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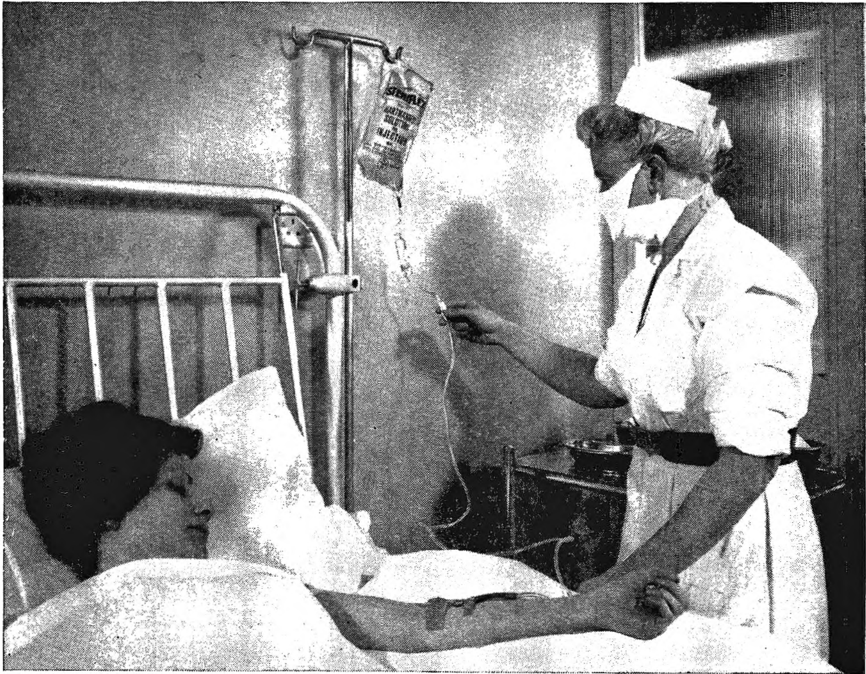

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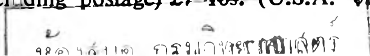

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Research Papers

The role of the nervous system in local inflammatory responses

H. J. FEARN, S. KARADY* AND G. B. WEST

Inflammation produced in rats by three means has been investigated. The response produced by the external application of xylol seemed to be the result of stimulation of the axon reflex in the area of skin where the substance was applied; it was prevented by chronic denervation of the sensory nerves. The inflammatory response produced by dextran involved the release of 5-hydroxytryptamine; it was prevented by the prior treatment with specific antagonists. The inflammatory response produced by heat treatment did not involve the axon reflex and appeared to be the result of another mechanism, probably involving bradykinin.

WHEN dextran is injected into rats, an acute inflammatory response results. This is called the anaphylactoid reaction and is characterised by pruritus and gross oedema of the extremities. When the hind-paws of rats are immersed in a water-bath at 45° for 30 min, a similar oedema develops below the tibio-tarsal articulation (Rocha e Silva & Antonio, 1960). The external application of xylol also produces oedema with leakage of blood proteins (Aschheim & Zweifach, 1964). We have now examined the role of the nervous system in these three inflammatory responses.

Experimental

METHODS

Groups of at least 5 male Wistar albino rats, 150–200 g, obtained from Bengers, Ltd., Holmes Chapel, were used in each experiment. Either they were injected intravenously with dextran (180 mg/kg) when their hind-paws were immersed in a water-bath at 45° for 30 min after light pentobarbitone anaesthesia (40 mg/kg intraperitoneally), or xylol (2 drops) was applied to each of their hind-paws. All animals received azovan blue dye (10 mg/kg intravenously) 10 min before each of these procedures. The rats were then carefully watched during the next 6 hr and records were made of the degree of oedema formation and of blueing, using an arbitrary scale of 0 to +++.

Surgical procedures. For the local acute denervation experiments, the sciatic and femoral nerves of the animals under ether anaesthesia were cut above the knee and the animals were used either immediately afterwards or about 3 hr later. Chronic denervation experiments were similarly made and the animals were used 3 weeks after the operation when visible signs of degeneration of the nerve fibres such as loss of muscular tone and of claws were prominent.

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Decerebration was carried out by the dorsal approach under ether anaesthesia; artificial respiration was not necessary and the blood pressure was well maintained at about 80 mm Hg. Pithing of the central nervous system was done by inserting a metal probe through the right eye under ether anaesthesia and then maintaining life with artificial respiration. Spinal transection was done under ether anaesthesia, after exposing the spinal cord at the required level, by cutting the cord with scissors; the blood pressure in these rats was also well maintained, the average value being 70 mm Hg.

Adrenalectomy was done under ether anaesthesia using the dorsal approach; 4 days later, the animals were either tested with the inflammatory agents or they were decerebrated or pithed. In a few experiments, the spinal cord of adrenalectomised animals was transected. Adrenaline (0·5 mg/kg) or noradrenaline (4 mg/kg) was injected subcutaneously 30 min before the inflammatory response was initiated. Other animals received the prior injection of adrenaline, 1 μg, or noradrenaline, 10 μg, intradermally, or of cocaine, 5 mg/kg, either subcutaneously into the paws to anaesthetise the sensory nerve endings or intramuscularly high up the thigh to produce infiltration block, or of cortisone (25 mg/kg subcutaneously).

Results

DEXTRAN RESPONSE

This response was maximal about 10 min after the injection, both oedema and blueing giving maximal scores (+++ results). It was modified by some of the surgical procedures; for example, decerebration, pithing or spinal transection each prevented its development when the dextran was injected immediately after the surgery, yet no inhibition was found when the response was tested 3 hr after surgery. As these surgical procedures did not prevent the dextran response in adrenalectomised rats even when the injection was made as soon as possible after the surgical procedure, it is possible that stimulation of the sympathetic nervous system and of the adrenal cortex occurred during and immediately after the surgery, the released adrenaline, noradrenaline and cortical hormones preventing the reaction. Furthermore, subcutaneous injections of adrenaline, noradrenaline or cortisone prevented the dextran reaction. Neither anaesthesia of the sensory nerve endings by cocaine nor infiltration nerve block modified the response. These results are shown in Table 1.

THERMAL RESPONSE

This inflammatory reaction developed during the heating and was maximal about 1 hr later. Many petechiae and much salivation were observed and oedema was intense. As with the dextran response, decerebration, pithing and spinal transection, each inhibited the thermal response when this was tested immediately after the surgical procedure.

Again, this inhibition may have been the result of stimulation of the sympathetic nervous system and of the adrenal cortex since it was not found when the test was made 3 hr later. Furthermore, no inhibition was found in adrenalectomised animals. Subcutaneous injections of adrenaline, noradrenaline or cortisone prevented the thermal reaction as effectively as they did the dextran response. Cocaine was without effect (see Table 1).

TABLE 1. EFFECT OF VARIOUS PROCEDURES ON THE LOCAL INFLAMMATORY RESPONSES PRODUCED BY DEXTRAN, HEAT OR XYLOL. Groups of 5 rats were used in each experiment. Results and expressed as 0 (= no effect) and + (= prevention of response).

Procedure	Agent producing local inflammatory response		
	Dextran	Heat	Xylol
Acute denervation	0	0	0
Chronic denervation	0	0	+
Cocaine (subcutaneously)	0	0	+
Cocaine (intramuscularly)	0	0	0
Acute decerebration	+	+	Reduced
3 hr after decerebration	0	0	0
Acute pithing	+	+	Reduced
3 hr after pithing	0	0	0
Acute spinal transection	+	+	Reduced
Spinal transection (3 hr later)	0	0	0
Adrenalectomy	0	0	0
Adrenalectomy + decerebration	0	0	0
Adrenalectomy + pithing	0	0	0
Adrenalectomy + spinal transection	0	0	0
Adrenaline or noradrenaline	+	+	+
Cortisone	+	+	0

XYLOL RESPONSE

Within 5 min of application of the xylol, the blueing response was maximal although the oedema reaction did not reach maximal values until about 15 min later. In contrast to the dextran and thermal responses the response resulting from xylol was prevented by chronic denervation and by local anaesthesia of the sensory nerve endings by cocaine (see Table 1). This evidence shows that the peripheral nervous system plays a role in this response but only so far as the intactness of the axon reflex (Jancsó, 1964). The xylol response was unaffected by adrenalectomy (made 4 days previously), but intradermal (and occasionally subcutaneous) adrenaline or noradrenaline inhibited it at the injection sites. Acute decerebration, pithing or spinal cord transection only slightly reduced the response whereas intramuscular cocaine had no action.

Discussion

Inflammation from chemical injury such as the external application of xylol probably depends upon the intactness of the sensory nerve endings, and the initial leak of protein from the blood vessels which is followed some 10 min later by the leak of fluid appears to be the result of stimulation of the axon reflex. The evidence for this is that the xylol reaction is prevented by chronic denervation of the sensory nerves and by cocaine

infiltration of the sensory nerve endings, but not by acute local denervation of the area to which the solvent is applied, by cocaine infiltration of the sensory nerve trunks, or by the procedures of pithing, decerebration or transection of the spinal cord. Intradermal injections of adrenaline or noradrenaline also prevent the xylol response but the mechanism of this antagonism is not clear since noradrenaline, the more potent pressor amine, is nearly 10 times less active than adrenaline. Similar relative activities have recently been reported by Brown & West (1965) who described the antagonism of the intradermal bradykinin and dextran responses in rats.

The topical application of xylol produces, in addition to the inflammatory response, an immediate and pronounced rise in blood pressure. This pressor response is further evidence of the involvement of the nervous system as it is abolished by acute and by chronic denervation of the hind-paws, by pithing, decerebration and spinal section, and by subcutaneous cocaine. Adrenalectomy does not modify this pressor response.

Inflammation from thermal injury or dextran shock, on the other hand, did not depend on intact sensory nerve endings, and the axon reflex probably is not involved. Cocaine infiltration of the sensory nerve ending or chronic denervation are without effect. Nevertheless, in acute experiments, the surgical procedures of pithing, decerebration or transection of the spinal cord much reduced and often completely prevented these two inflammatory responses, although both were well elicited 3 hr later. It is possible that these procedures stimulated the sympathetic nervous system and the adrenal cortex. Both the thermal and dextran responses are prevented by prior treatment with adrenaline, noradrenaline or cortisone.

The primary event in chemical injury is therefore a stimulation of the axon reflex, resulting firstly in a leak of protein from the blood vessels, and later, a leak of fluid after the permeability of the capillaries has been increased by released substances. On the other hand, the primary event in both dextran and thermal shock is the formation and release of vaso-active substances such as bradykinin, histamine and 5-hydroxytryptamine (5-HT), which act directly on the blood vessels leading to a leak of blood protein and fluid into the tissue spaces. Evidence has already been presented that 5-HT is involved in dextran shock (Parratt & West, 1957) and further observations during the present work confirm this result. For example, 2-bromolysergic acid diethylamide, a specific antagonist of 5-HT, in doses of 2 mg/kg intravenously; cyproheptadine, 2 mg/kg intravenously; and reserpine, a liberator of 5-HT, in doses of 2·5 mg/kg intraperitoneally, were all effective in preventing the dextran oedema reaction. On the other hand mepyramine, a specific antagonist of histamine, in doses of 10 mg/kg intravenously, was without effect. Atropine, 100 mg/kg intraperitoneally, and acetylsalicylic acid, 1000 mg/kg orally, also prevented the dextran response, but heparin 10 mg/kg intravenously, had no effect. All of these agents prevented the development of the thermal reaction which probably involves only bradykinin formation and release (Gecse, Karady, Starr & West, 1965).

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Note on a micromethod for the estimation of ascaridole

S. K. WAHBA,* N. Z. YOUSSEF, S. W. AMIN AND K. N. GIRGES

A micro stoichiometric method for the determination of ascaridole in a pure state and in oil of chenopodium is described. The determination is made either by direct or indirect titration of stannous chloride solution. A blank standardisation for the stannous chloride solution should be carried out consecutively.

ASCARIDOLE (1,4-epidioxy-2-*p*-menthene) is the main constituent of oil of chenopodium. Methods in the American (XII), British (1953) and Egyptian (1953) Pharmacopoeias are based on the fact that ascaridole liberates iodine from potassium iodide in concentrated acid medium. However, this method is empirically rather than stoichiometrically based.

In the last 25 years, several attempts have been made to modify the original Cocking and Hymas method. Lepetit (1943) stated that the factor in the official method (1 ml of 0·1 N sodium thiosulphate is equivalent to 0·00665 g ascaridole) should be reduced to 0·00605 g.

Böhme & Van Emster (1951) reported the titre to be affected by the concentration of ascaridole in the sample. Beckett, Donbrow & Jolliffe (1955) confirmed both Lepetit's and Böhme's findings. They gave a quadratic equation from which the amount of ascaridole can be calculated.

Said, Amer, Wahba & Ahmed (1962) described a method based on hydriodic acid as a reductant with heating. It was thought that it would be of interest to establish a simple and quick method for the evaluation of microquantities of ascaridole.

MATERIALS

Ascaridole (Bayer) was purified by partition chromatography on silica column, using *n*-hexane-ethyl acetate mixture as the eluent (Beckett & Jolliffe, 1955). Purity was confirmed by physicochemical methods. B.p. at 20 mm/Hg, 112°; $d_4^{20} = 0{\cdot}9980$; n_D^{20}, 1·4769; $[\alpha_D^{20}] \pm 0{\cdot}00$; $c_{20}^{20} = 1{\cdot}002$.

Oil of chenopodium (Rodofran Corporation) labelled to contain not less than 65% ascaridole, complied with the U.S.P. XII requirements.

Stannous chloride solution (1·5%): dissolve 1·5 g stannous chloride with heat in 50 ml concentrated hydrochloric acid, cool, adjust to 100 ml with freshly distilled water. The reagent should be recently prepared and kept under nitrogen.

Ascaridole solution, (0·5 and 5·0% w/v) in 90% acetic acid.

Iodine solution 0·05 N. The solution is stored in small air-tight bottles filled completely and kept in a cool, dark place.

Starch test solution as indicator.

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Experimental

The proposed method is based on the fact that ascaridole can be estimated by its reaction with a powerful reducing agent. Ascorbic acid, sodium sulphite, hydriodic acid in the cold, and stannous chloride were tried. The latter proved to be the most favourable reducing agent in determining ascaridole in its pure state and in oil of chenopodium. The method of analysis can be made either by direct or indirect titration.

Indirect titration. Into a stoppered test tube, of about 25 ml capacity, 5 ml of stannous chloride solution is measured accurately. From a burette, 1–5 ml of ascaridole solution is added, followed by three drops of starch solution. The mixture is shaken and titrated immediately with the standard iodine solution until the appearance of the blue colour. A blank is estimated under the same conditions.

Direct titration. Into a stoppered test tube, of about 25 ml capacity, 5 ml of stannous chloride solution is measured accurately. Three drops of starch solution and one drop of 10% potassium iodide solution is added and the mixture titrated with the ascaridole solution until the appearance of permanent blue colour. Another 5 ml of stannous chloride solution is titrated with iodine using starch test solution as indicator.

TABLE 1. ANALYSIS OF DIFFERENT QUANTITIES OF ASCARIDOLE

Weight mg	ml 0·05 N iodine consumed†	mg ascaridole equiv. to 1 ml 0·05 N iodine
5	1·20	4·255
10	2·35	4·166
15	3·60	4·166
20	4·85	4·125
25	6·05	4·132

† Means of three estimations. Ascaridole equivalent to one ml of 0·05 N iodine solution is 4·1688 mg.

Analyses of different concentrations of ascaridole solution were made to find the reproducibility of the method. The results are presented in Table 1. It is clear that 1 ml of 0·05 N iodine is equivalent to 4·1688 mg ascaridole. As one molecule of ascaridole contains two atoms of oxygen which can be reduced, one ml of 0·05 N iodine is theoretically equivalent to 4·2057 or 8·4114 mg of ascaridole. The experimental findings are in favour of the first figure (4·2057).

A comparison between official and the stannous chloride methods was made. Two concentrations (0·5 and 5% w/v in 90 % acetic acid) of pure ascaridole and of oil of chenopodium were analysed. The results are shown in Table 2.

Conclusions

The Pharmacopoeial methods for determining ascaridole involve the use of an empirical factor. The results we obtained were erroneous, especially at low concentrations. One molecule of ascaridole is equivalent to 4 atoms of iodine, indicating that the method is a stoichiometric

TABLE 2. COMPARISON BETWEEN THE OFFICIAL METHODS AND STANNOUS CHLORIDE METHOD

Ascaridole mg	Conc. of soln %	Found %*					
		Ascaridole			Oil of chenopodium		
		Official	Stannous chloride		Official	Stannous chloride	
			1·5%	5%		1·5%	5%
10		290·90	98·83	—	183·42	63·69	—
15		241·25	100·93	—	145·70	64·40	—
20	0·5	210·45	101·98	—	132·50	64·05	—
25		184·50	101·77	—	115·63	64·84	—
100		119·00	69·8	100·51	75·60	—	63·84
150	5·0	116·15	76·6	100·79	72·24	—	63·70
200		111·00	85·0	99·88	69·30	—	64·47
250		104·70	79·4	100·19	66·05	—	63·84

The indirect method of estimation was used for the comparison.
* Means of three estimations.
On estimating high concentrations, low results were obtained in spite of using 5 ml stannous chloride solution per ml 5% ascaridole solution. However, on increasing the concentration of stannous chloride to 5% the expected values were obtained.

one. Our findings agree with those of Böhme & Van Emster and Beckett & others (1955) that the titres in the official methods are affected by the concentration of ascaridole (Table 2). The proposed method will estimate as little as 5–25 mg ascaridole, it is simple, requires no special apparatus and takes a comparatively short time.

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The significance of an isoprenaline-like metabolite in the interpretation of the responses of blood vessels in skeletal muscle to adrenaline

D. J. ROBERTS

A depressor substance, which resembled isoprenaline in Rf value, was demonstrated after chromatography of plasma collected from chloralose-anaesthetised cats during the blood pressure responses to intravenous injections of adrenaline, but the results obtained did not substantiate the hypothesis of a functional metabolite. The amount of vasodilatation that the "metabolite" would have caused in the skeletal muscles of the hind limb could not, for example, be correlated with the increases in blood flow produced by the original intravenous doses of adrenaline. The fact that adrenaline was largely vasoconstrictor in acutely denervated hind limbs was taken to indicate that the original vasodilator effects were of reflex nervous origin. It is suggested that the afferent source of this and related reflexes involves the stimulation of chemoreceptors as opposed to mechanoreceptors and that increases in flow through denervated muscles in response to adrenaline are caused by a direct action on the walls of the blood vessels.

IT is now generally recognised that the intravenous or intra-arterial administration of small doses of adrenaline produce vasodilatation in skeletal muscles while larger doses cause vasoconstriction. A variety of explanations of these opposing biphasic actions of adrenaline have been proposed; these include different responses in separate segments of the vascular bed (Dale & Richards, 1918), stimulation of different receptors in one particular vessel (Ahlquist, 1948), "partial agonist" phenomena (Burn & Rand, 1958), the involvement of nervous reflexes (Hartman & Fraser, 1917; Duff & Swan, 1951; Gruhzit, Freyberger & Moe, 1954; Bowman, 1959a) and the liberation by adrenaline of substances in the body which have vasodilator action (Whelan, 1952), such as histamine (Staub, 1946), lactic acid (Lundholm, 1956) or isoprenaline (Cobbold, Ginsburg & Paton, 1960; Glover, Greenfield & Shanks, 1962).

A substance which resembles isoprenaline in pharmacological actions and Rf value has been demonstrated in extracts of adrenal glands (Lockett, 1954; Subrahmanyam, 1959), in extracts of cat blood collected either from the pulmonary veins after stimulation of the upper thoracic sympathetic chains (Lockett, 1957) or from the aorta following the administration of adrenaline (Eakins & Lockett, 1961), and in extracts of rabbit aortic blood collected during the intravenous infusion of adrenaline (Roberts & Lockett, 1961). Eakins & Lockett (1961) consider the substance to be formed principally in the liver as a metabolite of adrenaline. More recently, Roberts (1965a) has shown that elevation of the adrenaline content of cat blood by reflex stimulation of sympathetic nerves in response to acute terminal haemorrhage is alone sufficient to cause the appearance of this "metabolite" on chromatograms of plasma extracts,

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and that its isoprenaline-like nature may be extended to include vasodilatation in skeletal muscle.

