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Some bronchoconstricting and bronchodilating responses of human isolated bronchi: evidence for the existence of α-adrenoceptors

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Isolated strips of human bronchi obtained during thoracic surgery exhibited pharmacological responses very similar to those of other species (e.g. guinea-pig). Analysis of the action of some sympathomimetic amines indicated that the human bronchi also possess a sparse population of α-adrenoceptors. Propranolol (>1 μg/ml) had a direct bronchoconstricting action, whereas another β-adrenoceptor blocking agent, alprenolol, produced bronchodilatation. Phentolamine (>4 μg/ml) also produced a bronchodilatation of its own. Theophylline and dibutyryl cyclic AMP produced dose-dependent relaxations. The strips were effectively contracted by acetylcholine, histamine, prostaglandin $F_2\alpha$ ($PGF_2\alpha$), slow reacting substance (SRS) and bradykinin. Bradykinin, regardless of the dose, produced bronchoconstriction in some preparations and dilatation in others. 5-Hydroxytryptamine produced bronchodilatation but at very high concentrations a constriction was obtained.

The pharmacological responses of bronchial smooth muscle preparations from human lungs removed during surgical procedures have been investigated for some time in our laboratory (Mathé & Strandberg, 1971; Mathé, Strandberg & Åström, 1971). The present study describes the action of a number of sympathomimetic amines and some β-adrenoceptor blockers thought to be of particular interest since propranolol has been found to produce bronchoconstriction in animal experiments (Herxheimer & Langer, 1967) as well as in man (McNeill, 1964; Zaid & Beall, 1966; MacDonald, Ingram & McNeill, 1967). Furthermore, bronchoconstricting agents including prostaglandin $F_2\alpha$ ($PGF_2\alpha$), slow reacting substance (SRS) and bradykinin, and also 5-hydroxytryptamine were studied. The action of these substances seemed to be of interest in view of their possible involvement in bronchial asthma (Brocklehurst, 1962; Austen, 1965; Piper & Vane, 1969).

MATERIALS AND METHODS

Bronchi were obtained from macroscopically normal parts of human lungs which had been removed because of carcinoma. A total of 89 preparations from 33 different lungs were immediately dissected from the lung tissue and kept in Tyrode solution. Helical strips were prepared from approximately 3–5 mm wide bronchi cut spirally into about 3 cm long and 3 mm wide strips and suspended in a 25 ml bath of Tyrode solution of the following composition g/litre: NaCl 8, KCl 0·2, $CaCl_2$ 0·2, $MgCl_2$ 0·2, $NaHCO_3$ 1, NaH_2PO_4 0·05 and glucose 1. The solution was kept at 37°, pH 7·3 and aerated with a mixture of 6·5% carbon dioxide in oxygen. The strips were allowed to equilibrate under a tension of 0·5 g for at least 1 h. Changes in active tone were measured isometrically with a Grass force-displacement transducer

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(model FT 03) and recorded on a Grass polygraph. Three to four preparations were usually run simultaneously. Thus tests with various agents and doses could be performed in differing order and with adequate controls. After each test and washing of the bath, a 15 min rest was allowed. Before each new test the tension was checked and, if required, adjusted to the original 0·5 g. Good responses could as a rule be obtained for about 8 h after the preparation of the strips.

Drugs. Acetylcholine chloride, (—)-adrenaline bitartrate, alprenolol (Aptin, Hässle), atropine hydrochloride, bradykinin (BRS 640, Sandoz), histamine dihydrochloride, 5-hydroxytryptamine creatinine sulphate, (—)-isoprenaline sulphate, methysergide (Sansert, Sandoz), phenylephrine (Neosynephrine, Winthrop), (—)-noradrenaline bitartrate, phentolamine (Regitin, Ciba), propranolol (Inderal, ICI), prostaglandin $F_2\alpha$, mepyramine, theophylline (Teofyllamin, ACO Läkemedel AB), cyclic N^6-2′O-dibutyryl-adenosine-3,5-monophosphate (cyclic AMP, Mannheim-Boerhinger), purified slow reacting substance not containing any prostaglandins (Strandberg, 1969) and polyphloretin phosphate (PPP). Doses of adrenaline, noradrenaline, isoprenaline, acetylcholine and histamine refer to the free base. Other drugs were calculated as salts.

RESULTS

The bronchodilating effect of adrenaline was readily abolished by β-adrenoceptor blockade (propranolol 0·4–5 μg/ml, Fig. 1; alprenolol 1–10 μg/ml, Fig. 2). After β-adrenoceptor blockade adrenaline elicited bronchoconstriction. The dose required for the constrictor effect was usually ten times higher than that used to produce an effective relaxation before the β-adrenoceptor blockade. This constriction could be abolished by phentolamine (Figs 1 and 2). Results similar to those with adrenaline were obtained with noradrenaline and phenylephrine. The constriction elicited by sympathomimetic amines after blockade of the β-adrenoceptors was never more than 10–20% of the maximum response elicited by acetylcholine or histamine. The bronchodilating action of noradrenaline and phenylephrine was approximately only 1/10 and 1/300 that of adrenaline. Isoprenaline on the other hand was about 3 times as potent a bronchodilator as adrenaline. Its action was completely abolished after β-adrenoceptor blockade. In contradistinction to the other sympathomimetic amines studied, no reversal of the effect could be observed. The ED50 values in μg/ml for the different sympathomimetic amines were: adrenaline 0·1 ± 0·01 (n = 7), noradrenaline 1·1 ± 0·2 (n = 5), phenylephrine 31 ± 13 (n = 7) and isoprenaline 0·03 ± 0·003 (n = 5).

The adrenoceptor blocking agents propranolol, alphrenolol and phenotalmine, in most experiments, produced an action *per se*. The bronchoconstriction observed

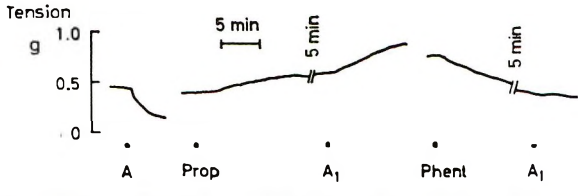

FIG. 1. Isolated strip of human bronchus. Dilating action of adrenaline (A 0·4 μg/ml, control) and constricting effect of a larger dose of A (A_1 4 μg/ml) after propranolol (Prop 4 μg/ml). The constriction caused by adrenaline (A_1 4 μg/ml) was abolished after phentolamine (Phent 16 μg/ml). Propranolol *per se* induced constriction and phentolamine relaxation.

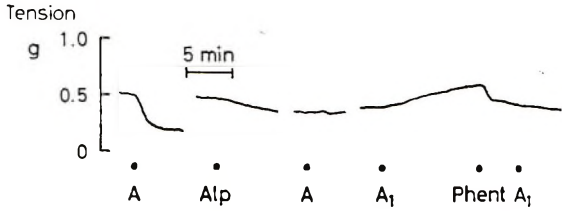

FIG. 2. Isolated strip of human bronchus. Dilating action of A (0·4 μg/ml, control). Alprenolol (Alp 4 μg/ml) in itself produced a dilatation and also abolished the dilating effect of the same dose of A (0·4 μg/ml). Ten times higher dose of A (A_1 4 μg/ml) elicited a constricting effect which was abolished after phentolamine (Phent 20 μg/ml).

with 1–5 μg/ml of propranolol was slow in onset and required up to 15 min to reach its maximum (Fig. 1). In contrast to propranolol, the β-adrenoceptor blocker alprenolol (1–10 μg/ml) produced a bronchodilatation (Fig. 2) confirming its previously reported β-adrenoceptor stimulating property (Åblad, Brogård & Ek, 1967). Phentolamine (5–20 μg/ml) elicited a bronchodilatation (Figs 1 and 2).

The bronchoconstricting effects of acetylcholine and histamine on the isolated bronchi were not modified by α-adrenoceptor blockade. The constriction produced by these agonists was readily abolished by atropine and an antihistamine (mepyramine). The ED50 value for acetylcholine was 0·4 ± 0·1 μg/ml (n = 24) and for histamine 0·5 ± 0·1 μg/ml (n = 12).

$PGF_2\alpha$, but not PGE_2, produced a dose-dependent bronchoconstriction as did SRS. The ED50 value for $PGF_2\alpha$ was 0·3 ± 0·1 μg/ml (n = 12) and for SRS 12 ± 3 units/ml (n = 9). In contrast to acetylcholine and histamine, the bronchoconstricting effect of $PGF_2\alpha$ and SRS took a longer time to reach its peak and lasted up to 30 min in spite of repeated washings with Tyrode solution. As reported elsewhere, polyphloretin phosphate (Diczfalusy, Fernö & others, 1953) antagonized the bronchoconstricting action of both $PGF_2\alpha$ and SRS (Mathé & others, 1971; Mathé & Strandberg, 1971).

Bradykinin (0·1–4 μg/ml) induced a bronchodilatation in 10 of 31 experiments (ED50 = 0·9 ± 0·3 μg/ml, n = 10) and a clear constriction in the other experiments. In no case were bronchoconstriction and bronchodilatation observed in the same preparation. Both types of action were dose dependent (Fig. 3). In a few instances the bronchoconstricting effect was diminished on repeated administration. The dilating effect was not antagonized by β-adrenoceptor blockade nor was the constriction modified by α-adrenoceptor blockade or by atropine.

5-Hydroxytryptamine (5-HT) in doses 2–10 μg/ml induced a bronchodilatation. This effect decreased with increasing dose, and at a concentration exceeding 20 μg/ml the response had either decreased to zero or been converted into a constricting effect. Methysergide (10–200 μg/ml) antagonized both types of action of 5-HT.

Dibutyryl cyclic AMP produced a dose dependent bronchodilatation. Although this compound is considered to reach its site of action more readily than the cyclic AMP, the doses required were high (50–500 μg/ml) and comparable with those used by Moore, Iorio & McManus (1968). The effect was gradual in onset and it often took 30 min or more for the preparation to return to the control level of tension in spite of repeated washings of the bath. In similarity with theophylline (Fig. 3B) the action of cyclic AMP was not modified by β-adrenoceptor blockade (Fig. 4).

The dose-dependent and readily reproducible bronchodilatation produced by

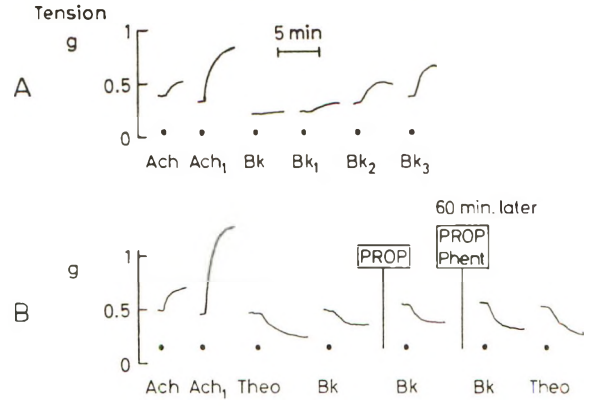

FIG. 3. Isolated strips of human bronchi from two different lungs. A. Bronchoconstricting responses produced by acetylcholine (Ach 0·04 and Ach_1 0·4 μg/ml) and different concentrations of bradykinin (Bk 0·005, Bk_{1-3} 0·02, 0·04, 0·4 μg/ml). B. Bronchoconstricting effect of acetylcholine (Ach 0·04 and Ach_1 0·4 μg/ml) and bronchodilating action of bradykinin (Bk 0·4 μg/ml). The bronchodilatation elicited by bradykinin was unchanged after β-adrenoceptor blockade (Prop 0·4 μg/ml). One h later, when the responses to both α- and β-adrenoceptors had been blocked, the bronchodilating response of bradykinin remained unchanged. The action of theophylline (Theo 40 μg/ml), used for comparison, was also unaffected by the α- and β-adrenoceptor blockade.

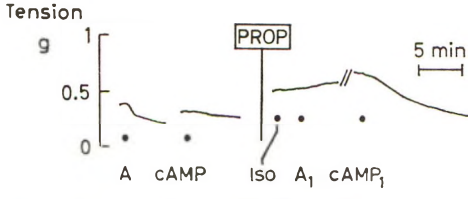

FIG. 4. Isolated strip of human bronchus. Dilating action of adrenaline (A 0·4 μg/ml) and dibutyryl cyclic AMP (cAMP 200 μg/ml). After β-adrenoceptor blockade (Prop 0·8 μg/ml) the dilating effect of isoprenaline (Iso 0·04 μg/ml) and adrenaline (A_1 4·0 μg/ml) was abolished but dibutyryl cyclic AMP ($cAMP_1$ 500 μg/ml) still elicited dilatation.

theophylline (10–100 μg/ml) was fully effective also in strips contracted with acetylcholine, histamine, $PGF_2\alpha$, SRS and bradykinin. The action of theophylline was not modified by β-adrenoceptor blockade (Fig. 3B).

DISCUSSION

The results obtained show that human bronchi respond in principally the same manner as those of guinea-pig, rabbit and dog. The observation that the relaxation normally produced by adrenaline, noradrenaline and phenylephrine, but not that by isoprenaline, could be converted into a constriction after β-adrenoceptor blockade is also in harmony with results obtained in animals (Castro de la Mata, Penna & Aviado, 1962; Persson & Johnsson, 1970; Fleisch, Maling & Brodie, 1970). On the basis of the results presented here it may be concluded that the human bronchi also possess α-adrenoceptors. However, the α-adrenoceptors do not seem to be prominent in the human bronchi, at least as studied in this investigation. Even with noradrenaline and phenylephrine the response at all dose levels tested was dilatation before β-adrenoceptor blockade.

The finding that the dilating effect of isoprenaline could not be converted into a constricting action by a β-adrenoceptor blocker supports the concept that this amine is a pure β-adrenoceptor stimulating agent, whereas phenylephrine seems to be

less pure in its α-stimulating property, since it regularly produced bronchodilatation before β-adrenoceptor blockade.

The bronchodilating effect of phentolamine *per se* has previously been demonstrated in the guinea-pig (Lish, Robbins & Dungan, 1968). It was suggested that this action resulted from a liberation of catecholamines. In our study, however, the bronchodilating action of phentolamine could be demonstrated also after β-adrenoceptor blockade.

Propranolol augments the response of guinea-pig lung *in vivo* to histamine, acetylcholine and $PGF_2\alpha$ (Collier & James, 1967; James, 1969). However, such a potentiation was not observed in our *in vitro* preparations. This discrepancy would seem to support the concept that constrictor responses of the bronchi *in vivo* are counteracted by an opposite action elicited reflexly via the sympathetic system as proposed by McCulloch, Proctor & Rand, (1967).

It has been observed clinically that propranolol causes an increased airway resistance in healthy subjects and even more so in asthmatic patients (McNeill & Ingram, 1966 MacDonald & others, 1967). In the former this effect of propranolol has been explained as being the result of a blockade of a normally existing β-adrenergic tone in the bronchial smooth muscle leading to a shift of the autonomic balance in the parasympathetic direction, whereas in the latter a hypothesis of a pre-existing partial β-adrenoceptor blockade in the lung has been proposed (Szentivanyi, 1968). The finding in this study that propranolol produces a bronchoconstriction also in isolated preparations, suggests that the bronchoconstriction *in vivo* may, at least in part, be elicited by a direct action on the bronchial smooth muscle. Whether this action is identical with the membrane-stabilizing property which is independent of the β-adrenoceptor effect (Fitzgerald, 1969) can not be answered by our study. It should be noted, however, that alprenolol, *per se*, causing a bronchodilatation, also possesses such a property. Hyper-reactivity of asthmatic bronchial smooth muscle to a variety of bronchoconstricting stimuli has been well documented (Tiffeneau, 1958). Thus the greater sensitivity of asthmatic patients to propranolol could alternatively be partially explained as an exaggerated response of the hyper-reactive bronchial smooth muscle to the bronchoconstricting effect of propranolol *per se*.

Bradykinin has been found to increase airway resistance to flow in the guinea-pig (Collier, Holgate & others, 1960; James, 1969). In this investigation bradykinin produced dilatation in some and a constriction in other preparations. In both cases the bronchi responded with constriction in the usual way when acetylcholine or histamine were added to the bath. It has been reported that bradykinin, administered as aerosol, elicits bronchoconstriction in patients with bronchial asthma but not in healthy subjects (Douglas, 1965). However, strips reacting with constriction in our experiments were not obtained from patients with bronchial asthma. The reason why some preparations reacted with constriction and others with dilatation therefore remains unknown.

The action of 5-HT on the bronchi seems to vary from one species to another. Guinea pig lung *in vivo* and also isolated trachea react with constriction (James, 1969). In a recent study (Booij-Noord, Orie & De Vries, 1969) it was found that about 25% of patients with chronic, nonspecific lung disease reacted to 5-HT inhalation with bronchoconstriction. However, the role of 5-HT, as well as that of bradykinin, in anaphylaxis is unclear (Austen, 1965; Piper & Vane, 1969). In the human isolated bronchi, 5-HT produced dilatation whereas constriction was obtained only at very

high concentrations (20–200 μg/ml).

As in experiments on the guinea-pig isolated trachea (Moore & others, 1968), the dibutyryl form of the cyclic AMP had to be used in order to elicit bronchodilatation. As expected, dibutyryl cyclic AMP produced a dose-dependent relaxation independent of β-receptor blockade.

The observation that theophylline regularly elicited bronchodilatation also in strips contracted with acetylcholine, histamine, $PGF_2\alpha$, SRS and bradykinin gives good pharmacological support to the clinical experience that it is effective in cases of bronchoconstriction of various etiology. The result that the dilatation was produced also after β-adrenoceptor blockade is in line with the current opinion that its action is exerted at a stage beyond that of the adrenoceptor.

Acknowledgements

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Antagonism by propranolol of the inhibitory effect of phenoxybenzamine on noradrenaline uptake *in vivo*

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The reduction of noradrenaline stores and [^{3}H]noradrenaline concentration in the heart of mice and rats induced by phenoxybenzamine-treatment, alone or in combination with cold-stress, was prevented by propranolol. Propranolol also antagonized a similar effect induced by phentolamine but not that induced by other noradrenaline uptake inhibitors, such as desipramine, cocaine, guanethidine and reserpine. Analysis of the time-course of antagonism by propranolol indicates that it was evident only when the β-adrenoceptor blocking agent remained in the body. The inhibitory effect of phenoxybenzamine on noradrenaline stores reappeared when propranolol was excreted. Propranolol alone did not change cardiac noradrenaline stores or [^{3}H]noradrenaline. It is concluded that the restoration of reflexly increased adrenergic discharge to normal, because of unmasking of spare α-adrenoceptors resulting from β-adrenoceptor blockade by propranolol rather than competition for binding at the active site of phenoxybenzamine, is responsible for the observed antagonism.

In addition to blockade of α-adrenoceptors, phenoxybenzamine (PBZ) has been shown to exert various effects on the adrenergic effector system. It inhibits neuronal uptake of noradrenaline (Hertting, Axelrod & Whitby, 1961; Stafford, 1963; Iversen, 1965; Iversen & Langer, 1969), decreases noradrenaline stores (Schapiro, 1958; Farrant, Harvey & Pennefather, 1964; Reid, Volicer & Brodie, 1969), causes an overflow of noradrenaline during sympathetic nerve stimulation (Brown & Gillespie, 1957; Kirpekar & Cervoni, 1963; Thoenen, Huerlimann & Haefely, 1964; Boullin, Costa & Brodie, 1967; Kirpekar & Wakade, 1970), increases its synthesis (Dairman, Gordon & others, 1968; Reid & others, 1969) and inhibits its extraneuronal uptake as well as its metabolism (Eisenfeld, Axelrod & Krakoff, 1967; Eisenfeld, Landsberg & Axelrod, 1967; Lightman & Iversen, 1969). PBZ also protects adrenergic neurons from the blocking effect of guanethidine (Thoenen, Huerlimann & Haefely, 1966; Matsumoto, 1966; Kirpekar, Wakade & others, 1969).

β-Adrenoceptor blocking drugs, including propranolol, have been shown to reverse α-adrenoceptor blockade induced by PBZ (Hull, Eltherington & Horita, 1960; Moreira & Osswald, 1965; Tuttle, 1965; Gulati, Gokhale & Udwadia, 1965; Eble & Rudzik, 1966; Garrett, Malafaya-Baptista & Osswald, 1966; Olivares, Smith & Aronow, 1967; Yamamura & Horita, 1968, 1969; Smith & Nash, 1969) and also to protect the α-adrenoceptor from irreversible binding with PBZ (Kohli & Ling, 1967; Patil, Tye & others, 1968; Mazurkiewicz-Kwilecki, 1970). The present paper describes other aspects of this antagonism between propranolol and PBZ. It will be shown that the inhibition of noradrenaline accumulation, as well as its reduction

in cardiac stores in mice and rats, caused by PBZ is prevented by the administration of propranolol.

METHODS

Long Evans rats of either sex weighing 180–250 g and NIH mice weighing 22–28 g were used.

Uptake of [^3H]noradrenaline

To each mouse 2 μCi (0·24 μg) of (±)-[1-^{3}H]noradrenaline (^{3}H-NA) in 0·2 ml was given intravenously in the tail vein, while for the rat, 25 μCi (3 μg)/kg of ^{3}H-NA was injected. After various intervals the animals were killed, the hearts rapidly excised and homogenized in a motor driven glass homogenizer with ice-cold 0·4N $HClO_4$. An aliquot (0·2 ml) of the supernatant was counted for its total radioactivity in a liquid scintillation spectrometer. The radioactivity was expressed as d min^{-1} g^{-1} wet tissue in the experiments using rats or as d min^{-1} per heart in the experiments using mice.

Assay of ^3H-NA

To an aliquot (2 ml) of the supernatant of the crude homogenate 0·2 g Al_2O_3 was added and the pH adjusted to about 8 by adding 90 mg $NaHCO_3$ and 0·5 ml 0·2M tris buffer. The catecholamines adsorbed onto Al_2O_3 were then eluted with 2 ml 0·1N HCl and the radioactivity contained in 0·2 ml of the eluate was counted. Since the amount of deaminated catechols in the heart is negligible (Eisenfeld & others, 1967) the count was taken to represent unchanged ^{3}H-NA.

Assay of endogenous noradrenaline

Eluates obtained from Al_2O_3 as described above were assayed for endogenous noradrenaline using a fluorospectrophotometric method (Chang, 1964).

Cold-stress

Rats, one each in a small cage, were put in a cold room at 6° for 60 min. After the cold-exposure, animals were killed immediately for assay of noradrenaline, total radioactivity and ^{3}H-NA.

The drugs used were metanephrine hydrochloride, phenoxybenzamine hydrochloride, propranolol hydrochloride, cocaine hydrochloride, desipramine hydrochloride, guanethidine sulphate, phentolamine mesylate, reserpine and (±)-[1-^{3}H]-noradrenaline (1400 mCi/mmol). The doses of the drugs except those of reserpine and noradrenaline refer to the salts.

RESULTS

Uptake and disappearance of ^3H-NA in the heart of mice

After intravenous injection, ^{3}H-NA was rapidly incorporated into the heart and then declined with two exponential components as reported previously by Montanari, Costa & others (1963). The half-life of the second exponential phase was about 7 h. The unchanged ^{3}H-NA appeared to be about 90% of the total radioactivity.

Pretreatment of the animals with metanephrine (25 mg/kg, s.c.), a potent inhibitor

Table 1. *Effects on the concentration of ^{3}H-NA in the heart of mice.* Each mouse received intravenously 2 μCi ^{3}H-NA and was killed 10 min or 5 h after injection. PBZ was given 2 h before, and propranolol or metanephrine 1 h before, ^{3}H-NA. The experiments were at room-temperature (18–25°).

Treatment	Dose (mg/kg, s.c.)	Total radioactivity (d min^{-1} per heart ± s.e.) 10 min	5 h
Control	—	24 900 ± 1350 (n = 31)	8690 ± 528 (n = 28)
Metanephrine	25	25 700 ± 4230 (n = 3)	8340 ± 1040 (n = 3)
PBZ	10	17 300 ± 406* (n = 13)	3760 ± 428* (n = 14)
Propranolol	5	24 100 ± 1030 (n = 25)	9920 ± 560 (n = 21)
PBZ + propranolol	10, 5	21 500 ± 689** (n = 7)	5670 ± 744** (n = 7)

* $P<0.05$ *vs* control.
** $P<0.05$ *vs* PBZ alone.

Table 2. *Effects of propranolol on the inhibition of noradrenaline uptake in the heart of mice caused by various agents.* All inhibitory drugs were given subcutaneously 2 h before ^{3}H-NA injection. Propranolol (5 mg/kg, s.c.) was administered 1 h after each inhibitory drug. Mice were killed at 5 h after ^{3}H-NA. The experiments were at room-temperature (18–25°).

Treatment	Dose (mg/kg)	Total radioactivity (d min^{-1} per heart ± s.e.) without propranolol	with propranolol
Control	—	8690 ± 528 (n = 28)	9920 ± 560 (n = 21)
Guanethidine	10	1920 ± 95 (n = 3)	2024 ± 111 (n = 5)
Reserpine	1	966 ± 187 (n = 4)	838 ± 82 (n = 4)
Desipramine	5	1820 ± 250 (n = 3)	1350 ± 258 (n = 3)
Cocaine	15	5160 ± 358 (n = 3)	5750 ± 510 (n = 5)
Phentolamine	15	5680 ± 484 (n = 10)	8870 ± 774* (n = 8)
PBZ	10	3760 ± 428 (n = 14)	5670 ± 744* (n = 6)

* $P<0.05$ *vs* without propranolol.

of extraneuronal noradrenaline uptake but without appreciable effect on the intraneuronal uptake (Iversen 1965; Eisenfeld & others, 1967), appeared to have no appreciable effect on the total radioactivity either when the animal was killed 10 min or 5 h after administration of ^{3}H-NA (Table 1). When compared with the marked effect caused by other inhibitors of neuronal uptake, such as guanethidine, desipramine and cocaine (Table 2), it may be inferred that the extraneuronal uptake of ^{3}H-NA contributed to a negligible extent to the observed total radioactivity in the present experiments.

Effect of PBZ on ^{3}H-NA concentration in mice

When mice were pretreated with a single dose of PBZ (10 mg/kg, s.c.) 2 h before ^{3}H-NA, the total radioactivity in the heart was about 30% lower than that in the control animals when the mice were killed 10 min after ^{3}H-NA injection, and about

Table 3. *Prevention by propranolol of the reduction of ^{3}H-NA caused by PBZ in the rat heart.* Rats, placed in a warm environment (27–30°), were treated with PBZ (10 mg/kg, i.v.) and /or propranolol (10 mg/kg, i.v.) 10 min before injection of ^{3}H-NA (25 μCi/kg, i.v.) and killed 60 min later.

Rats	Total radioactivity (d min^{-1} g^{-1} ± s.e.)		^{3}H-NA (% of total radioactivity)	
	without propranolol	with propranolol	without propranolol	with propranolol
Control	121 000 ± 5230 (n = 6)	119 000 ± 1470 (n = 4)	95 ± 1·8	92 ± 3·1
PBZ-treated ..	46 200 ± 7700* (n = 3)	120 000 ± 10 790** (n = 4)	93 ± 3·7	92 ± 3·4

* $P<0.05$ *vs* control.
** $P<0.05$ *vs* PBZ only.

60% lower when the animals were killed at 5 h (Table 1). This may indicate that, in addition to competitive inhibition of the neuronal uptake (Iversen & Langer, 1969), PBZ also increases the reflex sympathetic discharge and turnover of noradrenaline due to blockade of α-adrenoceptors (Reid & others, 1969). Inhibition by PBZ of extraneuronal noradrenaline uptake (Eisenfeld & others, 1967; Iversen & Langer, 1969) was probably not involved since little extraneuronal binding of the amine was involved under the present experimental conditions.

Antagonism by propranolol in mice

The inhibitory effect of PBZ on ^{3}H-HA concentrations was significantly reduced when the mouse was treated with propranolol (5 mg/kg, s.c.) 1 h after PBZ administration. The data in Table 1 show that the total radioactivity in the heart of the PBZ-treated mice was partially restored. Propranolol, when given alone, did not appreciably affect the ^{3}H-NA concentrations, or noradrenaline stores, of hearts either in the mouse (Table 1) or in the rat (Table 3).

Whether the inhibitory effect on ^{3}H-NA uptake by other drugs might also be prevented by propranolol was tested. The results illustrated in Table 2 indicate that none of the effects of reserpine, guanethidine, cocaine or desipramine was appreciably reduced by propranolol, whereas the inhibition caused by phentolamine was reversed in a similar manner to that of PBZ.

Antagonism by propranolol in rats

The antagonism between propranolol and PBZ appeared to be more evident in this species. When ^{3}H-NA was administered to rats 10 min after intravenous injection of PBZ (10 mg/kg), the radioactivity remaining in the heart 60 min after injection of ^{3}H-NA was about 60% lower than in the controls. Simultaneous treatment with propranolol (10 mg/kg, i.v.) completely antagonized this effect of PBZ (Table 3). No appreciable change in the proportion of ^{3}H-NA to total radioactivity in the heart was observed in PBZ- or propranolol-treated animals, or in animals given both drugs (Table 3). This indicates that the effect is not simply due to increased retention of metabolites.

Time-course of the antagonism by propranolol

To ascertain the mechanism involved in the antagonism by propranolol of PBZ, propranolol (10 mg/kg, i.p.) was administered to the rat at various times after pre-

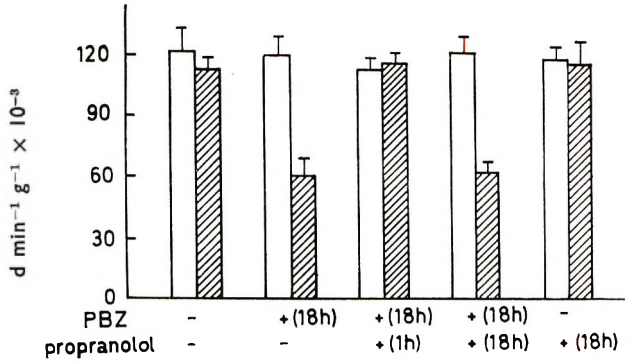

FIG. 1. Time-course of the antagonism by propranolol of the reduction of ^{3}H-NA concn in the rat heart induced by PBZ treatment and cold-stress. PBZ (10 mg/kg, i.p.) and propranolol (10 mg/kg, i.p.) were given as indicated in the bottom of the figure at the time shown in parentheses before injection of ^{3}H-NA. The rats were killed 60 min after ^{3}H-NA. Open columns show the mean d min^{-1} g^{-1} heart ± s.e. of control rats (n = 4–5) kept in 30° environment throughout the experiment; shaded columns show the values of rats kept in 6° environment for the last 60 min before death.

