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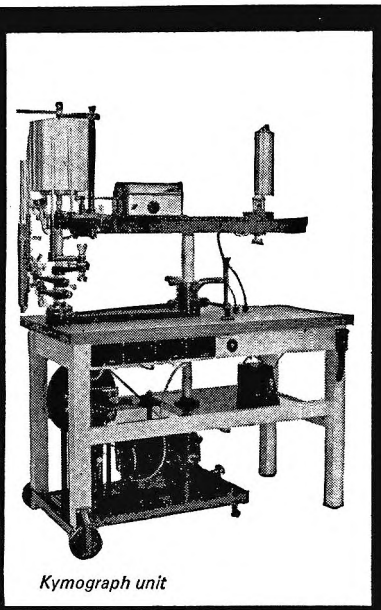

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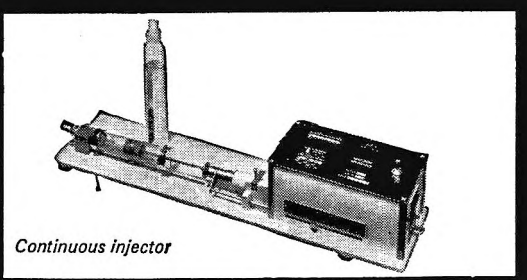

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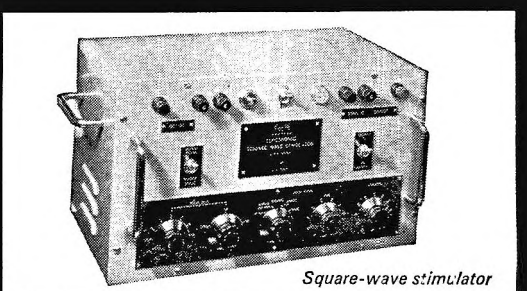

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Simulation of the apparent effects of mebanazine on growth hormone by pair-feeding of control animals

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The degree and duration of insulin hypoglycaemia was potentiated by chronic oral medication with mebanazine in rats. Hypophysectomy alone increased sensitivity to insulin but did not abolish the potentiating effect of mebanazine. Chronic mebanazine treatment (15 mg/kg/day) for 6 weeks markedly reduced weight gain, food and water consumption and pituitary growth hormone content, but the results were not significantly different from those in unmedicated pair-fed controls. Similarly, immature rats treated with mebanazine had a significant reduction in the width of the tibial epiphysial cartilage but this was not different from that in pair-fed animals. After 18 h of fasting, acute administration of mebanazine had little effect on food consumption in the 2 h period following dosing but a significant effect over 24 h. In fed rats mebanazine in a single oral dose significantly reduced eating in the following 6 h. Treatment with mebanazine at 2·5 mg/kg for 15 days significantly reduced food intake but did not potentiate insulin hypoglycaemia. From the results it would appear that previous suggestions that mebanazine specifically interferes with growth hormone release are incorrect and the findings emphasize the importance of measuring food intake in experiments of long duration.

Long-term administration of the monoamine oxidase inhibitor mebanazine to rats has been shown to potentiate the hypoglycaemic effects of both insulin and tolbutamide (Barrett, 1965). The results were compatible with the hypothesis that mebanazine treatment interfered with the adrenergically mediated mechanisms for combating low circulating glucose levels. It was proposed that the pattern of insulin potentiation and hypotensive episodes during the clinical use of mebanazine (Wickström & Pettersson, 1964; Cooper & Keddie, 1964) might have a common origin. However, it was recognized that an alternative explanation of the increased sensitivity to insulin could derive from an alteration in the balance between insulin and pituitary growth hormone after the production of hypoglycaemia. Experimental evidence purporting to demonstrate that mebanazine does inhibit the secretion of growth hormone has been reported (Zor, Dikstein & Sulman, 1965a,b). The work now reported presents the results of experiments designed to discover the relation between the effects of mebanazine on growth hormone and insulin potentiation. A preliminary account of this study has been presented to the British Pharmacological Society (Barrett, 1966).

EXPERIMENTAL

The animals were male albino rats from the specific pathogen-free strain bred at Alderley Park. In most experiments they weighed between 190–230 g except where

immature rats were used for tibial tests (35–45 g), and were maintained on a cubed diet and water *ad libitum*. In some experiments food intake was restricted for purposes of pair-feeding controls. Hypophysectomy was performed by the parapharyngeal approach with subsequent maintenance on 5% glucose solution in place of water. The animals were used 2 weeks after surgery and completeness of hypophysectomy was checked visually after death. Blood samples were obtained from the abdominal aorta after intraperitoneal pentobarbitone sodium anaesthesia. Each experiment involved groups of 5 animals except for the initial experiment where there were only 4. Blood glucose was estimated by a kit glucose-oxidase method, and growth hormone by the tibial test (Papkoff & Li, 1962).

Mebanazine oxalate was administered orally or intraperitoneally in aqueous solution and soluble insulin subcutaneously. The rats were weighed daily and food and water consumption recorded for groups of 5 rats.

RESULTS

The hypoglycaemic response to insulin was significantly potentiated and prolonged by oral pretreatment with mebanazine (15 mg/kg daily) for a period of 3 weeks. The results are summarized in Table 1. In the control animals, blood sugar levels had returned to pre-insulin levels within 3 h whereas those of the mebanazine-treated rats had only recovered to 50% of the initial values in the same time interval. Chronic administration of mebanazine did not significantly alter the resting blood sugar concentration.

Rats which have been hypophysectomized are more sensitive to the hypoglycaemic actions of insulin and it was found that they were unable to tolerate the same doses of mebanazine as intact rats. It was possible however, to administer mebanazine

Table 1. *Effects of insulin* (1 *unit/kg s.c.*) *on the blood sugar level over* 3 *h of control rats and rats pretreated with mebanazine* (15 *mg/kg daily for* 3 *weeks*) (*means* ±*s.e.*). Four animals per group. Blood glucose values are expressed as mg/100 ml of blood

Time after insulin (min)	Control rats	Mebanazine treated rats	*P* value
0	106 ± 6	92 ± 7	N.S.
60	42 ± 4	30 ± 4	N.S.
90	48 ± 4	26 ± 4	<0·01
120	51 ± 3	36 ± 6	<0·02
150	61 ± 7	42 ± 3	<0·02
180	110 ± 6	48 ± 4	<0·001

Table 2. *Effect of insulin on blood glucose levels in hypophysectomized rats with and without pretreatment with mebanazine daily* (10 *mg/kg*). Four rats in each group. Means ±s.e. expressed as mg/100 ml blood

Dose of insulin (units/kg)	Control rats	Mebanazine treated rats	*P* value
0	128 ± 2	115 ± 8	N.S.
0·125	106 ± 3	57 ± 11	<0·01
0·250	77 ± 7	51 ± 2	<0·02
0·50	61 ± 4	47 ± 6	<0·05

daily at 10 mg/kg for 15 days without any overt signs of toxicity. An insulin tolerance test was made in hypophysectomized animals and the results are summarized in Table 2. Blood sugar levels are given before and 90 min after insulin, corresponding to the time at which a maximal response was observed in the first experiment. As in intact rats, mebanazine-treatment alone did not significantly affect the resting blood sugar values. The sensitivity to insulin was, however, significantly greater than in control hypophysectomized rats.