It seemed possible, therefore, that the dilator effects of intravenously administered adrenaline might be due to its partial conversion to this isoprenaline-like substance; the experiments described here are a direct test of this hypothesis.

Some workers have been unable to record any vasodilatation with intra-arterially or intravenously administered adrenaline, and some confusion also exists over the effects of noradrenaline on the calibre of blood vessels (see Bowman, 1959a, for references). Because some of these discrepancies might have been due to factors such as the use of different species, different anaesthetics and different recording methods, it was decided to investigate the effects of intravenous and intra-arterial noradrenaline and adrenaline in parallel with those of isoprenaline, on blood flow through skeletal muscle as measured using the apparatus and conditions now described.

Experimental

Methods

Cats of either sex, 1·4–5·6 kg, were anaesthetised with chloralose, 7·5 ml/kg of a 1% w/v solution in 0·9% w/v saline intraperitoneally or into a femoral venous cannula, after induction with ether, and prepared to enable continuous recordings to be made of carotid arterial pressure and venous outflow from the skinned left hind limb, as previously described (Roberts, 1965a). In some experiments the left sciatic nerve was exposed high in the thigh to facilitate denervation of the muscles when required. In experiments in which the effects of intravenously administered noradrenaline, adrenaline and isoprenaline were examined, the same doses were administered before and after connecting a blood pressure stabiliser (Fig. 1) to the carotid cannula. Drugs were administered intravenously by a cannula in the right femoral vein or intra-arterially by means of the polyethylene tubing in a small branch of the left femoral artery, usually the profunda femoris (Roberts, 1965a). A semimicro syringe was used for all intra-arterial injections and solutions were adjusted to pH 6·8.

For the second part of the investigation arterial blood samples were required and these were collected by an additional polyethylene catheter ('Portex' Poly 49A) introduced through the right femoral artery so that its tip lay just beyond the inguinal ligament. In these experiments, after setting up the blood flow circuit, the following procedure was adopted.

Stage 1. Blood samples were collected from the right femoral artery at rest and after a minimum of two, and a maximum of five, doses of intravenously administered adrenaline (0·5–25 μg). In each instance the plasma was separated without delay and protein and lipid-free extracts were prepared for chromatography (Roberts, 1963b).

Stage 2. The blood cells from Stage 1 were mixed with aqueous 0·9% w/v sodium chloride and infused back into the cat to re-establish the blood volume.

Stage 3. The blood pressure stabiliser (Fig. 1) was connected to the carotid artery and the effects of intravenous injections of adrenaline

(0·5–25μg) were measured on the blood flow through the left hind limb of the same preparation.

Stage 4. Adrenaline and isoprenaline (0·001–1·0 μg) were injected intra-arterially by way of the profunda femoris and the blood flow responses in the hind limb were similarly recorded. Log dose response curves were then constructed for each of these two amines.

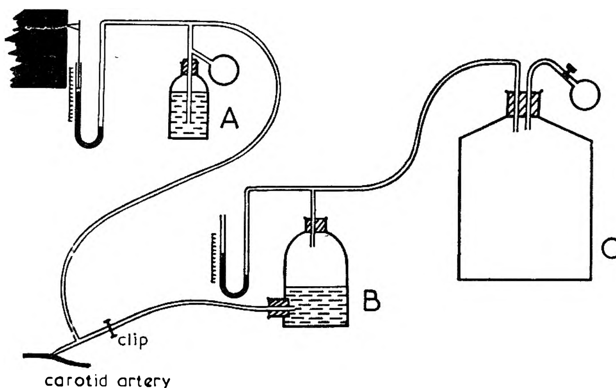

FIG. 1. Diagram of the blood pressure stabiliser. The cat's blood pressure is first measured on the mercury manometer connected to A with the clip on the tubing leading to B closed. The pressure in B, as measured on its attached manometer, is then raised to equal the blood pressure of the cat using the pump connected to C. The clip is then released and the blood pressure is effectively stabilised. A and B contain 0·9% w/v sodium chloride solution; C contains air.

Stage 5. The adrenaline and any isoprenaline-like metabolite present in the plasma extracts of Stage 1 were separated chromatographically on acid-washed papers treated with ascorbic acid (Lockett, 1957) by development in phenol containing 15% v/v 0·1 N hydrochloric acid using apparatus and materials previously described (Roberts, 1963a). Elution was conducted overnight using distilled water containing 1 mg ascorbic acid per 100 ml only as the eluant to reduce interference by 'blank' pharmacological activity (Roberts, 1964a). The eluates were then assayed on rat blood pressure preparations in terms of (–)-isoprenaline activity (pentobarbitone sodium anaesthesia) and (–)-adrenaline activity (the animals being pithed and treated with cocaine, 5 mg/kg, and pronethalol, 5 mg/kg, intravenously) respectively. A 50% recovery was assumed (Roberts, 1963b) and the assay results were adjusted accordingly.

The doses refer to the quantity of catecholamine calculated as base, but the amines were administered as salts. With the (–)-adrenaline acid tartrate and (–)-noradrenaline acid tartrate this involved simple calculations of molecular weights. In the case of (±)-isoprenaline sulphate the calculated amount of base was further divided by 2 to convert all doses in terms of (–)-isoprenaline base. The errors which must result from attributing the activity of (±)-isoprenaline solely to the laevorotatory component are not large since this isomer is several hundred times more

potent than the *dextro* form (Lands, Ludueña & Tuller, 1954). Quantitative measurements of absolute vasodilator and vasoconstrictor activities were calculated as previously described (Roberts, 1965b).

DRUGS USED

(–)-Adrenaline acid tartrate, (±)-isoprenaline sulphate, cocaine hydrochloride (Burroughs Wellcome & Co.), (–)-noradrenaline acid tartrate (L. Light & Co.), pronethalol (Alderlin, I.C.I. Ltd.), heparin (Pularin, Evans Medical Ltd.) and pentobarbitone sodium (Nembutal, Abbott Laboratories) were obtained commercially.

Results

INTRAVENOUSLY ADMINISTERED NORADRENALINE, ADRENALINE AND ISOPRENALINE

The influence of any one dose of any one amine on blood pressure and venous outflow varied from animal to animal but the effects of each amine were qualitatively similar in 4 out of 6 experiments. In these the resting mean arterial pressure was between 110 and 180 mm Hg and the results obtained were essentially similar to those described by Bowman (1959a). Typical records are shown in Fig. 2.

Small doses of adrenaline and noradrenaline (0·1–2·5 μg/kg) caused only increases in venous outflow, the responses increasing with increase in dose. After larger doses the increases in flow were followed by decreases in flow which became more prominent as the doses increased; the initial increases in flow then became progressively less. Whereas, weight for weight, noradrenaline produced a greater pressor response than adrenaline, the increases in blood flow were smaller.

When the blood pressure stabiliser was connected to the carotid artery, the increases in blood flow produced by intravenously administered adrenaline were reduced both in extent and duration and those produced by intravenously administered noradrenaline were abolished. In both cases the secondary decreases in flow now appeared at lower dose levels and increased with increase in dose. Intravenously administered isoprenaline caused decreases in blood flow through the skeletal muscles which were shown by means of the blood pressure stabiliser to be passive effects caused by the fall in blood pressure; when changes in blood pressure were prevented, isoprenaline caused only an increase in flow from the blood vessels of the hind limbs.

The responses to the amines were further modified by cutting the sciatic nerve high in the thigh. After this acute denervation of the skeletal muscles, and when the blood pressure had been stabilised, intravenously administered adrenaline caused a reduction in blood flow; this effect of adrenaline increased with increase in dose. When large doses (2·5 μg/kg and above) of adrenaline were injected, small increases in flow became apparent superimposed upon, and occuring 20–30 sec after, the onset of the decreases in flow (Fig. 3, upper record). Intravenous noradrenaline also decreased the venous outflow after acute

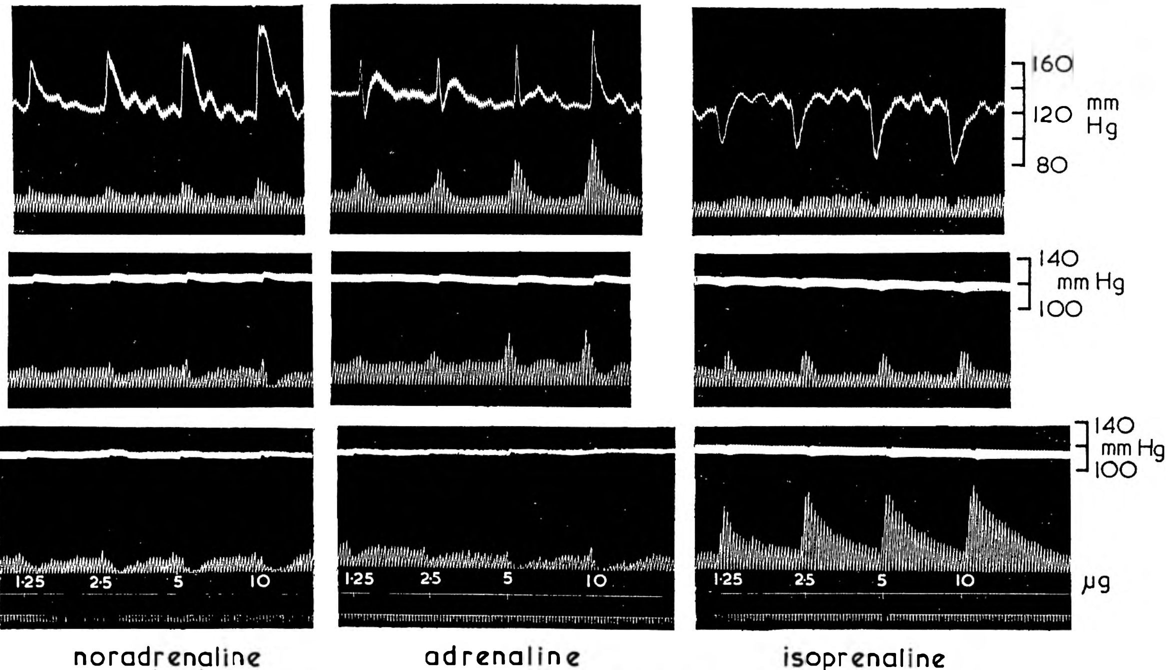

FIG. 2. Female cat, 3.3kg. The effect of stabilising the blood pressure and of acute denervation on the vascular responses in hind limb skeletal muscles to intravenously administered noradrenaline, adrenaline and isoprenaline. Upper records: innervated muscles; middle records: innervated muscles, blood pressure stabilised; lower records: acutely denervated muscles, blood pressure stabilised. On each record the upper trace is carotid arterial blood pressure and the lower trace is venous outflow. Time trace, 10 sec.

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denervation, the reduction for any given dose being greater than that obtained in the innervated muscle. Increases in flow superimposed upon the vasoconstriction were only seen occasionally. In contrast, intravenously administered isoprenaline, after sectioning of the sciatic nerve, produced on most occasions a much greater increase in venous outflow than that obtained from the innervated muscle, the increase being most marked at dose levels which produced maximal vasodilatation before denervation. In some experiments the effects of pronethalol (5 mg/kg) on the vasodilator responses to the catecholamines in denervated limbs were investigated. Under these conditions increases in flow other than those occurring passively as a result of increases in blood pressure were absent.

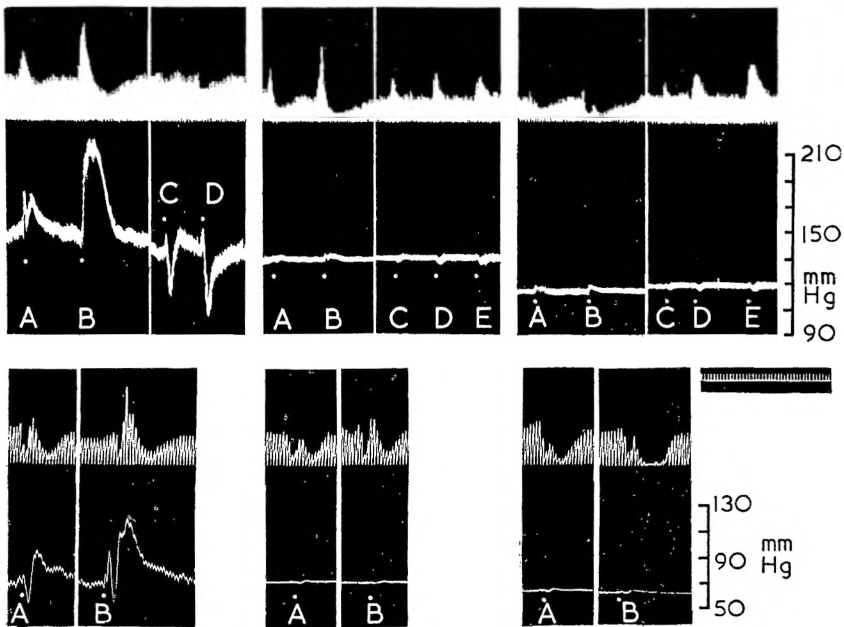

FIG. 3. Upper records, male cat 2kg; lower records, male cat 2·9kg. The effects of intravenously administered adrenaline (A, 1; B, 5 μg) and isoprenaline (C, 0·5; D, 2·5; E, 5 μg) on the blood flow through the skeletal muscles of the cat hind limb. Left-hand records: innervated muscles; centre records: innervated muscles, blood pressure stabilised; right-hand records: acutely denervated muscles, blood pressure stabilised. On each record: upper tracings, venous outflow and lower tracings, arterial blood pressure. Time trace, 10 sec.

In two experiments the resting blood pressure was low (70 mm Hg). In one of these no increase in blood flow, other than passive increases as the blood pressure rose, were observed following the administration of either noradrenaline or adrenaline. The vasoconstrictions that were observed instead increased with increase in dose and were intensified by acute denervation. The responses in the other experiment differed from the above in that increases in blood flow were observed in response

to adrenaline injected intravenously, but only as increases superimposed upon vasoconstriction. These increases were reduced by stabilising the blood pressure but persisted even after acute denervation (Fig. 3, lower record). In both experiments noradrenaline was more potent than adrenaline in decreasing the blood flow from the hind-limb and the responses to isoprenaline were essentially similar to those already described.

In all cases, the cutting of the sciatic nerve resulted in an initial increase in flow which subsequently subsided (30 sec to 5 min) to reach the initial presectional resting level.

INTRA-ARTERIALLY ADMINISTERED NORADRENALINE, ADRENALINE AND ISOPRENALINE

The resting systemic mean arterial pressure lay between 110 and 180 mm Hg in all experiments in which the effects of intra-arterially administered noradrenaline, adrenaline and isoprenaline were studied.

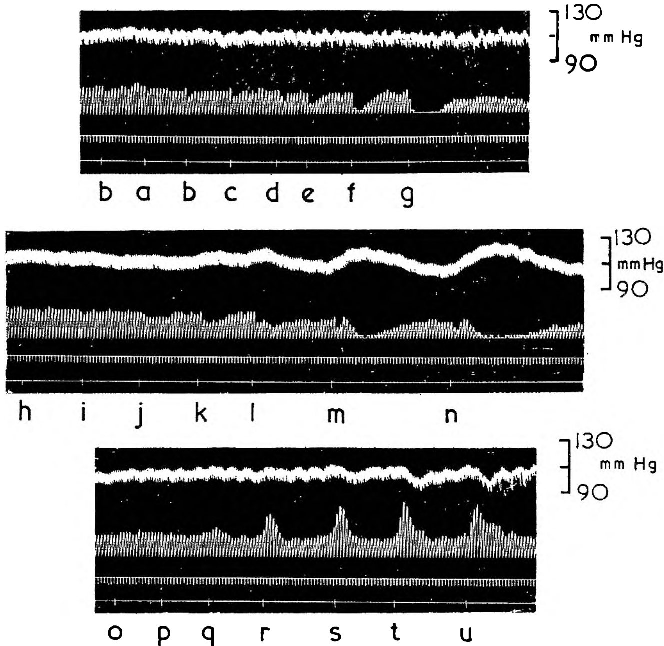

FIG. 4. Female cat, 3kg. The effect of intra-arterially administered noradrenaline (upper record), adrenaline (middle record) and isoprenaline (lower record) on the venous outflow from the innervated skeletal muscles of the cat hind limb. In each case traces show from above downwards: arterial blood pressure, venous outflow, 10 sec interval marker and injection of drug. At a, b, c, d, e, f and g, 0·001, 0·005, 0·01, 0·025, 0·05, 0·1 and 0·5 μg noradrenaline. At h, i, j, k, l, m and n, 0·001, 0·005, 0·01, 0·05, 0·1, 0·5 and 1 μg adrenaline. At o, p, q, r, s, t and u, 0·0005, 0·001, 0·005, 0·01, 0·05, 0·1 and 0·5 μg isoprenaline.

Minimal effective intra-arterial doses (0·001–0·01 μg) of adrenaline produced only a short lasting decrease in flow. This response increased as the dose was increased, but after larger doses (0·05–0·5 μg) a

short-lasting increase in flow sometimes occurred 20–30 sec after the onset of the reduction in venous outflow. On these occasions the overall picture was one of a constant increase in flow superimposed upon an increasing reduction in flow as the dose was raised (Fig. 4, middle record).

Minimal effective intra-arterial doses of noradrenaline (0·001–0·01 μg) caused only a decrease in blood flow from the skeletal muscles (Fig. 4 upper record) and this response increased with increase in dose (0·01–1·0 μg). In general, noradrenaline produced a greater reduction in venous outflow than that produced by adrenaline.

Minimal effective intra-arterial doses of isoprenaline (0·001–0·01 μg) caused only an increase in the venous outflow from the hind limb. This response increased with increase in the dose to reach a maximum at a dose of 0·1 μg and a further increase in dose merely prolonged the effect (Fig. 4, lower record). The increases in flow were abolished after treatment of the cats with pronethalol (5 mg/kg).

After acute denervation, the decreases in flow caused by intra-arterial doses of noradrenaline were slightly greater than, and the increases in flow caused by doses of isoprenaline slightly less than, those obtained in the innervated muscle.

Both the decreases and the superimposed increase in flow obtained in response to intra-arterial adrenaline were greater and longer lasting in the acutely denervated than in the innervated limb. In no experiment was the increase in flow abolished by acute denervation; by contrast, no superimposed increases in flow were demonstrable in cats treated with pronethalol (5 mg/kg).

Significance of the isoprenaline-like substance

The changes in blood flow resulting from the different intravenous doses of adrenaline were measured from the traces obtained during stage 3 of the experimental procedure described on page 770. From stages 1 and 5 the amounts of isoprenaline-like "metabolite" and adrenaline likely to be reaching the hind limb as a result of these different intravenous doses were calculated, and from the intra-arterial log dose response curves of Stage 4 the changes in blood flow that these amounts of isoprenaline-like "metabolite" and adrenaline would have caused were estimated. Sufficient information was now available to investigate whether or not the amount of isoprenaline-like "metabolite" reaching the hind limb following the intravenous injection of any one dose of adrenaline could account for the vasodilator effect of such a dose.

Unfortunately, the variations in the responses to any one intravenous dose of adrenaline in the different experiments were enormous and on occasions, particularly at the higher dose levels, very poor recoveries of amine were encountered following chromatography of the plasma extracts. The same clear general trends were apparent in all experiments, however, and these are accepted. These trends are well exemplified by the means of the observations as expressed in Table 1 and Fig. 5.