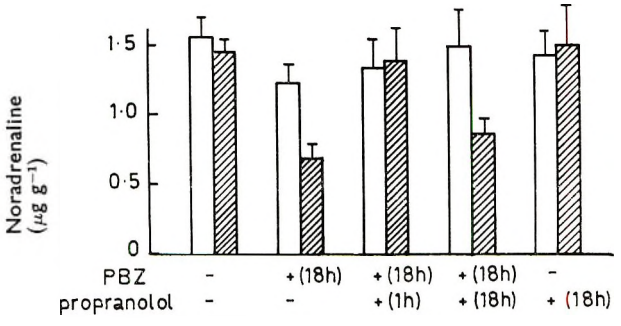

FIG. 2. Antagonism by propranolol of the PBZ-induced depletion of noradrenaline stores in the rat heart. Experimental conditions were the same as those shown in the legend to Fig. 1. The data are mean ± s.e. (n = 4–9).

treatment with PBZ. It was found that PBZ (10 mg/kg), administered intraperitoneally to animals kept in a warm environment (30°), did not cause any change in the amount of ^{3}H-NA in the heart. However, when the animals were placed in a refrigerator (6°) for 1 h, a marked reduction in the ^{3}H-NA concentration in the heart was observed. This effect of PBZ persisted, being still evident 18 h after PBZ administration. Advantage of this persistent effect was taken to test whether the antagonism by propranolol involved competition for binding at the active site, or that the pharmacological effect of propranolol *per se* was responsible for the antagonism. The experiments shown in Fig. 1 reveal that propranolol was effective when given shortly before the injection of ^{3}H-NA even when PBZ had been administered 18 h previously. In contrast, propranolol was ineffective when given simultaneously with PBZ.

Effect on noradrenaline stores

PBZ has been shown to increase the turnover rate of noradrenaline and decrease the amount of it in stores (Schapiro, 1958; Farrant & others, 1964; Reid & others,

1969). We found that this effect of PBZ in the heart could be negated when the rat was placed in a warm environment (30°), but was markedly enhanced when placed in a cold environment (6°, for 1 h). As illustrated in Fig. 2, this depleting effect of PBZ on heart noradrenaline stores was completely antagonized by propranolol provided it was administered shortly before the cold-stress.

DISCUSSION

The results of the present experiments demonstrate that the reduction of ^{3}H-NA accumulation, as well as that of cardiac noradrenaline stores, induced by PBZ treatment, was prevented by propranolol. The time-course of this antagonism indicates that it is not due to prevention, by propranolol, of the binding of PBZ with the active site. Rather, it is likely that a pharmacological action of the propranolol *per se* is responsible for the effect.

PBZ may cause reduction of noradrenaline cardiac stores, or of accumulation of ^{3}H-NA, *in vivo* either by inhibiting the uptake or by reflexly increasing cardiac adrenergic discharge resulting from α-adrenoceptor blockade. The findings that propranolol also antagonized the inhibitory effect on ^{3}H-NA accumulation by phentolamine but none of those induced by uptake inhibitors, such as desipramine, cocaine, guanethidine or reserpine, suggest that it is the reflexly increased adrenergic discharge which is antagonized by propranolol since both PBZ and phentolamine are inhibitors of α-adrenoceptors. Indeed, our recent experiments show that, in rats, the reduction of noradrenaline stores or decreased accumulation of ^{3}H-NA in the heart *in vivo* by PBZ is not due to inhibition of noradrenaline uptake but rather to increase of turnover rate (Chang & Lee, in preparation). It may be that the reversal by propranolol of PBZ-induced α-adrenoceptor blockade (Olivares & others, 1967; Yamamura & Horita, 1968, 1969; Smith & Nash, 1969; Mazurkiewicz-Kwilecki, 1970) restores the reflexly increased adrenergic discharge to normal and consequently removes the cause of decline of noradrenaline stores.

Two hypotheses have been proposed to explain the reversal by propranolol of PBZ-induced α-adrenoceptor blockade. Olivares & others (1967), observing that the antagonistic effect of propranolol persisted longer than its β-adrenoceptor blocking activity, concluded that the antagonism was due to displacement of PBZ from the receptor. Yamamura & Horita (1968, 1969), however, were not able to confirm this. Smith & Nash (1969) and Yamamura & Horita (1968, 1969) concluded, on the basis that the reversal occurred only with (−)-propranolol and that the reversal was observed only when α-adrenoceptor agonists which also had β-agonist properties were used, that the reversal was due to unmasking of unblocked 'spare' α-receptors resulting from β-receptor inhibition by propranolol. Our experiment on the time-course of the antagonism of the effect of PBZ on noradrenaline storage seems to support this conclusion.

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The effects of sympathetic nerve stimulation and guanethidine on parasympathetic neuroeffector transmission; the inhibition of acetylcholine release

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The effect of noradrenaline released either by sympathetic nerve stimulation or guanethidine added to the organ bath has been studied on acetylcholine release from parasympathetic nerve terminals and compared with the effect of exogenous noradrenaline. Sympathetic nerve stimulation, guanethidine and noradrenaline reduced the release of acetylcholine from resting rabbit intestine by up to 70%. Sympathetic stimulation and guanethidine failed to reduce acetylcholine release in preparations previously depleted of noradrenaline. Noradrenaline added to the bath still remained effective. The fact that noradrenaline released is capable of inhibiting acetylcholine release supports the concept that noradrenaline physiologically controls the release of acetylcholine.

It has been shown by Paton & Vizi (1969) and Vizi (1968) that noradrenaline and adrenaline inhibit the acetylcholine release from the parasympathetic nerve terminals of longitudinal muscle strip of guinea-pig ileum, particularly during rest periods or when stimulation frequency was low (0·1 to 2·0 Hz) and that this inhibitory action is mediated through α-adrenoceptors. It has further been shown (Paton & Vizi, 1969; Vizi, 1968) that the reduction of sympathetic outflow by reserpine or guanethidine pretreatment increases the acetylcholine which thus indicates a continuous sympathetic control on parasympathetic transmitter release. Norberg (1964), Jacobowitz (1965), and Norberg & Sjöqvist (1966) presented evidence that the adrenergic fibres embrace the ganglia in gut without directly innervating the smooth muscle. The inhibitory action of noradrenaline on acetylcholine release was also observed by Kosterlitz, Lydon & Watt (1970). In addition, Beani, Bianchi & Crema (1969) presented evidence that noradrenaline reduced the release of acetylcholine from colon. It was confirmed (Kosterlitz & others, 1970; Beani & others, 1969) that the inhibitory effect of noradrenaline prevails when the cholinergic fibres were stimulated at low rates. However, Knoll & Vizi (1970, 1971), using intermittent (trains of 2–10 shocks, with intervals of 50–1000 ms, repeated at intervals of 10 s) high rate stimulation of parasympathetic nerves of the longitudinal muscle strip of guinea-pig ileum, have established that under these conditions noradrenaline is able to reduce the acetylcholine release also.

The experiments now described were made to study the effect of noradrenaline released either by sympathetic nerve stimulation or by guanethidine on the release of acetylcholine due to parasympathetic nerve stimulation of different frequency and at resting condition.

Some of the present findings have been communicated to Hungarian Physiological Society (1968 Meeting; Vizi, 1970).

METHODS AND MATERIALS

The preparation of the rabbit isolated intestine was essentially similar to that described by Finkleman (1930). Rabbits, 2 to 3 kg, were killed by blow on the head and as long a length as possible of periarterial sympathetic nerve was dissected out together with 3–4 cm of the intestine. The preparation was suspended in Krebs solution at 36° in a 10 ml organ bath, aerated with 5% carbon dioxide in oxygen. The composition of the Krebs solution was (mM): NaCl 113; KCl 4·7; $CaCl_2$ 2·5; KH_2PO_4 1·2; $MgSO_4$ 1·2; $NaHCO_3$ 25 and glucose 11·5. Mesenteric artery was drawn through an insulated platinum ring electrode as far away from the intestine as possible. This was to reduce current spread that might cause acetylcholine release and so overshadow the effect of sympathetic nerve stimulation on acetylcholine release. The pulse duration was 0·3 ms and the strength of stimulation 5 to 10 V, was supramaximal. In other experiments the preparation was also stimulated by a square-wave pulse of 1 ms duration through two platinum electrodes one in the top and one in the bottom of the organ bath (Field stimulation) giving a potential drop of 8–12 V/cm. This also was supramaximal. The stimulation was so arranged that one electrode was used for stimulation, the other was without earth connections. Except during actual stimulation, the electrodes were short-circuited. During single field stimulation the simultaneous sympathetic stimulation was stopped for 1 s to avoid any passing of current.

Contractions of the intestine were recorded auxotonically (Paton, 1957) using a frontal writing lever with a magnification of 10-fold and exerting a tension of 1 g.

Longitudinal muscle strip of guinea-pig ileum prepared according to Paton & Vizi (1969) was set up in a 3·5 ml organ-bath filled with Krebs solution, bubbled with 5% carbon dioxide in oxygen at 37°. Supramaximal field stimulation (8–12 V cm), was used. The contractions were recorded either by means of an auxotonic writing lever and kymograph or by an isometric recording system.

The output of acetylcholine from rabbit intestine and guinea-pig ileum was collected in the presence of eserine sulphate (2×10^{-6} g/ml) and assayed using a guinea-pig ileum suspended in 3·5 ml Krebs solution at 36°. A polythene cannula was inserted into the distal end of the gut to drain off intraluminal contents and in this way to maintain a gut highly reliable for the assay. Control responses to a standard solution of acetylcholine were obtained in the presence of the same concentration of test drug that was produced when the test sample was added to the assay bath. In few experiments "intermittent" train stimulation was used (Knoll & Vizi, 1970).

The resting acetylcholine output and the output per volley were calculated according to Paton & Vizi (1969) and expressed in ng/g min, and ng/g per volley, respectively. Drugs used were: acetylcholine iodide (BDH), (—)-noradrenaline bitartrate (Koch-Light Laboratories Ltd.), guanethidine sulphate (CIBA), physostigmine sulphate (Macarthys Ltd.), cocaine HCl, phentolamine methane sulphonate (CIBA) tetrodotoxin (Sankyo). The drugs were dissolved in distilled water or distilled water saline. Concentrations are expressed in terms of the drug salts or in molar concentration.

Statistical calculations were made according to conventional procedures.

RESULTS

Table 1 shows the inhibitory effect of noradrenaline, sympathetic nerve stimulation and guanethidine on acetylcholine output from the Finkleman preparation. The

Table 1. *Reduction of acetylcholine release from rabbit jejunum by endogenous and exogenous noradrenaline.*

Expt No.	Condition	Rate of field stimulation or collection period in min	No. of shocks applied	Control ACh-output ng/g min s.e.	Change in ACh-output during treatment or sympathetic stimulation	Change in ACh-output reduction in percent s.e.	P
1.	Resting (23)	5–10	—	5·1 ± 0·9			
2.	Resting (4)	5	—	8·6 ± 1·1	sympathetic stim. 10 Hz for 5 min	40·2 ± 4·0	<·001
3.	Resting (3)	10	—	4·8 ± 0·7	(−)-NA $1·5 \times 10^{-6}$M	57·1 ± 6·0	<0·01
4.	Resting (3)	10	—	4·3 ± 0·6	guanethidine 4×10^{-5}M	41·3 ± 5·5	<0·05
5.	Stimulated (15)*	0·5 Hz	600	8·9 ± 0·7	—	—	—
6.	Stimulated (3)	0·5 Hz	600	10·2 ± 1·9	(−)-NA $1·5 \times 10^{-6}$M	66·5 ± 9·0	<0·01
7.	Stimulated (3)	0·5 Hz	600	7·1 ± 1·1	guanethidine 4×10^{-6}M	57·2 ± 6·2	<0·01
8.	Stimulated (5)	10 Hz	1200	26·5 ± 3·6	—	—	8:5 <0·10
9.	Stimulated (2)	10 Hz	1200	29·1	(−)-NA 3×10^{-6}M	5·5	n.s.

* The corresponding resting output is 5·0 ± 0·3 ng/g min and the difference is significant, $P < 0·01$. Number in brackets indicate the number of experiments.

Table 2. *The inhibitory action of noradrenaline released by guanethidine on acetylcholine output from the Auerbach plexus of longitudinal muscle strip of guinea-pig ileum.*

Expt No.	Condition	Rate of stimulation or collection period in min	Type and duration of stimulation	No of shocks	Total ACh output ng/g min s.e.	Volley output ng/g min	Change in ACh output reduction %	P
1.	Resting (25)	15	—	—	41·2 ± 2·0	—	—	—
2.	Resting + (−)NA 2×10^{-6}M	15	—	—	10·7 ± 2·1	—	74·3	2:1<0·01
3.	Resting (5) + guanethidine 4×10^{-5}M	15	—	—	18·7 ± 4·1	—	55·7	3:1<0·01
4.	Stimulated (3)	0·1 Hz	continuous 10 min	60	114·0 ± 5·6	10·6	—	—
5.	Stimulated (3) + (−)-NA $2·9 \times 10^{-6}$M	0·1 Hz	continuous 10 min	60	21·2 ± 5·6	1·8	81·4	5:4<0·01
6.	Stimulated (3) + guanethidine 4×10^{-5}M	0·1 Hz	continuous 10 min	60	50·7 ± 7·1	4·9	55·6	6:4<0·01
7.	Stimulated (5)	10 Hz	continuous 1 min	600	1025·0 ± 41·6	1·6	—	—
8.	Stimulated (5) + (−)-NA $2·9 \times 10^{-6}$M	10 Hz	continuous 1 min	600	1003·0 ± 60·1	1·6	—	no change
9.	Stimulated (3) + guanethidine 4×10^{-5}M	10 Hz	continuous 1 min	600	1102·0 ± 74·0	1·8	—	n.s.
10.	Stimulated (3)	10 Hz	inermittent*	300	1489† ± 92·4 ng/g 10 min	4·8	—	—
11.	Stimulated (3) + guanethidine 4×10^{-5}M	10 Hz	intermittent*	300	735·2† ± 65·2 ng/g 10 min	2·3	46·2	11:10<0·05

* Intermittent stimulation (5 shocks of 100 ms intervals in every 10 s = one train; 60 trains = 60 × 5 = 300 shocks).
† Total ACh output during the 10 min period. Numbers in parentheses indicate the number of experiments.

resting output, 5·1 ng/g min (18·8 pmol/g min), showed a large deviation, probably dependent on the density of sympathetic innervation. This is supported by the fact that the acetylcholine output in intestine from the rabbit pretreated with guanethidine (20 mg/kg, s.c., 6 h before testing), was higher (13·6 ng/g min; n = 3). The concomitant stimulation of the sympathetic nerve (10 Hz; 0·3 ms; for 5 min) reduced the resting output on average by 45%. The higher the control resting output, the

more effective was the sympathetic stimulation in reducing acetylcholine release. The data in Table 1 also show that when the sympathetic nerve stimulation was applied simultaneously with noradrenaline for 5 min, the increased acetylcholine output in response to the field (parasympathetic) stimulation was abolished. Noradrenaline ($1{\cdot}5 \times 10^{-6}$M) and guanethidine (4×10^{-5}M), reduced the acetylcholine output both at resting and at 0·5 Hz stimulation. However, the sympathetic stimulation and guanethidine proved to be ineffective in rabbit intestine pretreated with guanethidine (20 mg/kg, s.c., 6 h before dissection); moreover in one experiment the sympathetic stimulation increased the acetylcholine output by 16%. Noradrenaline, $1{\cdot}5 \times 10^{-6}$M, inhibited both the resting and stimulation (0·5 Hz) output, but failed to have an effect at a high rate of stimulation (10 Hz; see Table 1). There is an inverse relation between stimulation rate and output per volley. At a stimulation rate of 0·5 Hz the volley output was 0·13 ng/g per volley while at 10 Hz the output was only 0·04 ng/g per volley. This is in agreement with the findings of Paton (1963), Paton & Zar (1968), Paton & Vizi (1969) and Knoll & Vizi (1971), who observed this phenomenon in parasympathetic nerve terminals of longitudinal muscle strip of guinea-pig ileum.

Tetrodotoxin, 4×10^{-5}M, inhibited the increase of acetylcholine output produced by field stimulation, indicating the neural origin of acetylcholine.

Table 2 shows the effect of guanethidine on acetylcholine output from nerves of the longitudinal muscle preparation of guinea-pig ileum. Guanethidine (4×10^{-5}M), like noradrenaline, reduced the output both of resting gut and of gut stimulated at low frequency. At 0·1 Hz the amount of acetylcholine released per impulse was reduced from 10·6 to 4·9 ng/g per volley. Acetylcholine release produced by continuous stimulation of 10 Hz was not affected by guanethidine. However, using intermittent stimulation, where the trains of 5 pulses with intervals of 100 ms (10 Hz) were repeated once every 10 s, guanethidine, 4×10^{-5}M reduced the acetylcholine output from 4·8 to 2·3 ng/g per volley (Table 2).

In the longitudinal muscle strip of ileum from the guinea-pig pretreated with guanethidine (15 mg/kg, s.c. 6 h previous to preparation), guanethidine ($2\text{–}4 \times 10^{-5}$M) added to the organ bath, was less effective in reducing the acetylcholine volley output.

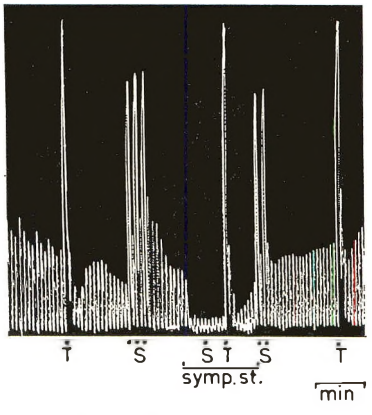

FIG. 1. Effect of sympathetic nerve stimulation on the contraction of rabbit jejunum evoked by "field" stimulation. Rabbit isolated jejunum prepared according to Finkleman. Sympathetic nerve stimulation: 10 Hz, 0·3 ms, 5 V. T = "field" stimulation of 10 Hz, 1 ms, 30 shocks, 8 V/cm. S = "field" stimulation with a single shock, 1 ms, 8 V/cm. Auxotonic recording. Krebs solution bubbled with a gas mixture of 95% O_2 + 5% CO_2. Organ bath, 10 ml.

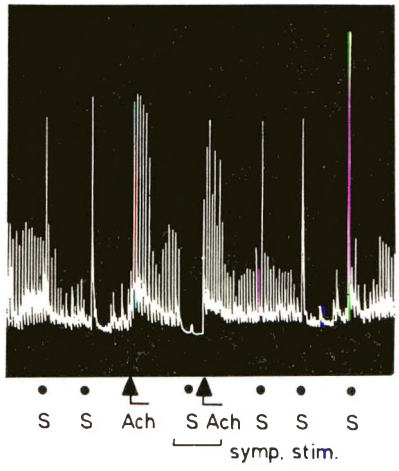

FIG. 2. Effect of sympathetic nerve stimulation on the responses to single "field" stimulation of rabbit jejunum and to acetylcholine. Finkleman preparation. Sympathetic stimulation (symp. stim.) = 10 Hz, 0·3 ms, 5 V. S = "field" stimulation with a single shock, 1 ms, 8 V/cm. ACh = acetylcholine iodide, 30 ng/ml. Contractions are recorded by auxotonic writing lever. Krebs solution. 95% O_2 + 5% CO_2. Organ bath, 10 ml.

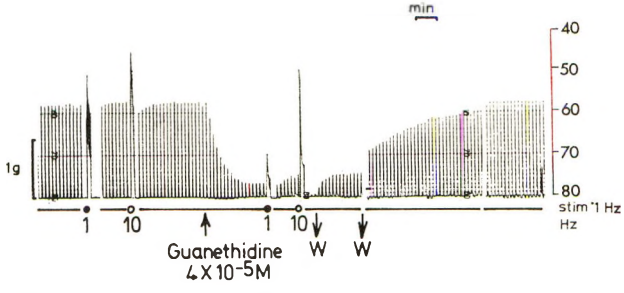

FIG. 3. The inhibitory action of guanethidine on the responses of longitudinal muscle strip of guinea-pig ileum to parasympathetic nerve stimulation with different frequencies. "Field" stimulation, 1 ms, 8 V/cm. The contractions were recorded isometrically. At 1 Hz 10 shocks, at 10 Hz 100 shocks were delivered. W = wash out. Krebs solution. 95% O_2 + 5% CO_2. Organ bath, 3·5 ml. "Overflow"-technique. The interval between first and second and second and third parts of trace was 5 min. Note the inhibitory action of guanethidine on the responses of longitudinal muscle strip of guinea-pig ileum to 0·1 and 1 Hz stimulation.

The above problems were also examined in the absence of a cholinesterase-inhibitor, the responses to the acetylcholine released were recorded auxotonically or isometrically. Fig. 1 shows the inhibitory action of sympathetic nerve stimulation on the response to single "field" stimulation. The contraction caused by high frequcncy of stimulation (10 Hz; 30 shocks) was not influenced. Noradrenaline (10^{-6} to 10^{-5}M) behaved similarly when added to the organ bath. The effect of acetylcholine added to the bath to increase pendular movement and to cause contraction of rabbit jejunum was not affected by sympathetic nerve stimulation; the contraction to single "field" stimulation, however, was still reduced (Fig. 2).

In longitudinal muscle strip of guinea-pig ileum, guanethidine ($2–4 \times 10^{-5}$M), added to the organ bath, like noradrenaline, reduced the twitch caused by stimulation of 0·1 and 1 Hz, without markedly reducing the contraction produced by 10 Hz stimulation (30 shocks were delivered; see Fig. 3). The effect was rapid in onset. After washing out, a fast recovery was observed. But if the guanethidine was left in

the bath for 15–45 min, a full recovery, and sometimes even an increase in size of contraction was observed. In any experiment in which repeated administration occurred, the strip at low frequency stimulation, developed tachyphylaxis to guanethidine. Exposing the strip to cocaine, $4{\cdot}7 \times 10^{-4}$M, or to phentolamaine $2{\cdot}2 \times 10^{-5}$M, for 10 min, reduced the effectiveness of guanethidine to abolish response to stimulation by 30–40%.

DISCUSSION

In the presence of the cholinesterase inhibitor, eserine sulphate, it was possible to measure the acetylcholine output from nerve elements of rabbit jejunum. The resting output varied from preparation to preparation (1·5–12·1 ng/g min), perhaps because of the density of sympathetic innervation. Noradrenaline released by sympathetic nerve stimulation reduced the acetylcholine output.

However, in Gershon's experiments (1967), stimulation of the sympathetic nerve at a frequency sufficient to produce relaxation (10 Hz) in an eserine-treated muscle failed to decrease the release of acetylcholine from rabbit jejunum. The discrepancy between these data and our results is probably due to the differences in stimulation. Gershon stimulated the sympathetic nerve for alternate 1 min periods for 10 min while we used continuous stimulation. Now Paton & Vizi (1969) have observed in longitudinal muscle strip of guinea-pig ileum an "overshoot" in acetylcholine release after withdrawal of noradrenaline i.e. the release exceeded the control level. Del Tacca, Soldan & others (1970) also found an "overshoot"-phenomenon in human isolated taenia coli. The relation of "overshoot" observed by Paton & Vizi (1970) to the response to noradrenaline was also inversely related to the time of exposure to noradrenaline (Paton & Vizi, 1970). This could explain the negative result obtained by Gershon. Nevertheless, Gershon did observe a reduction in acetylcholine output with sympathetic nerve stimulated at 30 and 40 Hz. In spite of the alternate stimulation, this reduction was probably due to release of an excess of noradrenaline, the effect of which was preserved between stimulation periods thus preventing the "overshoot"-phenomenon which would otherwise have overshadowed the depression of acetylcholine output.

In the Finkleman preparation the motor response to field stimulation is due to acetylcholine release since its output increased after stimulation.

Guanethidine interferes with the mechanism for noradrenaline-storage by releasing the amine (Cass, Kuntzman & Brodie, 1960) and, as Paton & Vizi (1969) have previously shown, it also effectively interferes with the release of acetylcholine by reducing noradrenaline content and output. In the present experiments guanethidine added to the organ bath reduced both the resting and the low rate stimulation ouptut of acetylcholine. This effect of guanethidine is probably due to release of noradrenaline. This explanation is supported by the fact that guanethidine proved to be ineffective in preparations previously depleted of noradrenaline. In addition, its release by guanethidine (Cass & others, 1963) is backed by the finding of Garret & Sousa (1963) and Harrison, Chidsey & others (1963) who provided evidence for the view that some acute biological responses, i.e. positive inotropic and chronotropic effects on isolated atria to guanethidine depend on the presence of releasable noradrenaline. Any effect on nerve conduction can also be excluded since the contractions of gut evoked by stimulation of low frequency (0·1 Hz) recovered in time in spite of the presence of the drug and since the response to sustained stimulation with a frequency greater than

5 Hz remained almost always unchanged. Moreover, nerve conduction in the vagus nerve of the rabbit was not influenced by guanethidine in concentrations of up to 10^{-3}M (Vizi & Knoll, unpublished observations). Chang, Chen & Cheng (1967) also failed to observe any change in nerve action potential with guanethidine. However, there is also no evidence that noradrenaline affects impulse transmission in the axon (Paton & Thompson, personal communication).

The evoked acetylcholine release per volley was reduced by guanethidine at the sustained stimulation of 0·5 Hz, however, at 10 Hz there was no reduction. Using high rate stimulation (10 Hz), but trains short in duration (5 shocks) and 10 s intervals between two consecutive trains, guanethidine reduced the volley output significantly. This result indicates that at high rate of stimulation the acetylcholine output caused by the first shocks is also sensitive to noradrenaline like that produced by low rate stimulation. Recently, the same effect was observed by Knoll & Vizi (1970; 1971) with noradrenaline added to the organ bath and by Cowie, Louise & others (1970) with morphine.

Since without a cholinesterase inhibitor it is not possible to measure acetylcholine release, the question arises as to how the presence of eserine influences the effect of noradrenaline or parasympathetic stimulation on acetylcholine release. All the data obtained during the study of the effect of sympathetic nerve stimulation, or of guanethidine, on contraction of intestine evoked by parasympathetic nerve stimulation are in agreement with the data obtained from direct measurement of acetylcholine output. Sympathetic nerve stimulation, guanethidine, by releasing noradrenaline, and noradrenaline added to the bath affected in a similar way both the responses to parasympathetic nerve stimulation and resting and stimulated acetylcholine output.

The single field stimulation was sometimes followed by reduction of pendular movement. It is probably due to the noradrenaline, or some other inhibitory substance, released since the non-adrenergic inhibitory innervation is a general feature of mammalian intestinal smooth-muscle (Burnstock, Campbell & others, 1963, 1964; Bülbring & Tomita, 1967; Furness, 1969). After guanethidine pretreatment, stimulattion of the sympathetic extrinsic nerve caused a contraction in rabbit jejunum, as seen by Day & Rand (1961).

A detailed analysis of the inhibitory effect of noradrenaline or adrenaline on acetylcholine output from nerve elements of intestine at stimulation (Paton & Vizi, 1969; Vizi, 1968; Knoll & Vizi, 1970; 1971), and the similar observations by others (Kosterlitz & others, 1970; Beani & others, 1969; Del Tacca & others, 1970) also in gut, or the reduction of acetylcholine release by noradrenaline or adrenaline from ganglia *in situ* (Paton & Thompson, 1958) or *in vitro* (Dawes & Vizi, unpublished) present evidence that noradrenaline may play a role in transmission by reducing the acetylcholine release. Another interesting coincidence is that adrenaline (McIsaac, 1966) and guanethidine (Maxwell, Plummer & others, 1957) were each able to reduce ganglionic transmission at low frequency of stimulation (0·33 and 0·5 Hz respectively). These data and the fact that noradrenaline released either by sympathetic nerve stimulation or by guanethidine was capable of inhibiting acetylcholine release, support the concept that noradrenaline physiologically controls the release of acetylcholine. Nerve impulses in sympathetic nerve, on reaching the nerve endings liberate noradrenaline which, in turn, blocks the release of acetylcholine. This seems to be a more economic form of counteraction between acetylcholine and noradrenaline than that which takes place at the postsynaptic membrane.

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Myocardial necroses produced by intravenous infusion of isoprenaline into rats

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Intravenous infusion of isoprenaline in doses of 2, 20, 200, 2000 μg/kg per min over 20 min into heparinized Wistar rats results in myocardial necroses and a rise in SGPT activity, erythrocyte sedimentation rate and leucocytosis. A quantitative method for estimation of intensity and distribution of myocardial necrotic foci is described. The intensity of myocardial lesions is observed linearly related to the infused isoprenaline at 2, 20 and 200 μg/kg per min. Damaged areas are mainly found in subendocardial and apical regions of left ventricle.

Isoprenaline produces myocardial necroses in rats, but its dosage, routes of injection and the time of observed effects differ among reports. Isoprenaline has been injected subcutaneously (Moudgil, 1960) or intraperitoneally (Sorokina & Altshuler, 1966) in doses ranging from 5 μg/kg (Dorigotti, Gaetani & others, 1969) up to 500 mg/kg or higher (Wexler, Kittinger & Judd, 1967; Leszkovszky & Gal, 1967) in a single (Arigoni, 1969) or repeated doses (Judd & Wexler, 1969). The heart was examined within 15 min (Korb, 1965) and up to 14 days from the moment of the injection (Wenzel, Raymond & Chou, 1966).

We describe a model of myocardial necroses produced by continuous intravenous infusion of isoprenaline. In rats so treated, the distribution and the intensity of myocardial necroses were investigated. The correlation between morphological and biochemical disturbances were also observed. The proposed model may be used for the quantitative evaluation of cardioprotective properties of β-adrenoceptor blocking drugs.

METHODS

Male Wistar rats, 200–220 g, were injected intraperitoneally with heparin (1000 units/kg), narcotized with sodium amylobarbitone (80 mg/kg) and infused intravenously (0·1 ml/rat per min) during a period of 20 min with a solution of (±)-isoprenaline hydrochloride or with saline. Four dose-levels of isoprenaline 2, 20, 200 or 2000 μg/kg per min were administered into four groups of animals. Twenty h later, rats were again given the barbiturate, the animals were bled and hearts excised.

Blood was used to estimate the activity of serum glutaminate-pyruvate-transaminase (SGPT) according to Reitmann & Frenkel (1957), leucocytosis and erythrocytes sedimentation rate (ESR).