Since potentiation of insulin hypoglycaemia was observed in the absence of the pituitary gland it seemed unlikely that the phenomenon was a direct consequence of diminished reserves of growth hormone in the mebanazine-treated intact rats. In earlier experiments, it had been observed that rats receiving mebanazine daily at 15 mg/kg for a period of 6 weeks gained considerably less weight than animals receiving a daily oral administration of saline. Subsequent analysis of their pituitary glands showed that the treated group had only about 40% of the growth hormone content of the control group. It was possible that the decreased rate of growth in the mebanazine-treated rats was due to a decreased overall consumption of food.

In a new experiment, three groups of weight-matched rats (10 per group) were selected. One group received mebanazine daily at 15 mg/kg and the other groups were given saline, all by mouth. The drug treated group and one control group were allowed food *ad libitum* whilst the remaining control group acted as a pair-fed control for the mebanazine treated rats. Food and water consumption were recorded daily for 6 weeks when half the animals in each group were killed and various analyses performed. The growth curves for the animals not killed are illustrated in Fig. 1. Whereas the control rats showed a steady weight gain there was little change in the weight of animals receiving mebanazine or their pair-fed controls. When drug treatment was stopped and free feeding provided for all groups, the pair-fed animals gained weight more rapidly than the mebanazine-treated rats.

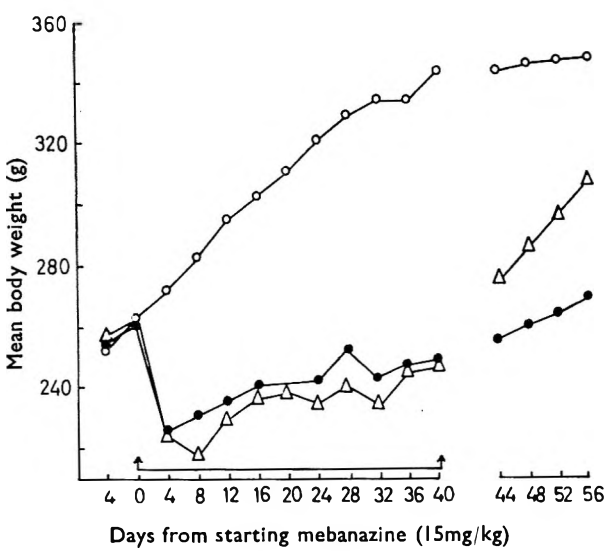

FIG. 1. Change in body weight for groups of 5 rats receiving saline (○—○), mebanazine daily 15 mg/kg (●—●) or saline with pair-feeding (△—△), during 40 days of treatment and 16 further days of free feeding.

Table 3. *Effects of mebanazine* (15 *mg/kg*) *and pair-feeding for* 6 *weeks in rats: body weight, food and water consumption, pituitary weight and growth hormone content* (*means* ±*s.e.*). Five animals in each group. An asterisk denotes a significant difference from control ($P < 0.05$)

Treatment	Change in body wt (g)	Daily consumption of food (g)	Daily consumption of water (ml)	Pituitary growth hormone Wt (mg)	Pituitary growth hormone (% control)
Control	+93 ± 6	19·9 ± 0·6	34·5 ± 1·6	8·1 ± 0·2	100
Mebanazine	−11 ± 12*	13·2 ± 0·4*	13·9 ± 1·1*	7·5 ± 0·4	60*
Pair-feeding	−10 ± 13*	13·2 ± 0·4*	24·0 ± 1·2*	8·2 ± 0·3	63*

Some of the results from the analyses of the rats killed after 6 weeks are summarized in Table 3. Control animals showed a net weight gain of 93 ± 6 g whereas both the mebanazine and pair-fed groups showed a net loss in weight. Both food and water consumption were significantly reduced by mebanazine-treatment although the pair-fed animals drank significantly greater volumes of water. Although there were no significant changes in pituitary weight, the growth hormone content was significantly reduced in both the mebanazine and pair-fed groups. Carcass analyses showed no significant differences between the mebanazine and pair-fed groups although both groups had approximately 50% less body fat than the control group. There were no significant differences between the mean weights of brain, spleen or adrenal glands for any of the groups.

Table 4. *The effects of mebanazine or amphetamine* (5 *mg/kg daily for* 5 *days*) *on body weight, food consumption and tibial epiphysial cartilage width in rats.* 5 rats per group

Treatment	Initial body wt (g)	Gain in wt (g)	Daily food intake (g)	Tibial cartilage width (μm)
Controls	40·7	13·0 ± 1·6	7·69 ± 0·75	352·8 ± 6·5
Amphetamine	41·9	13·3 ± 1·4	7·03 ± 0·75	348·3 ± 7·7
Pair-fed with amphetamine dosed	41·8	13·4 ± 0·8	7·03 ± 0·75	358·1 ± 12·8
Mebanazine	43·0	6·8 ± 0·6**	5·15 ± 0·67*	281·9 ± 6·1***
Pair-fed with mebanazine dosed	42·8	4·8 ± 1·2**	5·15 ± 0·67*	293·0 ± 8·1***

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

In an earlier study (Zor & others, 1965a) it was shown that treatment of immature rats with mebanazine significantly reduced the width of the tibial epiphysial cartilage whereas amphetamine did not. This experiment has been repeated but with the inclusion of pair-fed control groups for both drug-treated groups of rats. The results are summarized in Table 4. Amphetamine did not reduce weight gain, overall food consumption or tibial epiphysial width. In contrast, mebanazine induced a highly significant reduction in epiphysial width, a lower overall food intake and a smaller weight gain. The results were very similar in the pair-fed controls which did not receive any drug-treatment. These experiments suggested that the reduction in the width of the tibial epiphysial cartilage was an indirect consequence of reduced

Table 5. *Food consumption in fasting and fed rats during various time intervals after oral dosing with amphetamine or mebanazine.* Five animals per group. An asterisk denotes significant difference ($P < 0.05$) from control group.

		Fasted 18 h				Fed overnight	
		2 h		24 h		6 h	
Treatment	Dose (mg/kg)	Consumed (g)	% Reduction	Consumed (g)	% Reduction	Consumed (g)	% Reduction
Saline ..	0·5 ml/100 g	4·9 ± 0·5	—	20·8 ± 0·2	—	4·0 ± 0·3	—
Amphetamine ..	2·5	4·0 ± 0·3	18	22·8 ± 0·2	0	4·6 ± 0·4	0
	5·0	2·0 ± 0·3	59*	20·2 ± 0·1	3	3·2 ± 0·3	20*
	10·0	0·3 ± 0·1	94*	20·8 ± 1·0	0	2·2 ± 0·2	45*
Mebanazine ..	2·0	4·6 ± 0·4	6	21·2 ± 0·6	0	2·6 ± 0·4	35*
	7·5	4·1 ± 0·1	16	17·6 ± 0·5	15*	1·4 ± 0·1	65*
	15·0	4·0 ± 0·4	18	15·2 ± 1·1	29*	0·0 ± 0·0	100*

food intake rather than to a specific reduction in growth hormone production by mebanazine.

Most tests for anorexic activity only measure acute effects in the immediate time interval after dosing. Whereas amphetamine, however, produced an acute suppression of appetite, the overall food consumption in a 24 h period following a single dose exceeded that of undosed animals (Table 5). In contrast, mebanazine had very little effect in the acute phase but significantly reduced the 24 h food intake. These results were obtained in animals which had been fasted overnight. When rats were allowed food up till the time of dosing, treatment with mebanazine had a proportionately greater effect than did amphetamine in the following 6 h. At the 15 mg/kg dose level, which was used in the 6 week experiment, there was a complete suppression of eating activity in the succeeding 6 h period. Doubling the single acute dose to 30 mg/kg suppressed food consumption for 24 h. In these rats there was a progressive fall in blood sugar level reaching its nadir at 24 h, the curve being superimposable on that for rats deprived of food but without drug.