In 7 out of 10 experiments intravenously administered adrenaline caused increases in venous outflow which increased with increase in

dose to reach a maximum (5–10 μg); thereafter increase in dose resulted in smaller overall increases in flow as vasoconstriction became predominant. In the remaining three experiments minimal effective doses of intravenously administered adrenaline produced only vasoconstriction which increased in intensity with increase in dose. These experiments have been omitted from the calculations of means and standard errors expressed in Table 1 and Fig. 5.

TABLE 1. THE CONCENTRATIONS OF ADRENALINE AND ISOPRENALINE-LIKE SUBSTANCE REACHING THE HIND LIMB AS A RESULT OF INTRAVENOUS INJECTIONS OF ADRENALINE; THE RESPECTIVE VASOCONSTRICTOR AND VASODILATOR EFFECTS THAT THESE AMOUNTS OF AMINE WOULD HAVE CAUSED IN THE SKELETAL MUSCLE OF THE HIND LIMB; THE RESULTANT MEAN VASCULAR EFFECT OF THE ADRENALINE AND THE ISOPRENALINE-LIKE SUBSTANCE REACHING THE HIND LIMB AND THE VASODILATION PRODUCED BY THE ORIGINAL INTRAVENOUS DOSE OF ADRENALINE. (Figures quoted are means ± standard errors)

Adrenaline i.v.(μg)	Conc.(μg) of amine reaching hind limb		Adrenaline† (constriction)	'Isoprenaline'† (dilatation)	Mean effect† + = dilatn − = constr.	Dilatn† i.v. adrenaline
	Adrenaline	'Isoprenaline'				
None (8)*	0·005 ± 0·0009	0·003 ± 0·0005				
0·5 (4)	0·01 ± 0·002	0·004 ± 0·0007	10·25 ± 3·15	13·25 ± 6·95	+ 3 ± 4·38	3·25 ± 0·95
1 (4)	0·034 ± 0·003	0·013 ± 0·003	22·5 ± 5·85	30·75 ± 6·75	+ 8·25 ± 3·32	5·25 ± 1·33
2·5 (3)	0·04 ± 0·12	0·018 ± 0·002	29 ± 10·69	40·67 ± 5·46	+ 11·67 ± 8·47	15·33 ± 4·06
5 (6)	0·106 ± 0·021	0·057 ± 0·009	48·83 ± 10·64	66·5 ± 15·99	+ 17·77 ± 8·51	32·71 ± 5·79
10 (4)	0·255 ± 0·056	0·114 ± 0·034	78·25 ± 32·87	75·75 ± 27·07	− 2·5 ± 7·84	115·5 ± 28·5
25 (5)	0·433 ± 0·061	0·143 ± 0·081	127·8 ± 25·34	82·4 ± 22·28	− 45·4 ± 8·47	120·4 ± 44·5

*No. of observations.
†The figures expressing vasodilatation and vasoconstriction are those obtained using the equation described by Roberts (1956b) multiplied by 100.

In all experiments the eluates prepared from strips taken at the isoprenaline Rf value had depressor activity on rat blood pressure preparations and with few exceptions the isoprenaline-like activity found became progressively greater as the intravenous dose of adrenaline was increased. The amounts of isoprenaline-like "metabolite" found were sufficient to have caused vasodilatation in the cat hind limb, but these calculated increases in blood flow produced by the "metabolite" could seldom be correlated with the vasodilatation produced by the "parent" doses of intravenously administered adrenaline. Furthermore, the calculated effects of the amounts of adrenaline reaching the hind limb (1–4% of the amount of adrenaline injected intravenously) were always vasoconstrictor, and since both the isoprenaline-like "metabolite" and this adrenaline must be considered to be reaching the hind limb together, the true expected response must be the mean of the two calculated effects. At dose levels of less than 5 μg of adrenaline intravenously these means represented increases in blood flow, but at higher dose levels the calculated vasoconstriction produced by the adrenaline reaching the hind limb counteracted and predominated over the calculated vasodilatation produced by the "metabolite". At no dose level could the calculated mean effect of the "metabolite" and the adrenaline reaching the hind limb be correlated with the effect of the intravenously administered adrenaline, although

at the lower dose levels an apparent correlation results from statistical treatment of all the observations (Fig. 5).

Blank eluates were not prepared during this series of experiments but the low resting levels of catecholamine (Table 1) indicated that activity other than that due to catecholamine was negligible.

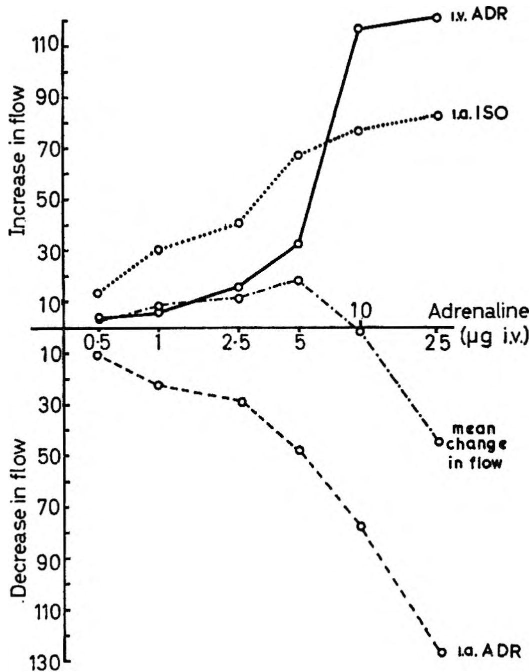

FIG. 5. The significance of the isoprenaline-like "metabolite" in the vasodilator responses to intravenously administered adrenaline. i.a. ISO and i.a. ADR respectively represent the changes in blood flow that would have been obtained from the amounts of "isoprenaline-like substance" and adrenaline reaching the hind limb following the intravenous administration of different doses of adrenaline. The difference between i.a. ISO (vasodilatation) and i.a. ADR (vasoconstriction) is the mean change in flow. The increases in blood flow caused by the original doses of intravenously administered adrenaline (measured with the blood pressure stabilised) are represented by i.v. ADR. The points plotted at each dose level are the means of several observations; the number of observations and the standard errors of these means are given in Table 1.

Discussion

The experiments described are an attempt to test directly the proposal (Cobbold & others, 1960; Glover & others, 1962) that the vasodilator effect of intravenously administered adrenaline may be due to the formation of an isoprenaline-like metabolite (Eakins & Lockett, 1961).

Intravenous injections of adrenaline in the cat do give rise to the appearance of a depressor substance at the isoprenaline Rf value on chromatograms of extracts of femoral arterial blood (Table 1) and this substance, similarly formed from the adrenaline released during haemorrhage

(Roberts, 1965a), equates fairly well with isoprenaline in assays on the blood pressure, the rat uterus and the cat hind limb blood flow. Also, the concentrations of this depressor substance reaching the hind limb usually increase with increase in the intravenous dose of adrenaline and are sufficient to cause vasodilatation. The concentrations of adrenaline reaching the hind limb are, by contrast, vasoconstrictor and at intravenous dose levels in excess of 5 μg the decreases in flow are sufficient to counteract the increases in flow due to the "metabolite" and the resultant mean effect is vasoconstriction. On the other hand, a mean effect of vasodilatation is arrived at when the lower doses, perhaps more representative of expected 'physiological' levels, are considered.

The results of other experiments indicate that most of the vasodilator effect of intravenously administered adrenaline is not mediated via the formation of an isoprenaline-like vasodilator metabolite but, in confirmation of the work of Bowman (1959a), via the nerves. Thus, in the innervated preparation with the blood pressure stabilised, intravenous doses of adrenaline produce a greater degree of vasodilatation than similar doses of isoprenaline. Also, doses of intravenously administered adrenaline causing increases in blood flow in the innervated limb result in marked decreases in flow after the sciatic nerve has been cut. Under the same conditions, the vasodilator response to intravenously administered isoprenaline is potentiated. Similarly, the results of the three experiments in which intravenously administered adrenaline caused uncomplicated vasoconstriction do not support the idea of a functional metabolite, because the intra-arterial dose-response curves and the concentrations of adrenaline and isoprenaline-like substances found in these experiments were no different from those found in the experiments where adrenaline was vasodilator.

In contrast, the increases in flow that were sometimes seen superimposed upon vasoconstriction when adrenaline was given intravenously to animals in which the blood pressure had been stabilised (Fig. 3), must be independent of nervous reflexes as they also occurred in denervated preparations. The observation that similar responses are often obtained after intra-arterial administration (Fig. 4) is compatible with a direct vasodilator action of adrenaline (Bowman, 1959a) being responsible for the superimposed increases in flow, especially as such an action has been demonstrated both in the denervated (Dale & Richards, 1918) and the isolated (Dale & Richards, 1927) perfused hind legs of cats. In this respect it is significant that the intra-arterial dose levels of adrenaline required to cause the transient vasodilatation, correspond to the concentrations of adrenaline that would be reaching the hind limb as a result of the intravenous dose levels required to produce the same effect. The absence of the superimposed increases in flow in the presence of pronethalol is also consistent with a direct vasodilator action.

Since both intra-arterial and intravenous injections of noradrenaline cause more intense vasoconstriction, but fewer superimposed increases in flow, than adrenaline, reactive hyperaemia in response to the reduction in blood flow cannot be the cause of the increases. Similarly, the fact that

the transient vasodilatations were seen at all following noradrenaline administration is inconsistent with the idea of an isoprenaline-like metabolite being responsible, because noradrenaline does not give rise to such a substance (Eakins & Lockett, 1961).

In none of the experiments described did a permanent hyperaemia result from cutting the sciatic nerve, suggesting that the vasomotor tone in the muscles may have been low (Bowman, 1959a). This does not detract in any way from the significance of the results obtained showing the importance of intact nervous connections in the vasodilator response to intravenously administered adrenaline. An "isoprenaline-like substance" is demonstrable on paper chromatograms of plasma extracts, and since it cannot be considered to be the cause of the vasodilator effects of adrenaline, its function might be to antagonise any local constrictor action of adrenaline so leaving the vasodilator reflex free to exert its full effect. Alternatively, it may be an artifact and the observation that adrenaline in the presence of hydrochloric acid can form multiple spots, one of which has an Rf value similar to that of isoprenaline (Roberts, 1964b), indicates a possible way by which the use of hydrochloric acid during extraction and chromatography of plasma samples containing adrenaline might give rise to such an artifact.

Secondary to the main investigations, the experiments described yielded information about the mechanism of the nervous reflexes influencing the blood flow responses to the catecholamines. In preparations where blood pressure changes are prevented by the use of the stabiliser, the vasodilatation caused by intravenously administered adrenaline is abolished by acute denervation while that caused by similar administration of isoprenaline is potentiated. With the blood pressure stabilised the reflex vasodilatation and reflex vasoconstriction required to explain the observations cannot originate from elevation or reduction of the pressure in the carotid sinuses. Similarly, since the inotropic cardiac action of isoprenaline is greater than that of adrenaline (Lands & Howard, 1952) any abolition of a reflex vasodilatation mediated via an inotropic cardiac effect (Gruhzit & others, 1954) would be expected to reduce the vasodilator response to isoprenaline and not potentiate it. This interpretation is, of course, subject to the limiting sensitivity of the apparatus used to produce and measure a stabilised blood pressure.

Bowman (1959b) has suggested that it is stimulation of chemoreceptors rather than mechanoreceptors that provides the afferent sources of the reflexes and the experiments of Taylor & Page (1951) have indicated that such receptors could exist in the cephalic circulation. To explain the effects of acute denervation described in this present investigation, however, two types of chemoreceptor are required. The first type must respond to adrenaline and to noradrenaline causing reflex vasodilatation, while the second type must respond to isoprenaline and cause reflex vasoconstriction. While it is tempting to classify these receptors as α-adrenotropic and β-adrenotropic respectively (Ahlquist, 1948) there is as yet no evidence for the existence of cephalic chemoreceptors responding to isoprenaline with a reflex vasoconstriction.

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The action of sympathomimetic amines on circular and longitudinal smooth muscle from the isolated oesophagus of the guinea-pig

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The responses to sympathomimetic amines of circular and longitudinal preparations of smooth muscle from the isolated oesophagus of the guinea-pig have been investigated. Evidence is provided for the existence of α-excitatory and β-inhibitory adrenergic receptors in both circular and longitudinal smooth muscle preparations.

THERE are two macroscopically distinct muscle layers in the guinea-pig oesophagus, an outer striated and an inner layer of smooth muscle. Separation of the two layers is a simple process, enabling the pharmacology of the different types of muscle to be investigated independently. The present experiments concern the responses to sympathomimetic amines of longitudinal and circular strips from the smooth muscle of the guinea-pig oesophagus.

Experimental

Adult guinea-pigs were killed by stunning and bleeding. The oesophagus was removed and pinned on a cork mat under Krebs solution. The point of a pair of scissors was then inserted between the layers at the gastric end and the outer muscle coat was cut longitudinally and gently peeled away leaving an inner tube. This tube was opened by longitudinal incision and pinned flat with the mucosal surface uppermost. Longitudinal strips were cut parallel to the long axis to produce a length of 3 cm with a width of 3 mm.

Circular strips were prepared as described by Harry (1963) for the guinea-pig ileum; 5–6 cuts were necessary to produce a preparation 3 cm long, the width being 3 mm.

The preparations were set up in Krebs solution, aerated with oxygen 95% carbon dioxide 5% at 37°. Responses were recorded on a smoked drum using a light, side-writing lever with an initial load on the tissue of 300 mg for longitudinal strips and 200 mg for circular preparations. Magnification in all cases was 10.

Strips of either type were prepared from distal, medial and proximal regions of the oesophagus, no regional pharmacological variation being observed.

Some longitudinal preparations possessed inherent tone, which could be inhibited by drugs to give relaxation. Circular strips, however, always lacked tone and in order to demonstrate inhibitory responses it was necessary first to induce tone. Acetylcholine was suitable for this purpose, and was most effective when added to the bath to produce a final concentration of 1 μg/ml, 90 sec before the addition of the sympathomimetic amine, after which both drugs were washed out.

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DRUGS

Acetylcholine chloride (Hopkins and Williams). Adrenaline acid tartrate B.P. (Burroughs Wellcome). Dihydroergotamine methane sulphonate (Sandoz). Isoprenaline sulphate (Burroughs Wellcome). (–)-Noradrenaline bitartrate (Bayer). Pronethalol hydrochloride (I.C.I.). Concentrations are expressed as final bath concentration (μg/ml) in terms of the base.

The Krebs solution contained NaCl 0·692, KCl 0·0354, $CaCl_2$ 0·0282, $NaHCO_3$ 0·21, KH_2PO_4 0·0162, $MgSO_4.7H_2O$ 0·0294, glucose 0·2 g/100 ml.

Results

ADRENALINE, NORADRENALINE AND ISOPRENALINE ON THE LONGITUDINAL AND CIRCULAR MUSCLE STRIPS

Adrenaline in low doses (0·01–0·05 μg/ml) produced contraction of most longitudinal preparations. The response gradually changed to relaxation as the dose was increased until (at 4 μg/ml) relaxation was virtually maximal (Fig. 1). Noradrenaline produced a small contraction in some preparations at a concentration of 0·02 μg/ml. Relaxation responses appeared at a concentration of 0·04–0·1 μg/ml noradrenaline. Isoprenaline produced relaxation at 0·01 μg/ml (Fig. 1). Contraction was never observed with smaller doses.

In some preparations adrenaline failed to produce contraction. This phenomenon did not appear to be related to the initial tone of the preparation nor to the region of the oesophagus from which it was taken. Contraction to larger doses of adrenaline in the presence of pronethalol (see below) was typically obtained in these preparations.

All three sympathomimetic amines produced relaxation in circular muscle strips preparations with acetylcholine-induced tone. Approximately the same amount of inhibition was produced by 1 μg/ml noradrenaline, 2 μg/ml adrenaline or 0·1 μg/ml isoprenaline (Fig. 2).

EFFECTS OF ADRENERGIC BLOCKING DRUGS

Blockade of β-adrenergic actions: pronethalol. On longitudinal preparations pronethalol, at a concentration of 5 μg/ml, abolished isoprenaline relaxation and reversed the adrenaline or noradrenaline relaxation to contraction (Fig. 3). Similarly, on circular strips pronethalol antagonised the relaxation to the three sympathomimetic amines and in some experiments reversed the adrenaline response to a small contraction (Fig. 2).

Blockade of α-adrenergic actions. In longitudinal preparations piperoxan, at a concentration of 2 μg/ml, abolished the contractions to small doses of adrenaline (Fig. 1), or noradrenaline. Contractions to larger doses of adrenaline or noradrenaline, after reversal by pronethalol, were abolished by dihydroergotamine (10 μg/ml). Relaxation responses to adrenaline or noradrenaline and contractile responses to acetylcholine

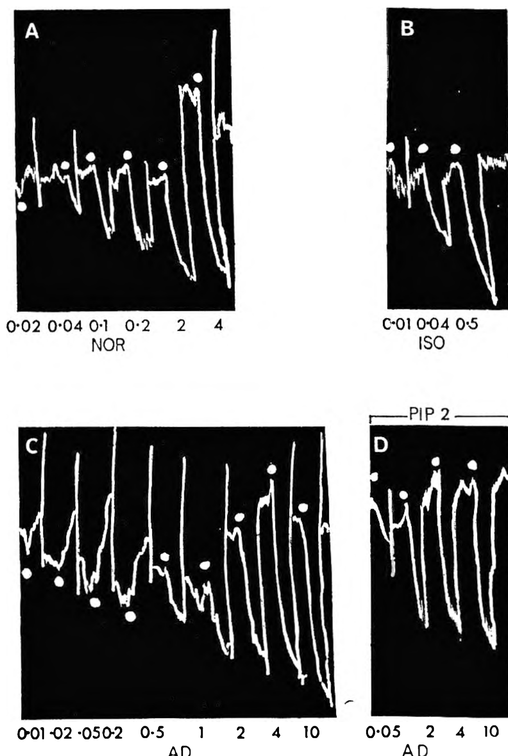

FIG. 1. Responses of the longitudinal smooth muscle preparation from the isolated oesophagus of the guinea-pig to noradrenaline (NOR), isoprenaline (ISO) and adrenaline (AD). The change from motor to inhibitory responses with increasing concentrations of noradrenaline in A or adrenaline in C is illustrated. Isoprenaline in B produced only inhibitory responses. Piperoxan (PIP 2 μg/ml) antagonised the motor but not the inhibitory responses to adrenaline in D. Concentrations are expressed as final bath concentrations (μg/ml) in terms of the drug base.

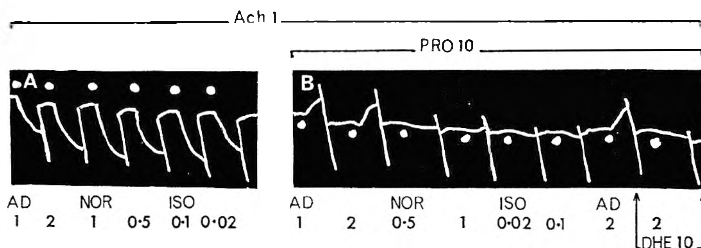

FIG. 2. The effect of sympathomimetic amines on the circular muscle preparation from the isolated oesophagus of the guinea-pig. Tone was induced with acetylcholine (Ach, 1 μg/ml). Adrenaline (AD), noradrenaline (NOR) or isoprenaline (ISO) produced inhibitory responses in A. In the presence of pronethalol (PRO 10 μg/ml in B) responses to noradrenaline or isoprenaline were abolished but adrenaline now produced a small motor response which was antagonised by dihydroergotamine (DHE). Concentrations are expressed as final bath concentrations (μg/ml) in terms of the drug base.

were not significantly modified by this concentration of dihydroergotamine (Fig. 4).

In circular muscle preparations dihydroergotamine antagonised the contraction produced by adrenaline in the presence of pronethalol (Fig. 2).