Hearts were fixed in 10% neutral formalin and then dissected transversely into four symmetrical thick slices (see Fig. 1), each used to prepare histological sections 5 μm thick. These were stained with hematoxylin and eosin or with oil-red, trichrom according to Masson, PAS according to McManus and acidic fuchsine according to Selye. Test cards with the standardized outlines of four cardiac slices were also prepared. Histological sections of each heart were examined under the microscope

FIG. 1. Test-cards containing the standardized outlines of four cardiac slices. The lesioned areas are marked black. LV—left ventricle, RV—right ventricle, a, b, c, are typical examples of test-cards from rats infused intravenously with isoprenaline 2, 20 and 200 μg/kg per min during 20 min.

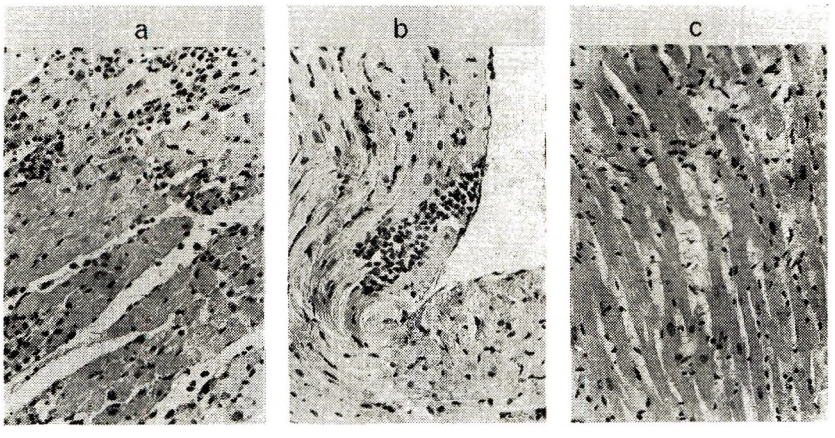

FIG. 2. Histological sections of rat myocardium stained with hematoxylin and eosin, magnification 180× after a 20 min infusion.

a. Subendocardial section of left ventricle of a rat infused with isoprenaline (200 μg/kg per min). Typical mosaic area contains few necrotic or degenerated muscle fibres and leucocytes. Proliferative swelling of stroma is composed of fibroblasts and histiocytes.

b. Endocardial surface of right ventricle of a rat treated with isoprenaline (200 μg/kg per min). Subendocardial necrotic focus is closely packed with granulocytes and histiocytes and surrounded by a monolayer of degenerating muscle fibres.

c. Section of left ventricle of a rat treated with isoprenaline (2000 μg/kg per min). Myocardial fibres have lost their cell structure. Sarcoplasma is swollen and vacuolized. Nuclei are also affected.

and affected areas were drawn into the standardized outlines of corresponding cardiac slices (Fig. 2). Then lesioned areas were cut out and weighed. The intensity of cardiac damage was expressed as the ratio of weight of the lesioned area to the total area in each slice. This gave a quantitative measure and a pattern of distribution of the cardiac lesions induced by different doses of isoprenaline.

The lesions were considered as mosaic areas surrounded by healthy myocardium: they usually contained few necrotic disintegrated muscle fibres but were abundantly

infiltrated with granulocytes and histocytes. In these areas the degenerated muscle fibres could be visualized by trichrom or by PAS staining. Some fibres was oil-red positive. The borderline between healthy and lesioned myocardium were not distinct but the approximate size and shape of the affected area was obvious.

RESULTS

Intravenous infusions of isoprenaline in doses 2, 20 and 200 μg/kg per min for 20 min cause myocardial lesions in rats, and 20 h after the infusion the lesioned area has a mosaic appearance because it consists of necrotized, degenerated and disintegrated muscle fibres mixed with healthy fibres that are embedded in a fibroblastic swelling and infiltration composed of granulocytes and histiocytes (Fig. 2a, b). Isoprenaline, 2 mg/kg per min results in a 50% death rate, the animals showing myocardiocytolysis (Fig. 2c).

The intensity of myocardial lesions is linearly dose dependent over the range 2–200 μg/kg min^{-1}.

The distribution of cardiac lesions is characteristic. All major myocardial lesions are found in the subendocardial area of the left ventricle. The septum is less affected and the right ventricle is almost free, except after 200 μg/kg per min (Fig. 1). About 80% of lesions are in the lower apical half of the hearts. At the 200 μg/kg per min dose some necrotic lesions are noted towards the base of the heart.

Biochemical and haematological changes accompanying heart damage are given in Table 1. The most sensitive but the least specific test for detection of myocardial micronecroses is the estimation of erythrocyte sedimentation rate (ESR). The least sensitive test is SGPT activity. Determination of leucocytosis seems to be fairly reliable as a measure of the severity of morphological disturbances in myocardium.

Pretreatment of rats with heparin is essential to avoid inducing disseminated lung infarcts, which strongly interfere with the development of myocardial lesions.

DISCUSSION

We introduced the intravenous route of administration of isoprenaline because only this route assures the steady concentration of isoprenaline in blood that reaches myocardium and a known duration of drug action.

The period of 20 min of an excessive β-adrenoceptor stimulation proved to be sufficient to induce the damage of myocardium in 100% of animals, even at 2 μg/kg per min of isoprenaline.

We consider that the small scatter of intensity of myocardial lesions within groups of rats and the good linear relation between dose and necrotic response are due to standardized conditions of β-adrenoceptor stimulation that were achieved by use of the intravenous route.

Hearts examined 20 h after an infusion showed the morphological alterations in myocardium precisely the changes correlated well with biochemical and haematological changes in blood (Kowalczykowa, Gryglewski & others, 1971; Gryglewski, Kowalczykowa & others, 1971).

The distribution of cardiac lesions in subendocardial areas of apical region of left ventricle is the most striking observation. The lack of myocardial damage in right ventricle proves that isoprenaline does not pass through the endocardium, but has to be transferred via the coronary circulation to the myocardium. The abundance

Table 1. *The effect of the 20 min intravenous infusion of isoprenaline on serum glutaminate-pyruvate transaminase (SGPT), leucocytosis, sedimentation rate of erythrocytes (ESR) and myocardial lesions in rats after the relapse of 20 h from the moment of isoprenaline infusion.* Figures represent mean value ±s.e. for n animals in a group.

Treatment μg/kg per min	SGPT Wróblewski U	Leucocytosis $\times 10^{-3}$ mm^3	ESR mm 2h	Cardiac damage % of affected area
Control ..	22·4 ± 1·13 n = 10	27·3 ± 1·12 n = 80	4·16 ± 0·16 n = 40	0 n = 10
Isoprenaline 2 ..	20·9 ± 2·2 n = 6	31·9 ± 5·4 n = 7	16·0 ± 3·4+ n = 6	6·22 ± 3·58+ n = 6
Isoprenaline 20 ..	24·7 ± 1·51 n = 12	40·5 ± 6·6+ n = 8	13·0 ± 2·6+ n = 8	27·57 ± 5·5+ n = 12
Isoprenaline 200	32·25 ± 3·8+ n = 9	52·2 ± 7·5+ n = 8	11·0 ± 2·4+ n = 8	46·23 ± 1·98+ n = 12
Isoprenaline 2000	18·10 ± 1·6 n = 16	26·6 ± 2·5 n = 8	13·0 ± 2·6+ n = 11	15·69 ± 3·38+ n = 12

+ statistically significant at $P < 0{\cdot}01$.

of coronary blood supply to left ventricle makes the isoprenaline more accessible than right ventricle is.

Subendocardial distribution of micronecroses in the apical region suggests that metabolic and mechanical components are involved in the necrotizing activity of isoprenaline on the myocardium. Isoprenaline results in an "oxygen-wasting effect" in the whole heart (Raab, 1963) but it increases the pressure mostly inside the left ventricle (Boerth, Covell & others, 1969). Jedeikin (1964) found the highest concentration of glycogen and phosphorylase at the endocardial surface of the left ventricle. Therefore it may be assumed that this region of the myocardium is biochemically prepared to be the most reactive to β-adrenoceptor stimulation by isoprenaline. The endocardial surface of the left ventricle reacts most to isoprenaline, but at the same time this region becomes deprived of macroenergic phosphates and oxygen (Boerth & others, 1969), moreover tissue perfusion is decreased and mechanical pressure increased to much higher extent than at the epicardial surface of both ventricles (Myers & Honig, 1966; Hefner, Sheffield & others, 1962). The above facts explain our observation on the susceptibility of endocardial layers to damaging effect of isoprenaline.

There is a practical significance of the data. The prevention of isoprenaline-induced myocardial micronecroses by cardioprotective drugs is usually evaluated semi-quantitatively (Dorigotti & others, 1969; Fleckenstein, 1970). Our method offers a quantitative approach. For such purposes the method can be simplified by using only one slice for histological examination, the second slice from the apex (Fig. 1) being fairly representative for the evaluation of the intensity of cardiac damage.

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The action of azapropazone, oxyphenbutazone and phenylbutazone on lysosomes

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Azapropazone, oxyphenbutazone and phenylbutazone have a stabilizing action on isolated lysosomes over a wide concentration range but a lytic action at high concentrations. The lytic action of phenylbutazone was greater than the other drugs. Phenylbutazone at a high concentration was found to accelerate the breakdown of lysosomes in isolated stomach preparations *in vitro*. Phenylbutazone was found to have a greater ulcerogenic action than azapropazone in rats and rabbits. Tissues removed from rats and rabbits dosed with phenylbutazone showed evidence of lysosomal damage when examined histochemically for acid phosphatase. In contrast, tissues from control and azapropazone-treated rats showed no evidence of lysosomal damage. Sections of rat gut incubated with azapropazone and phenylbutazone *in vitro* showed similar results. The possibility is discussed that drugs in high concentration may damage lysosomes in the gastrointestinal tract. It is suggested that lysosomal damage may contribute to the ulcerogenic action of the drugs *in vivo*.

The actions of anti-inflammatory drugs on lysosomes are of interest since lysosomal enzymes are suspected mediators of inflammation (Weissman, 1967). The actions of some anti-inflammatory aromatic acids and anti-inflammatory steroids are concentration-dependent (Lewis, 1970; Lewis, Symons & Ancill, 1970). This investigation was concerned with the effect of concentration of azapropazone, oxyphenbutazone and phenylbutazone on lysosomes. The actions of the drugs on lysosomes was compared with that of prednisolone since the anti-inflammatory action of steroids has been related to their stabilizing actions on lysosomes (Weissmann & Dingle, 1961). The ulcerogenic action of phenylbutazone in rats was also investigated to examine the possibility that lysosomal damage at high drug concentrations may be involved in the development of ulcers *in vivo*.

METHODS

Isolated lysosome experiments

Lysosomes were isolated from rat and rabbit liver by methods previously described for rabbit liver (Weissmann, 1965). An identical procedure was used to prepare lysosomes from rat ileum. The lysosomes were finally suspended in 0·05M-tris-acetate buffer (pH 7·4), sucrose (0·25M) and the protein concentration determined (Lowry, Rosebrough & others, 1951). The drugs were dissolved in dimethyl sulphoxide and 0·1 ml portions added to 5 ml of the lysosome suspension. Dimethyl

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sulphoxide was added to the controls. The suspensions were incubated with shaking at 37° for 90 min. After incubation the lysosomes were removed and the acid phosphatase (EC 3.1.3.2) and β-acetylglucosaminase (EC 3.2.1.30) activity of the supernatants were determined (Symons & others, 1969; Lewis & others, 1970).

Isolated tissue experiments

Six female rats (200 g) were killed by a blow to the head and the stomachs removed and placed on cracked ice. Each stomach was slit open and placed in 22 ml of oxygenated Ringer-Tyrode buffered at pH 6 with 0·1M phosphate containing *p*-nitrophenyl phosphate (0·015M) in stoppered flasks. The flasks were gently shaken and samples of the medium (1 ml) taken at intervals. The samples were centrifuged and 0·5 ml portions of the supernatants transferred to test tubes and 5 ml of 0·1N sodium hydroxide added. The absorbances of the solutions were then determined at 420 nm. After the stomachs had been incubating for 75 min, phenylbutazone dissolved in dimethyl sulphoxide was added to three flasks to give a final concentration of $4{\cdot}4 \times 10^{-3}$M. The same volume (0·5 ml) of dimethyl sulphoxide was added to the three control flasks. The flasks were incubated for 6 h, samples of the medium being taken at intervals and assayed for acid phosphatase. In a second experiment female rats (250–300 g) were killed by a blow to the head and 5 cm ileum segments removed. After slitting longitudinally these were placed in oxygenated Ringer-Tyrode solution buffered at pH 6 with 0·1M phosphate, and containing either phenylbutazone or azapropazone at a concentration of 4×10^{-3}M. The flasks were incubated at 37° for 90 min, after which time the segments were removed and plunged into acetone-solid CO_2 at −70°. The segments were then examined histochemically for acid phosphatase by the Gomori (1941) procedure except that the incubation time with sodium β-glycerophosphate was reduced to 2 h instead of 4 h.

Whole animal experiments

Rabbits. Azapropazone and phenylbutazone were separately granulated and mixed with animal meal and subsequently pelleted for feeding to rabbits. Preliminary experiments had established that a rabbit would eat 200–250 g of pellets each day. During the experimental period of 5 days they were given 100 g of the pellets containing drugs and it was found that this was completely eaten. This was equivalent to a drug dose of 500 mg/kg per day. The rabbits selected were of similar weight (1 kg). Two were treated with phenylbutazone and two with azapropazone. After five days they were killed and the stomach and gut removed for examination. Segments were frozen in acetone-solid CO_2 and subsequently examined histochemically as described by the Gomori (1941) procedure for acid phosphatase.

Rats. Azapropazone or phenylbutazone was suspended (20%, w/v) in syrup B.P. and separately administered to Wistar rats (250 g). In one experiment drug, 500 mg/kg was administered daily for five days. In a second experiment drug, 250 mg/kg, was administered at 48 h intervals. In this experiment the rats were dosed on four occasions. Six h after the rats had their final dose they were killed and the digestive systems were examined for signs of ulceration.

In the second experiment segments of the ileum were plunged into acetone-solid CO_2 at −70° and then examined for acid phosphatase (Gomori, 1941).

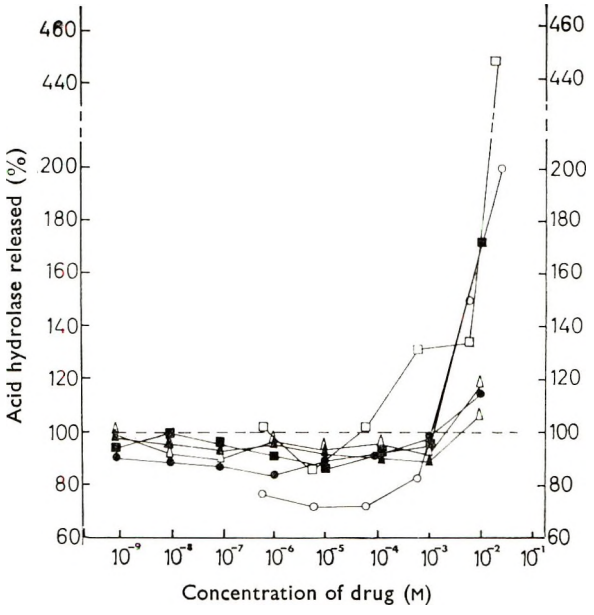

FIG. 1. Action of azapropazone, oxyphenbutazone and phenylbutazone on the release of acid phosphatase from lysosomes. The control valves have been adjusted to 100. Values below 100 represent a stabilizing action and values above 100 represent a lytic action by the drugs. The results represent the mean values of four determinations for the liver lysosomes and two determinations for the ileum lysosomes. Legends. ●—Azapropazone; ▲—oxyphenbutazone; ■—phenylbutazone; △—prednisolone (rabbit liver lysosomes); ○—azapropazone; □—phenylbutazone (rat ileum lysosomes). Although not plotted the standard error of the mean did not exceed ±2·4 for any value. Most values were less than ±0·5.

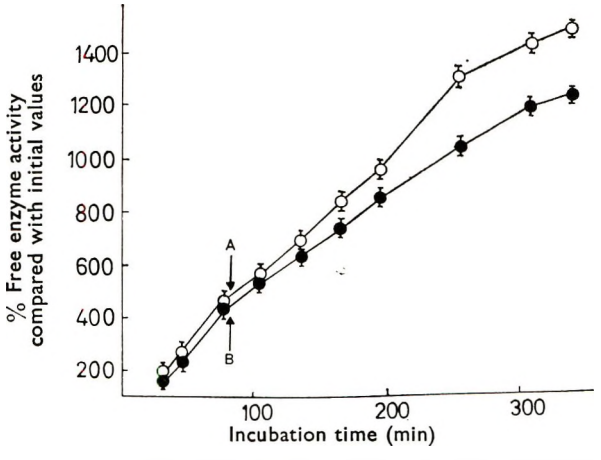

FIG. 2. The effect of phenylbutazone on the release of acid phosphatase from rat stomachs *in vitro*. Each value is the mean ± standard error of the mean of three experiments. ○—experimental; ●—control. Initial values have been adjusted to 100%. A, addition of DMSO to controls. B, addition of phenylbutazone to tests.

RESULTS

Effects of the drugs on lysosomes

The action of the three drugs on rabbit liver lysosomes and rat ileum lysosomes is shown in Fig. 1. Clearly the drugs have stabilized the lysosomes at the lower

concentrations; the % stabilizing action on liver lysosomes is similar to that of prednisolone. At higher concentrations the drugs have a lytic action on lysosomes, phenylbutazone being the most lytic. A similar type of stabilization-lysis curve was obtained when rat liver was used in place of rabbit liver and where β-acetyl glucosaminase activity was plotted in place of acid phosphatase.

The protein content of the ileum and liver lysosome suspensions was 3·5–6 mg/ml.

Effects of the drugs on isolated tissues

The results of the experiments where stomachs were incubated with *p*-nitrophenyl phosphate in the presence of phenylbutazone is shown in Fig. 2. It is clear that phenylbutazone has accelerated the rate of hydrolysis of *p*-nitrophenyl phosphate.

Evidence that the drugs damage lysosomes in tissues was provided by the experiment where gut segments were incubated *in vitro* with phenylbutazone and azapropazone. Segments that had been incubated with phenylbutazone stained heavily and diffusely in patches, for acid phosphatase, whilst the controls and azapropazone-treated segments were only lightly stained in discrete 'point-like' granules.

Whole animal experiments

Rats. Phenylbutazone was found to have a greater ulcerogenic action in rats than azapropazone. Four out of six rats survived the phenylbutazone treatment, and five out of six rats survived the azapropazone treatment. This was at a dose of 500 mg/kg. Numerous ulcers were present in the ileum and caecum of the phenylbutazone-treated rats and stomach ulcers were found in two rats. A few ulcers were found in the caecae of two rats treated with azapropazone but the ilea and stomachs were not ulcerated. At the lower dose the ilea and caecae of the phenylbutazone-treated rats were still ulcerated although the ulcers were much smaller and fewer than those found at the higher dose. The stomachs were free from ulcers. No ulcers were found where the rats were treated with azapropazone at the lower dose. Ilea segments were examined by the Gomori procedure for acid phosphatase. Tissue taken from a non-ulcerated region of a segment of ileum removed from a rat that has been dosed with phenylbutazone shows the staining to be heavy and diffuse. In contrast the ileum from azapropazone-treated rats and the controls were only slightly stained and no diffused staining was observed. When segments were incubated with sodium β-glycerophosphate for 4 h periods some diffuse staining was found in the control as well as the experimental segments. Presumably this was due to the breakdown of the lysosomes over the longer period. Although a large number of controls were incubated for 2 h with sodium β-glycerophosphate no lysosomal damage was observed in these specimens. It is therefore unlikely that the procedure described was responsible for the lysosomal damage observed in the phenylbutazone-treated specimens.

Rabbits. A similar result was found with rabbits. Both drugs induced stomach ulceration in the pyloric region and segments of this area, when examined by the modified Gomori (1941) procedure for acid phosphatase, stained heavily and diffusely compared with a light staining pattern for controls.

The duodenum of phenylbutazone dosed rabbits was also ulcerated but not that of the azapropazone-treated animals or the controls. The ulcerogenic action of phenylbutazone in rabbits was greater than that of azapropazone.

DISCUSSION

Azapropazone, oxyphenbutazone and phenylbutazone clearly stabilize lysosomes at concentrations of physiological interest, and it is possible that this property may be the basis of their anti-inflammatory action. At high concentrations the drugs induced lysis of the lysosomes; phenylbutazone has a much stronger lytic action than the other drugs. Phenylbutazone also appears to have a much stronger ulcerogenic action than azapropazone in rats and rabbits. In addition, phenylbutazone, at high concentrations *in vitro* appears to damage lysosomes in the stomach. Some additional histochemical evidence that phenylbutazone may damage lysosomes was found where the phenylbutazone-treated ileal sections showed evidence of lysosomal damage whereas the azapropazone and control sections did not. Although histochemical evidence is often difficult to evaluate since the staining process probably damages lysosomes it was consistently found that when the incubation time with the substrate was reduced to 2 h the phenylbutazone-treated sections stained heavily but the control and azapropazone sections did not. This suggests that the lysosomes in the phenylbutazone-treated tissues were more fragile than in the other sections. Lysosomal damage was observed in the ileal sections taken from rats dosed with phenylbutazone. No lysosomal damage was observed in the sections taken from the azapropazone and control rats. However, since inflammation is associated with lysosomal damage it may be that the lysosomal damage observed histochemically was part of the inflammatory response associated with ulceration. It is also possible that it was caused directly by the phenylbutazone. The diffuse staining was general and not specifically located in the ulcers and this may have represented a pre-ulcerogenic condition. In a previous paper (Lewis, 1970) it was suggested that lysosomal damage induced by high concentrations of drugs may result in the intracellular release of acid hydrolases such as cathepsins with consequential damage to surrounding tissue. Such local high concentrations may occur after oral administration. Therefore lysosomal damage may be involved, at least in part, with gastrointestinal ulceration.

The additional evidence in this paper is consistent with this suggestion.

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The excretion of hydroxyamylobarbitone in man after oral administration of amylobarbitone and hydroxyamylobarbitone

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The excretion of hydroxyamylobarbitone in man has been measured over six days after an oral dose of 200 mg of sodium amylobarbitone. The biological half-life of hydroxyamylobarbitone determined from "Sigma-minus" plots ranged between 16·8 and 22 h in seven subjects, in another subject the half-life was 34·4 h. The effects of increasing urine flow on the amount of hydroxyamylobarbitone excreted after ingestion of 200 mg of sodium amylobarbitone were assessed. A subject normally excreting 34% of the dose as hydroxyamylobarbitone excreted 45% of the dose as metabolite while taking chlorothiazide as a diuretic. With the same subject taking increased fluids to produce a greater urine flow 41% of the dose was excreted as hydroxyamylobarbitone. Hydroxyamylobarbitone is not bound to plasma proteins and when an aqueous solution of 50 mg of hydroxyamylobarbitone was taken by mouth, 57% of the dose was eliminated in the first 8 h and 91% in the first 24 h. The half-life for ingested hydroxyamylobarbitone was 5·7 h, showing that the rate of elimination of this metabolite is faster than its rate of formation when amylobarbitone is ingested.

Information about the formation and elimination of hydroxyamylobarbitone, derived from amylobarbitone, is incomplete in man because of lack of sensitive analytical techniques. Measurements of hydroxyamylobarbitone in urine have been made using ^{15}N labelled amylobarbitone (Maynert & Van Dyke, 1950), ultraviolet spectrophotometry (Moss, 1965) and gas-liquid chromatography (Kamm & Van Loon, 1966; Balasubramanian, Mawer & Rodgers, 1969). Kamm & Van Loon (1966) after giving their subjects amylobarbitone followed the excretion of the hydroxyamylobarbitone in the urine for five days by chromatographing the chlorotrimethylsilane derivative of the metabolite, but the limit of detection was only 5 μg/ml of sample. A more sensitive gas chromatographic assay, described by Grove & Toseland (1970), employs the new stationary polar phase FFAP and allows unchanged hydroxyamylobarbitone to be chromatographed with a limit of detection of 2 μg (total) from an extract of either plasma or urine.

Mawer & Lee (1968) have suggested that "rapid removal of metabolites might increase the rate of barbiturate hydroxylation" and, if forced diuresis increased the excretion of metabolites, a case be made for this treatment in the management of overdosage. We therefore investigated the effect of increasing the urine flow on the amounts of hydroxyamylobarbitone excreted, using the method of Grove & Toseland (1970).

METHODS

Excretion of hydroxyamylobarbitone after oral administration of sodium amylobarbitone

Each of eight healthy adult volunteers were given a capsule containing 200 mg of

sodium amylobarbitone. Complete urine collections were made for the six ensuing days and 20 ml of blood withdrawn into heparinized tubes at intervals over the first 15 h. The blood was centrifuged and the plasma was separated. The plasma samples and an aliquot from each 24 h urine was then analysed for hydroxyamylobarbitone by the method of Grove & Toseland (1970).

Excretion of hydroxyamylobarbitone after oral administration of sodium amylobarbitone with a diuretic

Sodium amylobarbitone (200 mg) was taken by one volunteer who also was given 500 mg of chlorothiazide to take at 8, 12, 16 and 20 h for the first and second day, the sodium amylobarbitone capsule being taken at 10 h on the first day. This resulted in a urine output for the two days of 6200 ml compared with 2840 ml without a diuretic. Urine was collected for six days and aliquots analysed as before.

Excretion of hydroxyamylobarbitone after oral administration of sodium amylobarbitone with increased fluid intake

The experiment was repeated a month later on the same volunteer, who was given sodium amylobarbitone (200 mg), and who increased his fluid intake over the first two days without taking the diuretic. The urine output for the 2 days was 4490 ml. All the urine was collected for six days and an aliquot taken from each 24-h sample for analysis.

Plasma binding of hydroxyamylobarbitone

Dialysis bags were made by knotting one end of each of a 25 cm length of 26/32 visking tubing (Scientific Supplies Ltd., Vine Hill, London, E.C.4) previously soaked in 1% acetic acid for 5 min. Three ml of fresh, heparinized plasma and 0·1 ml of a 0·4 mg% of hydroxyamylobarbitone in Tyrode solution (equivalent to 40 μg of hydroxyamylobarbitone) were added and the other end of the tube knotted. The bags were equilibrated with 3 ml of Tyrode solution for $\frac{1}{2}$, 1, 2, $2\frac{1}{2}$ and 3 h respectively in a metabolic shaker at 37°. The whole of the plasma and 2 ml of the Tyrode solution were analysed for hydroxyamylobarbitone.

Oral administration of hydroxyamylobarbitone

A healthy adult volunteer ingested a single dose of 50 mg of hydroxyamylobarbitone in water. All the urine was collected over two days and 20 ml of blood withdrawn into heparinized tubes at intervals over the first 5 h. Aliquots of urine were analysed as before. The blood samples were centrifuged, the plasma separated and analysed for hydroxyamylobarbitone.

Calculation of the half-life of hydroxyamylobarbitone

The excretion data obtained from the eight subjects ingesting 200 mg of sodium amylobarbitone and the one subject ingesting 50 mg of hydroxyamylobarbitone were examined using the graphical treatment suggested by Cummings, Martin & Park (1967). This treatment, known as a "Sigma-minus" method, assumes first-order elimination and is based on the following equation:

$$M_{u_\infty} - M_u = \frac{k_f D_0 e^{-Kt}}{K} + M_B$$

Where M_{u_∞} = Amount of the metabolite in the urine when excretion is complete; M_u = Amount of metabolite in the urine at time t; M_B = Amount of metabolite

in the body at time t; k_f = First order rate constant governing the formation of the metabolite; K is the overall elimination rate constant of the drug by all routes, i.e. unchanged drug and metabolites; D_0 is a constant related to the drug at zero time.

A plot of $\ln(M_{u\infty} - M_u)$ against time has a slope of $-K$. The value of the biological half-life may also be obtained from this graph since $t_{0.5} = 0.693/K$.

RESULTS AND DISCUSSION

Plasma concentrations of hydroxyamylobarbitone never exceeded 0·5 μg/ml in hourly samples taken over the 15 h after amylobarbitone had been taken. Attempts to measure the plasma half-life of the metabolite from these data were, therefore, unsuccessful.

The excretion pattern of hydroxyamylobarbitone in the urine of eight volunteers is shown in Table 1. These results are in good agreement with those findings of Kamm & Van Loon (1966). The $t_{0.5}$ for seven of our volunteers ranged from 16·8–22 h, an eighth volunteer with a $t_{0.5}$ of 34·4 h (subject B) had had her gall bladder removed some years previously but whether this might have interfered with biliary excretion is uncertain. There were no other clinical abnormalities in this subject.

Although our results showed that to obtain a more accurate slope ideally the urine collections should have been taken for smaller periods in the initial stages, the difference between the derived $t_{0.5}$ and that for hydroxyamylobarbitone (see later) was felt sufficient not to warrant repetition of the early analyses.

Effect of increased urine flow on the excretion of hydroxyamylobarbitone

Table 2 shows the excretion pattern of volunteer A with a normal urine flow, a diuretically augmented urine output and a urine output increased by extra fluid intake. A greater urine output is seen to be accompanied by a greater excretion of hydroxyamylobarbitone, lending some support to the proposition of Mawer & Lee (1968) that forced diuresis may be of value in cases of barbiturate overdose.

Protein binding

Since hydroxyamylobarbitone was excreted over a period of six days, we considered the possibility that the metabolite was bound to the plasma proteins. The distribution of hydroxyamylobarbitone between fresh plasma and Tyrode solution after equilibration for intervals up to 3 h revealed that complete equilibrium occurred after 2 h, indicating that protein binding of the hydroxyamylobarbitone had not taken place.

Excretion of hydroxyamylobarbitone after oral administration of the metabolite

Table 3 gives the hydroxyamylobarbitone concentration of the urine and plasma samples, collected after one volunteer had taken 50 mg of hydroxyamylobarbitone in a solution of 50 ml water. Clearances for hydroxyamylobarbitone and creatinine are also shown.

As expected, hydroxyamylobarbitone is rapidly excreted with 57% of the dose being eliminated in the first 8 h and 91% in the first 24 h.

Since the measurement of clearances depends on a constant plasma concentration, comparison between that of hydroxyamylobarbitone and creatinine is not strictly correct. Nevertheless, it would appear from our figures that, in the initial stages,

when a large amount of hydroxyamylobarbitone is still present in the body, it is excreted at the glomerular filtration rate. It was not possible to do this experiment on more subjects owing to the lack of pure hydroxyamylobarbitone.