It was observed in the previous experiment that even at the lowest dose of mebanazine tested (2·0 mg/kg orally) there was a statistically significant reduction in the food intake of fed rats. It was of interest therefore to determine whether or not this dose would also potentiate insulin hypoglycaemia. The results of an experiment utilizing three dose levels of mebanazine are summarized in Table 6. There was little change in body weight at 2·0 mg/kg per day compared with an increase of 49 ± 6 g in the controls. At the higher dose levels the animals showed a net loss in body weight. Food consumption was depressed in all the treated groups as was water intake. There were no significant differences in the resting blood sugar values after 15 days treatment at any dose level when compared to the controls. The fall in blood glucose 90 min after insulin was greater in all the treated groups but that seen after 2·0 mg/kg per day was not statistically greater than in the control group. The hypoglycaemia was potentiated significantly in the two higher dose-level groups. The results suggest that reduction in food and water intake alone does not entirely account for the potentiation of the hypoglycaemic response to insulin.

Table 6. *The effect of different doses of mebanazine on body weight, food and water consumption and response to insulin after* 15 *days oral treatment.* Five rats in each group: an asterisk denotes significant difference from controls.

	Saline 0·5 ml/100 g	Mebanazine 2·5 mg/kg	Mebanazine 7·5 mg/kg	Mebanazine 15·0 mg/kg
Body weight (g)				
Initial	240 ± 4	250 ± 3	251 ± 6	252 ± 1
After 15 days	289 ± 4	253 ± 7	236 ± 9	227 ± 10
Change	+49	+3	−15	−23
Food intake (g)				
Total	276 ± 19	192 ± 26*	153 ± 21*	137 ± 26*
% controls	100	70	56	50
Water intake (ml)				
Total	390 ± 21	259 ± 30	241 ± 41	161 ± 21
% controls	100	66	62	41
Blood sugar level (mg/100 ml)				
On day 15	121 ± 9	124 ± 3	114 ± 5	122 ± 12
90 min after insulin (10 u/kg s.c.)	66 ± 3	63 ± 3	38 ± 6	43 ± 3
Change	−55	−61	−76	−79
% control response	100	111	138	144

DISCUSSION

Potentiation of the hypoglycaemic response to insulin by mebanazine has been reported in rats (Barrett, 1965; Zor, Mishkinsky & Sulman, 1965; Adnitt, 1968a,b), in rabbits (Cooper & Ashcroft, 1966) and man (Wickström & Pettersson, 1964; Cooper & Keddie, 1964). A direct hypoglycaemic effect of mebanazine was observed by Zor & others (1965) but not by the other investigators. The dose of mebanazine used by Zor and his colleagues was 35 mg/kg, the maximum effect being at 24 h when the blood sugar averaged 72 mg/100 ml compared with a control level of 113 mg/100 ml. In the present study it has been shown that this dose of mebanazine is sufficient to depress food intake for 24 h and it may be concluded that the "direct" hypoglycaemic effect of the drug was, in fact, a secondary consequence of drug-induced fasting.

Subsequently Zor & others (1965a) demonstrated that mebanazine reduced the tibial epiphysial cartilage width whereas amphetamine did not. The results implied an inhibitory effect on growth hormone rather than on food intake. A further paper (Zor & others, 1965b) extended these findings in that mebanazine was shown to depress growth, that the effect was potentiated by hydrocortisone and only partially overcome by concomitant injection of growth hormone. It was implied that mebanazine specifically inhibited some enzyme involved in the release of growth hormone. In 1966, Zor, Winer & others found that mebanazine decreased hepatic DNA and total liver protein in immature rats. More specific evidence came from experiments in which chronic treatment of rats with mebanazine was observed to decrease glucose-6-phosphate dehydrogenase activity in the pituitary (Zor, Shore & others, 1967). They also noted a reduction in both pituitary RNA and DNA content, although the RNA : DNA ratio was unaltered. In none of these studies from Sulman's laboratory was there an adequate control allowing for the reduction in food intake demonstrated in this study. Physical limitation of food intake has been shown to have very similar effects both on weight gain and tibial epiphysial width to that of surgical excision of

the pituitary gland (Thompson & Crean, 1963). The results from the present experiments clearly show marked parallels between effects of mebanazine and pair-feeding and do not support the contention that the drug exerts a specific effect on growth hormone production, release or activity. The mechanism of potentiation of insulin hypoglycaemia is not solely dependent on the anorexic actions of mebanazine.

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The pharmacology of 5-(2-t-butylamino-1-hydroxyethyl) salicylamide (AH 3474), a β-adrenoreceptor blocking agent

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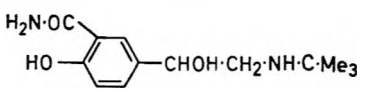
AH 3474 is a specific β-adrenoreceptor antagonist, devoid of stimulant activity. When given by mouth to conscious guinea-pigs and dogs, AH3474 and propranolol are equiactive in antagonizing isoprenaline-induced tachycardia. In anaesthetized animals AH 3474 was 2–4 times less active than propranolol when given intravenously. A similar potency ratio was found in volunteer studies in which the drug was taken orally. On isolated tissues AH 3474 was much less active than propranolol. AH 3474 had 1/10th the activity of propranolol in blocking the inhibitory action of isoprenaline on the rat uterus and was at least 100 times less active in antagonizing the tachycardia induced by adrenaline on the guinea-pig atria. *In vitro*, equilibrium conditions for AH 3474 were obtained in 15 min, whereas 45 min were required for propranolol. AH 3474 antagonized the cardiac arrhythmias induced by ouabain in the anaesthetized dog. The amount required far exceeded the β-adrenoreceptor blocking dose. AH 3474 possessed no "quinidine-like" actions on cardiac muscle of dog or guinea-pig. The local anaesthetic activity of AH 3474 was 400 times less than that of propranolol.

The first drug shown to antagonize the effects of catecholamines at β-adrenoreceptor (β-receptor) sites was dichloroisoprenaline (Powell & Slater 1958). This drug, like its successor pronethalol, possessed some β-stimulant activity (Black & Stephenson, 1962). Later, propranolol, a close analogue of pronethalol, was shown to be a potent β-adrenoreceptor blocker without sympathomimetic activity (Black, Crowther, & others, 1964). Propranolol has also potent quinidine-like and local anaesthetic actions (Morales-Aguilera & Vaughan Williams, 1965).

AH 3474 [1;5-(2-t-butylamino-1-hydroxyethyl)salicylamide] is a β-adrenoreceptor blocking agent devoid of quinidine-like, local anaesthetic and β-stimulant properties. The compound was prepared in the Chemical Research Laboratories of Allen & Hanburys Ltd. Its structure is given below:



I

EXPERIMENTAL

Anaesthetized animals

Cats and dogs. Cats of either sex weighing 2–3 kg were anaesthetized with chloralose (80 mg/kg intravenously) after induction with 3% halothane in nitrous oxide and oxygen (3:1). Beagles of either sex weighing 7–11 kg were anaesthetized with pentobarbitone sodium 30 mg/kg intravenously. Arterial blood pressure was recorded

from a cannula in the right femoral artery by a Devices blood pressure transducer coupled to a Devices polygraph recorder. Respiration was recorded via a Magill cuffed endotracheal tube and Statham low pressure transducer. Heart rate was measured by a Nielson instantaneous ratemeter triggered from the pulse pressure sensed by the blood pressure transducer or triggered by the QRS complex of the electrocardiogram. In some experiments the cervical vagus and cardiac accelerans or cervical sympathetic nerves or splanchnic nerves were cut and the peripheral ends stimulated with trains of rectangular pulses of 1 ms duration and supramaximal voltage. Contractions of the cat nictitating membrane were recorded using an isometric strain gauge. Drugs were injected or infused through a cannula in the left femoral vein.