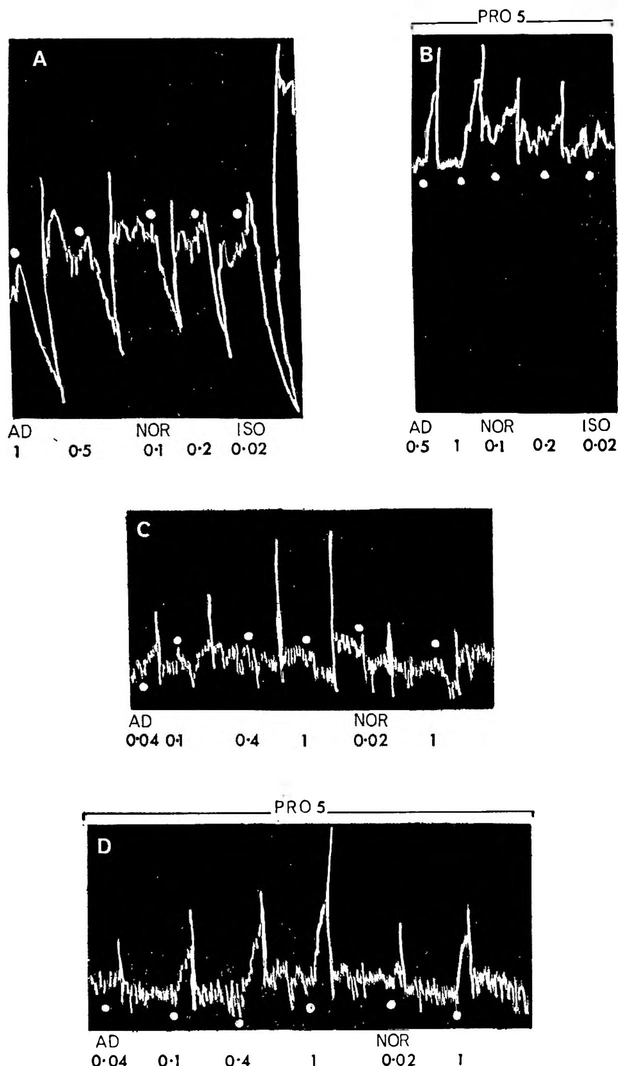

FIG. 3. The effect of pronethalol (PRO) on the responses of the longitudinal smooth muscle preparation from the isolated oesophagus of the guinea-pig, to sympathomimetic amines. In the preparation with inherent tone (see A) the inhibitory responses to adrenaline (AD) or noradrenaline (NOR) were reversed in the presence of pronethalol (5 μg/ml in B); the inhibitory response to isoprenaline was antagonised. In another preparation, with little tone (see C), adrenaline or noradrenaline produced little or no inhibitory effect; but after pronethalol in D, both drugs produced motor responses. Concentrations are expressed as final bath concentrations (μg/ml) in terms of the drug base.

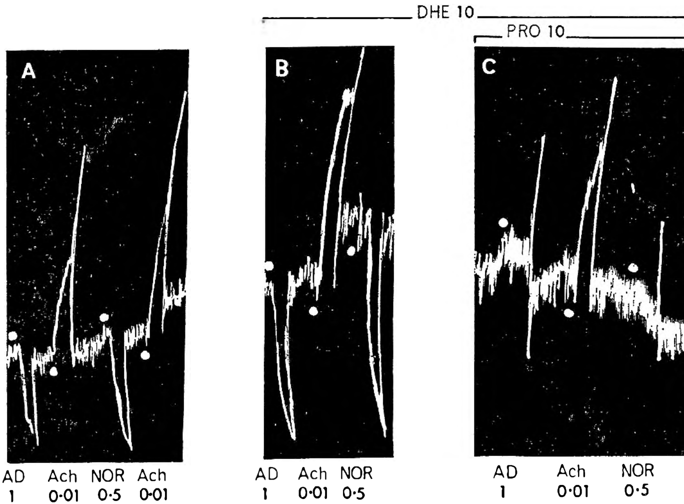

FIG. 4. The effect of dihydroergotamine (DHE) and pronethalol (PRO) on the responses of the longitudinal smooth muscle preparation from the isolated oesophagus of the guinea-pig to adrenaline (AD), noradrenaline (NOR) or acetylcholine (Ach). Responses in the absence of an antagonist are shown in A. In the presence of dihydroergotamine (10 μg/ml in B), the responses to the three drugs were not antagonised. Pronethalol (10 μg/ml) in the presence of dihydroergotamine in C abolished the inhibitory response to adrenaline or noradrenaline while the motor response to acetylcholine remained. Concentrations are expressed as final bath concentrations (μg/ml) in terms of the drug base.

Discussion

These experiments provide evidence for the presence of two kinds of adrenergic receptors in the smooth muscle of the oesophagus of the guinea-pig. These receptors correspond to the α- and β-actions defined by Ahlquist (1948, 1962). The receptor associated with the inhibitory response, whether of longitudinal or circular muscle strips, is most responsive to isoprenaline. The responses associated with this receptor are antagonised by pronethalol, a β-blocking agent (Black & Stephenson, 1962).

The receptor associated with contractile responses to sympathomimetic amines in both longitudinal and circular preparations, and which is unmasked by pronethalol, is most responsive to adrenaline. Furthermore, this receptor is blocked by the α-receptor antagonists dihydroergotamine or piperoxan. Thus there seem to be α-excitatory and β-inhibitory receptors in both circular and longitudinal components of the smooth muscle of the guinea-pig oesophagus.

Turning to the effect of concentration of the sympathomimetic amines, it was noticeable that on the longitudinal muscle preparation the α-effects were seen with lower concentrations, yet the β-effects, although initiated only at a higher threshold, predominated. A change from excitation to inhibition with increasing doses of adrenaline is an effect apparently not hitherto reported for the gastrointestinal tract. Various workers have

reported adrenaline-induced motor responses of muscularis mucosa from differing areas of the gut. Thus, Burnstock (1960) demonstrated adrenaline contractions of the muscularis mucosa of the domestic pig oesophagus. Walder (1953) found that adrenaline produced a contraction of some regions of the muscularis mucosa of human stomach which was antagonised by ergotamine. Similarly with preparations of the muscularis mucosa of the small intestine of the dog, King & Robinson (1945), and also King, Glass & Townsend (1947), observed contraction of the circular and longitudinal preparations, induced by adrenaline which could be antagonised by ergotamine but not by atropine. Adrenaline motor effects have also been reported in isolated segments of the terminal ileum of the guinea-pig (Munro, 1951).

In the gastrointestinal tract, adrenergic receptors, whether of α- or β-type reaction, are generally associated with inhibitory responses. However, in many other tissues the occurrence of α-excitatory and β-inhibitory receptors has been also demonstrated. Vascular smooth muscle is an obvious example; others include the isolated nictitating membrane of the cat (Thompson, 1958), the uterus of the guinea-pig (Hermansen, 1960), the bronchioles of the anaesthetised dog (Castro de la Mata, Penna & Aviado, 1962), the isolated junction of the bile duct and the duodenum (Crema & Berté, 1963) and the isolated vas deferens hypogastric nerve preparation (Holman & Jowett, 1964; Large, 1965).

It may be noted that the relative predominance of α- or β-effects varies from preparation to preparation. α-Excitation is predominant in the vas deferens, the nictitating membrane and the bile duct. β-Inhibition predominates in the bronchioles and also, as reported here, in the oesophagus.

Acknowledgements. I am grateful to Professor G. Brownlee for his helpful advice. This work was supported by a scholarship from the Medical Research Council.

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Macusine B: further pharmacology

B. E. LEONARD

Macusine B reduced the induction time and potentiated the barbiturate sleeping time of mice, possibly as a consequence of the hypothermia produced by the alkaloid. There was no evidence to suggest that macusine B affects the synthesis or metabolism of noradrenaline, dopamine, 5-hydroxytryptamine or γ-aminobutyric acid in the rat brain, nor was there any evidence to suggest that it affected the binding of noradrenaline and 5-hydroxytryptamine within the brain. There is an indication that macusine B may act on central adrenergic receptors. The slight depression of the behavioural activity which occurs in rats and mice after the injection of macusine B was probably due to the hypothermia induced by the alkaloid.

IN a previous investigation, macusine B was found to act on both adrenergic and tryptamine receptors (Leonard, 1965). This alkaloid also caused convulsions when injected into rats or mice. The present study was therefore undertaken to determine whether it was possible to explain the effect of macusine B on the brain by its effect on amine levels or on adrenergic receptors within this organ.

Experimental

EFFECTS ON THE BARBITURATE SLEEPING TIME OF MICE

Amylobarbitone sodium (100 mg/kg) was injected intraperitoneally into albino mice, 18–22 g, of the same sex. This produced a sleeping time of approximately 15 min in the control group, the members of which were also injected with 0·9% saline (10 ml/kg i.p.). The experimental groups were pretreated with macusine B, 25 mg/kg i.p., for 0, 1, 2½, 4, 8, 13 and 24 hr before being injected with amylobarbitone sodium. The mice were kept at room temperature (approximately 21°) during the course of the experiment and the induction time and sleeping time recorded. The induction time was taken as the period between the injection and the loss of righting reflex, and the sleeping time as the period between the loss and the return of the righting reflex.

EFFECT ON THE BODY TEMPERATURE OF MICE UNDER ANAESTHESIA

Groups of 10 albino mice (18–26 g) were injected with amylobarbitone sodium (100 mg/kg i.p.) after their rectal temperature had been recorded using a clinical thermometer. The control group was injected with 0·9% saline (10 ml/kg i.p.) and the experimental group with macusine B (10 mg/kg i.p.). The animals were kept at room temperature (approximately 21°) during the experiment and the sleeping time determined. The rectal temperature of the mice was taken at 5–10 min intervals for 1 hr, after the injection of the alkaloid, by means of a hypothermic thermometer*. The experiment was then repeated, using other mice, at an ambient temperature of 35° by placing the mice in a well ventilated oven immediately after they had lost their righting reflex.

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EFFECT ON BRAIN AMINE LEVELS

Groups of 5 albino rats (90–100 g) were injected with macusine B (10 mg/kg i.p.) and decapitated ½, 1, 2, 4 and 8 hr after the injection. The brain and heart were removed rapidly and blotted free from excess blood. A piece of cerebral cortex (approximately 100 mg) was removed from the left hemisphere, weighed, ground in a glass mortar with 0·01 N hydrochloric acid and the γ-aminobutyric acid content estimated fluorimetrically by the method of Lowe, Robins & Eyerman (1958) as modified by Uchida & O'Brien (1964). The remainder of the brain and the heart were weighed and dropped into a container of liquid oxygen. The tissues were crushed in a metal anvil and extracted with 0·4 N perchloric acid. Noradrenaline and dopamine were estimated in the supernatant by the methods of Anton & Sayre (1962; 1964). A similar group of animals was used for the estimation of the effect of macusine B on 5-hydroxytryptamine (5-HT) content of the brain. The brains were frozen in liquid oxygen and crushed in an anvil before being transferred to a salt-saturated butanol-hydrochloric acid mixture. The 5-HT was then estimated, mainly by the method of Shore & Olin (1958), but owing to the finding that macusine B has a fluorescence peak in the same region of the spectrum as 5-HT when assayed in dilute hydrochloric acid, the fluorophor was developed in 3 N hydrochloric acid as described by Bogdanski, Pletscher, Brodie & Udenfriend (1956). The amine content was also estimated in the brains and hearts of a group of 10 control rats which had been injected with 0·9% saline.

EFFECT ON THE DEPLETION OF BRAIN 5-HT AND NORADRENALINE INDUCED BY RESERPINE

Two groups of 10 albino rats (90–100 g) were injected with reserpine (2 mg/kg i.p.) and decapitated 4 hr later. Group 1 was injected with reserpine alone and group 2 was injected with reserpine and macusine B (10 mg/kg i.p.). The control group was injected with the polypropylene glycol, benzyl alcohol and citric acid vehicle used to dissolve the reserpine (Leyden, Pomerantz & Bouchard, 1956). The noradrenaline and 5-HT content of the brains were determined as described above.

EFFECT ON THE ACTIVITY OF RATS

The activity was recorded using an activity cage of dimension 13 × 12 × 30 cm in which a photoelectric cell was activated every time the rat intercepted a beam of light. The photoelectric cell was connected to a post office counter and the number of counts were recorded at hourly intervals. Six albino rats, 90–100 g, were put singly into the activity cages 1 hr before the start of the experiment. At the start of the experiment 3 animals were injected with saline, 10 ml/kg, and 3 with macusine B, 10 mg/kg i.p. Each animal acted as its own control and therefore on the second day of the experiment the rats were injected with either saline or macusine B.

EFFECT ON THE CONDITIONED AVOIDANCE RESPONSE OF RATS

Some 4 days before the start of the experiment, 5 rats were trained to jump over a barrier at the sound of an electric doorbell. This was an

avoidance reaction to the electric shock which was applied to the feet 5 sec after the bell should the animal fail to show the positive response. Two min later the bell was rung again and this was repeated for each rat 10 times per daily run. Three of the rats conditioned easily and gave approximately 9 positive responses by the fourth day of the training schedule, but 2 of the rats only gave 3 or 4 positive responses when subjected to the same schedule. Each day of the training period the animals were injected with saline (10 ml/kg i.p.) 10–15 min before being put into the conditioning cage. On the 5th day, all the animals were injected with macusine B (10 mg/kg i.p.) and the experiment was repeated.

EFFECT ON THE KNEE JERK REFLEX OF THE CAT

Two cats were anaesthetised with chloralose (100 mg/kg i.p.), the common carotid artery was cannulated and the knee jerk was elicited by an electrically driven hammer once every 10 sec. Macusine B (0·5–1·0 mg/kg) was injected into the femoral vein and the effects on the blood pressure and knee jerk reflex were recorded.

Results and discussion

Macusine B significantly reduced the induction time and increased the sleeping time to barbiturate anaesthesia in mice. The induction time was 5·81 $\pm$ 0·64 (standard error) min for the control group and 4·08 $\pm$ 0·04 min for the experimental group which had been injected with macusine B at the same time as the barbiturate ($P < 0·001$). There was a similar reduction in the induction time for anaesthesia between the control group and the other experimental groups in which macusine B was found to potentiate the sleeping time. The potentiation of the sleeping time was found to be greatest (280% of the control value) in the group of mice which had been treated 4 hr previously with macusine B, but there was no significant difference between the group of mice pretreated with the alkaloid for 13 hr and the control group (Fig. 1).

In the previous investigation of macusine B (Leonard, 1965) it was found that the alkaloid had marked convulsant properties and it was therefore of interest to determine in what way this alkaloid potentiated the barbiturate sleeping time. Mullen & Fouts (1965) in their investigation of several adrenergic blocking drugs found that those compounds potentiating the barbiturate sleeping time of mice did so either indirectly, by causing vasodilatation and hence hypothermia, or directly by blocking the breakdown of the barbiturate by the liver microsomes. To determine whether mascusine B, which also blocks some adrenergic receptors, potentiated the sleeping time in one of these ways, the effect of this alkaloid was investigated on the rectal temperature of mice under barbiturate anaesthesia. When the mice were at an ambient temperature of 20° the experimental group had a significantly lower rectal temperature than the control group, this difference being first apparent 15 min after injection of macusine B (Fig. 2). There was also a significant increase in the sleeping time of the experimental group compared with the control

group. However, when the experimental and control groups were kept at an ambient temperature of 35° the difference between the rectal temperatures and the sleeping time of the control (26·4 ± 1·5 min) and the experimental group (22·4 ± 0·5 min) was not significant. These results are therefore consistent with the view that macusine B potentiates the barbiturate sleeping time as a consequence of hypothermia and in this respect is comparable with another α-adrenergic blocking drug tolazoline which Mullen & Fouts (1965) found to potentiate the sleeping time in a similar way.

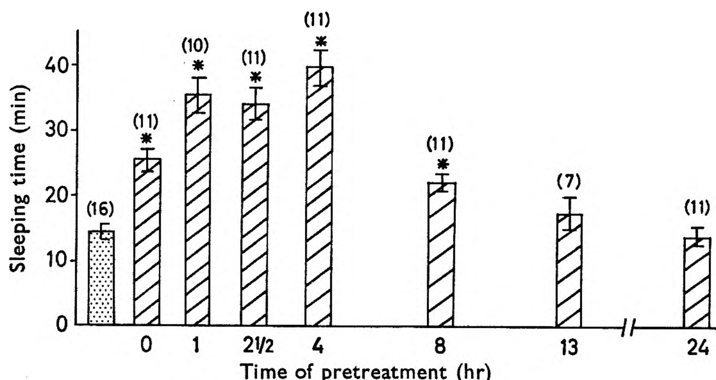

FIG. 1. Effect of macusine B on the barbiturate sleeping time of mice. Groups of mice injected with amylobarbitone sodium alone as controls (area stippled) or with amylobarbitone and macusine B (25 mg/kg) (areas hatched). The latter were pretreated with macusine B at the times indicated before injection of the barbiturate. The histograms represent the mean sleeping time ± standard error for each group. The number of animals per group is given in parentheses. * Difference between the experimental and control group significant at $P < 0{\cdot}001$ level.

The structure of macusine B and the similarity of its pharmacological properties to the ergot alkaloids (Leonard, 1965) suggests that it may have some effect on the synthesis, breakdown or binding of amines within the brain. The results of the experiments in which 5-HT, γ-aminobutyric acid, noradrenaline and dopamine were measured in the brain, and noradrenaline and dopamine in the rat heart, following the injection of macusine B, showed that this alkaloid had no apparent effect on the levels of these amines irrespective of the time of pretreatment with the alkaloid. Furthermore, when rats were injected with reserpine and macusine B, 4 hr before killing, there was no significant difference between the extent of the depletion of 5-HT and noradrenaline in the brain of the animals injected with reserpine alone (5-HT was 66% and noradrenaline was 40% of the control value) and the animals injected with macusine B and reserpine (5-HT was 60% and noradrenaline was 34% of the control value). These results suggest that, at least at the dose level used, macusine B does not affect the synthesis, binding or breakdown of these amines in the rat brain. The possibility exists that the alkaloid has a direct action on central adrenergic receptors and one method of investigating this possibility is by the effect of the alkaloid on the hyperactivity induced in mice by (±)-amphetamine. Preliminary results from this laboratory show that

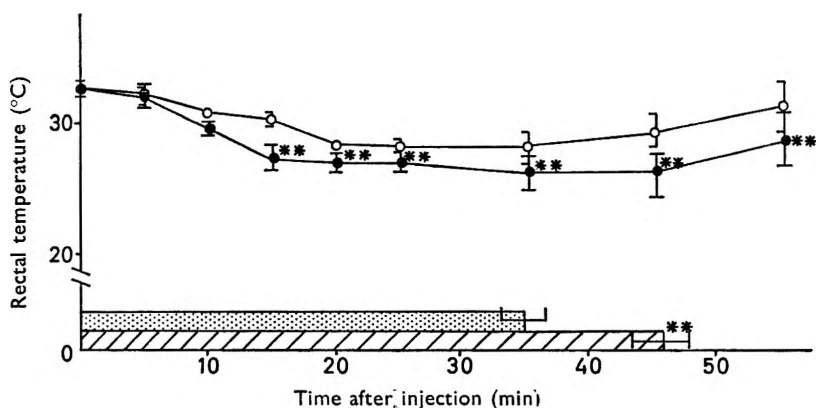

FIG. 2. Effect of macusine B on the rectal temperature of mice under barbiturate anaesthesia. Ambient temperature 20°. Groups of 12 mice injected with amylobarbitone sodium alone (○) or with amylobarbitone sodium and macusine B (10 mg/kg) (●). Each point represents the mean ± standard error. ** Difference between the experimental and control group significant at $P < 0{\cdot}05 > 0{\cdot}02$. The sleeping time of the control (area stippled) and experimental groups (area hatched) indicated in the histograms.

mascusine B partially blocks the amphetamine-induced hyperactivity suggesting that it has some action on central adrenergic receptors. In this respect, macusine B resembles the action of such α-adrenergic blocking drugs as tolazoline which have a similar effect on the amphetamine-induced hyperactivity (Tripod, 1952). Nickerson (1959) has suggested that one of the difficulties of attributing these effects of tolazoline to a specific central adrenergic blockade is that the effect is not quantitatively correlated with the peripheral adrenergic blocking activity, so that caution must be shown in attributing the blocking of the amphetamine induced hyperactivity by macusine B to a specific action on central adrenergic receptors.