Table 1. *% dose of sodium amylobarbitone (200 mg) excreted as hydroxyamylobarbitone (HO-A) by eight subjects.*

Subject	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Total % dose as HO-A	$t_{0.5}$ (h)
A	12·32	11·87	5·73	2·66	1·40	0	33·98	19·4
B	9·68	9·26	6·63	3·97	3·17	2·46	35·17	34·4
C	18·36	19·21	5·88	1·51	2·59	0·95	48·50	22·0
D	13·06	13·69	9·41	4·15	2·42	2·33	45·06	18·4
E	10·26	13·43	10·01	4·67	1·14	0·67	40·18	20·0
F	17·21	16·01	5·15	1·46	1·30	—	41·13	16·8
G	9·93	10·11	8·60	2·70	1·90	0·29	33·56	22·0
H	18·42	13·07	4·19	6·70	2·02	0·81	45·21	21·6
Mean	13·66	13·33	6·95	3·48	1·99	1·07	40·35	21·8
± Standard deviation	±3·81	±2·98	±1·99	±1·65	±0·22	±0·26	±5·32	±4·99

Table 2. *The concentration of hydroxyamylobarbitone (HO-A) in the urine of subject A after ingesting 200 mg of sodium amylobarbitone with no diuretic, chlorothiazide and extra fluid.*

	No diuretic			500 mg chlorothiazide qds for first two days			Fluid intake increased		
HO-A on day:	Urine volume (ml)	μg/ml	% Dose	Urine volume (ml)	μg/ml	% Dose	Urine volume (ml)	μg/ml	% Dose
1	1000	22·46	12·32	4000	9·12	20·01	2150	14·0	16·51
2	1840	11·76	11·87	2200	6·62	8·0	2340	10·6	13·61
3	1630	8·7	5·73	1250	16·4	11·25	1750	5·12	4·91
4	1200	4·15	2·66	1110	7·2	4·38	2200	2·98	3·60
5	1170	2·0	1·40	1400	0·8	0·61	2550	1·02	1·43
6	1260	0	0	1580	0·64	0·55	1900	0·72	0·75
Total % dose as HO-A			33·98			44·8			40·81

Table 3. *Excretion of hydroxyamylobarbitone (HO-A) after ingestion of a 50 mg dose.*

	Urine			Blood		Clearance (ml/min)	
Time (h)	Volume (ml)	μg/ml	% Dose	Time (h)	μg/ml plasma	HO-A	Creatinine
0–2	157	47·3	14·9	¾	0·55	112	107
				1¼	0·81	76	73
2–4	91	61·0	11·1	2¼	0·91	51	84
				2¾	1·25	37	61
4–6	236	40·0	18·9	4¼	1·0	79	50
6–8	288	20·4	11·7				
8–10	271	16·3	9·0				
10–12	432	10·6	9·1				
12–14	220	14·2	6·2				
14–24	475	10·4	9·9				
24–48	1180	3·2	9·5				
			100·3				

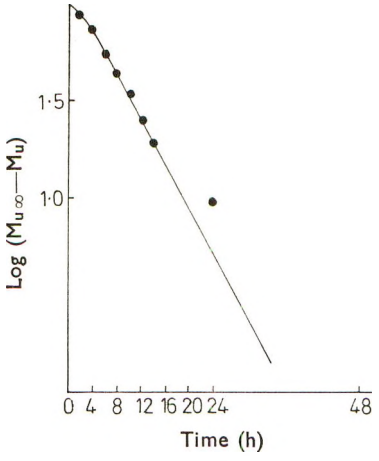

FIG. 1. The "Sigma-minus" plot of the excretion of hydroxyamylobarbitone after ingestion of 50 mg of hydroxyamylobarbitone. $t_{0\cdot5}$ = 5·7 h.

When this data was plotted as a Sigma-minus function (Fig. 1) the rapid elimination is emphasized. The $t_{0\cdot5}$ for hydroxyamylobarbitone is seen to be 5·7 h.

We can infer from the difference in the $t_{0\cdot5}$ for ingested hydroxyamylobarbitone and the $t_{0\cdot5}$ for hydroxyamylobarbitone derived from sodium amylobarbitone that the rate of elimination of this metabolite is much faster than its rate of formation. This explains the negligible accrual of the metabolite in the blood of patients ingesting amylobarbitone, indeed hydroxyamylobarbitone concentrations of any significance may only be found in the blood of patients in coma or renal failure. For these reasons we would not expect to find evidence of the conjugation of hydroxyamylobarbitone, although Balasubramanian, Lucas & others (1970) have reported three subjects excreting hydroxyamylobarbitone partly as a conjugate readily hydrolysed in acid. We have not been able to substantiate their findings. Analysis of twenty different urines before and after acid hydrolysis with 4N HCl failed to produce any increase in the hydroxyamylobarbitone concentration.

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The influence of methyl substitution on the *N*-demethylation and *N*-oxidation of normethadone in animal species

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N-Monodemethylation and *N*-oxidation were shown to be the major routes of metabolism of normethadone, (—)-methadone and (—)-isomethadone *in vitro* by hepatic microsomal preparations from rat, rabbit, guinea-pig, mouse and hamster. The rate of *N*-oxidation was decreased and the rate of *N*-demethylation was increased by the introduction of the methyl substituent into normethadone; the configuration of the methyl substituent influenced these processes. K_m and V_{max} values were determined for liver microsomal *N*-demethylation of normethadone, (—)-methadone and (—)-isomethadone by rat and guinea-pig and for the *N*-oxidation of normethadone by guinea-pig. The use of selective inhibitors showed that *N*-demethylation was not preceeded by *N*-oxidation in these compounds.

Rat, rabbit and guinea-pig liver microsomal preparations demethylate methadone (Axelrod, 1956), and (Beckett, Taylor & others, 1968) established that methadone was *N*-demethylated in man to a secondary amine which spontaneously rearranges to a pyrroline derivative. Subsequently, methadone, isomethadone and normethadone were shown to be converted to *N*-oxides and to be demethylated to their corresponding pyrroline derivative by guinea-pig microsomal preparations (Beckett, Mitchard & Shihab, 1971).

It has been suggested that *N*-dealkylation proceeds via an *N*-oxide intermediate which rearranges to form a biochemically unstable *N*-alkylcarbinol (Fish, Johnson & others, 1955; Fish, Sweeley & others, 1956; Petitt & Ziegler, 1963). Stabilized *N*-hydroxymethyl intermediates have since been isolated (McMahon, 1966; Gorrod, Temple & Beckett, 1970), but the formation of the precursor *N*-oxide is disputed by McMahon & Sullivan (1965).

To provide more definitive information, the influence of α- or β-methyl substitution on the rates of *N*-oxidation and demethylation of normethadone by liver microsomes of different species and the effect of selective inhibitors on those processes have been investigated.

METHODS

Young adult male animals (Wistar rats, 250–300 g; Duncan Hartley guinea-pigs, 350–400 g; New Zealand white rabbits, 1·5–2·0 kg; Swiss albino mice, 25 g; golden hamsters, 90–100 g) were killed, the livers rapidly removed and homogenized with an Ultra-turrax (Janke & Kunkel K.G., Stanfeni. Br., Germany) and fortified incubation mixtures prepared as described previously (Beckett & others, 1971).

Substrate studies. (—)-Methadone (National Institute of Health, Bethesda); (—)-isomethadone (Sterling Winthrop Research Institute) and normethadone

(Janssen Pharmaceutica) (5 μmol) were each incubated for 80 min at 37° in a mixture containing, (i) whole liver homogenate, (ii) 10 000 *g* supernatant, (iii) washed and resuspended microsomes and (iv) 140 000 *g* supernatant. In each case the liver preparations contained the equivalent of 0·5 g liver preparation in isotonic KCl (2ml), and the respective preparations were added to separate incubation mixtures to give a final volume of 6 ml. (+)-Methadone and (+)-isomethadone (National Institute of Health, Bethesda) (5 μmol) were incubated with 10 000 *g* supernatant. Methadone *N*-oxide, isomethadone *N*-oxide and normethadone *N*-oxide (prepared by Beckett & others, 1971) (5 μmol) were separately incubated with (i) whole liver homogenate, (ii) 10 000 *g* supernatant or (iii) heat inactivated 10 000 *g* supernatant respectively.

Kinetic studies. The K_m and the maximum rates of metabolism (V_{max}) for microsomal *N*-demethylation of normethadone, (−)-isomethadone and (−)-methadone by rat and guinea-pig and for the *N*-oxidation of normethadone by guinea-pig were determined over the concentration range 5×10^{-5} to 2×10^{-3}M using the washed microsome preparation. The K_m values were calculated from Lineweaver and Burke double reciprocal plots.

Inhibitors studies. The metabolism of normethadone (5 μmol) in incubates containing 10 000 *g* supernatant preparations from guinea-pig liver was investigated in the presence of the following inhibitors: 2-mercaptoethylammonium chloride (cysteamine HCl), *p*-chloromercuribenzoic acid, *N*-ethylmaleimide, 1,10-phenanthroline hydrate (all obtained from BDH), dithiothreitol (P.L. Biochemicals Inc.), SKF 525A (Smith Kline and French) and sodium cyanide (in all cases [I] = 10^{-3}M). Normethadone and the inhibitors were incubated together for 60 min at 37°.

Assay procedure. Normethadone, isomethadone and methadone, their cyclic metabolites and *N*-oxides were extracted and assayed as before (Beckett & others, 1971).

RESULTS AND DISCUSSION

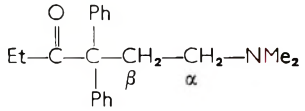
Preliminary studies with fractions from guinea-pig liver indicated that maximal *N*-demethylation and *N*-oxidation of normethadone and its methyl analogues occurred in the 10 000 *g* supernatant (Table 1). Whole liver homogenate and washed microsomes, both fortified with the same co-factors, were less active, whilst no *N*-oxidation and very little *N*-demethylation occurred with the 140 000 *g* supernatant (soluble fraction). The lower activity of the whole homogenate was probably due to the presence of inhibitors since Mitchard (1970) has shown that *N*-demethylation of different substrates by liver microsomal fractions increased as the fraction was purified. The apparent lower activity of the washed microsomes compared with the 10 000 *g* supernatant is due to the recording of results as total activity; purification procedures only increase specific activity.

The influence of α- and β-methyl substitution on the metabolism of normethadone. The 10 000 *g* supernatants of guinea-pig, hamster, rat, mouse and rabbit livers were used to study the effect of species variation on the rate of *N*-dealkylation and *N*-

Table 1. *The percentage recovery of normethadone, (—)-isomethadone and (—)-methadone* (U) *and their respective cyclic metabolites* (C) *and* N-*oxides* (O), *after incubation* (80 *min*) *with different fractions of guinea-pig liver microsomal preparations.* Determination using different livers gave comparable order of results.

	Normethadone			(—)-Isomethadone			(—)-Methadone		
Liver fraction	U	C	O	U	C	O	U	C	O
Whole liver homogenate	51·0	28·5	8·0	48·0	36·0	4·0	41·0	40·0	1·5
Soluble fraction	94·0	3·0	1·0	87·0	2·0	0·0	86·5	5·5	0·0
10 000 *g* supernatant	10·5	39·5	27·0	8·0	63·0	8·5	13·0	65·0	2·5
Washed microsomes and resuspended in isotonic KCl	47·5	20·0	15·0	50·0	27·0	5·0	46·0	34·5	2·0

oxidation of methadones. Methyl substitution reduced the rate of *N*-oxidation in all species; a β-methyl substituent (isomethadone) significantly reduced the rate whilst



Normethadone

an α-methyl substituent (methadone) almost abolished activity (Fig. 1). Conversely, with the exception of the rat, methyl substitution slightly increased the rate of *N*-demethylation, an α-methyl being more effective than a β-methyl substituent. The total metabolism of these compounds by other routes in different species cannot be correlated with the effect of methyl substitution (Fig. 1).

The effect of configuration of the methyl substituents. *S*-(+)-Methadone and *R*-(+)-isomethadone were *N*-monodemethylated (Fig. 2) by the 10 000 *g* rat liver supernatant slightly more rapidly than their enantiomorphs. However, the 10 000 *g*

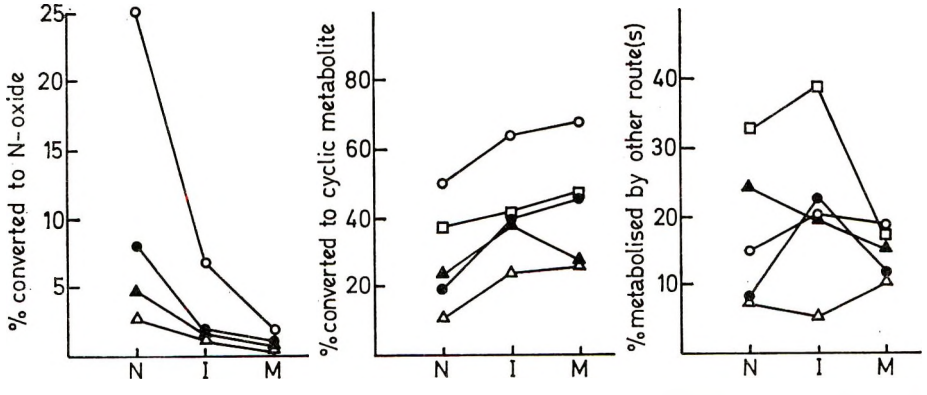


FIG. 1. The effect of methyl substitution in normethadone on *N*-oxidation, *N*-monodemethylation and metabolism by other route(s), using liver microsomal preparations (10 000 *g*) from guinea-pig —○—, hamster —●—, rat —▲—, mouse —△— and rabbit —□—. The linking lines have no significance and serve only to emphasize the trends seen in each species. *N*-oxidation as measured by *N*-oxide formation, *N*-monodemethylation as measured by cyclic metabolite formation. Incubation time 80 min. N = normethadone, I = (—)-isomethadone, M = (—)-methadone.

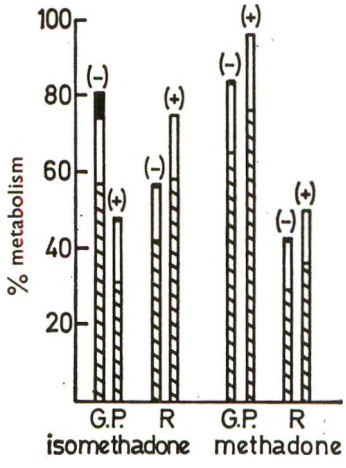


FIG. 2. The effect of stereochemistry of the methyl group in methadone and isomethadone on *N*-oxidation (solid columns), *N*-monodemethylation (hatched columns) and metabolism by other routes (open columns) using liver microsomal preparations (10 000 *g* supernatant) of rat and guinea-pig. Incubation time 80 min. GP = guinea-pig, R = rat.

guinea-pig liver supernatant, although *N*-dealkylating *S*-(+)- more than *R*-(−)-methadone, demethylated *S*-(−)-isomethadone more rapidly than its isomer.

The differences between the rates of metabolism of the isomers were greater with a β- than with an α-methyl substituent. *N*-Oxidation of methadone was only a minor route of metabolism. More *N*-oxide was formed with *S*-(−)- than with *R*-(+)-isomethadone using guinea-pig and to a lesser extent rat liver preparations.

Enzyme substrate characteristics. The guinea-pig and rat enzyme-substrate characteristics (V_{max} and K_m) are presented in Table 2. It was possible only to determine the K_m value ($5{\cdot}4 \times 10^{-4}$M) for the *N*-oxidation of normethadone with the guinea-pig liver preparation because, in all other cases, rates of *N*-oxidation were too slow to permit accurate measurement; this value is very similar to the corresponding K_m value ($5{\cdot}0 \times 10^{-4}$M) for the *N*-demethylation of normethadone.

Metabolism of the N-*oxides.* There was negligible metabolism of the *N*-oxides by the 10 000 *g* supernatants from rat, guinea-pig, mouse and hamster, incubated at 37° for 80 min. The heat inactivated preparations gave similar results. Although

Table 2. *Enzyme-substrate characteristics* (K_m *and* V_{max}) *for* N-*monodemethylation and* N-*oxidation of normethadone,* (−)-*isomethadone and* (−)-*methadone by guinea-pig and rat liver microsomes* (140 000 *g*). The rates were linear up to 15 min; V_{max} represent nmol g^{-1} liver min^{-1}.

	N-Monodemethylation of						*N*-Oxidation of	
	Normethadone		(—)-Isomethadone		(—)-Methadone		Normethadone	
Species	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
Guinea-pig ..	$5{\cdot}0 \times 10^{-4}$M	38	$2{\cdot}5 \times 10^{-4}$M	43	$3{\cdot}5 \times 10^{-4}$M	56	$5{\cdot}4 \times 10^{-4}$M	27
Rat	$2{\cdot}5 \times 10^{-4}$M	33	$2{\cdot}2 \times 10^{-4}$M	45	$2{\cdot}1 \times 10^{-4}$M	38	—	—

Table 3. *The effect of various inhibitors on* N-*monodemethylation and* N-*oxidation of normethadone by guinea-pig* (10 000 *g*) *liver microsomes.* Incubation time was 60 min at 37°C. Metabolism in absence of inhibitors considered as 100%.

1×10^{-3}M inhibitors	Exp.	Percentage metabolism by different routes *N*-Monodemethylation	*N*-Oxidation	Other route(s)
SKF 525A	1	15	105	43
	2	13	105	40
2-Mercaptoethyl ammonium chloride	1	60	10	90
	2	65	12	87
Sodium cyanide	1	76	105	125
	2	80	100	125
-Chloromercuribenzoic acid	1	82	76	150
	2	75	68	140
N-Ethylmaleimide	1	77	97	100
	2	83	92	100
1,10-Phenanthroline hydrate	1	39	63	100
	2	41	62	100
Dithiothreitol	1	88	17	120
	2	90	16	125

some *N*-oxide (12%) was metabolized by the whole homogenate, this observation alone is insufficient to implicate *N*-oxide formation in the process of *N*-dealkylation particularly as neither cyclic metabolites nor tertiary amines were detected when the *N*-oxides were incubated with the 10 000 *g* supernatant or heat inactivated 10 000 *g* supernatant.

Inhibitors studies. Enzyme inhibitors studies provided further evidence that *N*-oxidation and *N*-dealkylation are independent processes. The percent *N*-oxidation and *N*-demethylation of normethadone after incubation with a number of inhibitors is shown in Table 3. The enzymic reactions were inhibited to different extents by all the inhibitors studied, but in particular SKF 525A specifically inhibited *N*-dealkylation and was without effect on *N*-oxidation whereas dithiothreitol was a specific inhibitor of *N*-oxidation and only slightly (10%) inhibited *N*-dealkylation. Selective inhibition of *N*-oxide and cotinine formation from nicotine has also been observed (Gorrod & Keysell, unpublished observation). Although the data obtained from inhibitor studies with SKF 525A do not preclude the possibility of the *N*-oxide forming as an intermediate in the process of *N*-dealkylation, data obtained from studies with dithiothreitol can only be explained if the two processes of *N*-oxidation and *N*-dealkylation occur independently.

N-*Oxidation and* N-*dealkylation as separate metabolic pathways*

The above isomeric substrate studies showed that the steric features which promoted *N*-oxidation reduced the rate of *N*-dealkylation. Methyl substitution in the vicinity of the nitrogen atom of normethadone significantly inhibited *N*-oxidation but the same substituent slightly increased *N*-dealkylation. *N*-Dealkylation but not *N*-oxidation reaction showed stereo-selectivity. The kinetic data therefore indicate that the two reactions proceed independently.

The metabolic studies with *N*-oxides as substrates failed to demonstrate demethylation and the inhibitor studies established different inhibitor profiles for *N*-oxidation and *N*-demethylation.

The results support therefore the conclusion that *N*-dealkylation of normethadone and its analogues is not preceded by *N*-oxidation in accord with similar conclusions of McMahon & Sullivan using propoxyphene (1965).

Acknowledgements

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The effect of ethanol on phenobarbitone and pentobarbitone absorption into rat blood and brain

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Male rats administered [^{14}C]phenobarbitone (50 mg/kg) or [^{14}C]-pentobarbitone (30 mg/kg) simultaneously with either 15% ethanol (3 g/kg) or saline intraperitoneally were killed 5, 10 or 20 min after injection. The radioactivity in the blood, whole brain and different brain areas was measured. Phenobarbitone was absorbed more slowly into the blood and brain than pentobarbitone. Ethanol-treated rats had significantly higher phenobarbitone concentrations than the saline-treated controls in the blood, whole brain, cerebrum and cerebellum up to 10 min after injection. Pentobarbitone concentrations were not significantly altered by ethanol. Barbiturate concentrations in the cerebral cortex were lower than in other regions of the brain. The brain:blood barbiturate ratios were not appreciably changed by ethanol. It is concluded that ethanol (15%) given intraperitoneally aided the transport of phenobarbitone across the peritoneum and hence increased the rate of its absorption into the blood and brain.

The reasons for the severe toxicity resulting from the simultaneous ingestion of ethanol and barbiturates remain obscure. Recently, ethanol has been shown to inhibit the degradation of several drugs, including pentobarbitone, in microsomal preparations and in liver slices and *in vivo* slows the rate of disappearance of drugs from the blood (Rubin & Lieber, 1968; Rubin, Bacchin & others, 1970; Rubin, Gang & others, 1970). It appears unlikely that the immediate pharmacological effects of ethanol–barbiturate interaction are due to changes in metabolism since they are most pronounced with the least metabolized barbiturates (Wiberg, Coldwell & Trenholm, 1969). Seidel (1967) observed with mice that pentobarbitone concentrations in several tissues including blood and brain, were enhanced by ethanol but phenobarbitone concentrations remained unaffected. In rats, ethanol increased the brain concentrations of phenobarbitone and barbitone, whilst those of pentobarbitone appeared unaffected (Coldwell, Wiberg & Trenholm, 1970). Subsequently, using [^{14}C] compounds, it was found that the concentrations of phenobarbitone and pentobarbitone in several tissues including the brain, were elevated in the presence of ethanol (Coldwell, Trenholm & others, 1971). This paper describes the results of a detailed investigation of the effect of ethanol on the distribution of [^{14}C]phenobarbitone and [^{14}C]pentobarbitone in the rat brain during the immediate post-injection period.

MATERIALS AND METHODS

Male rats of the Wistar strain, 175 to 225 g, were housed 8–10 per cage and acclimatized one week to the environment. After overnight starvation the animals were randomly divided into 4 groups of 36 each and treated intraperitoneally (20 ml/kg)

with either pentobarbitone, (30 mg/kg) or phenobarbitone, (50 mg/kg) in saline or 15% ethanol (3 g/kg). The barbiturates were ring-labelled with ^{14}C in the two position; the specific activity of each dose of pentobarbitone was 500 μCi/g and of phenobarbitone was 175 μCi/g.

Twelve animals of each group were decapitated 5, 10 and 20 min after drug injection. The brains were removed and sectioned into the half-brain, cerebellum, pons plus medulla, thalamus and cerebral cortex. Triplicate specimens of the half-brain and all of the other sections were weighed immediately and added to Soluene (1 ml/100 mg, Packard Instrument Co.) in scintillation counter vials. The blood was collected and triplicate 10 μl aliquots were treated similarly. After incubation at 37° for 18 h, a toluene-based scintillation fluid containing 0·6% 2,5-diphenyloxazole (PPO) and 0·02% *p*-bis-(2-4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP) was added to each vial, in the proportion of 10 ml/ml of Soluene. The radioactivity was measured with a liquid scintillation counter. All vials were counted three times. Standards were similarly processed to obtain measurements of machine efficiency, background count, and specific activity of the injected drug. Quenching was corrected by the external standard method. All calculations were made on an IBM-360 computer. The radioactivity was expressed as unchanged barbiturate.

RESULTS

The concentrations of [^{14}C]pentobarbitone in the blood, brain, and the various component areas of the latter, in the presence and absence of ethanol, are shown in Fig. 1A. Peak concentrations were reached in all tissues, except the half-brain, within 10 min of the drug being given. Ethanol had no significant effect on pentobarbitone uptake. Brain concentration of pentobarbitone reflected those in blood

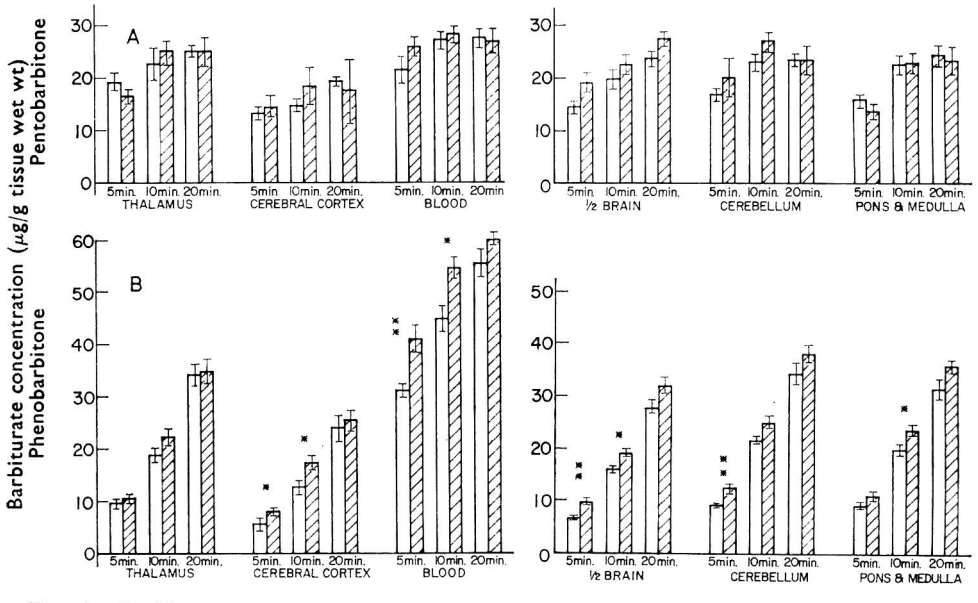

FIG. 1. Barbiturate concentrations in blood and brain tissue at 5, 10 and 20 min intervals after administrtion (i.p.) of (A) pentobarbitone (30 mg/kg), (B) phenobarbitone (50 mg/kg) (open columns) in saline and in ethanol (3 g/kg, 15% w/v soln) (hatched columns). * $P<0.05$; ** $P<0.01$.

and the brain: blood pentobarbitone ratios were not appreciably altered by ethanol. Concentrations in the cerebral cortex were significantly lower than in other areas of the brain.

Phenobarbitone concentrations in the tissues increased continuously during the 20 min after injection (Fig. 1B), however, that in the brain ($27{\cdot}7 \pm 1{\cdot}6$ μg/g) lagged behind that in the blood ($55{\cdot}7 \pm 2{\cdot}8$ μg/g) at 20 min. The uptake of the barbiturate in blood and brain was more rapid in the presence of ethanol, especially during the first 10 min after injection. This was reflected in all areas of the brain except the thalamus. Concentrations in the cerebral cortex were significantly lower than in the thalamus and cerebellum 20 min after drug administration. The brain:blood phenobarbitone ratios were unaffected by ethanol.

DISCUSSION

Since pentobarbitone is more lipid soluble than phenobarbitone, under physiological conditions it is more rapidly absorbed by tissues (Goodman & Gilman, 1965). At physiological concentrations ethanol slightly increased the lipid solubility of both barbiturates (Thomas, Coldwell, Trenholm & Wiberg, unpublished); in 15% w/w ethanol their solubilities are several times greater than in water (Breon & Paruta, 1970). Our findings suggest that ethanol increased the *in vivo* lipid solubility of phenobarbitone and its rate of transport across the peritoneum and uptake into the blood. This effect would be less pronounced on pentobarbitone uptake because of its inherent high lipid solubility and rapid absorption.

There was no indication that ethanol (3 g/kg) caused a perturbation of the blood-brain barrier. Unpublished data from our laboratory has established that pentobarbitone metabolites are absent from rat brain 3 h after dosing the animals with the barbiturate in saline or 15% ethanol. The similarity in the brain:blood barbiturate ratios in the presence and absence of ethanol and the non-specific nature of the higher tissue concentration of barbiturate when ethanol is administered simultaneously (Coldwell & others, 1971) indicate that in the rat the blood-brain barrier is relatively unaffected by intoxicating amounts of ethanol.

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Determination of *N*-(2-benzoyloxyethyl)-norfenfluramine (JP 992) and its metabolites in urine

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A gas-liquid chromatographic method of determination of norfenfluramine and *N*-2-hydroxyethylnorfenfluramine is described. Also a method is reported to determine *N*-(2-benzoyloxyethyl)norfenfluramine and its metabolites containing the *m*-trifluoromethylbenzyl and -benzoyl moieties in urine, by oxidation of these to m-trifluoromethyl benzoic acid followed by methylation and gas-liquid chromatography.

Preliminary investigations indicated that *N*-(2-benzoyloxyethyl)norfenfluramine (JP 992; compound 1d) is metabolized in man to *N*-2-hydroxyethylnorfenfluramine (compound 1c), norfenfluramine (compound 1a) and other metabolites, while no unchanged drug was detected in urine.

A method was therefore sought to determine quantitatively in urine, the metabolites of compound 1d containing the *m*-trifluoromethyl-benzyl and -benzoyl moieties, as well as the above bases (compound 1c and a).

Bruce & Maynard Jr. (1968) described a method of oxidizing these compounds to *m*-trifluoromethyl benzoic acid, but in our hands the method only gave a 55% conversion of compound 1d to the acid in solution in urine.

The possibility of rearrangement of compound 1d upon changing the pH of an aqueous solution of the compound was also investigated, since it is known that esters of β-aminoalcohols in acid solution rearrange to the amides upon making the solution alkaline (Immediata & Day, 1940; Kanao, 1928; Phillips & Baltzly, 1947; Fodor, Bruckner & others, 1949) as under conditions of extraction of the above bases into organic solvents.

MATERIALS AND METHODS

Apparatus

Perkin-Elmer Model F.11 Gas Chromatograph (F.I.D.), Hitachi Perkin-Elmer Model 159 recorder.