The hind-limb of the anaesthetized dog was perfused as follows: the skin was removed from the right hind-limb and a torniquet applied to the right ankle. A catheter was placed in the right femoral artery so that the tip lay in the abdominal aorta. Ties were placed on the right deep circumflex iliac, external and internal iliac and the deep femoral artery. Blood was sampled from this catheter and pumped (Watson-Marlow peristalic pump) into the right hind limb at a constant rate via a catheter placed in the femoral artery distal to the entry of the sampling catheter. The flow rate was adjusted so that the perfusion pressure approximated to the systemic blood pressure. Perfusion pressure was measured by Devices blood pressure transducer connected to a T-piece proximal to the entry of the catheter into the femoral artery. Drugs were injected into a rubber junction in the sampling catheter.

Rat. Female rats of a weight range 200–300 g were pretreated with 0·1 mg/100 g stilboestrol 24 h before use. Animals were anaesthetized with pentobarbitone sodium, 3–6 mg/100 g intraperitoneally. The trachea was cannulated and the uterus exposed by a mid-line abdominal incision. One horn of the uterus was mobilized and attached to a strain gauge transducer. The external jugular vein was cannulated and oxytocin infused (2 units/kg h^{-1}). Drugs were injected into the venous cannula by a 3-way tap system.

Conscious animals

Dog. Heart rate was determined in normotensive dogs using a Nielson instantaneous ratemeter triggered by the QRS complex of the ECG. The ECG was recorded from plate electrodes attached to the limbs (an area of skin was shaved). The dogs were trained to lie quietly on their right side. Isoprenaline was given intravenously; the β-receptor blocking drugs were given by mouth in hard gelatin capsules.

Hypertensive dogs were prepared as described by Cullum, Farmer & Handley (1967).

Guinea-pig. Heart rate in guinea-pig was determined by the method of Farmer & Levy (1968a). Guinea-pigs, 250–500 g, were trained to stand unrestrained on four plate electrodes, the ECG obtained was used to trigger the instantaneous ratemeter. Drugs were given orally in solution except isoprenaline which was given subcutaneously.

Local anaesthetic activity of the β-receptor blocking drugs was determined in the guinea-pig by the intradermal wheal method of Bülbring & Wajda (1945).

Hypertensive rats. Male Wistar rats, 100–150 g, were made hypertensive by unilateral nephrectomy and subcutaneous implantation of desoxycorticosterone acetate. Heart rate and blood pressure were measured indirectly from the tail (Farmer & Levy, 1968b). Drugs were given orally or subcutaneously in solution.

Isolated tissues

Rat uterus. Uterine horns taken from rats pretreated with 0·1 mg/100 g stilboestrol were suspended in a physiological salt solution at 37° and gassed with 5% carbon dioxide in oxygen. The composition of the salt solution was; g/litre, NaCl 9·0; $NaHCO_3$ 1·0; KCl 0·42; $CaCl_2$ 0·24; glucose 1·0. Contractions were recorded using an isometric strain gauge.

Guinea atria. The hearts of guinea-pigs were removed and placed in chilled McEwen solution (1956). The blood was gently squeezed from the heart and the atria separated, cleared of fat and suspended in McEwen solution maintained at 32° and gassed with 5% carbon dioxide in oxygen. Contractions were measured using an isometric strain gauge. The relative refractory period of the heart muscle was measured by the method of Dawes (1946). The atria were anchored directly to the terminals of a bipolar electrode and were driven with rectangular impulses of 1 ms duration. The maximum rate at which the atria could be driven in absence and presence of a drug was determined. The reciprocal of the maximum rate gives a measure of the relative refractory period. In some experiments the effects of drugs on the force of contraction of spontaneously beating atria were determined.

Drugs. AH 3474 [5-(2-t-butylamino-l-hydroxyethyl)salicylamide], propranolol hydrochloride (ICI), oubain (BDH), isoprenaline sulphate (BW), papaverine hydrochloride (Hopkin & Williams) and quinidine sulphate (BDH). Doses of drugs refer to the bases.

RESULTS

The cardiovascular effects of AH 3474 and propranolol on the anaesthetized dog Blood pressure, heart rate, carotid occlusion reflex and respiration

AH 3474, 0·1–5 mg/kg given intravenously, produced a 10–30 mm Hg fall in systolic and diastolic blood pressures and a bradycardia of 10–40 beats/min. The intensity and duration of these effects varied considerably, and graded responses to the drug were not obtained. However, the bradycardia lasted much longer (2–3 h) than the effect on blood pressure (40–80 min). The response to bilateral occlusion of the common carotid arteries was reduced by 30–50% but recovered in parallel with the blood pressure (Fig. 1). Rate and depth of respiration were not affected by AH 3474. Similar effects were observed with 0·05–1·0 mg/kg propranolol.

Isoprenaline-induced tachycardia and depressor responses

AH 3474, 1·6 mg/kg given intravenously, considerably reduced the depressor response and tachycardia caused by intravenous injection of isoprenaline for 2–3 h. Propranolol, 0·4 mg/kg, produced a similar reduction of the responses to isoprenaline. Antagonism of the depressor response was more prolonged than antagonism of the tachycardia. At equiactive doses, the durations of the action of the drugs were similar. In other experiments tachycardia was produced by infusing isoprenaline, with 5 min intervals between infusions. When a reproducible tachycardia was obtained AH 3474 or propranolol were infused at increasing rates. Infusions lasted for 10 min at any given rate and were started 5 min before infusion of isoprenaline. The rate of infusion which caused a 50% reduction of the tachycardia was calculated. For AH 3474, this was 9·5 μg/kg min^{-1} and for propranolol, 7·0 μg/kg min^{-1}.

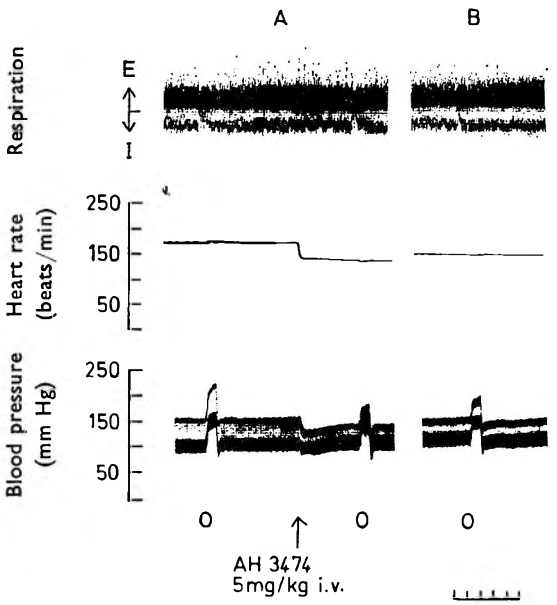


FIG. 1. The effects of AH 3474, 5 mg/kg given intravenously, on respiration, heart rate and blood pressure of anaesthetized dog. ○ = carotid occlusion for 30 s; 70 min elapsed between panels A and B.