On many occasions during the investigation of the general pharmacology of macusine B it was observed that rats and mice became behaviourally depressed when injected with subconvulsive doses of the alkaloid. As a result of this observation, the general activity of rats was measured over the 24 hr period after the injection of the alkaloid. The results show that animals injected with the alkaloid had a significantly lower activity over the first 12 hr period (226 ± 3·2 counts/12 hr) compared with the control animals (287 ± 7·8 counts/12 hr; $P < 0{\cdot}05 > 0{\cdot}02$). However there was no significant difference in the activity of these groups during the second 12 hr period of the experiment. From this it seems reasonable to conclude that the slight depression in activity produced by subconvulsive doses of the alkaloid was possibly due to hypothermia, but, in order to exclude the possibility of macusine B having any tranquillising activity, its effect on the conditioned avoidance response of rats was tested. The rats showed a total of 33 positive responses to the auditory stimulus after the fourth day of training. On the fifth day, after the same rats had been

injected with a subconvulsive dose of macusine B, the animals also gave a total of 33 positive responses to the stimulus. This experiment indicates that macusine B does not produce the depression in behaviour activity by a tranquillising action and therefore presumably produces this effect as a consequence of hypothermia.

In the previous investigation of the pharmacology of macusine B it was found that this alkaloid did not have any apparent effect on the neuromuscular junction *in vitro* even when present in high concentrations. However in the present study, when macusine B was injected *in vivo*, it was found to produce a transient depression of the knee jerk reflex accompanied by an appreciable fall in the blood pressure (approximately 40 mm mercury). When lower doses of the alkaloid were injected which did not affect the blood pressure, the knee jerk reflex was also unaffected. This seems to suggest that macusine B depressed this reflex as a consequence of its effect on the blood pressure. The general conclusion can also be reached that although macusine B is a convulsant alkaloid not structurally dissimilar to strychnine, it is evident from these studies that it is quite unlike strychnine in its pharmacological activities.

In conclusion, macusine B resembles other adrenergic blocking drugs in its pharmacological activities and, apart from its convulsant activity, it seems likely from the present investigation that its actions on the central nervous system are a consequence of its effect on the vascular system. It is possible that the alkaloid may have an effect on specific regions of the brain which are unlikely to be discovered by the methods used in the present study. It is of interest in this respect to find that lysergic acid diethylamide, while having little apparent effect on many of the conventional pharmacological screening tests used to investigate the actions of drugs on the central nervous system, is an inhibitor of cortical synapses (Marrazzi & Hart, 1955), a potent hallucinogen and furthermore does inhibit some peripheral (Hornykiewicz & Obenaus, 1958) and central (Goldstein, 1962) adrenergic receptors.

Acknowledgements. The author wishes to thank Professor A. R. Battersby for providing the sample of macusine B.

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The action of arsenic on *Bacillus cereus**

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Sodium arsenite (0·4 mM) inhibited the growth of exponentially growing *Bacillus cereus*, whereas sodium arsenate was growth-inhibitory only at 10 mM concentration. When the phosphate concentration was reduced sharply, 3·3 mM sodium arsenate inhibited growth, whereas the inhibitory effect of arsenite was independent of phosphate concentration. Neither arsenite nor arsenate produced any specific effects on the incorporation of precursors into ribonucleic and deoxyribonucleic acids, protein or cell wall, in support of the concept that their actions were at stages of energy utilisation rather than biosynthesis of macromolecules. Although some ^{74}arsenate may have been bound by cells during inhibition of growth, ^{74}arsenite was selectively and potently concentrated in micro-organisms. There was no evidence for the formation of arsenic-containing nucleic acids nor were labelled organic intermediates found. No instance of interconversion between pentavalent and trivalent states of arsenic could be established, and it was concluded that both compounds inhibited the growth of *B. cereus* by separate mechanisms.

THE chemical similarity between arsenic and phosphorus is believed to be responsible for many of the biochemical properties of the former element, particularly when in the pentavalent form. One example of this relationship is the process of arsenolysis (reviewed by Cohn, 1961), in which arsenic substitutes for phosphorus in intermediary metabolism. Although none of the low-molecular arsenic-containing analogues hypothesised as intermediates has yet been isolated, the possibility exists that if arsenic acts as a nucleic acid phosphorus analogue by replacement, stable cell constituents containing arsenic might be identified.

The use of radioisotopic arsenic with high specific activity offered a most sensitive technique for measuring such incorporation in arsenic-sensitive bacteria. Labelled arsenite was prepared from ^{74}arsenate, and information on the uptake of these drugs and their interconversions by exponentially growing cells was sought in relation to the effect of the drugs on growth, since pentavalent arsenicals are generally believed to become growth-inhibitory only after reduction to the trivalent form.

The *Bacillus cereus* system has served previously to demonstrate dissociations between various cell processes occurring during growth-inhibition produced by analogues of nucleic acid bases. Thus, 8-azaguanine specifically blocked the incorporation of amino-acids into proteins (Roodyn & Mandel, 1960a); 6-mercaptopurine and 6-thioguanine reduced the uptake of precursors into nucleic acids (Carey & Mandel, 1961; Mandel, Latimer & Riis, 1965); and 5-fluorouracil blocked the synthesis of deoxyribonucleic acid (DNA) (Reich & Mandel, 1964). Both 8-azaguanine and 5-fluorouracil have been shown to be incorporated extensively into polyribonucleotide derivatives in this system (Mandel, 1957; Reich & Mandel, 1964) and a trace of thioguanine was present in

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nucleic acids (Mandel & others, 1965). Evidence for similar effects was therefore sought for arsenic.

A preliminary report has been presented (Mandel & Mayersak, 1962).

Experimental

MATERIALS AND METHODS

Growth and fractionation of bacteria. *Bacillus cereus* 569H cultures were grown as described previously (Mandel, 1961). When the growth medium was required to be low in phosphate, 95% of the potassium phosphate was replaced by 9 mM potassium sulphate and 16 mM trishydroxymethylaminomethane (TRIS) buffer. Growth was measured turbidimetrically in a Beckman spectrophotometer model DU at 540 mμ. Cultures were sampled by removing 2 ml aliquots, measuring turbidity, and mixing with 2 ml saline, 10% trichloroacetic acid (TCA) or N potassium hydroxide before filtration through Schleicher and Schuell coarse membranes. For cells grown in the presence of radioisotopes, radioassay after cold TCA-washing measured radioactivity in all cell fractions except the acid-soluble pool. Correspondingly, filtration of cells washed in hot TCA led to recovery on the filter of protein and cell wall. For cells grown with labelled uracil, the potassium hydroxide treatment at room temperature, resulting in loss of the ribonucleic acid (RNA) from cells, allowed the measurement of radioactivity in DNA on the filter (Roodyn & Mandel, 1960b).

To isolate and identify radioactive compounds in cell fractions, 25 ml samples of cells were harvested when suspensions had an optical density of 0·4 at 540 mμ (corresponding to about 0·4 mg dry weight of cells per ml), washed in 80% ethanol at 85° to remove the soluble pool and lipid fraction, and incubated with N potassium hydroxide overnight to hydrolyse RNA into mononucleotides. The ethanol-soluble fraction was chromatographed on paper using aqueous isopropanol (Markham & Smith, 1952) or ammonium acetate: ethanol (Paladini & Leloir, 1952). Mononucleotides were separated at pH 3·5 by paper electrophoresis (Markham & Smith, 1952).

Total activities in cells were estimated by centrifuging cell suspensions and counting aliquots of medium and packed cells. Radioactivity was readily lost upon washing the cells, and therefore it needed to be measured for unwashed bacteria.

For the fractionation of cell-free preparations, all at 0°, washed cells suspended in 4·5 mM magnesium acetate and 1 mM TRIS buffer at pH 7·4 were disintegrated in a Mickle apparatus, and remaining cells and cell walls removed by centrifugation. The supernatant solution was extracted twice with saturated aqueous phenol to precipitate proteins (Kirby, 1956) and with ether to remove phenol; the nucleic acids were then precipitated with 2 volumes of ethanol. The precipitate was dissolved in water and the solution dialysed overnight against the TRIS buffer.

Respiration studies. Oxygen uptake was assessed by the usual manometric technique (Umbreit, Burris & Stauffer, 1957).

Radioisotopes. Guanine-8-^{14}C, uracil-2-^{14}C, and DL-leucine-1-^{14}C were obtained from Isotopes Specialties Co., Burbank, California, U.S.A. Diaminopimelic acid-^{3}H was a gift from Dr. J. Strominger, Washington Univ., St. Louis, Mo., U.S.A.

A procedure was developed for the preparation of trivalent arsenic by reduction of ^{74}arsenate purchased from Radiochemical Centre, Amersham, Buckinghamshire, England (1 mc in approximately 10 μg).

To convert pentavalent arsenic to the trivalent state, 4 mg sodium arsenate, containing 160 μc ^{74}As, was mixed with 90 mg sodium metabisulphite in 0·65 ml water and 0·02 ml concentrated hydrochloric acid, and was placed in a water-bath at 60° for 1 hr. The product, together with 200 μg arsenic trioxide carrier, was streaked on Whatman No. 3 MM paper, neutralised with dilute ammonia, and subjected to descending chromatography in 90% aqueous isopropanol. The area of the paper corresponding to arsenite was identified by radioautography, eluted with water and concentrated by evaporation *in vacuo*.

Arsenic analytical procedures. Arsenic trioxide was dissolved in 1% sodium carbonate. Trivalent arsenic was detected on paper chromatograms as a yellow spot after dipping the paper in a solution of diphenylthiocarbazone (dithizone) in carbon tetrachloride and air-drying. Arsenic trioxide had virtually no mobility during paper electrophoresis at pH 3·5. At pH 7 or above (by treatment of the origin area of the paper with dilute ammonia) the compound exhibited an Rf of about 0·5 during descending chromatography on Whatman 3 MM paper in isopropanol: water (9:1). In isopropanol:water (7:3) its Rf was 0·6. A trace of arsenic always remained at the origin.

Arsenic pentoxide was dissolved by heating in diluted hydrochloric acid or potassium hydroxide. It was detected on chromatograms as a brown spot by spraying with a saturated solution of potassium iodide containing hydrochloric acid. Sodium arsenate, dissolved in water at equivalent concentrations, provided identical results and could be used interchangeably. At pH 3·5 the pentavalent ion migrated towards the anode during electrophoresis, and at pH 7 or above, remained at the origin during chromatography in the 90% isopropanol system. In aqueous 70% isopropanol arsenate had an Rf of 0·3.

In vitro complex formations. Dimercaprol and thioctic acid (6,8-dithioloctanoic acid) were from Calbiochem, Los Angeles, Calif., U.S.A. Sulphydryl compounds were detected by the nitroprusside method (Smith, 1960). The sulphur-containing compounds were dissolved in ethanol, diluted in water, and arsenite-containing ^{74}As marker was added. Chromatography of the mixture in water rather than organic solvents provided the best separation of the components.

Results

EFFECT OF ARSENITE AND ARSENATE ON GROWTH OF *Bacillus cereus*

Concentrations of 40 μg/ml arsenic trioxide (equivalent to 0·4 mM arsenite) produced inhibition of growth for about 30 min, after which

partial recovery took place (Fig. 1). Cells present during the inhibitory or recovery phases were indistinguishable in size and appearance from control cells after Gram staining. Whereas 0·1 mM arsenite produced practically no effect on growth, 1 mM arsenite essentially abolished any increase in turbidity of the bacterial suspension for at least 2 hr. These results were independent of the concentration of phosphate in the two growth media used.

Sodium arsenate, on the other hand, only became inhibitory to growth of *B. cereus* at 3 mg/ml (10 mM). When the phosphate buffer of the medium was replaced by TRIS, growth inhibition was observed at considerably lower arsenate concentrations. With only 5% of the usual concentration of phosphate, 3·3 mM arsenate produced inhibition of growth corresponding to that observed with 0·4 mM arsenite. At 1 mM arsenate in the TRIS medium inhibition was either brief or non-detectable.

UPTAKE OF RADIOACTIVITY BY CELLS GROWING IN THE PRESENCE OF LABELLED ARSENATE

Cultures of *B. cereus* were grown in phosphate medium with arsenate at subinhibitory concentrations of $1{\cdot}2 \times 10^{-4}$ mM (no carrier added), 0·16 mM or 2·6 mM, or in TRIS medium during inhibition by 3·3 mM arsenate. Up to $1{\cdot}3 \times 10^{6}$ cpm of ^{74}arsenate were present per ml of medium. Filters containing cells after washing with cold TCA usually contained 0·05% of the radioactivity of the medium filtered, apparently due to adsorption by the filters. During growth of the culture in the presence of arsenate the radioactivity on the filters did not increase, implying that no appreciable progressive accumulation of ^{74}As had taken place.

To determine the quantity of ^{74}arsenate in the whole cell, growth of a bacterial suspension was inhibited with 3·3 mM arsenate, the cells centrifuged off and their radioactivity counted. The radioactivity calculated per mg wet weight of cells suggested that there was some selective uptake of arsenate by cells. The results varied partially because of unavoidable contamination with traces of radioactive medium. Any selective absorption of arsenate was minor compared to that of the corresponding arsenite binding (Table 1).

Upon extraction of inhibited cells with hot 80% ethanol, only unchanged radioarsenate was recovered in the extract as determined by paper electrophoresis and chromatography. When carrier arsenite was added to the cells before the ethanol extraction (to exclude the possibility of loss by oxidation of any trace of radioarsenite during the extraction procedure) and the arsenite re-isolated and radioassayed, no evidence of reduction of radioarsenate to arsenite by the micro-organisms could be demonstrated in this fraction. Incubation of the ethanol-extracted cell residue with potassium hydroxide and separation of the liberated RNA mononucleotides by paper electrophoresis revealed no radioactive metabolite containing arsenic.

Lack of incorporation of radioarsenic into nucleic acids was confirmed using cell-free preparations, thus avoiding the ethanol and alkali

treatments. Cells from 300 ml of suspension inhibited by arsenate for 1 hr were disintegrated to destroy cell walls, and the supernatant cell-free fraction extracted with phenol to precipitate proteins. Almost all of the radioactivity was recovered in the aqueous phase containing nucleic acids. Dialysis against TRIS buffer removed most of the residual activity, but a trace (0·1%) of the original isotope resisted dialysis and was concentrated inside the dialysis bag. When normal cells were disintegrated as above, and then dialysed against ^{74}arsenate-containing buffer and then non-radioactive buffer, a small amount of isotope again was associated with the undialysed fraction. This trace of radioisotope was thought not to be incorporated during the biosynthesis of nucleic acids but to be adsorbed to some non-dialysable cellular component, though not necessarily a nucleic acid.

TABLE 1. RELATIVE ACCUMULATION OF ^{74}ARSENITE OR ^{74}ARSENATE IN CELLS

^{74}Arsenic Compound	Expt.	Radioactivity			Growth	
		Cells (cpm/mg wet weight)	Medium (cpm/μl)	Ratio, cells: medium	Effect	Generation time ratio
Arsenate	1	102	63·5	1·6	inhibition	
	2	1887*	98	19	inhibition	20
		1340*	102	13·1	recovery	1·5
		283	108	2·6	recovery	1·3
Arsenite	1	416	3·64	114	inhibition	
	2	227	1·50	151	inhibition	11·5
		66	1·56	42	recovery	2·2
		51	1·74	29	recovery	1·4
	3	1170	0·82	1430	inhibition	17
		322	1·33	242	recovery	2·8
		209	1·54	136	recovery	1·8

Growth of *B. cereus* in radioactive medium inhibited by 3·3 mM arsenate and 0·3 mM arsenite, followed by the usual spontaneous recovery of growth. Bacterial suspension was centrifuged, and one ml samples of supernatant solution plated on planchets, desiccated and radioassayed. Packed cells were plated directly on tared planchets, desiccated, weighed and radioassayed. Wet weight assumes 80% water content in cells. Growth was measured before harvest of cells and is expressed as ratio of generation times of drug-treated and control cultures.

* These values probably represent some entrainment of radioactivity from medium.

UPTAKE OF RADIOACTIVITY BY CELLS GROWING IN THE PRESENCE OF LABELLED ARSENITE

Cells growing in the presence of radioarsenite supplemented with carrier to 0·4 mM and 1 mM were filtered and washed with cold TCA before measuring incorporation of arsenic by the membrane technique. The radioactivity on the filters did not increase progressively during 2 hr of incubation. Usually about 0·1% of radioactivity, present in the medium filtered, was present on all membranes, including the zero-time sample, apparently due to adsorption.

Measurement of radioactivity in cells after centrifugation revealed a selective uptake of arsenite by cells. On a weight basis, cells contained hundreds of times the concentration of radioarsenite of the surrounding medium (Table 1). As growth recovered in the presence of arsenite (Fig. 1) the relative accumulation decreased. Extraction of this radioactivity with hot 80% ethanol or water, followed by paper chromatography, revealed only unchanged arsenite. No metabolites of arsenite could be

found in the nucleic acid fraction, as measured after electrophoresis of the mononucleotides.

FORMATION OF CHEMICAL COMPLEXES WITH TRIVALENT ARSENIC

Since the possibility existed that a complex of trivalent arsenic and thioctic acid might be present in cells, attempts were made to synthesise this compound. The interaction *in vitro* between these two compounds by varying pH, concentration, temperature, solvents and periods of interaction followed by chromatography led only to the recovery of the two reagents. By contrast, the interaction between dimercaprol and trivalent arsenic resulted in the immediate formation of an insoluble product with an Rf in water between that of dimercaprol and arsenite.

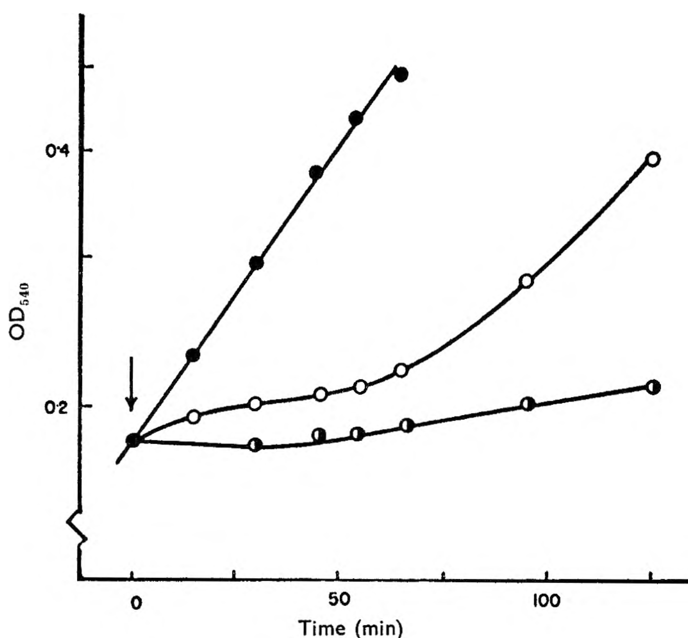

FIG. 1. Effect of arsenite on growth of exponentially growing *Bacillus cereus*. Concentration of arsenite: ●, 0; ○, 0·4 mM; and ◑, 1 mM.

RECOVERY FROM ARSENITE GROWTH INHIBITION

Cells inhibited by arsenite resumed growth after a lag period, the duration of which was related to the concentration of arsenite. The new rate of turbidimetric increase was less than that of control cells (Fig. 1), suggesting some residual cell effect which gradually was diluted out during subsequent growth. Cells which had adapted to grow in 0·4 mM arsenite could tolerate the addition of another 1 mmole of arsenite per litre without a significant decline in the growth rate. Greater additions again produced slowing of the rate of turbidimetric increase (Fig. 2).