Materials and reagents

Compounds Ia, b, c, d, e hydrochlorides and *m*-trifluoromethyl benzoic acid were supplied by Selpharm Laboratories Ltd., England; Aletamine HCl by National Drug Co., Philadelphia; m-toluic acid by Hopkin and Williams Ltd., England. Freshly distilled Analar diethylether. 5N HCl, 20% NaOH, 10% $NaHCO_3$. Freshly prepared diazomethane in ether (Vogel, 1957) saturated $KMnO_4$ solution ($\simeq$0·4M)

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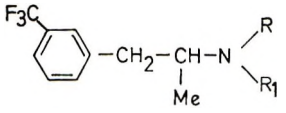

I

Ia	R=H; R_1=H	Norfenfluramine
Ib	R=H; R_1=Et	Fenfluramine
Ic	R=H; R_1=C_2H_4OH	*N*-2-Hydroxyethyl norfenfluramine
Id	R=H; R_1=C_2H_4OCO 	*N*-(2-Benzoyloxyethyl)-norfenfluramine (JP 992)
Ie	R=CO  R_1=C_2H_4OH	*N*-(2-Hydroxyethyl)-*N*-benzoyl norfenfluramine (Rearranged from of JP 992).

Gas-liquid chromatography. The chromatographic columns and conditions of chromatography are described in Table 1.

Recovery of m-*trifluoromethyl benzoic acid by oxidation of compound 1d*

(a) Urine or water (5 ml) containing known quantities (4–50 μg/ml) of *m*-trifluoromethyl benzoic acid was placed in Quickfit tubes, the solution acidified, internal standard added and the organic acid analysed as in the general procedure below.

(b) Urine (5 ml) containing known quantities (10 μg/ml and 50 μg/ml) of compound 1d was placed in Quickfit tubes, NaOH (0·5 ml), and saturated $KMnO_4$ solution (2·5 ml to 12 ml) were added, the solution oxidized, and the organic acid analysed as below.

(c) Urine (5 ml) containing known quantities (4–50 μg/ml) of *m*-trifluoromethyl benzoic acid was oxidized with $KMnO_4$ (2·5 ml) and the solution analysed as below.

Reserve capacity of $KMnO_4$ *solution for oxidation in the general procedure*

To urine samples (5 ml) collected from five subjects at different times of the day was added 100 μg/ml of compound 1d and the solution then oxidized with $KMnO_4$

Table 1. *Gas-liquid chromatographic conditions and retention times of* N-(2-*benzoyloxyethyl*)*norfenfluramine, metabolites and related oxidation products.*

Column	Support material	Stationary phase	Operating temp. °C	Column length	Hydrogen pressure lb/in²	Air pressure lb/in²	Nitrogen flow rate at room temp.	Retention times (min) of
A	Chromosorb G A/W, DMCS treated 80–100 mesh	10% Apiezon L 10% KOH	140	1 metre S.S. ⅛″ o.d.	15	26	27 ml/min	NF = 2·3 JPA = 15·2 ALT* = 8·4
B	Chromosorb G A/W, DMCS treated 80–100 mesh	2% SE 30	195	2 metre glass ¼″ o.d.	20	30	60 ml/min	JP992 = 6·3 JPR = 9·2
C	Gas-chrom Q A/W, DMCS treated 100–120 mesh	10% Carbowax 20M	110	2 metre glass ¼″ o.d.	15	25	80 ml/min	TMB = 4·0 MB′ = 7·0 MMT* = 11·9

NF = norfenfluramine, JPA = *N*-2-hydroxyethylnorfenfluramine, JP922 = N-(2-benzoyloxethyl)norfenfluramine, JPR = JP992 rearranged form, TMB = methyl *m*-trifluoromethyl benzoate, MB′ = methyl benzoate, MMT = methyl *m*-toluate, ALT = Aletamine, A/W = acid washed, DMCS = dimethylchlorosilane. S.S. = stainless steel. Injection block temperature *ca* 250°C. * Used as markers.

as in the general procedure. After cooling, dilute H_2SO_4 (10 ml) and potassium iodide (3 g) were added to the solution and the liberated iodine titrated with 0·1N sodium thiosulphate.

General procedure

Analysis of compound 1*d in urine by oxidation to* m-*trifluoromethyl benzoic acid.* Urine (5 ml) to which was added compound 1d was placed in a Quickfit tube, NaOH solution (0·5 ml) and saturated $KMnO_4$ solution (2·5 ml) were added and the solution refluxed in a water bath (90°) for 2 h. The solution was cooled, HCl solution (1·5 ml) added and the solution then refluxed for a further half hour.

The above solution was allowed to cool and the internal standard (1 ml, 20 μg/ml *m*-toluic acid in water) was added. The organic acids were extracted with ether (3 × 4 ml) and then repartitioned into $NaHCO_3$ solution (5 ml). The solution was made acidic with HCl and re-extracted with ether (3 × 2·5 ml). The bulked ether extracts were concentrated (*ca* 5 ml) on a water bath (42°), cooled and excess of ice-cold ethereal solution of diazomethane was added. After mixing for 2 min, the solution was washed with distilled water (3 × 5 ml). The ethereal layer was separated and concentrated in an evaporating tube (*ca* 50 μl) and 2 μl was injected on column C.

Calibration factor. Urine (5 ml) containing known quantities (4–50 μg/ml) of compound 1d was placed in Quickfit tubes, oxidized and assayed as above. The calibration factor (μg base/ml urine ÷ the drug to marker ratio) was calculated.

Reproducibility of general procedure. Urine (5 ml; 10 samples) from a bulked 24 h collection from a subject, who was given a dose of compound 1d, was oxidized and assayed as above.

Specificity of general procedure. "Blank" urine (5 ml; 10 subjects) was oxidized and assayed as above without incorporating the internal standard, to check for the absence of peaks chromatographing in the vicinity of the *m*-trifluoromethyl benzoic and *m*-toluic acid peaks.

Analysis in urine of compound 1*d*

Urine or water (5 ml) containing known quantities (0·2–10 μg/ml) of compound 1d was placed in centrifuge tube, NaOH (0·5 ml) or dilute ammonia (0·5 ml) internal standard (1 ml; 2 μg base/ml tripelennamine hydrochloride in water) added and extracted with ether (3 × 2·5 ml). The ether extracts were concentrated (*ca* 50 μl) on a water bath (42°) and 2 μl injected on column B.

Analysis in urine of compounds 1*a and c*

Urine or water (5 ml) containing known quantities (0·2–10 μg/ml) of compounds 1a and c was placed in a centrifuge tube, NaOH (0·5 ml) and internal standard (1 ml, 10 μg/ml aletamine hydrochloride in water) added and extracted with ether (8 × 2·5 ml). The ether extracts were concentrated (*ca* 50 μl) on a water bath (42°) and 5 μl was injected on column A.

Stability of compound 1*a and c in urine*

Compound 1a and c, 1 μg/ml, in acidic (pH 0·5), alkaline (pH 8·1) and neutral

urine (pH 7·2) were stored at 4° and the drug content determined every third day for two weeks.

Analysis of compounds 1a *and* 1c *in the presence of* 1d *and the total measured as* m-*trifluoromethyl benzoic acid by the general procedure*

Urine (5 ml) containing known quantities (2–12 μg/ml) of compounds 1a, 1c and 1d was placed in centrifuge tube, NaOH (0·5 ml) and the internal standard added and analysed for 1a and 1c as above.

A second sample (5 ml) was oxidized and analysed by the general procedure to determine the total as *m*-trifluoromethyl benzoic acid.

RESULTS AND DISCUSSION

Oxidation of compound 1d

The analysis by gas-liquid chromatography (g.l.c.) of the ethereal extracts of blank urine samples oxidized by the general procedure, showed no major interfering peaks in the vicinity of *m*-trifluoromethyl benzoic and *m*-toluic acids when chromatographed as their methyl esters, the retention times are recorded in Table 1. Benzoic acid formed in relatively large quantities on oxidizing urine gave a peak well separated from the peaks of the above acids (Fig. 1). Freshly prepared diazomethane solution ensured non interference with the g.l.c. analysis.

Compound 1d was chosen as a model in the oxidation studies and it was assumed that the *m*-trifluoromethyl-benzyl and -benzoyl compounds present in the urine

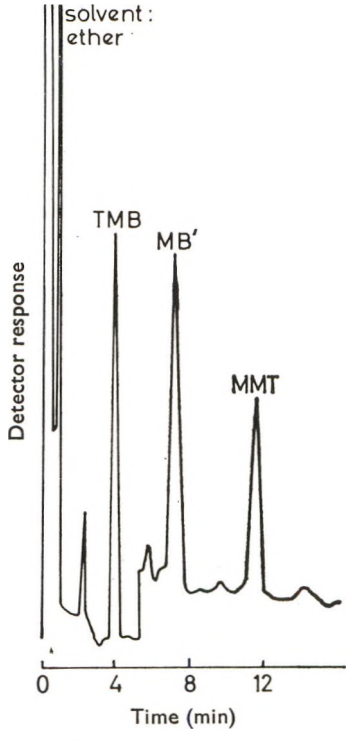

FIG. 1. Chromatogram of an ether extract of oxidized urine from a subject after a dose of JP992, showing *m*-trifluoromethyl benzoic acid (TMB), benzoic acid (MB') and *m*-toluic acid (MMT; internal marker) chromatographed as methyl esters.

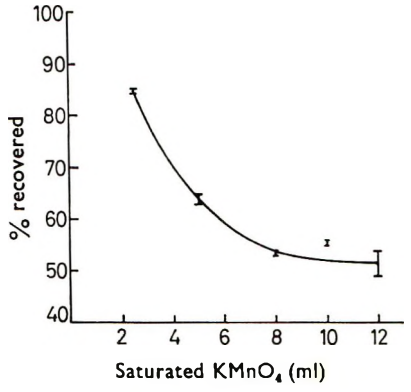

FIG. 2. The effect of concentration of $KMnO_4$ on the recovery of *m*-trifluoromethyl benzoic acid by oxidation of JP992 (compound Id) in urine.

Table 2. *Determination of compounds Ia and Ic in urine in the presence of Id and the total by oxidation to* m-*trifluoromethyl benzoic acid.*

Amount of compound added to urine (μg/ml)				Amount of compound determined in urine (μg/ml)			
Ia	Ic	Id	*Total in terms of Id	Ia	Ic	Id	Total by oxidation
2·0	2·0	6·0	12·26	1·80	2·14	ND	10·85
6·0	2·0	2·0	15·14	6·12	2·20	ND	15·30
2·0	6·0	2·0	13·90	2·12	5·83	ND	15·03
4·0	4·0	12·0	24·52	4·21	3·91	ND	23·72
12·0	4·0	4·0	30·28	11·70	4·20	ND	30·29
4·0	12·0	4·0	27·81	4·10	11·88	ND	27·14

ND—not determined.
*—calculated on molar basis.

following a dose of this compound would not be more difficult to oxidize to *m*-trifluoromethyl benzoic acid than was the parent drug. The product of oxidation, *m*-trifluoromethyl benzoic acid was not oxidized further under the conditions of the general procedure.

On oxidizing compound Id, increasing amounts of *m*-trifluoromethyl benzoic acid were produced as the amount of excess $KMnO_4$ was reduced (Fig. 2). Oxidizible substances are found in urine and inter subject variation in these occur; a compromise was therefore sought between the amount of excess $KMnO_4$ required for oxidizing compound Id quantitatively and the need for excess $KMnO_4$ to deal with the inter-subject variation in oxidizable substances; 2·5 ml/5 ml of urine for normal doses of drug (compound Id) represents this compromise in the general procedure. When varying amounts of compound Id (4–50 μg/ml) were present in the urine, analysis by the general procedure gave linear calibration graphs. Comparison of the results obtained by adding compound Id and *m*-trifluoromethyl benzoic acid to urine samples indicated that there was 85% conversion of the former to the latter (co-efficient of variance (c.v.) ±4%).

When known amounts of compounds Ia, Ic and Id were added to urine, Ia and Ic could be recovered quantitatively and the general procedure gave quantitative

conversion of the total of these compounds to *m*-trifluoromethyl benzoic acid (Table 2).

Analysis of a urine sample from a subject who had taken a dose of compound 1d was reproducible (c.v. $\pm$ 3·8%).

Analysis of compound 1*a and c*

Linear calibration graphs were obtained for compounds 1a and c over the range 0·2–10 μg/ml in urine and water (c.v. $\pm$ 2·2% and 1·8% respectively); retention times are shown in Table 1. No substance interfering with the determination of the amines was found in urine. Both the amines were stable in urine at 4° for at least two weeks.

Analysis of compound 1*d*

When compound 1d was placed in urine, the solution made alkaline and immediately extracted into ether, some conversion to compound 1e occurred. Storage of the solution at an alkaline pH increased this conversion but prolonged storage did not result in complete conversion.

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Infrared identification of sulphonamides using attenuated total reflection

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The infrared spectra of ten sulphonamides have been determined by attenuated total reflection and compared with transmission spectra. No major differences were found in the positions and shapes of the main absorption peaks and it was possible to use ATR to identify sulphacetamide sodium in eye-drops and in eye ointments.

The ready identification of sulphonamides in the solid state by infrared absorption spectra can be complicated by the occurrence of polymorphic forms which give rise to different infrared spectra (Mesley & Houghton, 1967; Moustafa & Carless, 1969). To allow for these different forms the sample for identification and the Authentic Specimen must both be converted to the same crystalline form before spectra can be compared.

Since the introduction of attenuated total reflection (ATR) (Fahrenfort, 1961) this technique has found many applications for obtaining spectra of substances difficult or impossible to obtain by transmission (Katlafsky & Keller, 1963; Wilks, 1965; Pawlak, Fricke & Szymanski, 1967; Wilks & Hirschfeld, 1967). Its use for the identification of sulphonamides has been investigated since the ATR spectra of drugs can be rapidly determined after applying the sample in acetone solution to the reflector plate and evaporating. It was expected that, following this treatment, the same polymorphic form of the drug would always be produced. In addition, transmission and ATR spectra of the same sulphonamides in Nujol mulls were recorded for comparison. ATR spectra of sulphacetamide eye ointments and eye-drops were also examined.

MATERIALS AND METHODS

Apparatus

Infrared spectra of Nujol mulls were recorded on a Hilger H900 Infrascan spectrophotometer. For ATR spectra a Wiltek Model 9T multiple internal reflection attachment was used, with a 45° KRS-5 (thallous bromide-iodide) reflector plate (50 × 20 × 2 mm) held in a M1R-1 Teflon holder. A variable beam attenuator in the reference beam was used to balance the energy levels between sample and reference beam.

Materials

B.P. Authentic Specimens of sulphacetamide sodium, sulphadiazine, sulphadimethoxine, sulphadimidine, sulphadimidine sodium, sulphafurazole, sulphamethizole, sulphamethoxydiazine, sulphamethoxypyridazine and sulphapyridine were used. In several cases ATR spectra were obtained from commercial samples of sulphonamides for comparison. Proprietary samples of sulphacetamide eye oint-

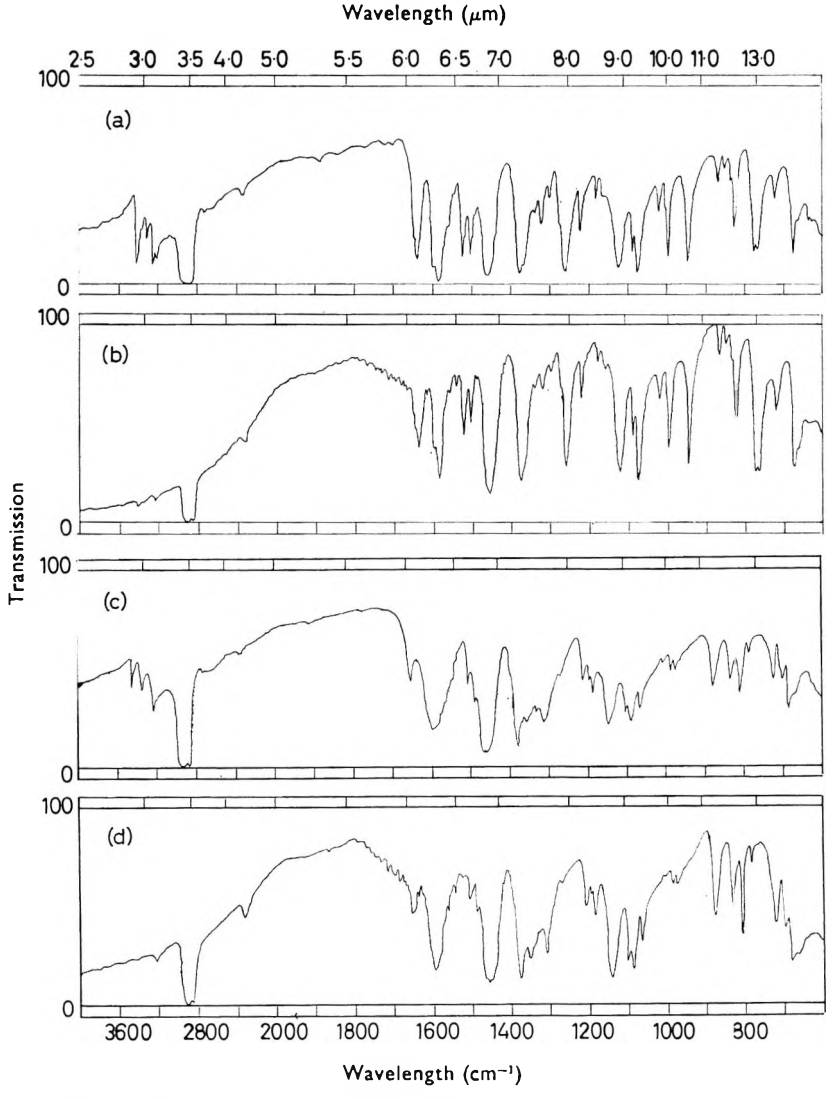

FIG. 1. Infrared spectra in Nujol mulls: (a) transmission and (b) ATR of sulphapyridine; (c) transmission and (d) ATR of sulphadimethoxine.

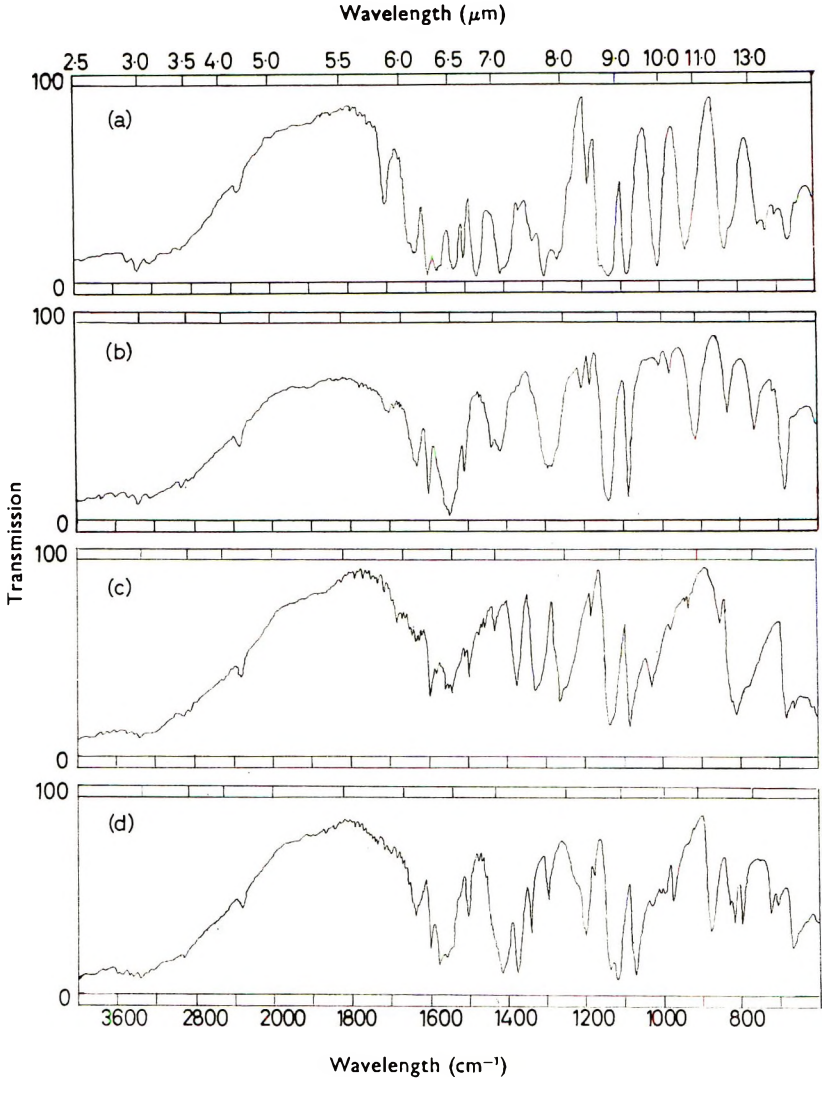

FIG. 2. ATR spectra of evaporated acetone solutions of (a) sulphamethoxypyridazine and (b) sulphamethizole. ATR spectra of evaporated acetone suspensions of (c) sulphacetamide sodium and (d) sulphadimidine sodium.

ments and eye-drops were used in addition to aqueous solutions of varying strengths, from 5 to 30%.

Methods

The ATR spectra of sulphonamides were obtained after allowing 2–3 drops of an acetone solution (about 3 mg/ml) to evaporate on one side of the KRS-5 plate. To obtain a satisfactory spectrum the sample covered the short dimension of the plate and normally about one third of the long dimension. Sodium salts were either used as an acetone suspension or were dissolved in the minimum of water and the aqueous solution diluted with about eight volumes of acetone.

The ATR spectra of water and aqueous solutions were obtained by placing 1 drop on the KRS-5 plate and covering with a rectangular glass cover slip of suitable size.

Ointments and Nujol mulls were smeared on one side of the plate, about one third of the area of one side being covered for mulls and from one third to the complete area of one side for ointments.

RESULTS AND DISCUSSION

Although Potts (1963) states that ATR spectra may not be completely identical with transmission spectra and for solids they can be so distorted as to be nearly useless unless contact between sample and plate is extremely intimate, in the present experiments no great variations between transmission and ATR spectra were obtained with any of the sulphonamides investigated (see Fig. 1). In general it was found that in all spectra the positions and shapes of the main peaks in the region 4000–650 cm^{-1} were identical whether they were obtained by transmission or ATR. However, peaks due to NH stretching vibrations in the range 3500 to 3200 cm^{-1} were all less intense in ATR spectra especially in Nujol mulls where frequently the weaker peaks disappeared completely.

In Fig. 2a and b are shown examples of ATR spectra obtained with evaporation of acetone solutions of the sulphonamides on the reflector plate. Fig. 2 c, d show the spectra obtained using acetone suspensions of sulphacetamide sodium and sulphadimidine sodium respectively. Solution of these sodium salts in the minimum of water followed by dilution with acetone gave spectra identical to those shown in Fig. 2 c and d, apart from additional absorptions in the 3300 and 1640 cm^{-1} regions due to traces of water.

Although no attempt was made to obtain different polymorphic forms of any of the sulphonamides for comparison of their ATR spectra after treatment with acetone as above, two different commercial samples of sulphadiazine, sulphadimidine, sulphadimidine sodium and sulphacetamide sodium gave spectra identical to those obtained from the respective Authentic Specimens.

In Fig. 3 the ATR spectrum of distilled water shows that there is sufficient transmittance in the region 1550–900 cm^{-1} to enable identification of the spectra of sulphacetamide sodium and sulphadimidine sodium in aqueous solutions. Katlafsky & Keller (1963) used a 40° Intran-2 prism for analysis of aqueous solutions and found that concentrations of at least 20% were required to obtain suitably intense spectra. They also found that this particular prism greatly decreased the intensity of the HOH deformation at 1640 cm^{-1}, allowing detection of aromatic absorption in the 1600 cm^{-1} region. With concentrations of 10% sulphacetamide sodium and

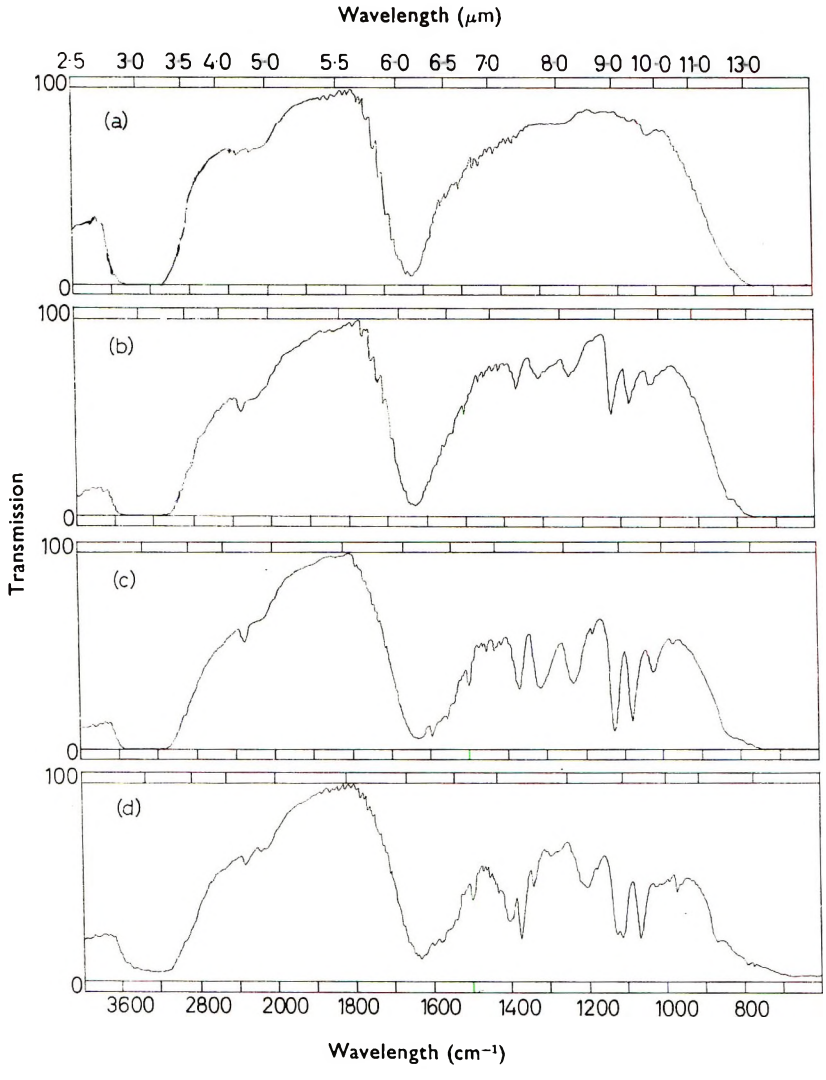

FIG. 3. ATR spectra: (a) distilled water, (b) aqueous solution of sulphacetamide sodium (5%), (c) aqueous solution of sulphacetamide sodium (20%), (d) aqueous solution of sulphadimidine sodium (10%).

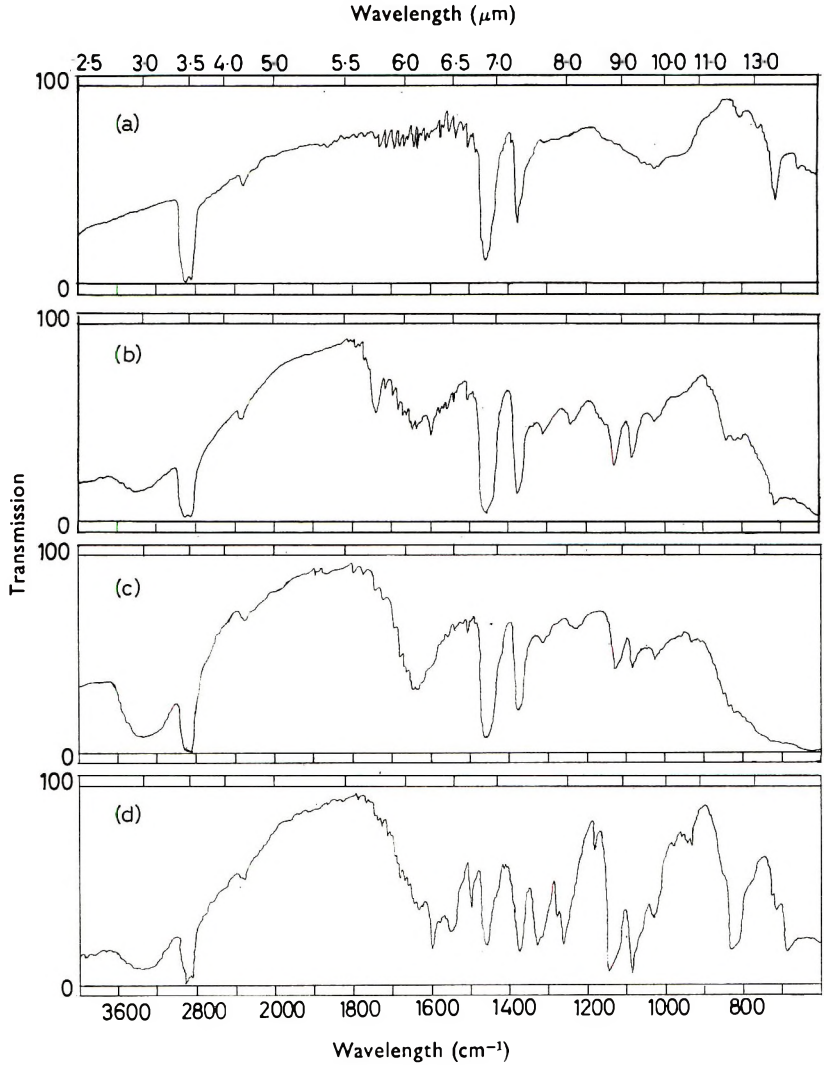

FIG. 4. ATR spectra: (a) ointment base, B.P., (b) sulphacetamide eye ointment, B.P., and proprietary ointment (c, 2½%: d, 10%).

using a 45° KRS-5 plate this absorption usually appears as a shoulder in the strong 1640 cm^{-1} absorption band, but even with concentrations of 15% there is a distinct peak. With 5% solutions the six main bands in the 1400–1000 cm^{-1} region are still distinct and characteristic enough for identification (see Fig. 3).

Identification of sulphacetamide sodium in eye-ointments was found to be easy in concentrations of 6% (B.P. sample) and 10%, but much less so in 2½% concentration where only the three peaks in the 1150–1000 cm^{-1} range are obvious (Fig. 4). However the complete absence of these latter peaks (and others) in a proprietary sample of sulphacetamide eye ointment B.P. examined was taken as proof that little or no sulphacetamide sodium was present.

The present investigation has shown that use can be made of attenuated total reflection to rapidly identify the sulphonamides investigated, either by using an Authentic Specimen for comparison or by direct comparison with standard transmission spectra. The ease of obtaining spectra of sulphacetamide sodium in ointments or aqueous solutions could be extended for ointments to give some quantitative estimate of the amount of drug present.