Ouabain-induced cardiac arrhythmia

The antiarrhythmic activities of AH 3474, propranolol and quinidine were compared in anaesthetized dogs. After a control ECG reading was obtained repeated intravenous doses of ouabain were given according to the following schedule; 40 μg/kg immediately, 20 μg/kg after 30 min and 10 μg/kg each 15 min thereafter until cardiac arrhythmia occurred. In control experiments the arrhythmias produced by ouabain usually lasted 2–3 h. In other experiments AH 3474, propranolol or quinidine was infused at increasing concentrations for 5 min periods until the ECG record became normal. The total doses of AH 3474 required to correct the arrhythmias in three separate experiments were 4·5, 11·85 and 11·85 mg/kg. For propranolol the doses were 1·85, 3·85, 3·85 mg/kg and for quinidine the dose was 8·85 mg/kg. Thus AH 3474 under these conditions is approximately equipotent with quinidine but about one-half to one-third as active as propranolol.

Vascular resistance in hind limb

Intra-arterial injection of AH 3474 or propranolol into the perfused hind limb of the dog caused a transient fall in perfusion pressure. This effect was produced by 1–4 mg/kg AH 3474 and by 0·05 to 0·8 mg/kg propranolol. The response to AH 3474 was small and not dose-dependent; that to propranolol was marked and dose-dependent.

In other experiments intra-arterial injections of AH 3474 or propranolol were found to reduce the vasodilation caused by intra-arterial injection of isoprenaline. AH 3474 was 4 times less potent than propranolol.

Acute toxicity of AH 3474 and propranolol

AH 3474 was infused continuously into the femoral vein of the anaesthetized dogs, and blood pressure, heart rate, respiration and ECG were recorded. AH 3474 (1 mg/kg

min^{-1}) reduced the blood pressure and heart rate but did not prove lethal after 100 min of infusion. AH 3474 (4 mg/kg min^{-1}) and propranolol (1 mg/kg min^{-1}) produced slow falls in blood pressure and heart rate which resulted in cardiovascular collapse and death. AH 3474 reduced the depth of respiration but increased the rate, whereas propranolol reduced both depth and rate. The dose infused at the time of death was 200 mg/kg for AH 3474 and 20 mg/kg for propranolol. Infusion of propranolol caused sinus arrhythmia and the ECG showed an increase in the PR and QT intervals and reduction in the QRS complex. These effects were not seen with AH 3474 (Fig. 2).

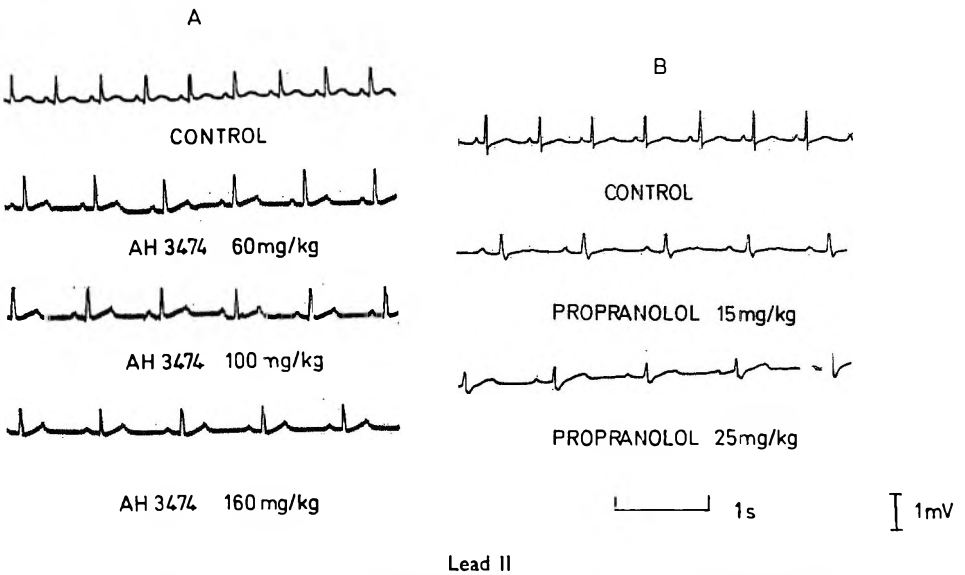


FIG. 2. The effects of AH 3474 (A) and propranolol (B) on the ECG of anaesthetized dog. Both drugs were given as an infusion, AH 3474 at 4 mg/kg min^{-1} and propranolol at 1 mg/kg min^{-1}.

The cardiovascular effects of AH 3474 *in the anaesthetized cat*

(i) *Blood pressure, heart rate and respiration.* AH 3474 in doses of 1, 2 and 5 mg/kg given intravenously, caused a 15–50 mm Hg fall in blood pressure of 10–45 min duration. The heart rate decreased by 5–50 beats/min for a period of 1–2 h. AH 3474 has no effect on heart rate in cats pretreated with reserpine. In the cat, the effect of AH 3474 on blood pressure and heart rate varied considerably and graded responses could not be obtained. The bradycardia lasted much longer than the hypotension. Rate and depth of respiration were not affected by AH 3474.

(ii) *Responses of the blood pressure and heart rate to vasopressor agents and stimulation of peripheral autonomic nervous system.* AH 3474 (5 mg/kg intravenously) augmented the intensity and duration of the pressor responses to noradrenaline and adrenaline. The response to intravenous angiotensin was augmented but that to tyramine was slightly reduced. AH 3474 (1 and 2 mg/kg) had no effect on the biphasic response of the blood pressure to stimulation of the splanchnic nerve.

AH 3474 (1 and 5 mg/kg) had no significant effect on the response of the nictitating membrane to preganglionic stimulation of the cervical sympathetic nerves, but the response to injected adrenaline was slightly potentiated. The response of the heart

rate to accelerans or vagal nerve stimulation was determined before and after infusion of 150 and 750 μg/kg of AH 3474 or propranolol over a 15 min period. The frequencies of nerve stimulation were 1, 2, 5, 10 and 20 Hz. AH 3474 and propranolol reduced the response of the heart rate to accelerans but not vagal nerve stimulation. In some experiments, the response of the heart to vagal stimulation appeared to be enhanced by β-adrenergic blockade. AH 3474 was 2–3 times less active than propranolol in reducing the increase in heart rate caused by stimulating the accelerans nerve.

The effects of AH 3474 *and propranolol on the uterus of the anaesthetized rat*

Intravenous injection of AH 3474, 0·5 mg/kg, or propranolol 0·125, 0·25 and 0·5 mg/kg, had no effect on the spontaneous motor activity of the rat uterus but both drugs reduced the inhibitory response of the uterus to isoprenaline. AH 3474 was 2–4 times less active than propranolol.

The effects of AH 3474 *and propranolol on isolated tissues*

Isolated uterus of the rat. AH 3474 (5 and 10 μg/ml) had no effect on the spontaneous contractions of the rat uterus; isoprenaline (1 μg/ml) abolished the contractions. AH 3474 (5 μg/ml) blocked the inhibitory effect of isoprenaline but had no effect on the inhibitory response to papaverine. Similar effects were observed with propranolol at 1/10th the concentration of AH 3474.

Guinea-pig isolated atria. Cumulative dose response curves for adrenaline were determined on spontaneously beating atria by adding geometrically increasing doses without changing the bath fluid, leaving each concentration to exert a maximal effect (60 s) before the addition of the next dose. Dose response curves for increased force of contraction were obtained before and after the addition of AH 3474 or propranolol. The antagonists were allowed 45 min contact with the tissue. This contact time was allowed since it was observed that equilibrium conditions were not obtained earlier with propranolol. Both AH 3474 and propranolol caused dose dependent shifts of the dose response curve to adrenaline. pA_2 values for AH 3474 and propranolol were calculated by the method of Arunlakshana & Schild (1959). Results for each drug were 6·13, 6·02 and 6·5 for AH 3474 and 8·02 and 7·78 for propranolol.