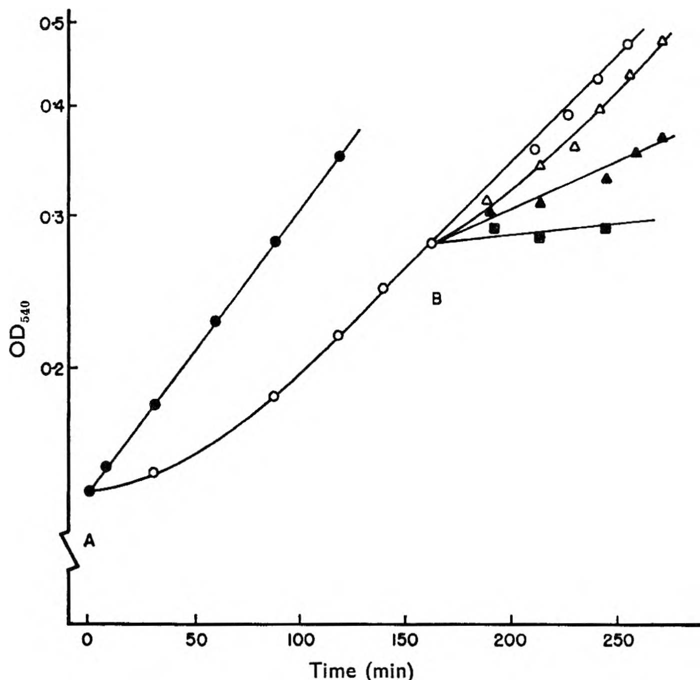

FIG. 2. Adaptation of cells of *B. cereus* to arsenite. At A, one culture received arsenite (0·4 mM) and was incubated until growth had recovered. At B, the culture was subdivided and more arsenite added. Final concentration of arsenite: ●, 0; ○ 0·4 mM; △, 1·4 mM; ▲, 2·4 mM; ■, 3·4 mM.

One possible explanation for the adaptation to arsenite would be its oxidation to arsenate, which does not inhibit growth at equimolar concentrations. Examination by chromatography and paper electrophoresis of growth medium of bacterial suspensions recovering from inhibition by ^{74}arsenite indicated the absence of labelled arsenate or any compound other than arsenite. There was still an inhibitory concentration of arsenite present in the medium in spite of the uptake of the drug by the cells, as also demonstrated by the inhibition of growth of fresh cells added to this medium. Thus, oxidation, which with less refined techniques at first appeared likely (Mandel & Mayersak, 1962) was not responsible for the adaptation. More recent experiments (Reich, M. & Mandel, H. G., unpublished) have suggested that cells in the presence of arsenite produce in the medium a substance which forms a dissociable complex with arsenite and thus reduces the effective concentration of the inhibitory arsenite.

EFFECT OF ARSENITE AND ARSENATE ON BIOSYNTHESES OF *B. cereus*

To determine whether arsenic treatment resulted in a specific effect on one of the major chemical components of cells, bacteria were grown in the presence of labelled precursors of various cell fractions in the presence

and absence of arsenite and arsenate at partially inhibitory concentrations. In Fig. 3, the uptake of radioactivity per ml of bacterial culture has been plotted against the progressive increase in bacterial turbidity. Such a parameter minimises the slower growth in the presence of the inhibitor and allows comparisons of relative utilisation of each isotope. It has been established from previous work that exogenous guanine-^{14}C labels RNA and DNA exclusively, leucine-^{14}C is taken up only by protein, uracil-^{14}C serves to measure exclusively DNA pyrimidines when cells are subsequently treated with potassium hydroxide, and diaminopimelic acid-^{3}H represents incorporation selectively into cell wall (Roodyn & Mandel, 1960b). Inhibition of growth by arsenite produced no specific effect on any of the reactions and the uptake of all of the isotopes was directly related to the formation of new cellular material. Correspondingly, when growth was completely inhibited by higher concentrations of arsenite, incorporation of any of the isotopic precursors was not evident.

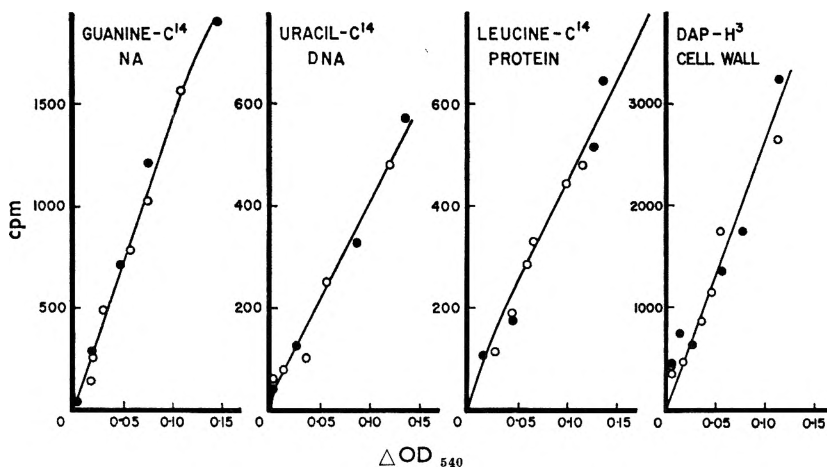

FIG. 3. Effect of arsenite on biosynthetic processes as a function of growth. Incorporation of ^{14}C-guanine measured labelling of nucleic acids, that of ^{14}C-uracil (followed by KOH digestion of RNA) for DNA formation, that of ^{14}C-leucine for protein synthesis, and that of ^{3}H-diaminopimelic acid for cell wall biosynthesis exclusively. Arsenite concentrations: ●, 0; ○, 0·4 mM.

Inhibition of growth by arsenate, similarly, did not involve a selective action on a particular cell fraction, and no dissociation of RNA, DNA or protein could be observed.

EFFECTS OF ARSENITE AND ARSENATE ON RESPIRATION

The rate of uptake of oxygen by cultures of cells growing in a Warburg apparatus in the presence of varying concentrations of arsenite (Fig. 4) and arsenate was found to be depressed when growth was inhibited. When comparisons were made after equivalent growth of the cultures, the consumption of oxygen exceeded that of a control culture, indicating that biosynthesis of macromolecules was curtailed to a greater extent by

the inhibitors than was oxygen uptake, and supporting the generally held concepts that the compounds act at early stages of energy utilisation rather than macromolecular biosynthesis.

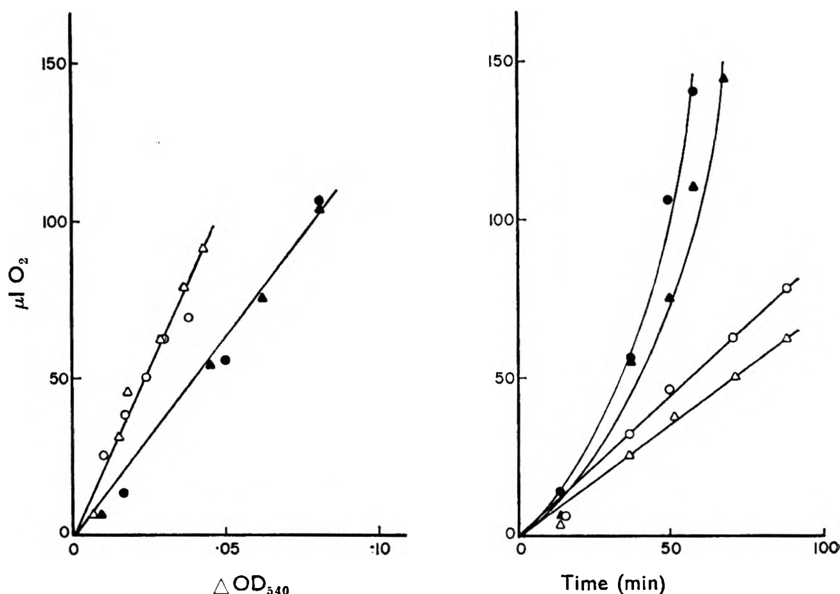

FIG. 4. Effect of arsenite on oxygen uptake of growing *B. cereus* cultures. Arsenite concentrations: ●, 0; ▲, 0·2 mM; ○, 0·4 mM; and △, 0·6 mM. Left, comparisons made with relation to increase in turbidity; right, rate of oxygen uptake.

Discussion

Effect of arsenite and arsenate on growth. The greater inhibitory action of arsenite than that of arsenate on growing cells has been reported for microbial and other systems (Loy, Schiaffino & Savchuck, 1961). The inverse relationship between arsenate action and phosphate concentration (Sussmann & Spiegelman, 1950; Rothstein, 1963) is in contrast to the independence of arsenite inhibition and phosphate concentration, as reported here.

Arsenite metabolism and binding. The interaction of trivalent arsenicals with sulphhydryl-containing tissue constituents, particularly the reduced form of thioctic (α-lipoic) acid in an enzyme-bound form, is believed to be extremely sensitive (Gunsalus, 1953; Sanadi, Langley & White, 1959). Although Reiss (1958) has reported evidence suggesting that γ-(*p*-arsenoso-phenyl)-n-butyrate *in vitro* formed a complex with dihydrolipoic acid, a complex between arsenite and thioctic acid was not detectable *in vivo* or *in vitro*, perhaps because of insufficient concentration of reduced thioctic acid, or the ready dissociation of such a complex. A complex between arsenite and dimercaprol could be prepared readily, however.

The trypanocidal action of particular organic arsenicals was observed to be related to the binding of the drugs to the parasite (Hawking, 1937),

and arsenosobenzene was found to be concentrated in susceptible trypanosomes 200 times more than its concentration in the supernatant solution (Eagle & Doak, 1951). In the present study with micro-organisms, the selective uptake of inorganic arsenite, but not that of arsenate, paralleled that of the organic derivatives, and appeared to be related to growth inhibition.

Arsenate metabolism and action. The elucidation of the metabolic alteration of arsenate has been severely limited by the suspected instability of organic arsenate esters in water, unlike that of the corresponding phosphates (suggested by Warburg & Christian, 1939). The inability to demonstrate any of the postulated radioactive organic arsenates in arsenate-treated *B. cereus* must be due to their instability, since the isotope technique is extremely sensitive. The observed arsenolysis of adenosine diphosphate tc adenosine monophosphate in the presence of arsenate and polynucleotide phosphorylase (instead of polyadenylic acid synthesis) (Singer, 1963), probably is related to the compound's inhibition of nucleic acid synthesis, and the instability of such intermediates as adenosine-monophosphate-monoarsenate may explain the lack of incorporation of arsenic into polynucleotides of growing *B. cereus* cultures.

The relative lack of trypanocidal effectiveness of pentavalent arsenicals and the long delay in onset of action in comparison with the trivalent compounds led Ehrlich (1909) to suggest that the pentavalent compounds are reduced by the host to the biologically active trivalent derivatives. The lack of any such conversion in arsenate-treated *B. cereus*, as indicated by the absence of arsenite in the soluble fraction of cells, implies that the growth inhibition of arsenate is not due to reduction to arsenite. The separate actions of arsenate and arsenite is also demonstrated in the different susceptibilities to variations in phosphate concentration in the medium. It must be concluded, therefore, that in these experiments arsenate is growth-inhibitory *per se*, probably because of its antagonistic action to phosphate.

Recently, a report by Kay (1965) has appeared documenting the incorporation of ^{74}arsenate into nucleic acids of Ehrlich Lettré ascites carcinoma *in vitro*. Each of the mononucleotides isolated from nuclear and cytoplasmic RNA was labelled, and DNA also contained radioactivity. The possible incorporation of arsenate into messenger RNA was suggested.

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On sympathetic regulation of carbohydrate metabolism in the liver

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Glycogenolysis in the liver produced by isoprenaline was inhibited by β-receptor blocking agents using the perfusion technique. Dichloroisoprenaline also inhibited spontaneous glucose release, as did α-receptor stimulation by noradrenaline.

REGULATION of glucose level, the main energy source of the organism, is under the influence of the sympathetic nervous system. Dale, in 1906, suggested that sympathetic nervous activity was mediated through two different receptors, thus explaining the modified response to adrenaline after the administration of ergot alkaloids. Ahlquist (1948) demonstrated that sympathomimetic agents exerted their effect on two different receptors, which he called alpha and beta. Since then several authors have examined the mode of action of sympathomimetic agents on the receptors and their effect on carbohydrate metabolism. Thus, van der Pool (1956) found both kinds of receptors were involved in the development of hyperglycaemia, since the α-receptor stimulator noradrenaline, given together with isoprenaline (a β-receptor stimulator), mutually increased the hyperglycaemic effect. Regulation of the glycogenolysis of the striated musculature is considered to be a β-receptor function (Ellis, Davis & Anderson, 1955; Vrij, Gho, de Groot & Weber, 1956; Furchgott, 1959), but in liver glycogenolysis an important role is attributed to the α-receptors. Thus, Vrij & others (1956) demonstrated *in vivo* that noradrenaline decreases the glycogen content of the rat liver, whereas isoprenaline given in similar doses does not. Again, Ellis (1951) and van Roy & Schulhof (1961) could not find any effect of isoprenaline on the glycogen content of liver slices. The experiments of Sutherland & Cori (1948) showed that adrenaline increased glucose release from liver slices more than did noradrenaline or isoprenaline. The present text is concerned with a re-examination of the problem using the perfused isolated liver of the rat.

METHODS

Albino rats of either sex, of the same stock, weighing 150–220 g and kept on standard diet, were used. The animals were bled and an isolated liver perfusion prepared (Issekutz, 1924). Cannulae were introduced into the portal vein and after washing through with Tyrode fluid for 15 min they were perfused with 100 ml of glucose-free Tyrode solution or with Tyrode solution containing 100 mg % glucose. Phenoxybenzamine, 10 mg % or dichloroisoprenaline, 0·7 mg %, was added to the perfusion fluid. Concentrations of pronethalol, 0·7 mg%, noradrenaline 0·05 mg% or (–)-isoprenaline, 0·002 mg %, were used. The liver was perfused at

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4 ml/min with a total volume of 100 ml Tyrode solution at 37° thermostatically controlled and also with constant oxygenation of the perfusion fluid. The glucose content of the fluid was determined every 30 min (Hagedorn & Jenson, 1923) for 2 hr, amino-acid nitrogen determinations were made 30 min after the 15 min preliminary period and after 2 hr (see Danielson, 1933). In the isolated organ-bath, according to Kline (1949) and Kaufmann & Wertheimer (1957), the increased nitrogen which is released secondary to cellular damage becomes negligible after the first 30 min, and the release of nitrogen which follows is a true indicator of protein metabolism. At the beginning and the end of the experiment the glycogen content of the liver was determined by the Good-Kramer-Somogyi method (1933). Each group, containing 10 livers, was analysed by the Student "*t*" test.

Results

Glucose released from the isolated perfused liver increased significantly during perfusion with glucose-free Tyrode solution and the decrease in glycogen content was accelerated significantly. Dichloroisoprenaline (Powell & Slater, 1958), a β-receptor blocking agent (Moran & Perkins, 1958), practically abolished the release of glucose. On the other hand, phenoxybenzamine, an α-receptor blocking agent, proved to be ineffective (Table 1). β-Receptor blockade also entirely inhibited the spontaneous release of glucose after perfusion with normal Tyrode solution containing glucose. β-Receptor blockade also increased the amino-acid nitrogen released from the liver, and this can perhaps be considered as an indicator of glyconeogenesis. In this respect the effect of dichloroisoprenaline was similar to that of the oral hypoglycaemic agent chlorpropamide (Pogátsa & Káldor, 1965).

TABLE 1. PERFUSION OF THE ISOLATED LIVER OF THE RAT. ALTERATIONS OF GLUCOSE RELEASED, GLYCOGEN CONCENTRATION AND AMINO-ACID NITROGEN RELEASED INTO NORMAL TYRODE SOLUTION OR INTO GLUCOSE-FREE TYRODE SOLUTION, AFTER THE ADMINISTRATION OF PHENOXYBENZAMINE OR DICHLOROISOPRENALINE IN GLUCOSE-FREE SOLUTION

	mg glucose/g liver/hr (mean ± s.e.) at times (min)				mg amino-acid nitrogen/g liver/hr (mean ± s.e.) at times (min)		Change of glycogen content %
	30	60	90	120	30	120	
Glucose-free Tyrode solution	18·4±1·4*	6·0±0·81	7·0±0·87*	7·3±0·89*	1·54±0·22	0·05±0·02	−77±4*
Tyrode solution ..	10·2±1·5	4·8±2·5	4·3±1·4	4·6±1·8			−49±9
Phenoxybenzamine	17·9±1·4	10·1±1·05*	9·0±1·0	6·0±0·8	1·56±0·11	0·07±0·02	−67±6
Dichloro-isoprenaline ..	3·0±0·5*	0·3±0·17*	0·2±0·14*	1·1±0·34*	1·41±0·12	0·25±0·05*	−36±6*

Phenoxybenzamine 10 mg/100 ml, and dichloroisoprenaline 0·7 mg/100 ml were each given at the beginning of perfusion. Significance* between groups 1 and 2: $P<0·001$; 3 and 1: $P<0·01$; 4 and 1: $P<0·001$.

The release of glucose from livers perfused with normal Tyrode solution containing isoprenaline (0·002 mg %) was increased significantly. Noradrenaline, in concentrations which produced selective α-receptor stimulation, inhibited the release of glucose. Dichloroisoprenaline inhibited

the increased release of glucose produced by isoprenaline and also inhibited the decrease of glycogen. Pronethalol (0·7 mg %) did not inhibit the spontaneous release of glucose (dichloroisoprenaline proved to be effective here) but abolished the effect of isoprenaline in releasing glucose (Table 2). The β-receptor blocking activity of pronethalol proved to be ten times more powerful than that of dichloroisoprenaline using the method of Smith (1963) on the nictitating membrane; the effect of pronethalol on the inhibition of glycogenolysis produced by isoprenaline is also stronger and more lasting.

TABLE 2. PERFUSION OF THE ISOLATED LIVER OF THE RAT. ALTERATIONS OF GLUCOSE RELEASED AND GLYCOGEN CONCENTRATION AFTER THE ADMINISTRATION OF NORADRENALINE, ISOPRENALINE, DICHLOROISOPRENALINE OR PRONETHALOL.

	mg glucose/g liver/hr (mean ± s.e.) at times (min)				Change of glycogen content %
	30	60	90	120	
Tyrode solution	10·2 ± 1·5	4·8 ± 2·5	4·3 ± 1·4	3·6 ± [illegible]·8	−49 ± 9
Noradrenaline	9·9 ± 0·9	−2·1 ± 0·5	−2·6 ± 0·5*	−5·1 ± [illegible]·2*	−64 ± 7
Isoprenaline	11·9 ± 1·8	16·0 ± 2·9*	10·6 ± 3·0	6·1 ± 3·7	−89 ± 3*
Dichloroisoprenaline + isoprenaline	3·6 ± 1·7*	−3·8 ± 1·7*	1·4 ± 2·6*	−1·4 ± 2·2	−29 ± 10*
Pronethalol + isoprenaline	8·2 ± 3·4	0·1 ± 1·3*	0·7 ± 2·5*	−6·3 ± 1·4*	−54 ± 35*

Dichloroisoprenaline 0·7 mg/100 ml and pronethalol 0·7 mg/100 ml were each given at the beginning of the perfusion and noradrenaline 0·05 mg/100 ml, isoprenaline 0·002 mg/100 ml after 30 min. Each group contained 10 livers. Significance (*) between groups 2 and 1: P<0·01; 3 and 1: P<0·001; 4 and 3: P<0·001; 5 and 3: P<0·001.

Discussion

The results of these experiments are in line with the observations of Sutherland & Rall (1960) which demonstrated that phosphorylase activation was most effective with isoprenaline, and this activation was inhibited by β-receptor blocking agents (Hornbrook & Brody, 1963). Furthermore, dichloroisoprenaline and pronethalol prevented the action of adrenaline or isoprenaline on carbohydrate metabolism in the heart and skeletal muscle (Mayer, Moran & Fain, 1961; Murad, Chi, Hall & Sutherland, 1962). Comparing our results on the liver and the observations of other authors on the muscle, it seems that receptors sensitive to isoprenaline distributed throughout the organism have a physiological function in carbohydrate metabolism, and receptors that are stimulated by isoprenaline may have a regulatory function in these metabolic processes.