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J. Pharm. Pharmac., 1971, **23**, 963

LETTERS TO THE EDITOR

Isolation from bovine plasma of a protein that enhances vascular permeability in rats

In 1969, Gecse, Karády & others isolated from bovine plasma a smooth muscle-stimulating substance which was predominantly α_2-globulin and had a molecular weight of about 800 000. It was obtained by fractionating the plasma proteins by Cohn's method and then identifying the active material in fraction IV–1 after purification using a Sephadex G 200 column. In the present study, the proteins of bovine plasma were fractionated by Cohn's method using cold ethanol and tested for their ability to increase vascular permeability in rat skin. It was found that only fraction II + III possessed this ability, the fraction being predominantly γ-globulin with a molecular weight of about 160 000.

Plasma was obtained from healthy oxen by centrifuging citrated blood at 4000 g for 15 min at 0° and then fractionating the proteins by Cohn's method and freeze-drying them. From each protein fraction, 250 μg in 0·1 ml saline was injected intradermally into the depilated skin of adult albino rats which had azovan blue dye (10 mg/kg) in their circulation. Thirty min later, the rats were killed, their dorsal skin was removed, cleaned and extracted with Germanin dissolved in methanol (Jancso-Gabor, Szolcsanyi & Jancso, 1967), and the amount of dye in solution determined in a spectrophotometer at 620 nm. Only fraction II + III exerted a pronounced effect on vascular permeability (49 $\pm$ 5 μg), the dye extracted being more than 5 times the control value of 9 $\pm$ 3 μg or any of the values obtained with the other fractions. γ-Globulin accounted for about 50% of the protein in fraction II + III when determined by gel electrophoresis. This fraction was then transferred to a Sephadex G 200 column (diameter 2 cm, length 63 cm) and eluted with tris buffer (pH 8·05) passing at a rate of 10 ml/h. Samples of eluate (1·5 ml) were tested for biological activity and for protein content by measuring light absorption at 280 nm and by studying gel electrophoresis. The active eluate enhancing vascular permeability contained more than 89% of γ-globulin and its rate of flow from the column corresponded with compounds of a molecular weight of about 160 000. The activity was significantly decreased 15 min after incubation with crystalline trypsin (1 mg/mg protein at 37° and pH 8·0) and was completely abolished at 50 min. Whereas soya bean trypsin inhibitor prevented the vascular permeability-increasing action of trypsin alone, it did not modify the activity of the Sephadex-separated active fraction from bovine plasma. When the active fraction was subjected to alkaline hydrolysis (pH 12, 100°), the activity significantly *increased* during the first 90 min of hydrolysis (exuded dye being 98 $\pm$ 8 μg) but then quickly decreased and by 180 min it was lost. Finally, on dilution 10-fold with saline, activity decreased and was finally lost when the fraction was diluted 100-fold, whereas undiluted guinea-pig serum was ineffective yet increased in activity as the serum was diluted (see Fig. 1).

At least two vascular permeability-enhancing factors have been previously isolated from the plasma and serum of guinea-pig, rat, rabbit and man. One of these has been shown to be identical with kininogenase whereas the other was called Permeability Factor (or PF, for short) and this was activated by dilution. According to Miles (1969) and Movat and his co-workers (1969 a, b), the activity of these two factors on

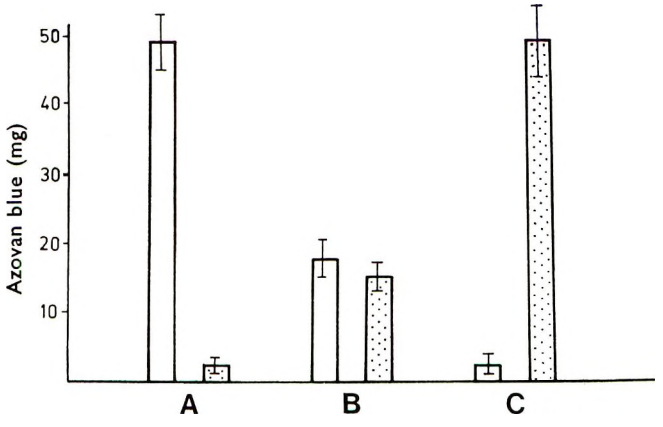

FIG. 1. The effect of dilution of the Sephadex-separated active fraction from bovine plasma (open columns) and of guinea-pig serum (dotted columns) on the vascular permeability action, as measured by the amount of azovan blue dye (μg, ordinate) exuded into rat skin after intradermal injections of 0·1 ml. Mean values ± s.e. of 20 determinations are recorded. A. Undiluted. B. Diluted 10-fold. C. Diluted 100-fold.

vascular permeability is suppressed by incubation with soya bean trypsin inhibitor, whereas in the present study that of the Sephadex-separated active fraction remained unchanged and further, it was not activated by dilution. On the basis of these findings, the plasma fraction that enhances vascular permeability is not kininogenase or PF. Its action, however, may lead to the leakage of plasma proteins into the extracellular compartments, thereby suggesting that kinin is being formed in the tissues. In pathological states where plasma protease levels are raised (such as in shock and anaphylaxis), the activity of the new permeability factor may be of importance, particularly in alkaline conditions. However, the optimum dose of the active protein was found to be in the range 100–200 μg, values which are to be compared with those of prostaglandin (10–100 ng) exerting a similar action.

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Potentiation by cocaine of responses of the guinea-pig isolated tracheal chain to ethylnoradrenaline and α-methylnoradrenaline

Ethylnoradrenaline and α-methylnoradrenaline (corbasil) differ in structure from noradrenaline only by substitution of an ethyl or methyl group on the α-carbon atom of the ethylamine side-chain. The potencies of these two compounds in relaxing the guinea-pig isolated tracheal chain preparation and the influence of 10^{-5}M cocaine on their potency has been compared with noradrenaline. The preparation of tracheal chain and determination of the mean concentration to give 50% of the maximum relaxation to isoprenaline (EC50) have been described previously (Chahl & O'Donnell, 1967; O'Donnell, 1968). Two preparations from each animal were set up so that noradrenaline could be examined on one chain and ethylnoradrenaline or α-methylnoradrenaline on the other. The same experimental design was used on both preparations, i.e. a control concentration–response line to the amine, a line after 10^{-5}M cocaine had been in contact with the tissue for 0·5 h, and a further line after 10^{-5}M cocaine and 10^{-6}M propranolol had been in contact for 1 h. In another series of experiments, the shift of the normal concentration–response line to each drug by 10^{-6}M propranolol, after 1 h contact with the tissue, was determined.

In the absence of cocaine, ethylnoradrenaline was more potent than α-methylnoradrenaline or noradrenaline, which were equipotent (Table 1). All three drugs were potentiated by cocaine but not to the same extent (Table 1). Thus, in the presence of cocaine, noradrenaline became more potent than the other two drugs, which were now equipotent.

An aim of the study was to assess the true potency of the three drugs on the β-adrenoceptors of the tissue. If it is assumed that cocaine blocks the loss of drug to neuronal uptake sites, then the potency found with cocaine present should be a truer representation of the potency on β-adrenoceptors. All three drugs were potentiated by cocaine. However, the study was made on the (−)-isomer of noradrenaline and racemic (*erythro*) ethyl- and methyl-noradrenaline. If the (−)-isomer is the active component of the racemic mixture and if the (+)-isomer contributes little to the response and does not antagonize neuronal uptake, then potentiation by cocaine of the racemic mixture and the (−)-isomer should be the same. On the other hand, potency values would require correction for the presence of inactive (+)-isomer. The simplest correction is to double the estimated potency of ethyl- and methyl-noradrenaline. This type of correction has been previously applied by Foster (1966).

Table 1. *Effect of cocaine* (10^{-5}M) *on noradrenaline, methylnoradrenaline and ethylnoradrenaline potency on the guinea-pig tracheal chain.*

	Negative mean log EC50		Potentiation by cocaine (log units)
	Control	Cocaine	
(−) Noradrenaline	6·16 ± 0·29* (36)†	7·34 ± 0·25 (11)	1·18 ± 0·29
(±) α-Methylnoradrenaline ..	6·23 ± 0·32 (17)	6·94 ± 0·31 (9)	0·71 ± 0·33
(±) Ethylnoradrenaline	6·60 ± 0·22 (32)	7·02 ± 0·13 (14)	0·42 ± 0·20

* Standard deviation.
† Number of experimental concentration-response lines contributing to the mean log EC50.

Table 2. *Effect of propranolol on noradrenaline, methylnoradrenaline and ethylnoradrenaline potency on the guinea-pig tracheal chain.*

	Negative mean log EC50			
	In presence propranolol (10^{-6}M)	In presence cocaine (10^{-5}M) and propranolol (10^{-6}M)	Degree of block by propranolol (log units)	
			No cocaine	Cocaine
(±) Ethylnoradrenaline	3·94 ± 0·14* (5)†	4·01 ± 0·23 (15)	2·66 ± 0·22	3·01 ± 0·20
(−) Noradrenaline ..	4·68 ± 0·19 (5)	4·87 ± 0·29 (14)	1·48 ± 0·29	2·47 ± 0·28
(±) α-Methylnoradrenaline	4·70 ± 0·16 (7)	4·74 ± 0·20 (10)	1·53 ± 0·30	2·20 ± 0·27

* Standard deviation,
† Number of experimental concentration-response lines contributing to the mean log EC50.

Application of this correction to the potencies obtained in the presence of cocaine showed that the three drugs became equipotent, suggesting that substitution of an ethyl or methyl group on the α-carbon in the *erythro* configuration of noradrenaline might have little effect on its potency on β-adrenoceptors in this preparation. In contrast, this substitution resulted in a decrease in the potentiation by cocaine which could be interpreted as a loss of affinity for the cocaine-sensitive uptake mechanism in this tissue.

The block of the concentration–response line by propranolol (10^{-6}M) was examined in the absence and presence of cocaine, and ethylnoradrenaline was always blocked more than α-methylnoradrenaline or noradrenaline (Table 2). If it is assumed that, when uptake is blocked by cocaine, the control concentration–response line to ethylnoradrenaline is positioned correctly, then the greater block might be explained

Effect of neuroleptics on brain amphetamine concentrations in the rat

Within certain dose ranges promazine, chlorprothixene (Halliwell, Quintin & William, 1964) and trifluprómazine prolong amphetamine-induced stereotyped behaviour (ASB). Perphenazine has no potentiating effect. We now report the effect of these four neuroleptics on brain amphetamine concentration.

Male Sprague-Dawley rats, about 150 g, were housed in pairs in wire cages, and groups of six injected with either trifluprómazine hydrochloride (Vesperin, Squibb), promazine hydrochloride (Sparine, Wyeth), chlorprothixene (Tarasan, Roche), each in a dose of 5 mg/kg, perphenazine (Trilafon, Schering), 1 mg/kg, or distilled water 2 h before (+)-amphetamine sulphate (Smith, Kline and French), 10 mg/kg containing 66 μCi/kg [^{3}H]amphetamine (New England Nuclear). Two animals in each group were killed at 30, 120 and 240 min and amphetamine concentrations measured in the brain. The procedure for extraction and measurement of [^{3}H]amphetamine was that of Lemberger, Witt & others (1970).

For behavioural effects, animals received similar treatment except that radioactive amphetamine was not added to the cold amphetamine. The duration of ASB was recorded by direct observation. Additional control rats received either distilled water or neuroleptic but no amphetamine. The pH of urine was found to range from 6·8–7·4.

All drugs were given in 1 ml aqueous solution intraperitoneally. Doses are expressed as the base.

The semilogarithmic plot in Fig. 1 shows the disappearance of amphetamine from brain under the different experimental conditions. The half-life for controls was

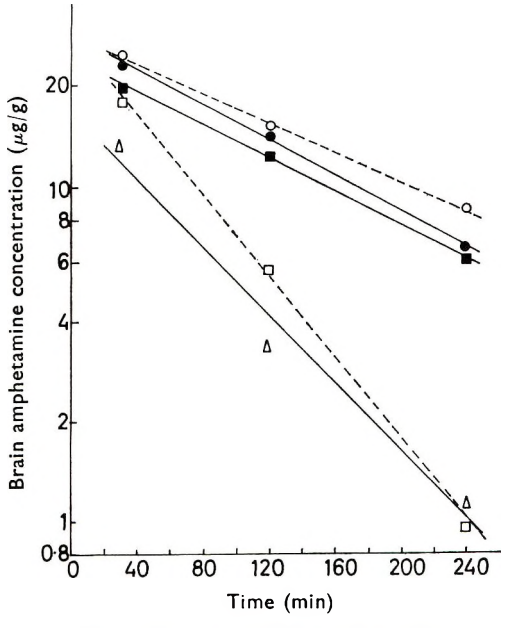

FIG. 1. Rats were treated with perphenazine (□) (1 mg/kg), trifluprómazine (○), promazine (●), chlorprothixene (■) (5 mg/kg) or distilled water (△) i.p. 2 h before amphetamine (10 mg/kg, i.p. containing 66 μCi/kg [^{3}H]amphetamine/rat). Animals were killed at 30, 120 or 240 min and brains assayed for [^{3}H]amphetamine. Each point represents the mean of 2 animals. The parameters of the regression lines were estimated by the method of least squares. The slopes for perphenazine and control-treated rats are not significantly different from one another; the slopes for the other three drugs are significantly different from those for perphenazine and controls.

Table 1. *Effect of neuroleptics on amphetamine-induced sterotyped behaviour in the rat.* Rats were injected with tranquillizing agent 2 h before amphetamine (10 mg/kg). All drugs were given i.p. Onset refers to time interval between injection of amphetamine and initiation of stereotypy. Termination refers to time interval from injection of amphetamine to termination of stereotypy. *** = $P < 0{\cdot}001$.

Pretreatment	Dose (mg/kg)	n	Onset (min)	Termination (min, $\bar{X} \pm$ s.e.)
Control	—	16	10	272 ± 9
Perphenazine	1	4	inhibition	—
Triflupromazine	5	4	30–480	878 ± 23***
Chlorprothixene	5	4	10–20	585 ± 9***
Promazine	5	4	10	570 ± 21***

59 min. This may be compared with the value of 52 min found by Lemberger & others (1970). When perphenazine was given before the amphetamine the initial brain concentration of amphetamine (estimated by extrapolation of the curves to time zero) was increased, but the half-life was only 50 min, indicating a more rapid disappearance of the labelled compound. The other drugs tested also increased the initial concentration of amphetamine to the same range of values, but the half-life for the radioactive amine was much extended (Fig. 1). The values for triflupromazine were 137, for promazine 112, and for chlorprothixene 125 min. Triflupromazine-treated rats at 30 or 120 min did not show ASB, even though brain concentrations of amphetamine were raised. Those at 240 min, however, had already initiated ASB about 30 min before.

In the second experiment, animals treated with amphetamine alone developed ASB within 10 min and this terminated about 4·5 h after injection of the amphetamine. Again, triflupromazine had a delaying, though somewhat variable, effect on the appearance of sterotyped behaviour, but ASB was markedly prolonged (Table 1). Promazine and chlorprothixene also prolonged ASB whereas perphenazine (1 mg/kg) completely inhibited it (Table 1).

The results show that prolongation of behavioural effects of amphetamine by neuroleptics is related to increased half-life of amphetamine in the brain. Chlorprothixene, like chlorpromazine and prochlorperazine, impairs hydroxylation of amphetamine (Lewander, 1969; Borella & Herr, 1971). Chlorpromazine, propericiazine and haloperidol increase brain amphetamine concentrations in the rat (Borella, Herr & Wojdan, 1969; Lemberger & others, 1970; Sulser & Dingell, 1968; Valzelli, Dolfini & others, 1968). This suggests that drug interaction at the level of hepatic drug-metabolizing enzymes is a general mechanism whereby tissue amphetamine concentrations are raised and hence behavioural effects of amphetamine are influenced by neuroleptics. The delayed onset of ASB with triflupromazine is presumably related to the competing central actions of amphetamine and tranquillizer on the striatal dopamine system in the brain which is believed to subserve ASB (Randrup & Munkvad, 1970). The failure of perphenazine to prolong the half-life of amphetamine may be related to the small dose used, relative to the other drugs. This dose may have been sufficient for blockade of dopamine receptor sites but insufficient to impair the biotransformation of amphetamine.

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Cross tolerance between methylamphetamine and morphine in the mouse

Recent work has attempted to explain the mechanism of narcotic agonist action in terms of interference with chemical transmission in the central nervous system. Evidence for the involvement of both cholinergic and monoaminergic systems has been reported (Harris, 1970).

Amongst the evidence in support of these hypotheses is the fact that both sympathomimetics (Colville & Chaplin, 1964) and cholinomimetics (Gross, Holland & others, 1948; Chen, 1958) possess antinociceptive activity.

The characteristics of morphine and sympathomimetic antinociception have been compared by Major & Pleuvry (1971). They showed that drugs known to cause changes in the content of putative transmitters in the central nervous system had a qualitatively similar effect upon the antinociceptive activity of morphine and methylamphetamine. Antinociception was increased when 5-hydroxytryptamine content was raised relative to noradrenaline, dopamine or both. Subsequent work in this laboratory has shown that whilst physostigmine antinociception has similar characteristics to that of morphine and methylamphetamine, oxotremorine antinociception has not.

Tolerance development is a characteristic of both morphine-like agonists and the sympathomimetics. In a further attempt to examine similarities between these various antinociceptive agents, the characteristics of tolerance to them has been compared.

Antinociception was estimated by the hot plate reaction time test (Bousfield & Rees, 1969). Drugs were administered twice daily for five days. The drugs were morphine sulphate (10 mg/kg, i.p.), methylamphetamine hydrochloride (10 mg/kg, i.p.), physostigmine salicylate (0·1 mg/kg, s.c.), oxotremorine (0·05 mg/kg s.c.) and saline (0·1 ml i.p. or s.c.). Single injections of the above doses of antinociceptive agents were approximately equipotent in the hot plate reaction time test. Reaction times in groups of 12 mice were measured at 5 min intervals for the first 30 min after the first injection each day and then at 10 min intervals until the reaction times were not significantly different from those of saline pretreated control mice.

In the afternoon of the fifth day, mice pretreated with methylamphetamine, physostigmine or oxotremorine were injected with 10 mg/kg morphine sulphate and the concurrently tested morphine-treated mice injected with either methylamphetamine, physostigmine or oxotremorine. Saline-pretreated mice were injected with either morphine, methylamphetamine, physostigmine or oxotremorine. The reaction

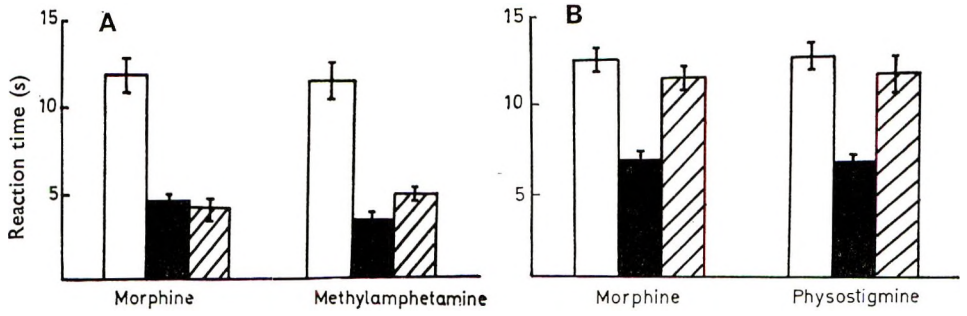

FIG. 1. A. Cross tolerance between the antinociceptive activity (ordinate) of morphine and methylamphetamine as morphine sulphate (10 mg/kg, i.p.) and methylamphetamine HCl (10 mg/kg, i.p.). The open columns show the effect on the first day of tolerance induction (see text), the closed columns show the effect on the fifth day of tolerance induction and the hatched columns show the effect in mice pretreated for five days with the alternate antinociceptive agent. The results are expressed as maximum mean reaction times ± s.e. of groups of 12 mice.

B. Absence of cross tolerance between the antinociceptive activity (ordinate) of morphine and physostigmine as morphine sulphate (10 mg/kg, i.p.) and physostigmine salicylate (0·1 mg/kg, s.c.).

times obtained with these agents in saline pretreated mice were not significantly different from those obtained with the same agent in non-pretreated control mice.

Tolerance developed to the antinociceptive activity of morphine, methylamphetamine and physostigmine during the pretreatment course. No tolerance developed to the effects of oxotremorine, there being no significant difference between the response on day 1 and day 5 ($P > 0{\cdot}30$).

The results obtained for morphine, methylamphetamine and physostigmine are shown in Fig. 1A and B.

Marked cross tolerance was detected between methylamphetamine and morphine (Fig. 1A). Mice pretreated with methylamphetamine, when challenged with morphine, responded in a quantitatively identical manner to those pretreated with morphine ($P > 0{\cdot}60$).

No cross tolerance existed between physostigmine and morphine, morphine having a similar effect in both physostigmine pretreated and non-pretreated control mice ($P > 0{\cdot}50$).

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Effect of dimethyl and monomethyl tricyclic antidepressants on central 5-hydroxytryptamine processes in the frog

We previously described a test for screening thymoleptic drugs. This test is based on measuring sedation (loss of the righting reflex) and of twitches of the extremities in the frog. Both effects are presumably related to activation of central 5-hydroxytryptamine (5-HT) processes (Lapin, Oxenkrug & others, 1970).

We now report our observations on the relation between chemical structure of tricyclic antidepressants and their activity in this test.

Male frogs (*Rana temporaria*) were used in our experimental procedure identical to that described earlier, except that the experiments were made in winter whilst the previous experiments were made in spring and summer. We used chlorimipramine, imipramine, amitriptyline and desipramine and nortriptyline. We measured the minimal doses that produced inhibition of the righting reflex and the appearance of twitches of the extremities 4 h after injecting reserpine.

Both sedation and twitches were observed when the elevation of brain 5-HT was 3-fold or more (Oxenkrug, Osipova & Uskova, 1970). Since an increase in brain 5-HT was produced by a combination of phenelzine (25 mg/kg) with reserpine (10 mg/kg) or more so by phenelzine alone (25 mg/kg) (Oxenkrug & others, 1970), one series of our experiments was done without reserpine (Table 1).

Table 1. *Potentiation of central 5-HT effects in the frog by dimethyl and monomethyl tricyclic antidepressants.*

	Minimal effective doses (mg/kg) for:							
	I*	T†	I	T	I	T	I	T
			after pretreatment with:					
Drug	Distilled water		Phenelzine (25 mg/kg) + reserpine (10 mg/kg)		Phenelzine (25 mg/kg)		Amylobarbitone (25 mg/kg)	
Dimethyl compounds								
Chlorimipramine ..	70	0	0·5	1·25	5	5	70	0
Imipramine	50	0	1·25	1·25	2·5	5	50	0
Amitriptyline	50	0	1·25	1·25	5	10	50	0
Monomethyl compounds								
Desipramine	50	0	20	>20‡	20	>20‡	50	0
Nortriptyline	40	0	20	>20‡	20	>20‡	40	0

* Inhibition of righting reflexes.
† Appearance of twitches.
‡ If any.

Dimethyl antidepressants are much stronger than their monomethyl derivatives in enhancing central 5-HT processes in the frog (Table 1). Difference in the strength of potentiation (in the test of inhibition of the righting reflex) is not related to the sedative action of tricyclic antidepressants, as it can be seen from the ratio of sedation to potentiation. The same is also shown in the inability of antidepressants to potentiate, or to enhance, the sedative action of amylobarbitone. The minimal sedative dose of amylobarbitone in our experiments was 60 mg/kg.

Our results are also consistent with observations of others that the tertiary compounds have more pronounced influence on metabolism of indolealkylamines than have the secondary tricyclic antidepressants measured on uptake of 5-HT by the presynaptic membrane of serotoninergic neuron (Carlsson, 1970) and by blood platelets (Todrick & Tait, 1969).

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The distribution of noradrenaline in the undivided spleen of the cat

Dearnaley & Geffen (1966) showed that it was more satisfactory to express the noradrenaline content of the cat spleen in terms of the deoxyribonucleic acid-phosphorus (DNA-P) content of that organ rather than in terms of tissue wet weight. Furthermore, when the relation between the noradrenaline contents, expressed per μmol DNA-P, of the anterior and posterior portions of the cat spleen was calculated, the posterior portion was found to contain an amount of noradrenaline that was equivalent to 90% of that in the anterior portion.

The distribution of the noradrenaline content of the anterior portion of cat spleens was found to be approximately $\log_{10}$ normal (Brown, Dearnaley & Geffen, 1967).

Experiments with the effects of several drugs on the noradrenaline content of the undivided spleen *in situ* (Abbs & Robertson, 1969, 1970; Robertson & Abbs, 1971) have been made and it was therefore reasonable to determine the normality of the distribution of noradrenaline in the whole spleen; the rankit method (Ipsen & Jerne, 1944; Bliss, 1967) was applied.

The experimental procedures have been previously described (Abbs & Robertson, 1970). Cat spleens were homogenized in an ice-cold sucrose medium and the noradrenaline and DNA-P contents were measured. Figures from twenty-six experiments were available for analysis.

Using the rankit method and an Elliot 4130 computer linked to a graph plotter, graphs were prepared and points plotted for the observations of the noradrenaline content of the spleen, expressed as ng per μmol DNA-P, without transformation and also with the following five transformations: x^2, $x^{\frac{1}{2}}$, x^0, $x^{-\frac{1}{2}}$, x^{-1}.

A straight line for each set of points was fitted by applying the method of least squares. The square root and logarithmic transformations gave reasonably linear trends but the best linear fit was found for those observations plotted without transformation (Fig. 1a). The mean and standard deviation for each form of the observations were interpolated from the six graphs using a procedure described by Ipsen & Jerne (1944) and were compared with calculated values (Table 1). Agreement between the interpolated and calculated means and standard deviations was closest with the untransformed figures but there was also good agreement for the square root and logarithmic functions.

As an attempt to differentiate between these possibilities, a second degree polynomial curve was fitted to each set of plotted points (Fig. 1b). In this series, the observations without transformation gave a graph which was almost rectilinear. The other graphs, however, were distinctly curvilinear indicating that the data in the transformations were not distributed normally.

Our results with the modified rankit method show that the criteria for normality

Table 1. *The effect of transformation of the data.*

Form of observation	Calculated Mean	Calculated s.d.	Interpolated Mean	Interpolated s.d.
x	88·6	25·8	88·6	26·7
x^2	8494	4742	8450	5130
$x^{\frac{1}{2}}$	9·31	1·39	9·31	1·45
x^0	1·929	0·135	1·928	0·141
$x^{-\frac{1}{2}}$	0·1099	0·0177	0·1098	0·0190
x^{-1}	0·01237	0·00413	0·01233	0·00459

Figures shown above are means and standard deviations (s.d.) relating to various forms of the observations of the noradrenaline content of the undivided spleen of the cat. The untransformed observations (x) are expressed in ng noradrenaline per μmol DNA-P. Interpolated values were derived from the graphs illustrated in Fig. 1a.

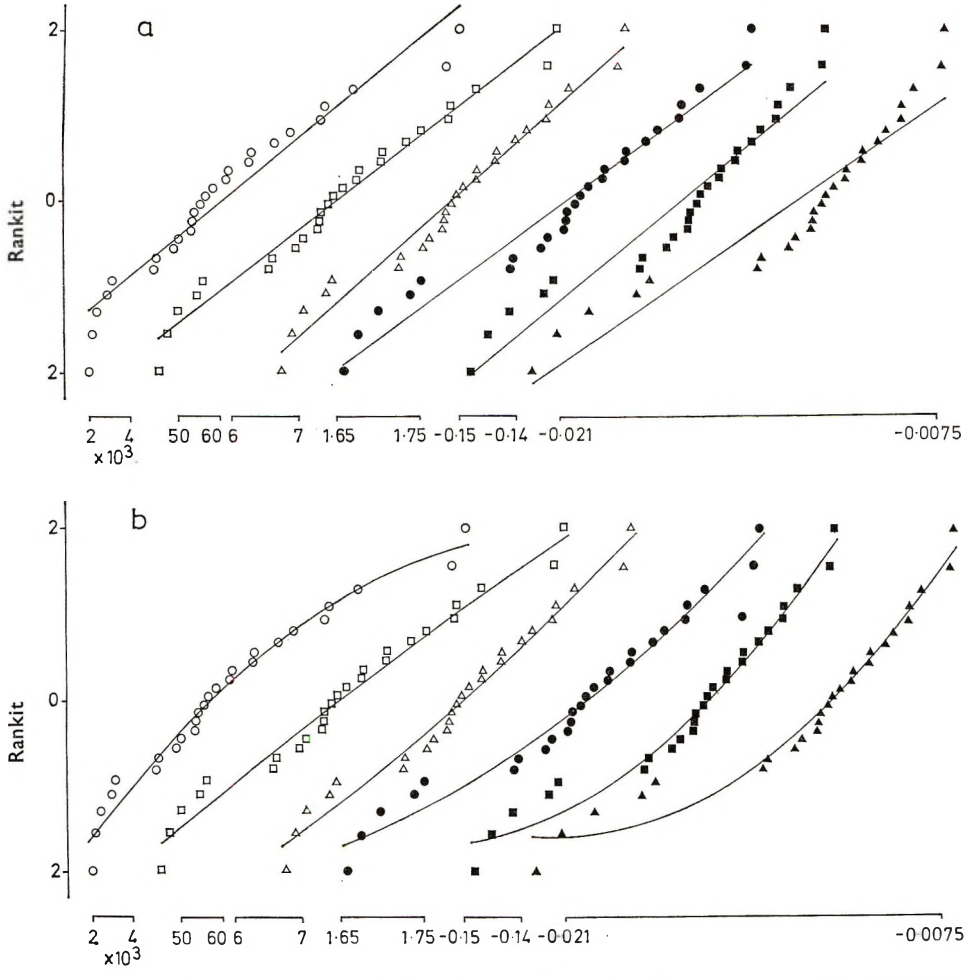

FIG. 1. Composite rankit diagrams to determine the normality of the distribution of noradrenaline in the undivided spleen of the cat. Untransformed observations, x (□), expressed in ng noradrenaline per μmol DNA-P, and the following transformations thereof—x^2 (○), $x^{\frac{1}{2}}$ (△), x^0 (●), $x^{-\frac{1}{2}}$ (■), and x^{-1} (▲)—were plotted against the appropriate rankits. Only parts of each scale are shown on the abscissa. a. A straight line was fitted to each set of points using the method of least squares. b. A second degree polynomial curve was fitted to each set of points.

of a distribution are met most satisfactorily when the observations of the noradrenaline content of the spleen are considered without transformation.