The effects of AH 3474, propranolol and quinidine on the relative refractory period of electrically driven guinea-pig auricles were determined. AH 3474 in concentrations up to 20 μg/ml had no effect but propranolol and quinidine, 0·1 to 10·0 μg/ml, caused concentration-dependent increases in the relative refractory period. At 10 μg/ml the percentage increase in the refractory period was 55 for propranolol and 45 for quinidine (Fig. 3).

AH 3474, propranolol and quinidine also reduced the spontaneous rate of contraction of isolated auricles but only propranolol and quinidine reduced the force of contraction.

Local anaesthetic activity of AH 3474 *and propranolol in the conscious guinea-pig*

Graded dose response curves for local anaesthetic activity were obtained for AH 3474, propranolol and procaine by the intradermal wheal test in the guinea-pig. Propranolol was 10 times more active and AH 3474 was 40 times less active than procaine.

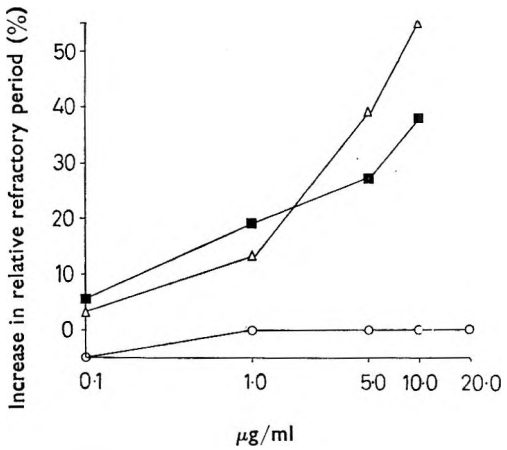


FIG. 3. The effects of AH 3474 (○—○), propranolol (△—△) and quinidine (■—■) on the relative refractory period (R.R.P.) of guinea-pig cardiac muscle *in vitro*, % increase in R.R.P. is plotted against log concentration of drug. Each point is the mean of two experiments.

The effect of AH 3474 *and propranolol on isoprenaline-induced tachycardia in conscious animals*

Guinea-pig. After two half-hourly heart rate determinations, groups of 4 animals were orally dosed with AH 3474 (50 mg/kg), propranolol (50 mg/kg) or saline. The effects of subcutaneous injections of isoprenaline (30 μg/kg) on heart rate were determined for each animal, 1, 3 and 5 h after administration of the β-receptor blockers. AH 3474 and propranolol had similar potencies and durations of action in blocking isoprenaline tachycardia (Fig. 4).

Dog. Propranolol or AH 3474, given orally, antagonized the tachycardia produced by repeated intravenous injection of isoprenaline 0·3 μg/kg. Typical results in the same dog are shown in Fig. 5. AH 3474 and propranolol (0·25 or 0·5 mg/kg.) were about equipotent in this test with maximal actions after 1 h. Both drugs at 0·5 mg/kg acted for longer than 4 h.

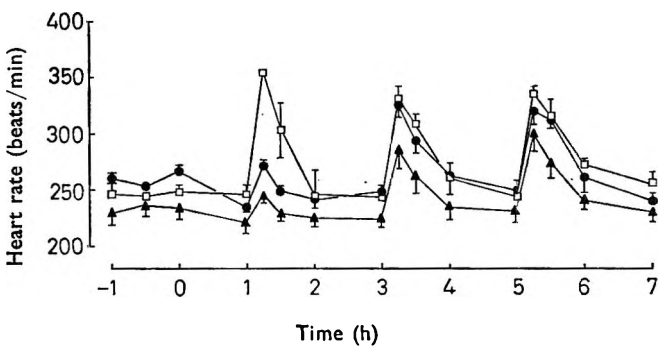


FIG. 4. The effects of AH 3474 (●—●) and propranolol (▲—▲), 50 mg/kg, given orally at time O, on isoprenaline induced tachycardia in guinea-pigs. Isoprenaline, 30 μg/kg, was given subcutaneously at 1, 3 and 5 h after drug administration. Each point is the mean response ± s.e. for a group of 4 guinea-pigs. □—□ saline control group.

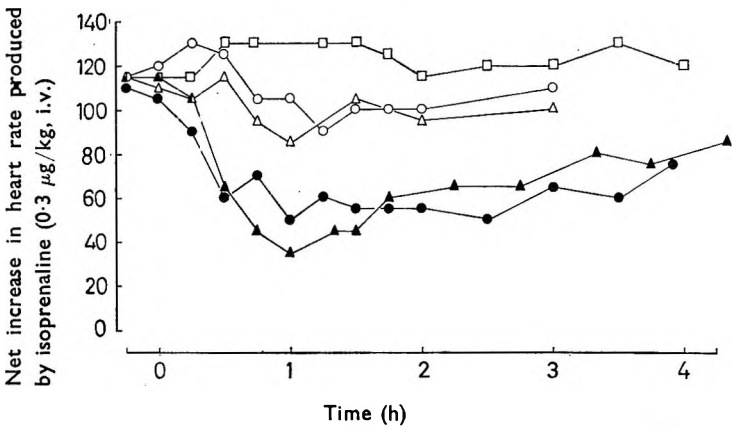


FIG. 5. The effects of AH 3474, 0·25 mg/kg (○—○) and 0·5 mg/kg (●—●), and propranolol, 0·25 mg/kg (△—△) and 0·5 mg/kg (▲—▲), on isoprenaline induced tachycardia in the conscious dog. □—□ saline control group. Drugs were given orally at time O. Isoprenaline, 0·3 μg/kg was given intravenously.

The effects of AH 3474 *on systolic blood pressure and heart rate of the conscious hypertensive rat*

The systolic blood pressures and heart rates of a group of hypertensive rats were determined daily. After control readings had been established supramaximal β-blocking doses of AH 3474 (50 mg/kg) were given subcutaneously twice daily for 2 days, 2 h before and 4 h after the blood pressure and heart rate determinations. The results are shown in Fig. 6. AH 3474 produced a fall in heart rate of some 50 beats/min without changing the systolic blood pressure. The heart rate took several days to recover after the last dose of AH 3474.

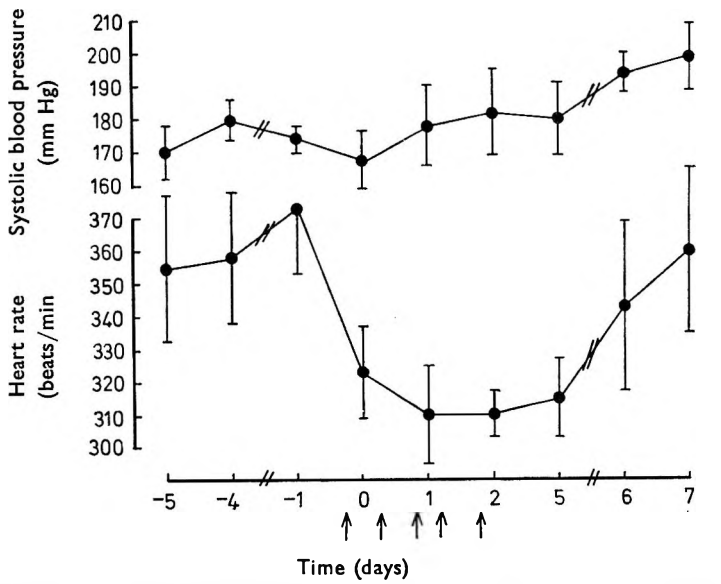


FIG. 6. The effects of AH 3474 on systolic blood pressure and heart rate of conscious hypertensive rats. AH 3474, 50 mg/kg, was given subcutaneously at ↑ 2 h before and 4 h after blood pressure and heart rate determinations.