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Preparation and fungicidal properties of (phenylthio)-acetohydroxamic acids and related compounds

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1-Chloro-4,5-dimethoxy-2-nitrobenzene and 1-bromo-2,5-dimethoxy-4-nitrobenzene condensed readily with thioglycollic acid in alkaline medium to give the corresponding sulphides. (4,5-Dimethoxy-2-nitrophenylthio)- and (2,5-dimethoxy-4-nitrophenyl-thio)acetic acid, as well as the corresponding amides and hydroxamic acids, have been prepared and tested for their fungicidal properties against *Aspergillus niger* and *Rhizoctonia solani*. The (phenylthio)acetohydroxamic acids were found to be more fungicidal than the corresponding carboxylic acids against the phytopathogenic fungus *Rhizoctonia solani*.

RECENTLY, phenoxyacetohydroxamic acids have been reported to possess fungicidal activity. Eckstein & others (1956, 1959, 1960, 1963) have studied the influence of substituents in the aromatic ring of phenoxy-acetohydroxamic acids on fungicidal properties. It seemed, therefore, of interest to investigate the properties of (arylthio)acetohydroxamic acids, as it is known that (arylthio)alkanecarboxylic acids are more fungicidal than the corresponding aryloxy derivatives (Crowdy & Wain, 1951; Fawcett, Spencer & Wain, 1955, 1957). In an attempt to prepare biologically active compounds from 1-chloro-4,5-dimethoxy-2-nitrobenzene, its condensation with thioglycollic acid has been investigated. The veratrole structure, a benzene ring carrying two adjacent methoxyl groups, is of interest since it is present in a number of physiologically active alkaloids including papaverine, gnoscopine and brucine.

(4,5-Dimethoxy-2-nitrophenylthio)acetic acid (Ia) and (2,5-dimethoxy-4-nitrophenylthio)acetic acid (IIa), as well as some of their derivatives, have been prepared. The carboxylic acids Ia and IIa, the hydroxamine acids Id and IId, and the amides Ic and IIc have been assessed for their fungistatic effect on *Aspergillus niger* and *Rhizoctonia solani*.

Thioglycollic acid condensed readily with 1-chloro-4,5-dimethoxy-2-nitrobenzene in alkaline medium to give Ia. For comparison, the other isomer (2,5-dimethoxy-4-nitrophenylthio)acetic acid (IIa), was similarly prepared from 1-bromo-2,5-dimethoxy-4-nitrobenzene. The methyl esters Ib and IIb were obtained from the corresponding acids by the action of ethereal diazomethane solution. These carboxylic acids and their methyl esters were easily oxidised with hydrogen peroxide in acetic acid to the corresponding sulphones. The methyl esters were converted into the corresponding hydroxamic acids by reaction with a freshly prepared alkaline hydroxylamine solution in methanol. The free hydroxamic acids were precipitated from the aqueous solution of their potassium salts by acidification. Hydroxamic acids corresponding to the sulphones of the methyl esters were unstable and could not easily be isolated.

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The acid chlorides of Ia and IIa were relatively stable crystalline compounds. They condensed readily with ammonia to give the corresponding amides.

Experimental

(4,5-*Dimethoxy-2-nitrophenylthio)acetic acid* (*Ia*) *and its sulphone* (*Ie*). To a solution of 1-chloro-4,5-dimethoxy-2-nitrobenzene (4 g) in hot ethanol (30 ml) 80% thioglycollic acid (3 ml) was added, followed by the dropwise addition of N sodium hydroxide (52 ml) to the refluxing mixture over 3 hr, and then further heating for 1 hr. The ethanol was distilled off and the remaining deep-red solution filtered. Compound Ia was precipitated from the cooled filtrate by the addition of dilute hydrochloric acid and was obtained as yellow needles from ethanol, m.p. 217°, yield 95%. Found: C, 43·9; H, 4·1; N, 5·4. $C_{10}H_{11}NO_6S$ requires C, 44·0; H, 4·0; N, 5·1%.

By oxidation of compound Ia, the sulphone Ie was obtained in 80% yield as colourless crystals from ethanol, m.p. 201°. Found: N, 4·4; S, 10·8. $C_{10}H_{11}NO_8S$ requires N, 4·6; S, 10·5%.

(2,5-*Dimethoxy-4-nitrophenylthio)acetic acid* (*IIa*) *and the sulphone* (*IIe*). 1-Bromo-2,5-dimethoxy-4-nitrobenzene (4 g) was allowed to react with thioglycollic acid in presence of sodium hydroxide as above. Compound IIa was obtained as yellow crystals from benzene, m.p. 167°, yield quantitative. Found: C, 44·3; H, 3·9; N, 4·9. $C_{10}H_{11}NO_6S$ requires C, 44·0; H, 4·0; N, 5·1%.

The oxidation of compound IIa gave the sulphone IIe, m.p. 188° (from ethanol). Found: C, 39·9; H, 3·7. $C_{10}H_{11}NO_8S$ requires C, 39·3; H, 3·6%.

Preparation of the methyl esters of compounds Ia and IIa and the sulphone If. The appropriate acid (1 g) was treated with an ethereal diazomethane solution (prepared from 2 g of nitrosomethylurea) and the mixture was left at room temperature for 12 hr. After evaporating off the ether the residue was crystallised from ethanol, yield quantitative.

Compound Ib formed orange crystals, m.p. 163°. Found: C, 45·9; H, 4·6. $C_{11}H_{13}NO_6S$ requires C, 46·0; H, 4·5%.

The sulphone If, obtained by oxidation of the methyl ester Ib with hydrogen peroxide, was identical with that obtained by esterification of the sulphonyl acetic acid Ie with diazomethane; it had a m.p. of 163° from dilute ethanol. Found: C, 41·2; H, 4·4. $C_{11}H_{13}NO_8S$ requires C, 41·4; H, 4·1%.

Compound IIb formed orange crystals, m.p. 117°. Found: C, 46·1; H, 4·7. $C_{11}H_{13}NO_6S$ requires C, 46·0; H, 4·5%.

Preparation of the hydroxamic acids Id and IId. A methanolic solution of alkaline hydroxylamine (prepared from 0·005 mole of hydroxylamine hydrochloride and 0·01 mole potassium hydroxide) was added to the methyl ester Ib or IIb (0·5 g) and the reaction mixture left at room temperature for 1 hr with occasional shaking. The methanol was then removed and water (10 ml) added. Acidification of the deep-red solution

with dilute sulphuric acid gave the corresponding hydroxamic acid Id or IId (orange crystals from ethanol) in a yield of 75–80%. Compound Id melted at 180°. Found: C, 41·4; H, 4·3; N, 10·1; S, 10·7. $C_{10}H_{12}N_2O_6S$ requires C, 41·7; H, 4·2; N, 9·7; S, 11·1%. Compound IId melted at 162°. Found: N, 9·5; S, 11·4. $C_{10}H_{12}N_2O_6S$ requires N, 9·7; S, 11·1%. Compounds Ic and IId were readily soluble in dilute sodium hydroxide solution and gave an intense violet colour with ethanolic ferric chloride solution.

Preparation of the acid amides Ic and IIc. The carboxylic acids Ia and IIa were converted into their acid chlorides by heating with an excess of thionyl chloride in carbon disulphide for 1 hr. The solution was filtered and the filtrate cooled, whereupon the acid chloride crystallised out. The acid chloride (1 g) was shaken vigorously with concentrated ammonia solution (5 ml) for 10 min, whereupon the amides separated at once. They were obtained in quantitative yields and were crystallised from ethanol. Compound Ic formed yellow crystals, m.p. 222°. Found: C, 43·9; H, 4·5; N, 10·0. $C_{10}H_{12}N_2O_6S$ requires C, 44·1; H, 4·4; N, 10·3%. Compound IIc gave yellow crystals, m.p. 186°. Found: C, 44·2; H, 4·4; N, 10·2. $C_{10}H_{12}N_2O_5S$ requires C, 44·1; H, 4·4; N, 10·3%.

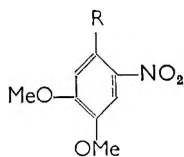
BIOLOGICAL RESULTS

The biological tests on the fungicidal activity were made on *Aspergillus niger* and the phytopathogenic mould *Rhizoctonia solani.* The effects were assessed with concentrations of the compounds of 10^{-4} and 10^{-5} M using the Dox liquid medium. Because of their slight solubility in water the compounds were dissolved in aqueous ethanol (final ethanol concentrations were 1 and 0·1% for the 10^{-4} and 10^{-5} M solutions respectively). The same ethanol concentrations were added to the nutrient media of the controls. Flasks were inoculated with a spore suspensionof *Aspergillus niger* or with discs of *Rhizoctonia solani* and incubated at 25° for five days. At the end of the incubation period, the mycelial mats produced were filtered, washed, dried at 100° and weighed. Replicate samples were used for each treatment.

TABLE 1. DRY WEIGHTS OF FUNGAL MATS OF *Rhizoctonia solani* PRODUCED ON DOX MEDIUM AND INHIBITION (%) EFFECTED BY THE CARBOXYLIC ACIDS Ia AND IIa, THEIR AMIDES AND HYDROXAMIC ACIDS AT 10^{-4} M CONCENTRATIONS

Substance	Compound	Dry weight* of the mycelium (mg)	% inhibition
Control	—	286	—
(4,5-Dimethoxy-2-nitrophenylthio)acetic acid	Ia	265	—
(2,5-Dimethoxy-4-nitrophenylthio)acetic acid	IIa	225	21
(4,5-Dimethoxy-2-nitrophenylthio)acetamide	Ic	70	75·5
(2,5-Dimethoxy-4-nitrophenylthio)acetamide	IIc	313	—
(4,5-Dimethoxy-2-nitrophenylthio)acetohydroxamic acid	Id	62	78·3
(2,5-Dimethoxy-4-nitrophenylthio)acetohydroxamic acid	IId	78	72·7

* The dry weight is the mean of three replicates.



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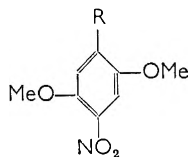

II

Ia, R = $S \cdot CH_2 \cdot CO_2H$
b, R = $S \cdot CH_2 \cdot CO \cdot O \cdot Me$
c, R = $S \cdot CH_2 \cdot CO \cdot NH_2$
d, R = $S \cdot CH_2 \cdot C(:N \cdot OH) \cdot OH$
e, R = $SO_2 \cdot CH_2 \cdot CO_2H$
f, R = $SO_2 \cdot CH_2 \cdot CO \cdot O \cdot Me$

IIa, R = $S \cdot CH_2 \cdot CO_2H$
b, R = $S \cdot CH_2 \cdot CO \cdot O \cdot Me$
c, R = $S \cdot CH_2 \cdot CO \cdot NH_2$
d, R = $S \cdot CH_2 \cdot C(:N \cdot OH) \cdot OH$
e, R = $SO_2 \cdot CH_2 \cdot CO_2H$

Statistical analysis of the results obtained showed that none of the chemicals tested possessed any fungicidal activity against *Aspergillus niger* at the above-mentioned concentrations. At a concentration of 10^{-4} M compounds Ic, Id and IId effected significant inhibition in the growth of *Rhizoctonia solani* (cf. Table 1). The other compounds showed less activity but none of the compounds had an effect at concentration of 10^{-5} M.

These results show that the hydroxamic acids Id and IId are more fungicidal to *Rhizoctonia solani* than the corresponding carboxylic acids Ia and IIa. The amide Ic, which contains an *o*-nitro-group, shows fungicidal properties comparable to those of the hydroxamic acids, while the *p*-nitro-amide IIc does not show such properties.

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Letters to the Editor

Reactivity of rats to dextran

SIR,—A single intraperitoneal injection of dextran produces in rats an inflammatory anaphylactoid reaction which is mediated chiefly through a release of 5-hydroxytryptamine and histamine (Parratt & West, 1957). Not all rats of the Wistar strain, however, react to this injection and also fail to respond to subsequent injections. The animals which do not respond to any of three weekly injections of dextran have been referred to as non-reactors (Harris & West, 1963), and non-reactivity has been shown to be a genetically-controlled recessive character.

Recently, the opportunity arose to test a large batch of male and female Wistar rats from a colony (Wellcome Research Laboratories, Beckenham) which had not previously shown non-reactivity. They were injected intraperitoneally with dextran (Intradex, Glaxo) at doses of 180 mg/kg, and scored for reactivity (gross oedema of the extremities) over the next 4 hr. Those which did not react were re-injected with dextran on the next day and again 5 days later. Ten out of 500 animals (2%) failed to respond after each of these 3 injections (see Table 1); they were classed as non-reactors, but this was modified

TABLE 1. THE REACTIVITY OF WISTAR RATS OF THE WELLCOME COLONY TO DEXTRAN

Injection No.	Number of rats injected	Number reacting	Number dead
1	500	417	25
2	83	50	0
3	33	23	0
4	10	10	0
5	10	10	0
6	10	10	0

when it was shown that these rats responded to the fourth, the fifth and the sixth injection of dextran, given at weekly intervals. The classification of non-reactors proposed by Harris & West (1963) appears therefore to require modification; failure to respond to at least four weekly injections of dextran is probably the better definition of non-reactivity. The reason why 25 rats (5%) in the present experiments died after the first injection of dextran is unknown but it may be due to dehydration after a long journey.

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Effect of metabolic poisons on anaphylactic shock

SIR,—The mechanism responsible for the release of histamine in anaphylaxis and the effect of various metabolic poisons on this process *in vitro* have received a good deal of attention (Moussatché & Prouvost-Danon, 1958; Rothschild, 1961; Chakravarty, 1962). The anaphylactic release of histamine can be prevented by metabolism inhibitors and by anoxia; the inhibition of metabolism may be counteracted by glucose. This would strongly suggest that the anaphylactic release of histamine is an energy requiring process, where the adenosine triphosphate (ATP) level in the tissue plays a decisive role.

As demonstrated earlier, dextran-induced anaphylactic oedema (Görög & Szporny, 1965a), as well as ultraviolet-induced erythema (Görög & Szporny, 1964) are effectively inhibited in guinea-pigs by some metabolic poisons.

The protective effect against anaphylactic shock of non-steroid anti-inflammatory agents, like phenylbutazone or salicylate, and some water-soluble glucocorticoids (Görög & Szporny, 1965b) is established and we now report the influence of some metabolic poisons on anaphylactic shock *in vivo*, and relate this to the action of the poisons on tissue metabolism.

Guinea-pigs of 300 to 400 g were sensitised by two intraperitoneal injections of 0·05 ml 5% ovalbumin solution at 24 hr intervals (Herberts, 1955). 21 to 30 days after sensitisation, the animals were made to inhale 5% albumin aerosol. The point when an animal exhibited signs of dyspnoea, by lying on its side and turning its head to right and left, was taken as the preconvulsion time. At this point, the animals were resuscitated by the inhalation of oxygen (Smith, 1961). Animals with shorter preconvulsion times than 100 sec, were used one week later. Each dose was given to six animals. The quotient of treatment time/control time indicates the inhibition. The preconvulsion time $\times 10$ of the daily untreated control group was regarded as the limit. The substances were administered intraperitoneally 30 min before the albumin aerosol. The inhibitory action on glucolysis was measured in homogenates of rat kidney (LePage, 1947). After incubation for 40 min at 37°, the amount of the lactic acid produced was measured spectrophotometrically (Barker & Summerson, 1948).

Of the investigated substances (Table 1), arsenate and maleate failed to avert shock. Upon comparing effective with toxic doses, the dose ratios were no worse than those in the case of shock inhibition by several established anti-inflammatory agents.

The facts in Table 2 illustrate *in vitro* inhibition of glucolysis. *In vivo*, 2-deoxyglucose accumulating within the cell as 2-deoxyglucose-6-phosphate, inhibits the glucolysis (Sols & Crane, 1954) and therefore the inhibitory effect cannot be proved by our homogenate technique. The first step in glucolysis, the phosphorylation of glucose, is inhibited by glyceraldehyde; hence to investigate this effect, fructose-1,6-diphosphate had to be omitted from the system, and glucose alone served as substrate. Under such conditions, the activity of the system was much weaker than in the presence of fructose-1,6-diphosphate. Our findings confirm the observation that malonate in high concentration inhibits glucolysis (Fawaz & Fawaz, 1962). Some parallelism can be noted in concentrations inhibiting shock and those inhibiting glucolysis *in vitro*. The protection against shock afforded by 2,4-dinitrophenol, is particularly marked at small doses; amounts approaching toxic doses have diminished action.

These experiments indicate that anaphylactic reaction can be successfully prevented *in vivo* by non-toxic doses of substances which inhibit metabolism.

TABLE 1. EFFECT OF METABOLIC POISONS ON ANAPHYLACTIC SHOCK IN GROUPS OF SIX GUINEA-PIGS

Substances	Dose mg/kg	Ratio†	Toxicity in rats intraperitoneally
Sodium monoiodo-acetate	20 10	8·8 5·4	MLD = 30–50
Sodium fluoride ..	5 4 2·5	8·7 4·4 1·1	**MLD = 28–35
Sodium malonate ..	300 200	7·0 6·0	LD50 = 1100*
2-Deoxyglucose ..	500	10·0	LD50 = 2000
DL-Glyceraldehyde ..	1000 500	10·0 8·6	LD50 = 2000
2,4-Dinitrophenol ..	20 10 5 2·5	5·6 7·1 10·0 3·9	LD50 = 20
Sodium arsenate ..	10	1·2	**MLD = 34·7–44·6
Sodium maleate ..	300	1·6	LD50 = 600

* The toxicity of malonate differs from the published value, i.e., LD50 = 2500. (*Handbook of Toxicology*, Editor, Spector, W., 1956. London: Saunders Co.).
** Data from: *Handbook of Toxicology*.
† Control preconvulsion time: 78·2 sec (30 animals).

TABLE 2. EFFECT OF METABOLIC POISONS ON GLUCOLYTIC ACTIVITY OF RAT KIDNEY *in vitro*

Substances	Final conc. M	Lactic acid μM produced/hr/mg nitrogen: Control	Lactic acid μM produced/hr/mg nitrogen: With substances	Inhibition %
Sodium monoiodo-acetate	1×10^{-4} 5×10^{-4}	13·80	7·40* 2·15	46 84
Sodium fluoride ..	1×10^{-4} 5×10^{-4}	14·20	6·25 1·32	56 91
Sodium malonate ..	2×10^{-2} 6×10^{-2}	11·70	8·60 3·20	26 73
DL-Glyceraldehyde ..	1×10^{-3} 5×10^{-3}	4·58	3·10 0·25	32 94
Sodium maleate ..	2×10^{-2}	13·80	13·10	5
Sodium arsenate ..	1×10^{-2}	14·50	14·25	2
2,4-Dinitrophenol ..	1×10^{-3}	13·65	12·90	5

* = Every value is the average of two parallels.

Thus the mechanism of anti-inflammatory action shows a close relationship to the mechanism responsible for the inhibition of anaphylactic shock. In both effects the inhibition of the ATP-generating system is involved. On the other hand, the difference between the two effects indicates that 2,4-dinitrophenol exerts no inhibitory action on experimental inflammation, with the exception of the anaphylactic dextran oedema in which its inhibitory effect is pronounced (Stenger, 1959).

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September 13, 1965

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An improved method for obtaining twitch responses to acetylcholine in the rat diaphragm

SIR,—Retrograde injection into the venous drainage of the right hemidiaphragm was originally developed by Burgen, Dickens & Zatman (1949) as a method for obtaining twitch responses to added acetylcholine. The method has more recently been described in detail by Paterson (1965) in studies on the pharmacology of denervated and innervated rat muscle.