It is possible that differences in the regional distribution of noradrenaline in the cat spleen underlie the difference between our results with undivided spleens and those of Brown & others (1967) relating to divided spleens. Certainly, differences in the densities of the adrenergic innervation of the lateral (thin or anterior) and medial (wide or posterior) ends of the cat spleen have been suggested (Green & Fleming, 1968).

This work forms part of a thesis presented by one of us (M.I.R.) in fulfilment of the requirements for the Degree of Doctor of Philosophy in the University of London.

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Effect of calcium on reserpine-induced catalepsy

High calcium pretreatment reverses the gross behavioral effects of reserpine in the guinea-pig as well as the reserpine-induced inhibition of pethidine analgesia (Radouco-Thomas, 1971). Furthermore, calcium pretreatment attenuates reserpine rigidity (Radouco-Thomas, 1970) and partially antagonizes the reserpine-induced depression of the conditioned avoidance response in the rat (Boyaner & Radouco-Thomas, 1971). These results prompted an investigation of the effect of high calcium pretreatment on reserpine-induced catalepsy in the rat.

Sprague-Dawley, male rats (260–300 g) were used to assess the intensity of catalepsy by carefully raising each leg of the animals in turn to a height of either 2 or 5 cm by placing it on a suitable block. If the rat did not remove its leg within 15 s, the catalepsy test was taken to be positive. The degree of catalepsy was expressed as the percentage of positive responses obtained in each group.

The rats were randomly divided into four treatment groups. Each group consisted of a minimum of 9 animals: group 1—placebo (0·9% NaCl); group 2—calcium chloride (3 × 100 mg/kg calcium); group 3—reserpine (1 mg/kg); group 4—calcium plus reserpine (3 × 100 mg/kg calcium +1 mg/kg reserpine).

All drugs were administered subcutaneously. The three injections of calcium were given at 15 min intervals. In calcium-reserpine treated rats, reserpine was injected 15 min after the last calcium injection.

Calcium-treated rats showed only a few positive responses (10% at 6 h) in the 2 cm block test and none in the 5 cm block test. The reserpine-treated group demonstrated the maximum intensity of catalepsy for the percentage of positive responses on both tests exceeded 50% at 3 h (56 and 59% respectively) and reached 100% at 6 h. On the other hand, in the calcium-reserpine treated group, the percentages of positive responses in the two tests were 36 and 19% at 3 h, and 44 and 53% at 6 h, respectively. Thus, calcium pretreatment resulted in a slower progression and an attenuation of the reserpine-induced catalepsy.

A dopamine deficiency in the extrapyramidal centres has been implicated in reserpine-induced catalepsy in animals and in man, for L-dopa readily reverses this syndrome (Carlsson, Lindqvist & Magnusson, 1957; Hornykiewicz, 1966; Blaschko & Chruschiel, 1960; Degkwitz, Frowein & others, 1960; Larochelle, Bedard & others, 1971). This attenuation of reserpine-induced catalepsy by exogenous calcium could be due to its inhibition of reserpine-induced depletion of cerebral dopamine as well as noradrenaline and 5-HT (Radouco-Thomas, Tessier & Lajeunesse, 1971).

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Diurnal variation of hepatic amphetamine concentrations in mice fed freely and fed single daily meals

Scheving, Vedral & Pauly (1968) demonstrated a circadian rhythm in studies of the mortality of rats maintained in light-dark, continuous light and continuous dark environments to (+)-amphetamine. This may represent a rhythm in the rate at which the drug is metabolized. The activities of several hepatic oxidative drug-metabolizing enzymes in mouse and rat liver have been shown to vary with the time of day (Radzialowski & Bousqet, 1969). The possibility that the cause of the rhythmic response to the drug is related to the rate at which amphetamine is metabolized has been examined.

Feeding schedules have been shown to influence metabolism (Fuller & Snoddy, 1968; Fuller & Diller, 1970; Fuller, 1970). We now report that hepatic amphetamine concentrations in mice fed freely vary significantly over a 24 h period, and that the concentrations in mice fed a single daily meal appear to be shifted 12 h out of phase.

Retired female breeder albino mice (Charles River Mouse Farm, Wilmington), 30–60 g, were housed three per cage in large ventilated room. Fluorescent lights were mechanically switched on at 7.00 a.m. and off at 9.00 p.m. daily. Thirty mice were fed freely, and 30 were given food from 8.00 a.m. to noon daily for 4 weeks before being killed.

Three mice from each feeding schedule were injected intraperitoneally with aqueous solutions of crystalline (+)-amphetamine sulphate (K & K Laboratories, Inc.) at a dose of 10 mg/kg every 3 h within the same 24-h period. 1 h after injection, the mice were decapitated and their livers were quickly removed and frozen on dry ice. Amphetamine concentration were measured (Dubnick, Leeson & others, 1963).

Hepatic amphetamine concentrations were highest during the night and lowest during the day in mice fed freely (Fig. 1). A second rhythm was found in the meal-fed

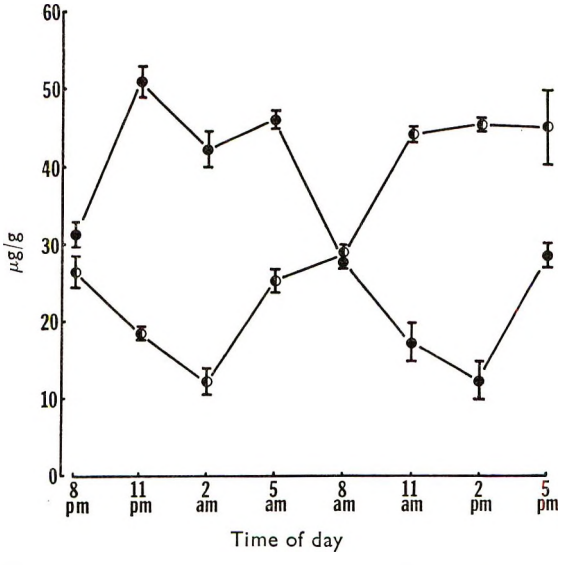

FIG. 1. Amphetamine concentration in mouse liver. The amount of drug in the 9000 *g* supernatant fraction of liver homogenates, expressed on the basis of wet tissue weight, is shown. Mean values and standard errors are shown for 3 mice per group (2 mice in the 2 a.m. and 5 a.m. groups allowed free access to food. ● Free access to food. ◐ Fed 8 a.m.–noon.

mice, indicating that a phase shift induced by meal-feeding had occurred. These results are consistent with those of Scheving & others (1968) in that high concentrations of amphetamine are correlated in time with high susceptibility to the drug. Since Radzialowski & Bousquet (1969) reported the activities of several oxidative drug-metabolizing enzymes in mice to be highest at night, the rate at which amphetamine is metabolized may not cause the rhythm. It remains to be determined that the rhythm represents a rhythm of metabolism rather than of uptake by the liver.

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Preliminary evidence that syrosingopine produces a selective depletion of central stores of sympathomimetic amines

The convulsive effects of leptazol are markedly facilitated by pretreating laboratory animals with reserpine (Jenney, 1954; Chen & Bohner, 1956; Kobinger, 1958; Lessin & Parkes, 1959; Pfeifer & Galambos, 1967). Reserpine is reported to produce non-selective depletion of both central and peripheral amine stores (Carlsson, 1964). These results have been confirmed in our laboratories.

Syrosingopine is a synthetic analogue of reserpine reported to produce a selective depletion of peripheral stores of sympathomimetic amines (Plummer, Barrett & others, 1959; Brodie, 1960; Orlans, Finger & Brodie, 1960).

Sixty male Porton Wistar albino rats, 200–250 g, were divided into groups of five. After pretreatment with syrosingopine at 0·4, 0·8 and 2·0 mg/kg (administered in 1 ml/kg dissolved in a mixture of 4% w/v propylene glycol, 4% ethanol and 2% lactic acid in distilled water into the penile vein of rats lightly anaesthetized with halothane), leptazol (65 mg/kg) was administered subcutaneously to the animals 4 h later and the number of clonic phases in the following 30 min period recorded, and expressed as a percentage maximum clonic convulsions (Spencer & Turner, 1969). Control animals received the vehicle intravenously under the same conditions. The mean and standard error of not less than three determinations was calculated. The results are shown in Fig. 1 (a). At 0·4 and 0·8 mg/kg there was no significant decrease of the leptazol threshold but at 2 mg/kg the number of clonic convulsive phases increased 100%. It seemed likely that at 0·8 mg/kg only peripheral amines were depleted and at 2 mg/kg there was a depletion of central amines which produced the marked decrease in leptazol threshold. Therefore, the effect of these two doses of syrosingopine on brain and cardiac amine concentrations was determined.

Thirty male Porton Wistar albino rats, 200–250 g, were divided into three groups of ten. Two groups received the syrosingopine at 0·8 or 2 mg/kg administered as before and the third group received the vehicle under identical conditions. Four h later the animals were killed and the brains and hearts removed for determination of

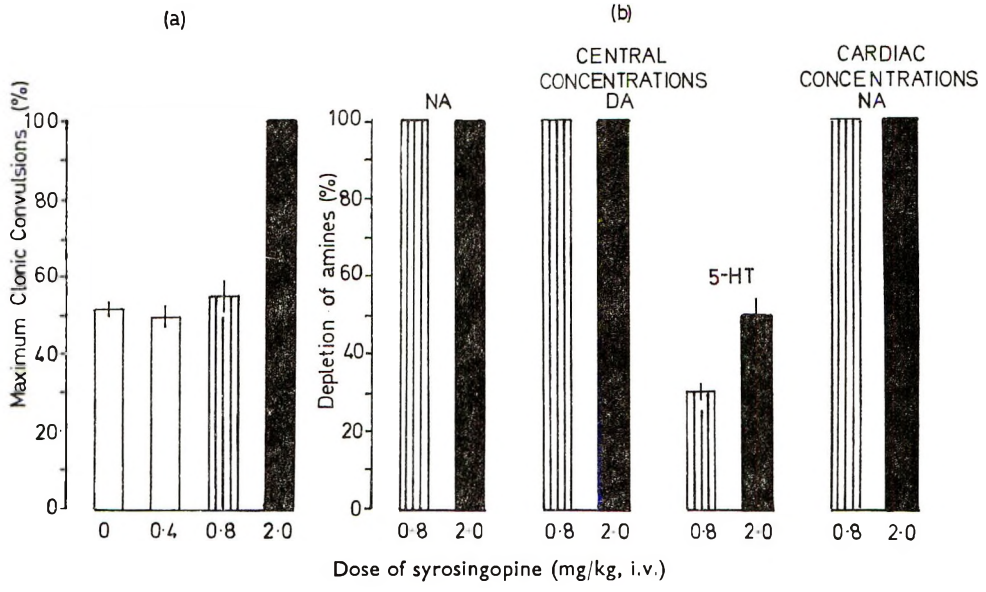

FIG. 1. Effect of 4 h pre-treatment with syrosingopine on (a) the incidence of leptazol-induced clonic convulsions and (b) the whole brain noradrenaline (NA), dopamine (DA) and 5-hydroxytryptamine (5-HT) concentrations and cardiac noradrenaline concentrations in the rat. Vertical bars indicate the standard error of the mean of not less than three determinations.

central noradrenaline, dopamine and 5-HT and cardiac noradrenaline concentrations (Spencer & Turner, 1969). The results are expressed as a percentage depletion of control concentrations. Each result is the mean of five determinations at each dose (Fig. 1 b). Both doses of syrosingopine produced total depletion of central noradrenaline and dopamine, and also cardiac noradrenaline. However, there was a less marked depletion of central 5-HT concentrations which appeared to be dose related.

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Differences in the locomotor activity of mice as measured by an Animex and photoresistor actometer

Recently, a new apparatus for measuring the locomotor activity of small animals has been introduced. The principle is the recording of changes in electromagnetic field (Svensson & Thieme, 1969). The apparatus (Animex) has been used by Svensson & Waldeck (1970), Strömberg (1970) and Corrodi, Fuxe & others (1970). We have noted that the apparatus gave qualitatively different results from those of the photoresistor actometer and we now report some significant differences between the results from the two instruments.

The experiments were made on male Albino Swiss mice, 18–23 g. The motor activity was measured using an Animex apparatus (Farad Electronics—Stockholm) and a photoresistor actometer (with one photoresistor and two light beams). The animals were placed individually in the instruments and their locomotor activity measured for the following 15 min. The experimental schedules and the environmental conditions were identical for both methods.

In reserpinized mice (5 mg/kg, s.c., 16 h before the test), L-dopa (500 mg/kg, i.p., 15 min before the test) increases the number of counts measured on the Animex apparatus at a sensitivity of 40 μA (as well as at 20 μA), but does not change the number of counts measured in photoresistor actometer (Table 1). Similar differences were noted in mice pretreated with α-methyltyrosine (250 mg/kg, i.p., 16 h before the test) and L-dopa (500 mg/kg, i.p., 15 min before test) (Table 1).

In reserpinized mice (5 mg/kg) amantadine (hydrochloride, 40, 80, or 160 mg/kg, s.c. 20 min before the test) markedly increased the number of counts in the Animex apparatus (sensitivity 40 μA) but did not change or only slightly increased (at 160 mg/kg) the number of movements recorded with the photoresistor actometer (Table 2). Similar differences between the results from these two instruments were found in reserpinized animals treated with phenoxybenzamine (20 mg/kg, i.p., 2 h before test) and amantadine (Table 2).

Strömberg, Svensson & Waldeck (1970) reported that the locomotor activity of reserpinized mice in groups of three measured by Animex was markedly increased by amantadine. We repeated this experiment using similar conditions and recording the locomotor activity with both methods. The only difference was that we used a dose of 5 mg/kg of reserpine instead of 10 mg/kg. Reserpine was given intraperi-

Table 1. *Effect of* L-*dopa on locomotor activity in mice pretreated with reserpine or* α-*methyltyrosine.* Drugs were given as follows: reserpine (s.c.) and α-methyltyrosine (i.p.) 16 h, L-dopa (i.p.) 15 min before the test. Locomotor activity was measured in individual animals for 15 min. Statistical significance was calculated with Student's *t*-test as follows: II/I; III/II; V/IV; VI/V; *—$P < 0.05$.

Group	Drug mg/kg	Activity counts Animex Sensitivity 40 μA	Animex Sensitivity 20 μA	Actometer
I	—	419·0 ± 37·1	100·2 ± 18·3	148·2 ± 20·0
II	Reserpine 5	14·0 ± 3·6*	1·2 ± 0·37*	1·6 ± 0·18*
III	Reserpine 5 + L-dopa 500	204·8 ± 82·3*	77·5 ± 30·6*	3·4 ± 2·18
IV	—	549·6 ± 42·8	139·0 ± 14·3	175·5 ± 9·6
V	α-Methyltyrosine 250	20·0 ± 9·60*	1·0 ± 0·21*	4·2 ± 1·32*
VI	α-Methyltyrosine 250 + L-dopa 500	204·1 ± 50·3*	90·1 ± 34·9*	18·0 ± 8·5

Table 2. *Effect of amantadine on locomotor activity in mice pretreated with reserpine or reserpine and phenoxybenzamine.* Drugs were given at following time intervals: reserpine (s.c.) 16 h, phenoxybenzamine (i.p.) 2 h, amantadine hydrochloride (s.c.) 20 min before the test. Locomotor activity was measured in individual animals for 15 min. Statistical significance was calculated with Student's *t*-test as follows: II/I; III, IV, V/II; VI/II; VII, VIII/VI; *—$P < 0.05$.

Group	Drug mg/kg	Activity counts Animex	Actometer
I	—	507·7 ± 7·5	175·5 ± 9·5
II	Reserpine 5	9·75 ± 3·9*	1·4 ± 0·36*
III	Reserpine 5 + amantadine 40	30·1 ± 7·1*	4·4 ± 1·6
IV	Reserpine 5 + amantadine 80	116·8 ± 44·8*	5·5 ± 1·9
V	Reserpine 5 + amantadine 160	240·7 ± 65·7*	19·3 ± 7·6*
VI	Reserpine 5 + phenoxybenzamine 20	6·5 ± 1·7	0·7 ± 0·3
VII	Reserpine 5 + phenoxybenzamine 20 + amantadine 40	89·0 ± 35·5*	5·3 ± 2·9
VIII	Reserpine 5 + phenoxybenzamine 20 + amantadine 80	190·7 ± 86·3*	7·1 ± 4·3

toneally 6 h 40 min before the test and amantadine (hydrochloride, 150 mg/kg, i.p.) 1 h 40 min before the test. The mice were put into the Animex or photoresistor actometer in groups of three, 1 h 30 min after amantadine. The locomotor was counted for 30 min beginning 10 min after the mice were put into the cages.

The results obtained with the Animex were similar to those described by Strömberg & others (1970). The number of counts after reserpine alone was 6·5 ± 1·5, after reserpine and amantadine—105·1 ± 39·9 ($P < 0.05$). The analogous values obtained in the photoresistor actometer were 0·7 ± 0·25 and 12·1 ± 3·6 ($P < 0.01$).

Visual observations revealed that in mice treated with L-dopa or amantadine there was some stimulation expressed as hyperexcitability, small movements, usually with single, uncoordinated jumps. In a light-beam actometer the animal usually does not cross the beam during such movements, and therefore they are not recorded. But in some experiments after amantadine we observed a not very large but statistically significant increase of counts by 30–40% compared with control values. This was probably caused by the fact that the mouse sat close to the light beam. Such an increase has been never observed after treatment with phenoxybenzamine.

With the use of the Animex, various types of movements are measured, including those which are not coordinated. The photoresistor actometer records primarily large, coordinated locomotive movements. The different results obtained with those two instruments may lead to different interpretations, which are exemplified by the experiments with amantadine.

It seems that different results are obtained if the balance between dopaminergic and noradrenargic neuron activity is shifted towards the former, particularly if dopaminergic neurons are stimulated with concomitant partial or total block of noradrenergic neurons.

The differences in the locomotor activity measured with two different automated methods indicate once again the importance of visual observation or animals tested.

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On the action of bombesin on the kidney of the rat and the dog

Bombesin is the tetradecapeptide isolated from extracts of the skin of the European discoglossid frogs *Bombina bombina* and *Bombina variegata variegata* (Anastasi, Erspamer & Bucci, 1971).

We now report the action of bombesin on the kidney of the rat and the dog, and also on the renin-angiotensin system.

Bombesin produced a reduction of urine flow in the rat. In animals anaesthetized with ethanol (5 ml of 15% ethanol per 100 g weight) and then given an intravenous infusion of 2% ethanol (50 μl/min) the threshold dose of bombesin capable of reducing diuresis by intravenous infusion was 50 ng/kg per min; in conscious rats given a water load by oral route the threshold subcutaneous dose was 20–50 μg/kg.

Reduction in urine flow was accompanied by a reduction in glomerular filtration rats (creatinine clearance) and in renal plasma flow (*p*-aminohippurate clearance). In a typical experiment in which bombesin was infused for 30 min at a rate of 100 ng/kg per min reduction of urine flow was 80%, of glomerular filtration rate 75% and of renal plasma flow 68%. Fractional sodium reabsorption (C_{Na}/C_{Cr} %) decreased during antidiuresis, which was apparently counteracted by high sodium intake.

In conscious rats, both hydrated and non-hydrated, 100 μg/kg of bombesin given subcutaneously produced an increase of blood pressure (10–30 mm Hg) lasting more than 2 h. In rats anaesthetized with ethanol, the intravenous infusion of 100 ng/kg per min of bombesin for 30 min initially caused a rise in blood pressure (20–30 mm Hg) which was followed by slow decline and then, after the infusion was discontinued, by a return to normal levels or slight hypotension (10–20 mm Hg). Thus, changes of blood pressure could counteract, but not favour, bombesin antidiuresis.

The effect of bombesin on the dog kidney was intense. In animals anaesthetized with sodium pentobarbitone and given a 5% water load by stomach tube, the threshold dose of the polypeptide producing antidiuresis by rapid intravenous injection was about 0·1 μg/kg, by intravenous infusion 0·5 ng/kg per min, and by subcutaneous route 0·5 μg/kg. The effect was of rapid onset and its duration depended on the dose given. Tachyphylaxis readily occurred, with conspicuous differences from one animal to another. With low doses a fair dose response relation could sometimes be observed; with large doses tachyphylaxis was more prompt and intense. Generally, antidiuresis paralleled the rise in blood pressure produced by bombesin, especially in its duration.

As in the rat, antidiuresis was accompanied by reduction in glomerular filtration rate (creatinine clearance) and in renal blood or plasma flow (washout of ^{85}Kr and ^{3}H-*p*-aminohippurate clearance). The urine eliminated during moderate bombesin antidiuresis (20–50% reduction of urine flow) had a reduced concentration of sodium.

Results obtained in some typical experiments were as follows. 1 ng/kg per min of bombesin infused for 30 min elicited a 50% reduction of urine volume accompanied by a 50% reduction of creatinine clearance and a 48% reduction of *p*-aminohippurate clearance. Component I of the ^{85}Kr washout curve (outer cortical flow) was reduced

by 40–50%, while component II of the same curve (inner cortical flow plus outer medullary flow) showed a 15% increase (see Thorburn, Kopald & others, 1963).

3 ng/kg per min of bombesin infused for 30 min produced reductions of 90% in urine volume, of 93% in creatinine clearance and of 85% in *p*-aminohippurate clearance. Component I of the ^{85}Kr washout curve was reduced by 60–70%, whilst component II was increased by 20%.

At the above dose levels the effect of bombesin on the kidney was prompt and, after discontinuing the infusion, renal circulation and function returned to normal within 40–50 min.

Compared with bombesin, the antidiuretic effect of Val^5-hypertensin was at least 100 times less.

Bombesin given by intravenous route (rapid injection and infusion) nearly always produced a rise of blood pressure which, to some extent, was dose-dependent and was maintained as long as the infusion was continued. After the infusion was discontinued, return to basal levels was prompt for low doses, but took 20–50 min for large doses. Once again the antidiuretic effect of bombesin was counteracted rather than favoured by the effect of the polypeptide on the systemic blood pressure.

Reduction in outer cortical flow was accompanied by a conspicuous renin release, measured by *in vitro* production of angiotensin I, immediately followed by an increase in angiotensin II in arterial plasma. Both events were demonstrated by means of radioimmunological methods.

During the intravenous infusion of 3 ng/kg per min of bombesin for 30 min arterial plasma concentrations of renin activity increased up to 2 times, and of angiotensin II up to 2·5 times. The effect began after 5 min, and reached a peak after 20 min. Discontinuation of the infusion often produced a second peak of renin activity and angiotensin II concentration after 15 min, with return to basal levels within 60 min. Under the above conditions, the renin secretion, as inferred from the difference in renin activity concentration between femoral artery plasma and renal venous plasma multiplied by renal plasma flow, was 8 times the normal.

If bombesin was infused at rates above 8–10 ng/kg min^{-1} a complete blockade of glomerular filtration was observed, together with a limited increase of renin secretion. For example, in an experiment in which bombesin was infused over 4 h at 10 ng/kg per min, renin secretion increased at first slightly but then, beginning at the second hour, fell below the control values. Discontinuing the infusion, a conspicious increase of renin secretion was apparent after 15–30 min, fading away within 120–150 min.

Thus bombesin appears to act on the kidney mainly through a constriction of the afferent glomerular bed in the cortex. This, of course, does not exclude the possibility that the polypeptide has other sites of action.

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Interaction of salicylate and cortisol on lysosomes

The stabilizing action of cortisol on lysosomes is abolished in the presence of salicylate (Lewis, 1970). In this communication we have examined the effect of salicylate on the uptake of cortisol by lysosomes.

The method employed has been described by Symons, Lewis & Ancill (1970). The procedure consists of immersing dialysis bags, containing rat or rabbit liver lysosomes in suspension in 0·05M tris-acetate buffered 0·25M sucrose (pH 7·4), into [^{3}H]-labelled steroid solutions prepared in the same sucrose buffer. In one set of experiments sodium salicylate to a final concentration of 5×10^{-4}M was added to the medium surrounding the dialysis bags.

Acid phosphatase and β-glucuronidase activity was measured in the supernatant obtained from centrifuging the contents of the dialysis bag at 20 000 *g* for 20 min at 4° (Symons, Lewis & Ancill, 1969). The protein concentration of the lysosome suspensions was determined by the method of Lowry, Rosebrough & others (1951).

The results (Table 1), show clearly that the presence of salicylate in the medium inhibited the uptake of cortisol by the organelles prepared from rabbit liver. A similar result was obtained with the organelles obtained from rat liver (Table 2).

Table 1. *The effect of salicylate on the uptake of cortisol by organelles in the dialysis bag.* The source of the organelles was rabbit liver. The results are the mean of two determinations.

Cortisol concn M	Salicylate concn M	Uptake of cortisol per mg protein n mole
5×10^{-3}	nil	35·2
5×10^{-3}	5×10^{-4}	23·9
5×10^{-5}	nil	0·660
5×10^{-5}	5×10^{-4}	0·318

Table 2. *The effect of salicylate on the uptake of steroids by organelles in the dialysis bag and the release of acid phosphatase from lysosomes.* Each result is the mean value ± s.e. of three determinations. The source of the organelles was rat liver.

Cortisol concn M	Salicylate concn M	Uptake of cortisol per mg protein n mol	% Release of lysosomal enzymes compared with control values adjusted to 100%	
			Acid phosphatase	β-Glucuronidase
$2{\cdot}5 \times 10^{-3}$	nil	68·0 ± 4·1	101·2 ± 1·3	102·8 ± 2·0
$2{\cdot}5 \times 10^{-3}$	5×10^{-4}	38·3 ± 3·1	92·7 ± 2·3	96·9 ± 1·1
$2{\cdot}5 \times 10^{-4}$	nil	5·3 ± 0·5	96·9 ± 1·8	91·4 ± 2·4
$2{\cdot}5 \times 10^{-4}$	5×10^{-4}	1·8 ± 0·2	100·3 ± 1·4	101·8 ± 1·1
$1{\cdot}25 \times 10^{-5}$	nil	0·38 ± 0·03	90·8 ± 3·1	91·4 ± 1·1
$1{\cdot}25 \times 10^{-5}$	5×10^{-4}	0·12 ± 0·03	100·3 ± 1·0	102 ± 2·7
$1{\cdot}25 \times 10^{-6}$	nil	0·042 ± 0·003	95·4 ± 2·1	92·6 ± 1·0
$1{\cdot}25 \times 10^{-6}$	5×10^{-4}	0·010 ± 0·002	103·1 ± 1·9	101·3 ± 2·1
$0{\cdot}63 \times 10^{-7}$	nil	0·0016 ± 0·0002	101·2 ± 2·0	100·5 ± 1·0
$0{\cdot}63 \times 10^{-7}$	5×10^{-4}	0·0004 ± 0·0001	102·2 ± 2·3	99·6 ± 1·6

0·63 × 10⁻⁷ 5 × 10⁻⁴ 0·0004 ± 0·0001

In addition, the presence of salicylate has clearly abolished the stabilizing action of the cortisol on the lysosomes.

It would appear that salicylate inhibits the action of cortisol on lysosomes by inhibiting the entry of the steroid into the membrane.

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Effect of eserine injected intraventricularly on behaviour and on activity of cholinesterase in some structures of the cerebral ventricles of the conscious cat

The role of cholinesterase of various brain structures in behavioural changes after intraventricular injection of drugs acting on cholinoceptors is ill-understood. We now report the inhibition of acetylcholinesterase activity in the structures underlying the cerebral ventricles and relate this with the appearance of gross behavioural changes after intraventricular injection of eserine.

Cats of either sex, 1·9–4·4 kg were anaesthetized with pentobarbitone sodium (30–40 mg/kg, i.p.) and a cannula (Feldberg & Sherwood, 1953) was screwed into the skull through which injections were made in conscious cats. Eserine was injected intraventricularly in a volume of 0·2 ml and saline, 0·2 ml, was injected into the cerebral ventricles of controls. Thirty min after injecting eserine or saline the animals were decapitated. Brains were removed and immediately chilled in ice. Acetylcholinesterase activity of slices (Milošević & Andjelković, 1966), 0·5 mm thick, of superficial layers of caudate nucleus, thalamus, anterior and posterior hypothalamus, was measured manometrically in the Warburg apparatus (Umbreit, Burris & Stuffer, 1957). Acetylcholine (0·01 mM, final concentration) was the substrate. Enzyme activity was expressed in μl carbon dioxide liberated per mg of fresh tissue.

By increasing the dose of eserine, the degree of the inhibition of acetylcholinesterase activity was simultaneously increased in the caudate nucleus (Table 1). Acetylcholinesterase activity was inhibited with the highest doses of eserine in the thalamus and hypothalamus.

With a small dose of eserine (0·02 mg), when the acetylcholinesterase activity was inhibited in the hypothalamus, itching and sometimes ataxia developed.

Table 1. *Acetylcholinesterase activity in superficial layers of the caudate nucleus, thalamus, anterior and posterior hypothalamus of conscious cats after intraventricular injections of eserine.* There were six experiments with each dose.

Eserine (mg)	Inhibition (%) in: Caudate nucleus	Thalamus	Hypothalamus anterior	Hypothalamus posterior
0·02	—	—	—	12
0·1	17	—	—	41
0·4	29	—	11	35
1·0	24	—	15	33
2·0	51	44	21	26

But, by increasing the dose of eserine (0·1–1·0 mg), when the symptomatology of autonomic nervous system (mydriasis, hippus, withdrawal of nictitating membrane, piloerection, salivation and lacrimation), akathisia, agitation with signs of fear and anger, rage, increase in depth and rate of respiration and motor disturbances (changes in gait and posture with myoclonic jerks, tremor, athetoid and choreiform hyperkinesias and circling movements) developed, the degree of the inhibition of the acetycholinesterase activity occurred to about the same extent (about 30%) as that in the caudate nucleus and hypothalamus. Moreover, when clonic-tonic convulsions developed after 2 mg of eserine, most of which experiments death occurred within 20 min, the highest degree of inhibition of acetylcholinesterase activity was found in the caudate nucleaus and thalamus.