The effects of AH 3474 on systolic blood pressure and heart rate in the conscious hypertensive dog

AH 3474 was given orally to 3 dogs on sixteen consecutive days. The animals received 1 mg/kg for 1 day, 2 mg/kg for 7 days and 20 mg/kg for 3 days. Doses of 2 to 5 mg/kg AH 3474 produced a bradycardia of 10–20 beats/min. At 10 and 20 mg/kg a small, probably insignificant fall in systolic blood pressure (10–15 mm Hg) was observed in 2 dogs.

DISCUSSION

AH 3474 has been shown to have β-receptor blocking activity in conscious and anaesthetized animals and on isolated tissues. In conscious dogs and guinea-pigs AH 3474 and propranolol, given orally, were about equipotent and had similar durations of action in antagonizing the tachycardia produced by isoprenaline. However, in anaesthetized animals the β-receptor blocking activity of AH 3474 was 2–4 times less than that of propranolol when assessed against the tachycardia produced by isoprenaline or accelerans nerve stimulation, but equiactive doses had similar durations of action. The second estimate of relative potency is close to that found in volunteer studies. Single oral doses of AH 3474, 100 mg, or propranolol, 40 mg, were about equally effective in suppressing exercise-induced tachycardia. AH 3474 acted for 3 to 4 h and propranolol slightly longer (W.T. Simpson, Personal communication).

AH 3474 was much less active than propranolol on isolated tissues. The β-blocking potency of AH 3474 on rat isolated uterus was 1/10th that of propranolol whilst on isolated atria, the activity was some 100 times less. It was also noted that AH 3474 took about 15 min to produce a constant reduction in the response of the isolated atria to adrenaline whereas propranolol took 45 min. There is an obvious discrepancy between the *in vivo* and *in vitro* potency ratios for the two drugs. The most likely explanation is that with propranolol the *in vitro* tests do not only measure β-receptor blocking activity, but also an intracellular quinidine-like action on the contractile mechanism. The latter action is clearly shown with higher concentrations of propranolol by the increase of the relative refractory period and the decrease in force of contraction of the atria. Propranolol, like most potent quinidine-like drugs, is easily lipid soluble and would therefore be expected to penetrate cell membranes and, given the right structural requirements, firmly associate with nonpolar receptors in the cells. perhaps in the contractile protein itself. AH 3474 did not increase the refractory period or decrease the force of contraction of heart muscle. It is probably significant that AH 3474 is much more polar than propranolol (the partition coefficient between water buffered to pH 7·2 and ethylene dichloride is 21·0 for AH 3474 and 0·18 for propranolol) and would not be expected to enter cells freely and there associate with nonpolar structures. The same properties may account for the lack of local anaesthetic activity in AH 3474.

AH 3474 given intravenously to anaesthetized animals caused bradycardia and hypotension. The bradycardia was attributed to blockade of resting sympathetic tone since AH 3474 did not affect the heart rates of cats pretreated with reserpine. The latter result also shows that AH 3474 is devoid of intrinsic β-receptor stimulant activity. This conclusion is confirmed by the lack of response of the rat uterus and guinea-pig atria to AH 3474. The fall in blood pressure with AH 3474 in anaesthetized

animals may be due to decreased cardiac ouput without change in peripheral resistance as suggested by Shanks (1966) for propranolol. Effective β-receptor blocking doses of AH 3474, like MJ 1999 and propranolol, failed to lower the blood pressure in conscious hypertensive rats and dogs. The much higher doses of propranolol which lower blood pressure in hypertensive rats do so by impairing cardiac function (Farmer & Levy, 1968b).

Propranolol is used in man as an antifibrillatory drug. From animal data, its effect might be mainly due to its β-blocking action or its quinidine like action or both (Morales-Aguilera & Williams, 1965; Howe & Shanks, 1966). In the present experiments AH 3474 was clearly shown to be devoid of a quinidine like action but it did correct ouabain-induced cardiac arrhythmias at about 12 times its β-blocking dose. Propranolol corrected these arrythmias at 4 times its β-blocking dose, a dose which did not greatly effect the force of contraction of heart muscle in anaesthetized dogs. These results indicate that ouabain reversal, at least in part, is mediated through β-receptor blockade.

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Prostaglandin E_1 action on canine isolated tracheal muscle

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Prostaglandin E_1 (PGE_1) inhibits contractions of dog isolated tracheal muscle stimulated by different agents, but the degree of inhibition varies with the agent used. Low concentrations of PGE_1 completely block the stimulant effect of 5-hydroxytryptamine, but even large concentrations of PGE_1 do not completely antagonize the contractions caused by acetylcholine. The inhibitory effect of PGE_1 is blocked by methysergide and not by propranolol, morphine or dihydroergotamine. PGE_1 does not relax depolarized smooth muscle, although bradykinin and isoprenaline do. It is concluded that in tracheal smooth muscle, PGE_1 interacts with cell membranes close to the 5-hydroxytryptamine D receptors. This causes activation of the smooth sarcoplasmic reticulum, leading to accumulation of calcium ions and relaxation.

Prostaglandin E_1 usually contracts isolated smooth muscle, but it relaxes tracheal muscle (see review by Bergström, Carlson & Weeks, 1968). We have found that when dog isolated tracheal muscle is contracted by acetylcholine or by 5-hydroxytryptamine (5-HT), the inhibition caused by prostaglandin E_1 (PGE_1) is quantitatively different. The present study deals with this difference and with the mechanism of action of PGE_1.

EXPERIMENTAL

Methods

Tracheae were obtained from normal mongrel dogs anaesthetized with sodium pentobarbitone (30 mg/kg i.v.). A tracheal ring was mounted in Tyrode solution ($NaCl$ 8·0; $NaHCO_3$ 1·0; KCl 0·2; $CaCl_2$ 0·2; $MgCl_2$ 0·1; Na_2HPO_4 0·05; and glucose 1·0 g/litre) in a 10 ml organ bath at 37° according to Akçasu (1959) and bubbled with O_2. Isomeric responses were measured with a Grass force-displacement transducer and recorded on a Beckman Dynograph Type RB. The muscle was allowed to equilibrate in Tyrode solution for 3–4 h while subjected to a passive stretch of 1 g.

In a few experiments the NaCl in Tyrode solution was replaced with isotonic KCl and in other experiments calcium content of the salt solution was varied. The following drugs were used: acetylcholine chloride, 5-hydroxytryptamine creatinine sulphate, methysergide, dihydroergotamine methyl sulphate, propranolol hydrochloride, ouabain and morphine sulphate. Prostaglandin E_1 was a gift from Upjohn. All drug concentrations were expressed as the free base.

RESULTS

Acetylcholine (10 ng/ml) and 5-HT (40 ng/ml) contracted the dog trachea to the same extent. The inhibitory action of PGE_1 was more marked on the 5-HT-contracted smooth muscle, 10 ng/ml of PGE_1 abolishing the response to 40 ng/ml of 5-HT, while

30 ng/ml of PGE_1 reduced response to 10 ng/ml of acetylcholine by about one-half (Table 1). In the presence of 10 ng/ml of acetylcholine, 512 ng/ml of PGE_1 was required to relax the muscle to 70% of its resting state, while in the presence of 40 ng/ml of 5-HT, only 8 ng/ml of PGE_1 was needed (Fig. 1).