In our quantitative investigations we experienced some difficulty in obtaining repeatable results using the preparation as described. Since the response of this preparation to retrograde injection is dependent not only upon the dose of drug, but also on the speed of delivery and volume of the injection, we have devised a method of mechanically delivering drug solutions to the diaphragm. The preparation was made as described by Paterson (1965) with the exception that the dead space of the injection cannula was reduced to 0·07 ml by using fine bore polythene tubing (Arnold Howell Ltd., ARH/3, hand drawn to 1 mm o.d.) as the cannula, and tied as low down into the thoracic vena cava as possible to minimise "ballooning" from the pressure of injection. After filling the cannula

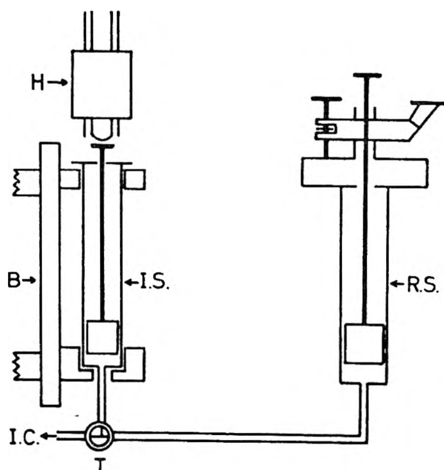

FIG. 1. Diagram of apparatus for the mechanical retrograde intravenous injection of acetylcholine into the isolated rat diaphragm. B. Brass holder for injection syringe. H. Knee jerk hammer. I.C. Injection cannula. I.S. Injection syringe. R.S. Reservoir syringe. T. Three way tap.

with the appropriate concentration of drug solution, 0·1 ml of the solution was injected from a 1 ml record type tuberculin syringe with a metal plunger and nozzle (injection syringe) held rigidly in a brass holder, and connected to a three way tap (C. F. Thackray Ltd., I.T. 4126) (Fig. 1). The plunger was mechanically actuated by a knee jerk hammer (Wright & Schweizer, 1937) supplied by C. F. Palmer, London, Ltd., mounted vertically above the syringe plunger. On connecting the coil to a 12V DC source, the hammer struck the plunger of the injection syringe, and the drug solution was injected. Accurate measurement of the drug solution volume was obtained by filling the injection syringe from a reservoir syringe capable of metering small volumes of fluid (Chance Repette syringe 2 ml).

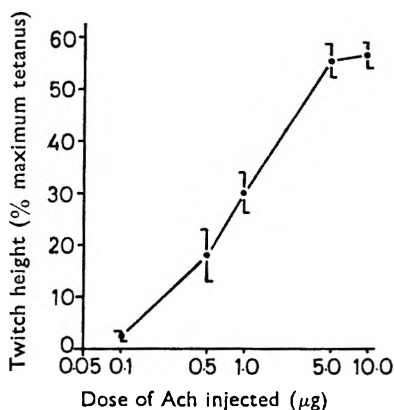

FIG. 2. Log dose-response curve of isolated rat diaphragm to retrograde intravenous injection of acetylcholine, showing standard errors of means of six diaphragm preparations. Abscissa: dose of Ach injected in 0·1 ml saline.

The advantages of these modifications to the described method may be judged from the results shown in Fig. 2, taken from a series of experiments in which responses to graded doses of acetylcholine were obtained.

The preparation was the right hemidiaphragm of the rat, suspended in Krebs solution at 27°, stimulated indirectly at a rate of 2 shocks/min. Retrograde injections of acetylcholine 0·1–10·0 μg were given at 5 min intervals, the indirect stimuli being suspended for 1 min periods before and after injection.

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Reversal by amphetamine of the protective effect of bretylium on reserpine-induced depletion of noradrenaline

SIR,—Bretylium has been shown to prevent the depletion of noradrenaline caused by many agents such as guanethidine (Kuntzman, Costa, Gessa & Brodie, 1962), reserpine (Callingham & Cass, 1962; Inesi, Pekkarinen, Hess, Shanfeld & Haugaard, 1962; Ryd, 1962; Arnold, McAuliff, Sobell & Archer, 1963) and metaraminol (unpublished observation). Release of noradrenaline on splenic nerve stimulation is also inhibited by bretylium (Boura & Green, 1959; Hertting, Axelrod & Patrick, 1962). On the other hand, the anti-hypertensive effect of bretylium is antagonized by amphetamine (Wilson & Long, 1960). Further, Matsumoto & Horita (1962) and Day (1962) have shown that the adrenergic neurone blocking effect of bretylium is reversed by amphetamine. It is interesting in this connection that Brodie, Chang & Costa (1965) showed that the uptake of bretylium in the rat heart was inhibited by amphetamine although it was not made certain whether the inhibition of bretylium binding is associated directly with the antagonism of adrenergic neurone blockade.

It seemed of interest, therefore, to see whether the effect of (+)-amphetamine extends to the protective action of bretylium on the drug-induced depletion of noradrenaline. Reserpine was used as a noradrenaline depleting agent since amphetamine itself does not interfere with the effect of reserpine (Table 1) while noradrenaline depletion by other agents, such as guanethidine (Matsumoto & Horita, 1963; Chang, Costa & Brodie, 1965) and metaraminol (unpublished observation), is interfered with. Noradrenaline in the rat heart was assayed by a modified trihydroxyindole method (Chang, 1964).

TABLE 1. EFFECT OF AMPHETAMINE ON THE PREVENTION BY BRETYLIUM OR GUANETHIDINE OF RESERPINE-INDUCED NORADRENALINE DEPLETION IN THE RAT HEART

Rats were treated (i.p.) with the drugs 30 min before administration of reserpine (i.v.) with or without 1·0 mg/kg of amphetamine (i.p.) given 30 min before the pretreatments. Rats were killed 5 hr after administration of reserpine.

Pretreatment	Dose of reserpine (mg/kg)	Noradrenaline as % of normal	
		Without amphetamine	With amphetamine
None	0·32	3 ± 0·8 (9)	5 ± 1·3 (3)
Bretylium, 10 mg/kg	,,	46 ± 1·2 (5)*	8 ± 2·2 (3)**
Guanethidine, 10 mg/kg	,,	10 ± 0·9 (4)*	6 ± 2·0 (4)
None	1·0	1 ± 0·4 (6)	1 ± 0·2 (3)
Bretylium, 10 mg/kg	,,	12 ± 1·2 (6)*	2 ± 0·2 (5)**
Guanethidine, 10 mg/kg	,,	9 ± 1·3 (5)*	2 ± 0·3 (4)**

* P < 0·05 vs reserpine alone. **P < 0·05 vs without amphetamine.

Fig. 1 shows that when the rat was pretreated with bretylium (10 mg/kg) the depletion of noradrenaline by reserpine was markedly inhibited, thus causing the dose-response curve of reserpine to be shifted almost in parallel to the right. It should be noted, however, that, in the animal pretreated with bretylium, it becomes impossible to have a complete depletion by reserpine even at higher doses (Fig. 1). This fact indicates a non-competitive nature of the antagonism between reserpine and bretylium. The broken line in Fig. 1 shows the effect of amphetamine (1 mg/kg) upon the action of bretylium. The dose-response curve affected by bretylium was shifted back to the left by amphetamine pretreatment and reserpine resumed its activity to induce a maximal depletion of noradrenaline. The data in Table 1 show that guanethidine also interfered with the action of

reserpine to some extent and the effect, like that of bretylium, was reversed by amphetamine.

Since bretylium appears not to act competitively with reserpine for the prevention of noradrenaline depletion, and, in contrast to amphetamine (Chang & others, 1965), prevents the noradrenaline depletion induced by guanethidine without inhibiting the binding of the latter drug (Brodie & others, 1965), it is likely that bretylium acts by a similar mechanism against divergent depleting agents. For such a common mechanism it may be assumed that bretylium interferes with the release from the nerve endings of the noradrenaline which is effected either by reserpine, guanethidine, metaraminol or possibly by nerve

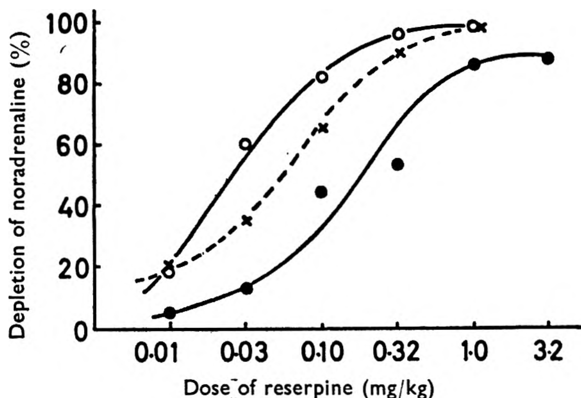

FIG. 1. Effects of amphetamine and bretylium on the noradrenaline depletion induced by various doses of reserpine in the rat heart. Rats were pretreated with bretylium (10 mg/kg; i.p.) and 30 min later with reserpine (i.v.). Amphetamine (1 mg/kg; i.p.) was given 30 min before administration of bretylium. Animals were killed 5 hr after the reserpine injection. Each point represents mean % depletion of heart noradrenaline from three to eight experiments. ○——○, reserpine alone; ●——●, bretylium plus reserpine; ×——×, amphetamine plus bretylium plus reserpine.

impulses. Amphetamine may interfere with the binding of bretylium to the target site and thus antagonizes the actions of bretylium; the site of binding, however, may be different from the site at which amphetamine and guanethidine compete (Chang & others, 1965; Brodie & others, 1965).

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Effects of certain tranquillisers on the level of homovanillic acid in the corpus striatum

SIR,—Earlier investigations have shown that chlorpromazine or haloperidol increase the levels of dihydroxyphenylacetic acid and homovanillic acid but not of 5-hydroxyindoleacetic acid in the corpus striatum of the rabbit (Andén, Roos & Werdinius, 1964). This increase occurs without any concomitant change in tissue monoamine levels. The first biochemical evidence of an influence on the monoamine metabolism *in vivo* by chlorpromazine and haloperidol was the observation that the accumulation of methoxytyramine and normetanephrine in brain after treatment with a monoamine oxidase inhibitor was enhanced by chlorpromazine or haloperidol (Carlsson & Lindqvist 1963). There is some evidence for the view that the elimination of the dopamine acid metabolites is retarded by the two drugs but other data support the suggestion that the synthesis of the acid metabolites is increased. Against the former and in favour of the latter hypothesis are the facts that the dihydroxyphenylacetic acid and homovanillic acid levels increase simultaneously and that 5-hydroxyindoleacetic acid is unchanged after chlorpromazine or haloperidol.

It is known that these drugs may block both peripheral and central effects of catecholamines. The blockade of the catecholamine receptors of the effector cells may have the effect of increasing the release of transmitter from the neurones with a compensatory stimulation of the catecholamine synthesis. In this instance it might be possible to assume that a stronger inhibition of the receptor may result in a greater increase of the levels of the phenolic acids in the brain. Homovanillic acid is formed from dihydroxyphenylacetic acid after the attack by the enzyme catechol-*O*-methyl transferase. This reaction is so far not known to be influenced by chlorpromazine or haloperidol.

The effect of 15 tranquillising substances has been investigated by giving them intravenously to rabbits. The homovanillic acid was measured 3 hr after the injection by the method developed in this laboratory (Andén & others, 1963) (Table 1). The animals were kept in a warm environment. Hypothermia can thus be excluded as a causative factor in the changes of the acid metabolites after these drugs.

It is interesting to note that major tranquillisers with a well-known antipsychotic effect, such as perphenazine, triflumethazine and clopenthixol, also strongly increase the homovanillic acid level. On the other hand, phenothiazines used as minor tranquillisers, for instance, prothipendyl or promazine hydrochloride, have only a slight effect, or none at all, on the level of the acid. The substances R6109 and R5147 are derivatives of the butyrophenone made by Janssen Ltd. in Belgium. Like haloperidol they seem to have a rather strong effect on homovanillic acid levels. Further work on the possible connection between the antipsychotic action and the homovanillic acid-increasing effects of tranquillising drugs is in progress.

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TABLE 1. LEVEL OF HOMOVANILLIC ACID IN RABBIT CORPUS STRIATUM 3 HR AFTER THE I.V. INJECTION OF TRANQUILLISER DRUGS

Drug	Dose mg/kg	Homovanillic acid µg/g	Drug	Dose mg/kg	Homovanillic acid µg/g
Controls	—	3·9 ± 0·9*	Prothipendyl HCl ..	10	5·0
Clopenthixol ..	10	10·6	Prothipendyl HCl ..	20	6·8
Triflumethazine ..	10	10·2	Prothipendyl HCl ..	30	6·4
Perphenazine	5	10·0	Azacyclonol ..	10	4·3
Dixyrazine	5	10·0	Azacyclonol ..	20	4·6
Prochlorperazine ..	0·5	4·6	Hydroxyzine ..	10	3·7
Prochlorperazine ..	2	9·5	Hydroxyzine ..	20	5·8
Methopromazine ..	10	8·4	Hydroxyzine ..	35	6·9
Thiroidazine ..	5	8·2	Earlier investigations		
Levomepromazine ..	0·2	4·4	(Anden & others,		
Levomepromazine ..	5	8·4	1964)		
R6109 Isospirilene ..	0·5	8·0	Controls		2·2 ± 0·6*
R5147 Spiroperidol ..	0·5	8·3	Chlorpromazine ..	5	4·9
Chlorprothixene ..	10	8·0	Haloperidol	0·5	5·1
Trimeprazine	5	3·5	Promethazine ..	20	2·2
Trimeprazine	10	7·2	Phenoxybenzamine ..	10	2·4

*Mean ± s.d

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Release of potassium chloride from tablets

SIR,—The occurrence of characteristic obstructive ulceration of the small intestine apparently associated with potassium supplemented thiazide therapy has recently been reported (Baker, Schrader & Hitchcock, 1964; Lindholmer, Nyman & Räf, 1964; Aberuzze & Gooding, 1965; Bennett & Davy, 1965; Buchan & Houston, 1965; Morganstern, Freilich & Panish, 1965).

Boley, Schultz, Krieger, Schwartz, Elguezebal & Allen (1965), working with dogs, and Diener, Shoffstall & Earl (1965), using olive baboons, have shown that tablets containing potassium chloride can give rise to the lesion, whereas controls without the salt gave no reaction. These workers suggest a correlation of the high local concentration of potassium chloride and the reported lesions.

Because large oral doses of potassium chloride can give rise to nausea and gastric irritation, its availability is usually modified to prevent or limit irritation in the stomach. We have measured the rate of release of potassium chloride from nine commercially available tablets containing the salt (either alone or in combination with diuretics (Table 1) by an *in vitro* method.

Method. Tablets were agitated by means of an apparatus conforming to the B.P. specification for disintegrating tests (B.P. 1963a) in beakers containing 1·0 litre of test solutions designed to simulate gastrointestinal environment. Acid pepsin solution or alkaline pancreatin solution (B.P. 1963b) freshly prepared for each determination was used. Solutions were stirred at a constant slow speed and maintained at 37 ± 0·1° in a thermostatically controlled water-bath.

Release of potassium chloride was measured by monitoring the conductivity of the solution continuously, using an immersion conductivity cell (Radiometer CD 104). Calibration curves of conductivity versus potassium chloride concentration were prepared using known increments of potassium chloride. The curves obtained were linear over the range studied.

Tablets equivalent to 10–12 g of potassium chloride were placed in the tubes of the disintegrator and a record of change in conductivity of the solution with time obtained. Experiments were run for 2 hr or to completion in the simulated gastric fluid, or to completion in the simulated intestinal fluid. Graphs of % total potassium chloride released versus time were prepared. Where potassium chloride was released, 100 ± 3% of the theoretical content was found in solution at completion of release, indicating that excipients and coating materials did not interfere with the measurements.

TABLE 1. DETAILS OF TABLETS AND MAXIMUM VALUES OF DW/DT* IN ACID PEPSIN AND ALKALINE PANCREATIN SOLUTIONS

Product	Remarks	Declared KCl content (mg)	No. of tablets used for test	dW/dt max.		Time from start at which dW/dt max. occurred	
				Pepsin	Pancreatin	Pepsin	Pancreatin
A	Manufacturer states "Slow release"	630	16	100	150	5 min	10 min
B	Manufacturer states "Enteric coated"	573	18	0	450	0	10 min
C	No statement	500	20	—†	—†	—†	—†
D	No statement	572	18	0	405	0	20 min
E	No statement	600	17	0	315	0	10 min
F	No statement	625	16	235	230	20 min	20 min
G	Manufacturer states "Slow release core"	600	17	80	115	10 min	10 min
H	Manufacturer states "Enteric sealed"	325	30	0	225	0	20 min
J	Manufacturer states "Slow release core"	400	25	65	70	10 min	10 min

* dW/dt = Average wt of potassium chloride in mg released by one tablet in time increment of 5 min.
† Lack of between tablet uniformity prevents valid assessment.

Results. These are expressed as the average amount of potassium chloride dW (in mg) released from one tablet in consecutive time increments dt (5 min), values for dW being read off % versus time curves. Histograms of dW/dt versus time were prepared, representative results being shown in Fig. 1. Maximum values of dW/dt are shown in Table 1 together with the time at which they occurred. This enables a comparison to be drawn between products which allows for inherent differences in tablet content. Reproducibility was assessed on an enteric coated tablet (Product D) and a controlled release tablet (Product G). Standard Error of the Mean for dW/dt max. was 5·4% for Product D and 6·2% for Product G.

From Table 1 it can be seen that products B, D, E and H can be classified as enteric coated and products A, F, G and J as controlled release preparations. Release from C was too erratic to allow evaluation.

The method measures the rate at which potassium chloride is released into solution from a tablet under standard conditions, results being expressed as the amount released in a given time, giving an indication of the local concentrations likely to arise. Thus, tablets showing higher values of dW/dt should give rise to higher local concentrations than those showing lower values, as more is released in a given time. Enteric coated tablets showed high values for dW/dt in the simulated intestinal fluid, and are therefore likely to give rise to high local concentration of potassium chloride. Controlled release tablets show appreciably lower values of dW/dt in simulated intestinal fluid, indicating that they are less likely to cause high local concentrations under the same conditions. In addition, these tablets showed release in simulated gastric fluid, further reducing the possibility of high dW/dt values occurring later under alkaline conditions.

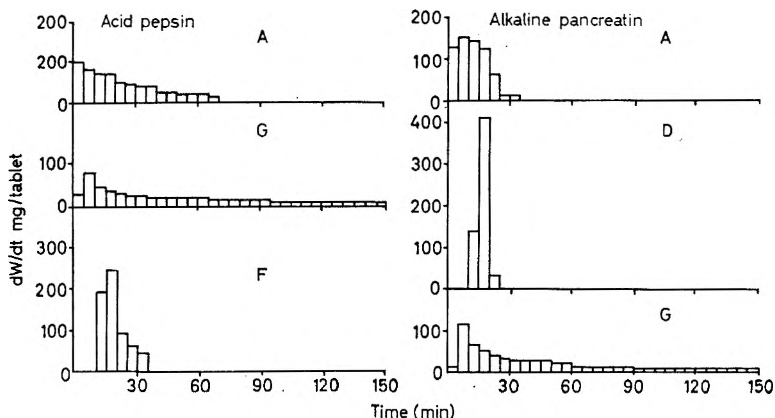

FIG. 1. Average wt in mg (dW) of potassium chloride released in 5 min increments (dt) versus time in acid pepsin solution and in alkaline pancreatin solution for products A, D, F, G.

Release patterns obtained using other simulated gastrointestinal fluids and buffer solutions gave a qualitatively identical overall picture, although different dW/dt max. values were found with different solutions.

A recently published report on experiments with olive baboons (Lister, 1965), shows that although enteric coated tablets gave rise to iatrogenic ulceration, a slow release preparation showed no lesions of the digestive tract. This suggests

a correlation between ulceration and rate of release of potassium chloride, and the method described here can be used as a comparative measure of this tablet parameter.

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Note: A key to products studied is available from the author on request.

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