were also effective in this respect. Reserpine was so persistent in its action that the tissue did not recover from its effects. Drug-induced contractions of arterial smooth muscle were also antagonised by cyanide and by anoxia.

Most /

Most of the intermediates of carbohydrate metabolism afforded protection against anoxia but not against cyanide. It was concluded that the use of hydrallazine is, in effect, analogous to rendering the tissue anoxic. The patterns of recovery of anoxic and hydrallazine-treated tissues were parallel - which reinforces the view stated.

Manometric studies of the effects of hydrallazine and reserpine on respiration of isolated rat brain, liver and kidney were made, using the Warburg "Direct" method. Preparations made from rabbit arterial smooth muscle were also used. The results of these experiments, which are presented in Chapter 6, show that reserpine had no effect upon the oxygen uptake of rat brain, liver or kidney, unless it was used in high concentrations. Hydrallazine depressed the respiration of all the tissues examined.

Studies on respiration were supplemented by estimations of adenosine nucleotides in rat brain, liver, heart and skeletal muscle. Reserpine was found to cause in vivo inhibition of oxidative phosphorylation in brain and liver, since the ratio ATP/ADP was significantly lowered. /

lowered. Hydrallazine behaved similarly in brain and liver, but not in skeletal muscle or heart. Reserpine had no effect upon the adenosine nucleotide levels in muscle and heart. On the basis of this evidence it has been suggested that hydrallazine may interfere with biological oxidation, and reserpine with oxidative phosphorylation.

Observations concerning the effects of reserpine on the catechol amine content of the rat suprarenal medulla have been presented in Chapter 7. It was found that reserpine depleted the suprarenal medulla of catechol amines and ATP. It was observed that both the amines and ATP disappeared in roughly the same proportions and no breakdown products of ATP were found. It was, therefore, suggested that reserpine had different actions in brain and the suprarenal medulla, since in the former depletion of ATP was accompanied by a corresponding rise in ADP while in the latter, depletion of ATP was not followed by a corresponding (and expected) rise of ADP levels. It was also pointed out that ATP might possess a specialised function in connection with the release or storage of catechol amines in the suprarenal medulla.

Evidence /

Evidence has been presented (Chapters 5 and 6) which suggests that reserpine and hydrallazine may interfere with normal cell metabolism. Metabolic inhibitors, such as cyanide and DNP, have marked effects on the transport of sodium and potassium ions in tissues. Experiments have been described in Chapter 8 in which the action of reserpine and hydrallazine upon the movements of these ions in frog skeletal muscle were studied, using isotopic tracer techniques. It was found that reserpine had virtually no effects either upon the release of potassium or on the uptake of potassium or sodium. Hydrallazine, however, increased potassium release, decreased potassium and increased sodium uptake. Cyanide and azide markedly increased potassium release but DNP and anoxia had virtually no effect. On the other hand, cyanide, azide, DNP and anoxia all decreased potassium and increased sodium uptake.

In Chapter 9, some other properties of hydrallazine have been described. Hydrallazine was shown to form chelates with different metals, and the iron chelate had been characterised both chemically and pharmacologically. Hydrallazine was shown to inhibit catalase slightly. It depressed the iron-catalysed oxidation of cysteine to cystine. /

cystine. The effect of high doses of hydrallazine on the blood picture of the rabbit was studied using isotopically labelled iron. The excretion of iron was also studied.

In the Discussion, it is suggested that interference with carbohydrate metabolism may result in a decreased availability of energy for muscular contraction. It was postulated that hydrallazine produced an "anoxia-like" condition in smooth muscle, while in oxidative phosphorylation reserpine acted as an "uncoupling" agent. Such an effect might explain the reduction of inherent tone in vascular smooth muscle. A reduction in the efficiency of the enzymes controlling metabolism may reduce the ability of muscle to retain potassium, resulting in the release of the potassium ion.

The possibility of a peripheral component in the effect of hydrallazine in hypertension is discussed. Reserpine also has a peripheral component in addition to its effects upon the central nervous system, which probably contributes largely to its main action. Reserpine causes the release of catechol amines and 5-hydroxytryptamine from different tissues, but it is still not clear whether this property explains its mode of action.