The results obtained in these experiments indicate that the acetylcholinesterase activity paralleled motor disturbances in the caudate nucleus. With the smallest dose of eserine, motor disturbances have been seldom seen and no changes in the activity of acetylcholinesterase occurred. By increasing the dose of eserine, motor disturbances strengthened and the acetylcholinesterase activity gradually decreased. In the cat, small, unilateral lesions, that damage exclusively the anteroventral region of the caudate nucleus, produce stable and permanent behavioral changes resembling human athetoid and choreiform hyperkinesias (Liles & Davis, 1969). Thus, it is possible that a neurohumoral imbalance produced by a cholinesterase inhibitor can cause motor disturbances mainly originating from the caudate nucleus. Finally, when the inhibition of acetylcholinesterase activity amounted to 50%, clonic tonic convulsions appeared.

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Excitatory effect of tetrodotoxin on an isolated smooth muscle organ

Tetrodotoxin has been shown to abolish action potentials by interfering with sodium conductance in nerve fibres (Narashi, Moore & Scott, 1964). Smooth muscle reactivity is not affected by this action and thus tetrodotoxin is recommended as a convenient "denervating" agent (cf. Gershon, 1967) in experiments on isolated innervated smooth muscle organs. The present report describes a stimulant action of tetrodotoxin in the isolated cat sphincter of Oddi.

It is possible to isolate the cat sphincter of Oddi from surrounding duodenal tissue and use it for experiments *in vitro*. The sphincter is used as a tubal preparation mounted longitudinally in oxygenated Krebs solution of 37° (Persson, 1971). The sphincter exhibits spontaneous rhythmic activity which is shown as simultaneous longitudinal activity and increased resistance to flow through the isolated sphincter (Fig. 1) when it is constantly perfused at a rate of 3 ml/h. The activity of the sphincter is not affected by atropine or by phenoxybenzamine and is present in the sphincter taken from reserpinized cats (Persson, 1971).

Tetrodotoxin (812093 B Sankyo Japan) (0·1–1 μg/ml) did not prevent the activity of the isolated sphincter of Oddi (Persson, 1971) but caused the sphincter to contract, increasing the amplitude and the frequency of contraction (Fig. 1). All ten investigated sphincter preparations reacted in the same way but to a various degree.

The effect of tetrodotoxin was dose-dependent and the response diminished and changed pattern after a few minutes while tetrodotoxin still was left in the organ bath (Fig. 1). Tachyphylaxis to repeated doses also developed but might at least partly be ascribed to the property of the preparation to show decreased reactivity to pharmacological agents together with decreased spontaneous activity with time (Persson, 1971). The spontaneous activity of the sphincter usually disappeared about 3 h after the mounting of the sphincter in the bath. Then tetrodotoxin (0·1–1 μg/ml) was able to transiently induce the activity again. Tetrodotoxin also potentiated the excitatory effect of noradrenaline and acetylcholine on the sphincter. This effect was most defined on the non-active sphincters and when tetrodotoxin was given in threshold active doses before addition of noradrenaline and acetylcholine (Fig. 2). Atropine and phenoxybenazmine in effective anti-acetylcholine and α-adrenoceptor

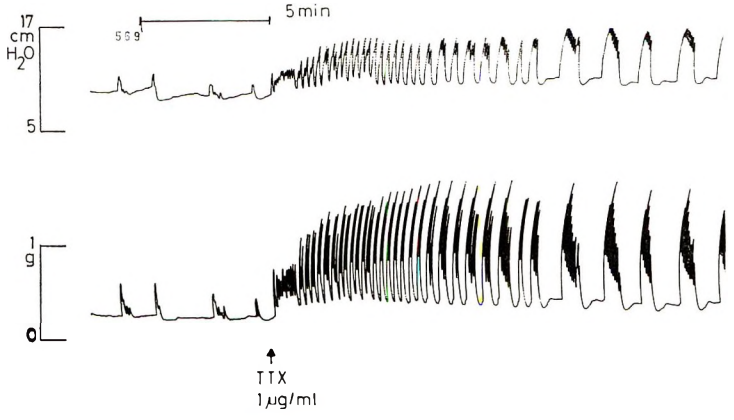

FIG. 1. Effect of tetrodotoxin (TTX) on the isolated sphincter of Oddi mounted longitudinally and perfused at a constant rate (3 ml/h). *Upper curve:* Resistance to flow thrugh the sphincter recorded as pressure changes in the perfusion catheter. *Lower curve:* Longitudinal tension changes. Tetrodotoxin is added to the bathing solution.

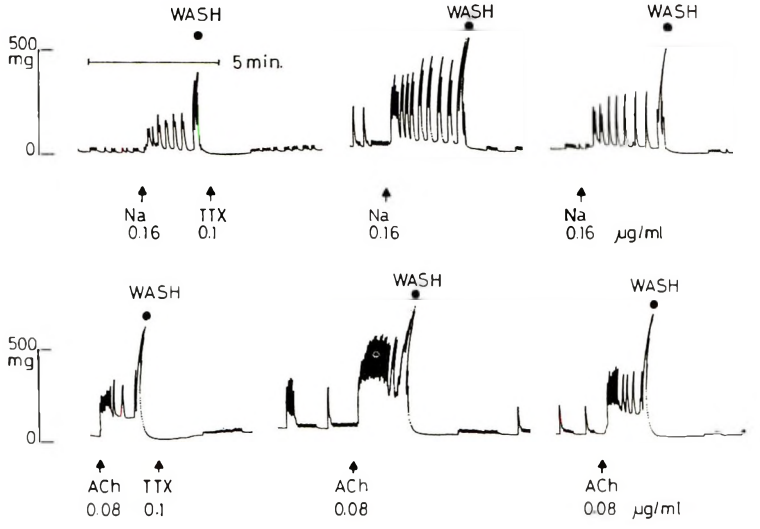

FIG. 2. *Upper curve:* Longitudinal activity of the isolated sphincter. Tetrodotoxin (TTX) 0·1 µg/ml potentiates the effect of noradrenaline (Na). *Lower curve:* Longitudinal activity of the isolated sphincter. Tetrodotoxin (TTX) 0·1 µg/ml potentiates the effect of acetylcholine (ACh).

blocking concentrations (0·1 µg/ml) did not prevent the excitatory action of tetrodotoxin. Atropine and phenoxybenazmine was left in contact with the sphincter for 10–40 min before tetrodotoxin was added to the bath.

Four other different smooth muscle preparations with similar myogenic spontaneous activity to the sphincter were included in the study: cat duodenum mounted as a 3 cm long whole piece (10 preparations) or as strips of the longitudinal muscle (5 preparations), rabbit jejunum (10 preparations) and uterus (6 preparations) from oestrogen-treated rats. In none of these isolated organs did tetrodotoxin (0·1–1 µg/ml) increase the spontaneous activity or affect the response to acetylcholine or noradrenaline.

Results from isolated smooth organs suggest that the sphincter has a unique tetrodotoxin sensitive property. Possibly the potentiation of cardiovascular response to noradrenaline and tyramine by tetrodotoxin as reported by Bell (1968) might be a similar effect. It has been stressed by Carter (1969), who showed that part of splanchnic nerves was resistant to tetrodotoxin, that tetrodotoxin should not be used blindly as a selective nerve-inhibiting agent in experiments with innervated smooth muscle organs. The effect of tetrodotoxin on the sphincter of Oddi is another effect of the agent that should be considered when utilizing it as a chemically "denervating" agent.

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Prolonged effects of reserpine administration on adrenoceptor activity in dogs

This report is a part of an investigation dealing with a group of mongrel dogs (10 to 12 kg) treated with 0·137 mg of reserpine (Serpasil-Ciba) twice a day for twelve months, and an equal number receiving an inert control substance. Femoral and mesenteric arterial strips were obtained from these animals under sodium pentobarbitone anaesthesia and prepared according to Furchgott & Bhadrakom (1953). The strips were suspended in Krebs-Hanseleit solution and isometric contractions measured after 2 h of stabilization with 4 g tension. Two cumulative dose-response curves for noradrenaline (base) were obtained by adding progressively higher doses at $1/2 \log_{10}$ intervals. The strips were exposed to phentolamine (base) ($1{\cdot}66 \times 10^{-6}$ g) for 15 min and cumulative dose-response curves to noradrenaline again were obtained. Regression line equations were calculated from the responses between 20 and 80%. The points corresponding to 25, 50, and 75% of maximum responses were then pooled and the mean value was used to plot Fig. 1. pD_2 for noradrenaline (negative log of molar concentration of drug producing 50% of the maximal effect) and pA_2 for phentolamine (negative log of the molar concentration of drug reducing the effect of a double dose of noradrenaline to that of a single dose) were calculated as described by van Rossum (1963) (Table 1).

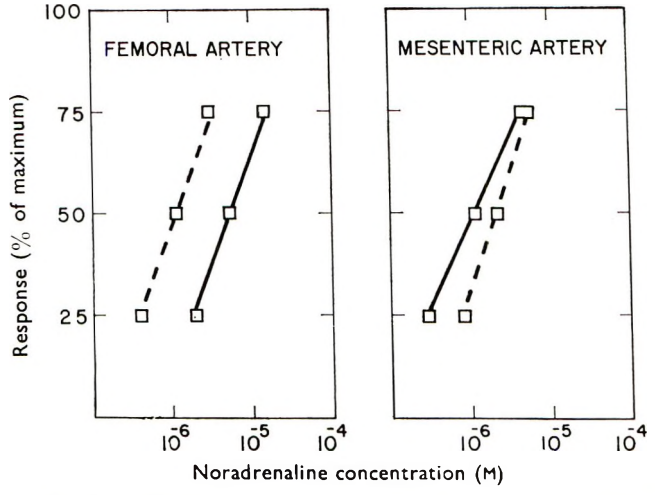

FIG. 1. Cumulative dose-response curves for femoral and mesenteric arterial strips obtained from reserpine-treated and control dogs. Percent of maximum responses to various molar concentrations of noradrenaline are represented. Broken line (□- - - - -□) indicates reserpine-treated group and the solid line (□——□) control group.

Table 1. *pD_2 for noradrenaline and pA_2 for phentolamine from cumulative dose-response curves of spirally cut femoral and mesenteric arterial strips on reserpine-treated and control dogs.*

	Mesenteric		Femoral	
	Reserpine mean ± s.e.	Control mean ± s.e.	Reserpine mean ± s.e.	Control mean ± s.e.
pD_2	5·66 ± 0·16	5·93 ± 0·20	5·96 ± 0·12*	5·27 ± 0·07*
pA_2	7·15 ± 0·10	7·23 ± 0·15	7·33 ± 0·13	7·29 ± 0·12

* Statistically different ($P < 0{\cdot}01$). A = 4.

Acute administration of reserpine has been reported to produce supersensitivity in structures innervated by the sympathetic nervous system. Enhanced α-adrenoceptor activity was noted in aortic strip of rabbit (Taylor & Green, 1971), in dog isolated carotid arteries (MacMillan, Smith & Jacobsen, 1962), in perfused femoral vessels of dogs (Carrier & Holland, 1965), and in the cat nictitating membrane (Fleming & Trendelenburg, 1961) after acute or subchronic administration of reserpine. We found a 5 fold increase in the sensitivity of the α-adrenoceptors in the femoral arterial strips of the reserpine-treated dog; but no such alteration was noted in the receptor sensitivity in the mesenteric strips from the same animals. Clarke, Adams & Buckley (1970) found there was a significant reduction in the responses of perfused mesenteric vessels of the treated dogs to sympathetic nerve stimulation, no significant changes were observed in α-adrenoceptor activity. It, therefore, seems that adrenoceptor sensitivity in vascular tissues or beds is not uniformly influenced by chronic reserpine treatment. Prolonged reserpine treatment did not produce any qualitative or quantitative alteration in receptors, as indicated by the pA_2 values for phentolamine obtained both in treated and control animals. This observation confirms the findings of Taylor & Green (1971).

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Evidence for a new type of dopamine receptor stimulating agent

It is well known that apomorphine is a shortlasting dopamine receptor stimulating agent (Ernst, 1967; Anden, Dahlström & others, 1967) an action which probably is the neurochemical basis for its marked stimulation of locomotion and stereotyped activities (see Randrup & Munkvad, 1968). It decreases dopamine turnover probably as a result of stimulating the dopamine receptor eliciting a feedback which causes a compensatory reduction in the activity of the dopamine neurons. Furthermore, in rats in which degeneration of one nigro-neostriatal dopamine pathway has been induced by 6-hydroxydopamine (Ungerstedt, 1968, 1971), apomorphine will cause rotation of the rats towards the unoperated side, probably as a result of development of supersensitivity of the dopamine receptors on the denervated side. Therefore, the operated side will become overactive compared with the intact side. Amphetamine, on the other hand, which is a catecholamine-releasing agent (Carlsson, Fuxe & others, 1966), will make the intact side overactive and cause rotation of the animal towards the operated side, since no dopamine release will occur on the denervated side. On the basis of its dopamine receptor stimulating property the potential usefulness of

apomorphine in treatment of Parkinson's disease has been pointed out (Cotzias, Papavasilou & others, 1970) and has, in fact, been shown recently (Düby, Cotzias & others, 1971). Therefore, for some time we have tested drugs in the turnover and rotation models mentioned above for a possible dopamine receptor stimulating property in order to develop new therapeutic tools in the treatment of Parkinson's disease. Apomorphine derivatives have so far proved to lack activity or to be only weakly active in the case of 7- and 11-hydroxy-apomorphines (Granchelli, Neumeyer & others, 1971). In the present paper evidence has been obtained that 7-(2''-pyrimidyl)-4-piperonyl-piperazine (ET495, Servier; Regnier, Canevari & others, 1968) when given systemically is a powerful dopamine receptor stimulating agent.

Male Sprague-Dawley rats (150–180 g) were used.

Chemical experiments. The effects of ET495 on dopamine and noradrenaline turnover in the brain have been examined with the help of the tyrosine-hydroxylase inhibitor α-methyl-tryosine-methylester (H44/68) (Corrodi & Hansson, 1966; Andén, Dahlström & others, 1966) biochemically and histochemically (Bertler, Carlsson & others, 1958; Carlsson & Waldeck, 1958; Carlsson & Lindqvist, 1962; Falck, Hillarp & others, 1962). ET495 was given intraperitoneally 15 min before the inhibitor. The biochemical determinations were made on whole brain (the numbers of rats used are given in Table 1) and the histochemical observations (made on some 40 rats) on the neocortex, the neostriatum and the hypothalamus.

Functional experiments. The effects of ET495 and apomorphine on dopamine receptor activity were evaluated in the rotometer model described (Andén, Dahlström & others, 1966; Ungerstedt, 1968, 1971). The rotations were registered quantitatively in a specially designed "rotometer" (Ungerstedt & Arbuthnott, 1970). Doses of ET495 were from 1–150 mg/kg (i.p) and 4 rats per dose were tested.

The biochemical results are summarized in Table 1. With a dose of 50 mg/kg of ET495 there was a reduction of H44/68-induced depletion of dopamine and an increase in the H44/68-induced depletion of noradrenaline. The histochemical results revealed similar changes. In a dose of 50 mg/kg, ET495 caused a reduction in the rate of H44/68-induced disappearance of dopamine fluorescence from the neostriatum and the limbic forebrain, and the rate of disappearance of noradrenaline fluorescence from the neocortical and the hypothalamic noradrenaline nerve terminals was increased. With a dose of 15 mg/kg of ET495, while a retardation of disappearance of dopamine fluorescence was observed there were no observed effects on the disappearance of noradrenaline fluorescence.

In the rotometer model it was found that ET495 induced rotations towards the unoperated intact side in a dose-dependent manner mimicking the action of apomorphine. The effects of ET495 were rapid in onset and lasted for 7–10 h at a dose of

Table 1. *Noradrenaline (NA) and dopamine (DA) concentrations in whole rat brain after treatment with ET495 i.p. followed by H44/68 treatment 15 min later (250 mg/kg, i.p.).* Four h after H44/68 the animals were killed and the catecholamines determined spectrofluorimetrically. Values in ng/g ± s.e. (n = number of experiments). Statistical evaluation by analysis of variance.

Treatment	Dose (mg/kg)	n	DA	NA
No drug treatment		10	515 ± 10	455 ± 10
ET495	50	4	541 ± 17	423 ± 13
H44/68		16	155 ± 6	225 ± 7
ET495 + H44/68	50	4	274 ± 16	115 ± 9
Significance of differences between H44/68 alone and ET495 + H44/68				$P < 0.001$

50 mg/kg in contrast to the short-lasting action of apomorphine. Clearcut rotations were observed at doses down to 1–5 mg/kg, and at 25 mg/kg a total of about 1000 rotations were observed. Apomorphine was effective in doses down to 0·1 mg/kg, but only short-lasting effects (30 min) were obtained (see Ungerstedt, 1971). With higher doses, stereotyped licking and biting behaviour was induced which abolished the rotation behaviour. This was not observed with ET495.

Experimental evidence has thus been given for the view that ET495 and/or an active metabolite is a new dopamine receptor stimulating agent with powerful and prolonged actions as revealed in both the amine turnover and the rotational model. In both these models the drug mimicks the effects of apomorphine, but not that of amphetamine, by reducing dopamine turnover and causing stimulation of the dopamine receptors in the denervated neostriatum. The reduction of dopamine turnover is probably the result of the dopamine receptor stimulation inducing a compensatory feedback to reduce activity in the dopamine neurons. In higher doses an increase in the noradrenaline turnover was found which may be secondary to the dopamine receptor stimulation (see Persson & Waldeck, 1970) or e.g. due to a direct action of ET495 on the noradrenaline neurons. The present results suggest ET495 has potential in the treatment of parkinsonism.

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Reduction of histamine in mouse brain by N^1-(DL-seryl)-N^2-(2,3,4-trihydroxybenzyl) hydrazine and reserpine

The monoamines, noradrenaline, dopamine and 5-hydroxytryptamine, are generally accepted to be involved in the functioning of the mammalian central nervous system. Histamine has also been suggested to be similarly involved, but the amount of evidence is less (see Green, 1970). In comparison to the monoamines, few drugs have been found to alter the concentrations of histamine in mammalian brains and wide species variations occur.

Reserpine is a classical depletor of the monoamines from mammalian brains. It blocks their granular uptake-storage mechanism and the resulting depletion approaches 100%. In contrast, only a 55–65% reduction of histamine in cat brain, and no reduction in rat brain, has been obtained after reserpine (Adam & Hye, 1966; Green & Erickson, 1964).

Inhibitors of the specific histidine decarboxylase enzyme have been widely used to deplete histamine in peripheral tissues, the two most commonly used being 4-bromo-3-hydroxybenzyloxyamine (NSD 1055) and α-hydrazinohistidine (MK 785). Using these drugs, Taylor & Snyder (1971) obtained a reduction of 35–40% in rat endogenous hypothalamic histamine.

Inhibitors of the aromatic amino-acid decarboxylase have been widely used to deplete monoamines, both in peripheral and central tissues. N^1-(DL-Seryl)-N^2-(2,3,4-trihydroxybenzyl) hydrazine (Ro 4-4602) has been frequently used, depletions of around 50% having been obtained in rat and mouse brain (see Pletscher, Gey & Burkard, 1965).

Here, the reduction of histamine in mouse brain by reserpine and Ro 4-4602 is reported.

Groups of 6 white female mice (NMRI), 18–24 g, were injected with reserpine, Ro 4-4602 or saline, alone or in combination, as indicated in Table 1. The animals were decapitated and each brain was rapidly excised and homogenized in ice-cold perchloric acid containing sodium metabisulphite and di-sodium ethylenediamine tetra-acetate. The extract of 6 pooled brains was used for the analysis of histamine. Histamine was isolated on a Dowex 50W-X4 cation exchange column and assayed spectrophotofluorimetrically using orthophthaldialdehyde as outlined by Atack & Magnusson (1970), except that, after the elution of the 5-hydroxytryptamine, histamine was eluted with 2 N aqueous HCl (4·5 ml). Spermidine has since been established to be completely separated from, and elutable after, histamine in this column procedure.

The effects of reserpine and Ro 4-4602 on the concentration of histamine in the entire mouse brain are indicated in Table 1. The significant depletion of histamine by reserpine alone is small (about 11%), but a similar depletion (about 15%) was

Table 1. *Histamine concentration in mouse brain after different drug treatments. The mice were kept at 30° for 5 h before death.*

Treatment	Histamine ng/g ± s.e. (n)
A. Controls, saline, i.p.	48·3 ± 3·344 (5)
B. Reserpine, 5 mg/kg, i.p., 7 h before death	42·8 ± 1.650 (5)
C. Ro 4-4602, 800 mg/kg, i.p., 2 h before death	32·0 ± 2·333 (5)
D. Reserpine + Ro 4-4602, doses and time intervals as above	27·3 ± 3·255 (5)

Statistical analysis of variance followed by *t*-test:
A–B, $P < 0.025$; A–C, $P < 0.001$; C–D, $P < 0.025$.

obtained after the combined administration of reserpine and Ro 4-4602 compared to the depletion caused by Ro 4-4602 alone (about 34%) which was highly significant. The possibility that this latter reduction is related to the concomitant lowering of the monoamines by the Ro 4-4602 is unlikely, since many drugs known to alter the concentrations of the monoamines in mouse brain were without effect on the histamine concentrations (Atack, unpublished data).

Probably, the reduction of histamine concentration was caused by the inhibition of an enzyme converting histidine to histamine. The specificity of inhibitors of the specific histidine decarboxylase and of the aromatic amino-acid decarboxylase has not been unequivocally established (see Aures, Håkanson & Clark, 1970). Schwartz, Lampart & Rose (1970) have identified an enzyme in rat brain with physical properties resembling those of the peripheral specific histidine decarboxylase and which was inhibited by NSD 1055 but not by α-methyldopa, the latter being a specific inhibitor of the aromatic amino-acid decarboxylase. Taylor & Snyder (1971), whilst obtaining the 35–40% reduction in rat endogenous hypothalamic histamine with NSD 1055 and MK 785, found no effect with α-methyldopa, thus suggesting the involvement of only the specific histidine decarboxylase.

Therefore, whilst Burkard, Gey & Pletscher (1964) found no effect of Ro 4-4602 on a bacterial specific histidine decarboxylase *in vitro*, the effect of this drug in reducing the concentrations of histamine in mouse brain is more likely to be the result of an inhibition of the specific histidine decarboxylase. The influence of α-methyldopa on histamine concentrations in mouse brain should help to elucidate this problem.

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Endogenous, possibly prostaglandin-mediated inhibition of the neuromuscular transmission in the vas deferens

Recently it was reported (Swedin, 1971) that the mechanical response of the rat and guinea-pig isolated, nerve stimulated vas deferens is divided into two separate phases when the usual 5 s period of stimulation is extended to 30 s (cf. Fig. 1). It was observed that repeated periods of prolonged nerve stimulation, either as field stimulation or via the hypogastric nerve (2–25 Hz, supramaximal voltage) depressed especially the initial, rapid phase ("twitch") of contractions of the organ (Fig. 1). Some features of this depression will be reported here.

The following findings led to the conclusion that the inhibition was induced by some substance, released together with the neurotransmitter upon nerve stimulation. (i) The inhibition was immediately abolished on washing of the organ (Fig. 1). (ii) The inhibition appeared faster and was more complete in an organ bath of small volume than in one of a greater volume (Fig. 2). (iii) The substance with its inhibiting properties could be transferred from one bath to influence an organ in another in a dose-dependent way (Fig. 2).

The inhibitory factor is probably acting at a prejunctional level, since tests with exogenous noradrenaline (0·1–2·0 μg/ml) revealed unaltered responses of the preparation after periods 30 s stimulation.

That the inhibitory agent is related to prostaglandins is supported by the following result. (i) The inhibitory agent influences both phases of contraction, the second

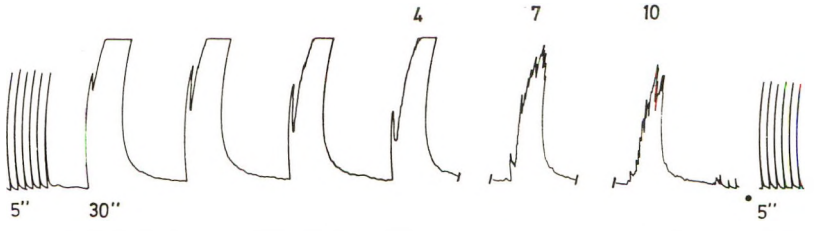

FIG. 1. Isolated field stimulated (5 and 30 s) guinea-pig vas deferens in 5 ml bath. 20 V, 1·5ms duration, 5 Hz. 1 min rest between stimulations. At dot washing. Number of 30 s contractions indicated above the contractions.

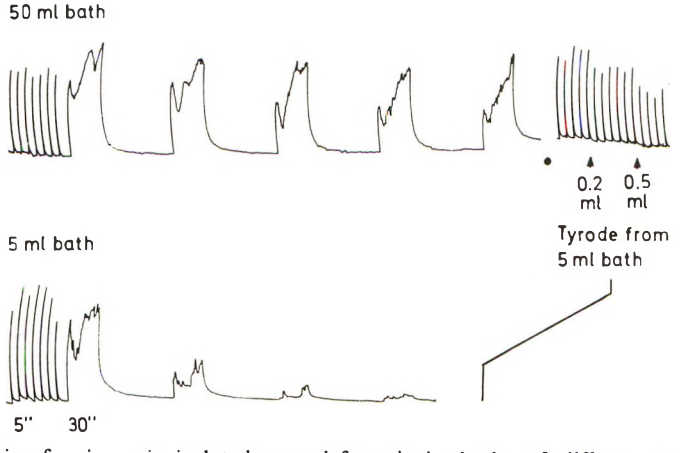

FIG. 2. A pair of guinea-pig isolated vasa deferentia in baths of different volumes. Same amplificiation. Hypogastric nerve stimulation at 25 V, 1·5 ms duration, 7 Hz. 1 min rest between stimulations. At dot washing. After four 30 s stimulations of the organ in the small bath, 0·2 and 0·5 ml of the Tyrode solution was transferred to the 50 ml bath as indicated.

phase, however, to a less extent, as do exogenously administered PGE_1 and PGE_2 (1–50 ng/ml). (ii) The ganglionic relay in the peripheral part of the hypogastric nerve was extremely sensitive to the inhibitory substance (cf. Fig. 2), and the same was found for exogeneous PG (0·1–0·4 ng/ml). (iii) Incubation of the vas deferens with an inhibitor of PG synthesis (5,8,11,14-eicosatetraynoic acid, ETA, 1–20 μg/ml) (Downing, Ahern & Bachta, 1970) led to a partial or total abolishment of the endogeneous inhibition. (iv) Release of prostaglandin-like material, mainly resembling PGE_2, from the vas deferens on nerve stimulation, was established by silicic acid thin-layer chromatography (Gréen & Samuelsson, 1964).

It can be concluded from the present results that the mechanical response of the isolated vas deferens to nerve stimulation is under a dual influence from excitatory and inhibitory agents. Several experimental data support the hypothesis that the endogenous inhibition could be due to release of prostaglandins that are acting by restricting the amount of noradrenaline liberated from adrenergic nerves by stimulation (cf. Hedqvist, 1970; Wennmalm, 1971). With regard to the effectiveness of the autoinhibition described even at very low frequencies of stimulation (2–5 Hz), it is tempting to suggest that this process might play a modulating role in the nerve-induced mechanical activity of this organ in *in vivo* conditions, as recently suggested by Wennmalm (1971) for the rabbit heart. Furthermore, the results provide the first indications of an inhibitory action of PG on ganglionic neuro-transmission.

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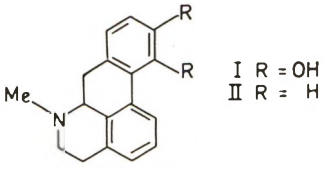
On the dopamine-like action of apomorphine

Apomorphine (I) elicits a gnawing compulsion in rats which is due to stimulation of inhibitory dopaminergic neurons in the corpus striatum (Ernst, 1967, 1969; Ernst & Smelik, 1966; Ungerstedt, Butcher, & others, 1969). It apparently reduces the impulse flow of these neurons by a negative feedback mechanism arising from dopamine receptor stimulation, although an indirect mechanism involving the release of dopamine from central stores has also been implicated (Fekete, Kurti & Pribusz, 1970). However, pretreatment with iproniazid does not potentiate apomorphine-induced gnawing (Ernst, 1967), and apomorphine both retards the utilization of brain dopamine (Andén, Rubenson, & others, 1967) and inhibits its biosynthesis (Goldstein, Freedman, & Backstrom, 1970). It is therefore likely that apomorphine acts directly



I R = OH
II R = H

on central dopamine receptors in producing gnawing effects, and it also appears to produce peripheral vasodilation by an action upon peripheral dopamine receptors (Yeh, McNay & Goldberg, 1969). From molecular orbital calculations, it has been concluded that the important part of the apomorphine molecule in its interaction with dopamine receptors is not the dihydroxytetrahydroaminonaphthalene moiety but the tetrahydroisoquinoline moiety (Kier & Truitt, 1970). We dispute this conclusion, and our results, and those of others, show it to be erroneous.

Gnawing was measured in male Porton rats (200–250 g) in metal cages with wire grid bottoms (Ernst, 1967). Apomorphine hydrochloride (5 mg/kg, i.p.) consistently produced a syndrome of stereotyped licking accompanied by periods of gnawing, in both normal and iproniazid-pretreated rats. Aporphine (II), tetrahydroisoquinoline, *N*-methyltetrahydroisoquinoline, or phenethylamine, administered (i.p.) as their hydrochlorides, produced no gnawing or licking movements at doses up to 20 mg/kg. The five compounds were also compared with dopamine for depressor effects on the blood pressure of urethanized rabbits (Burn & Rand, 1958). Apomorphine was twice as potent as dopamine (minimum effective dose, 0·05 mg/kg, i.v.) while aporphine had less than one-fifth the potency of dopamine; the other compounds produced pressor effects except for *N*-methyltetrahydroisoquinoline which was inactive.

If Kier and Truitt are correct, then one would have expected that aporphine and the two isoquinoline derivatives would have had apomorphine-like effects on dopamine receptors, while phenethylamine might also attain a folded conformation corresponding to that proposed for the dopamine pharmacophore. The structural requirements for dopamine-like renal vasodilatation are very specific (Goldberg, Sonneville & McNay, 1968), and phenethylamine and the two isoquinolines (Hanna & Shutt, 1953) do not have vasodilator properties in contrast to apomorphine. Our results support these findings and suggest that similar structural specificity exists in the central nervous system. Apomorphine is reported to be inactive on dopaminergic neurons in *Helix aspersa* (Woodruff & Walker, 1969) but the invertebrate nervous system may well differ from that in the mammal. Our thesis is therefore that if apomorphine, in producing dopamine-like effects, acts on dopamine receptors then it does so in a way which intimately involves the dihydroxytetrahydroaminonaphthalene moiety.

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