Table 1. *Inhibition of dog isolated tracheal muscle by prostaglandin* E_1

Concentration of PGE_1 (ng/mg)	% Inhibition on muscle contracted by acetylcholine (10 ng/ml) (mean ± s.e.)	% Inhibition on muscle contracted by 5-HT (40 ng/ml) (mean ± s.e.)
0·5	5·0 ± 0·2 (n = 5)	20·0 ± 0·8 (n = 10)
1·0	8·0 ± 0·4 (n = 6)	45·0 ± 0·7 (n = 10)
2·0	15·1 ± 0·8 (n = 5)	80·4 ± 0·6 (n = 10)
4·0	25·3 ± 0·5 (n = 7)	95·5 ± 0·8 (n = 10)
8·0	33·0 ± 0·8 (n = 6)	100 (n = 10)
16·0	41·0 ± 0·7 (n = 6)	
32·0	53·0 ± 0·9 (n = 8)	
64·0	58·0 ± 0·6 (n = 10)	
128·0	65·7 ± 0·7 (n = 10)	
512·0	70·0 ± 0·6 (n = 10)	

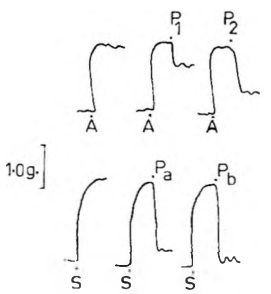

FIG. 1. Responses of isolated tracheal muscle. Initial tension 1·0 g. *Upper Panel:* A 10 ng/ml acetylcholine. P_1 8 ng/ml PGE_1. P_2 512 ng/ml PGE_1. *Lower Panel:* S 40 ng/ml 5-HT. P_a 2 ng/ml PGE_1. P_b 8 ng/ml PGE_1. Bar at left indicates muscle tension of 1·0 g.

Effect of drugs on the inhibitory action of PGE_1 in acetylcholine-constricted muscle

PGE_1 (128 ng/ml) reduced the submaximal contractions caused by acetylcholine (10 ng/ml) by 65·7 ± 0·7% (n= 10) of the control value. After exposure of the muscle to 1 ηg/ml of methysergide for 10 min, the inhibition caused by 128 ng/ml of PGE_1 was only 32·8 ± 2·0 (n = 6). At 10 μg/ml, methysergide completely blocked the effect of 5-HT and slightly potentiated the effect of acetylcholine on the tracheal muscle. PGE_1, in the presence of 10 μg/ml of methysergide, inhibited the acetylcholine-induced contraction by only 13·4 ± 1·07% (n = 10). When the muscle was contracted with 20 mM KCl instead of acetylcholine, PGE_1, 128 ng/ml, caused a relaxation to 23·7 ± 0·8% (n = 6). Contraction to 5-HT was not tested, because of antagonism by methysergide. Propranolol (5 μg/ml), morphine (10 μg/ml) and dihydroergotamine, given 10 min before and ouabain (5·5 μg/ml) given 2 h before PGE_1 had no effect on relaxation.

Effect of ions

Decreasing calcium ion concentration in Tyrode solution to 0·45 mM or addition of 2×10^{-3} M ethylenediamine tetra-acetate (EDTA) in a calcium-free Tyrode solution, had no effect on the relaxant response to PGE_1 in an acetylcholine-contracted muscle. The

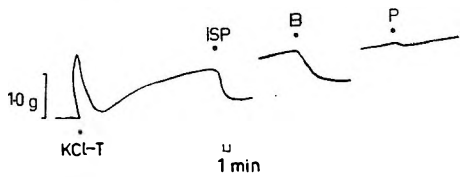

FIG. 2. Responses of dog isolated tracheal muscle depolarized by KCl. Initial tension 1·0 g. KCl-T replacing NaCl in normal Tyrode solution by isotonic KCl. ISP represents 10 ng/ml isoprenaline. B represents 50 ng/ml bradykinin. P represents 512 ng/ml PGE_1. Bar at left indicates muscle tension of 1·0 g.

degree of contraction was not different from that obtained in normal Tyrode. When the muscle was depolarized by replacing the salt solution with isotonic KCl Tyrode, the tracheal ring first contracted rapidly then partially relaxed. Addition of 512 ng/ml of PGE_1 had no further relaxant effect, while 10 ng/ml of isoprenaline and 50 ng/ml of bradykinin caused further relaxation (Fig. 2).

DISCUSSION

Prostaglandin E_1 relaxes dog tracheal muscle as it does tracheal muscle from other species (Horton & Main, 1965). However, the relaxation differs quantitatively when the muscle tonus is increased by equipotent amounts of acetylcholine or 5-HT. When these agonists were given to produce equal degrees of contraction, 8 ng/ml of PGE_1 produced 100% relaxation after 5-HT; while 512 ng/ml produced only a 70% relaxation after acetylcholine. These results indicate that the effect of PGE_1 is not a simple type of drug-response reaction.

In a study on 5-HT receptors in uterine and ileal smooth muscle preparations, Gaddum & Picarelli (1957) subdivided these into two classes; M receptors, possibly on nerve endings and blocked by morphine, and D receptors, possibly on smooth muscle membranes and blocked by dihydroergotamine and lysergic acid diethylamide. Gyermek (1962) reported that tracheal smooth muscle response to 5-HT was not blocked by morphine. Thus it has been concluded that only D receptors are present (Constantine & Knott, 1964).

Since the relaxing effect of PGE_1 is blocked by methysergide and not by morphine, this must indicate that the lipid is acting at the membrane surface, at or close to the specific 5-HT D receptor site, preventing the binding of 5-HT. This receptor site differs from α- and β-adrenergic receptors since dihydroergotamine and propranolol have no effect on responses to PGE_1. We have demonstrated an interaction between PGE_1. and 5-HT receptors in rat isolated duodenum (Khairallah, Page & Türker, 1967). PGE_1 relaxes rat duodenum owing to release of catecholamines, since the response was converted to a contractile one in the presence of α- and β-adrenergic blocking agents or after reserpine pretreatment. Only bromolysergic acid (BOL), another D receptor blocking agent, abolished the contractile response. In no other tissue has PGE_1 been reported to interact with 5-HT receptors. Using isolated mesenteric arterial strips, Strong & Bohr (1967), found that lysergic acid diethylamide had no effect on the response to PGE_1. Thus, interaction of the lipid with 5-HT D receptors may be limited to the muscle of rat duodenum and dog trachea.

The relaxant effect of PGE_1 is significantly decreased and then abolished when KCl replaces NaCl in the bathing medium. KCl-depolarized tracheal smooth muscle does not relax to PGE_1 but does relax to bradykinin and isoprenaline. The latter has

been shown by Schild (1964, 1967) to relax KCl-depolarized uterine muscle, an action which is antagonized by a β-adrenergic blocker, dichloroisoprenaline (DCI). The author concluded that isoprenaline acts independently of membrane potential. PGE_1, on the other hand, is not active in depolarized tracheal smooth muscle, and probably once it is bound to its receptor sites, it leads to depolarization of the normal membrane potential, but would have no effect in a previously depolarized membrane. PGE_1 also acts independently of external calcium ions. Even addition of a chelating agent, EDTA, to the physiological salt solution does not block the relaxing effect seen after PGE_1, although the relaxant effect of isoprenaline in depolarized uterine muscle is abolished in the absence of calcium (Schild, 1967). He has proposed a hypothesis explaining this. Isoprenaline activates a factor which leads to accumulation of calcium ions, thus lowering the concentration of free sarcoplasmic Ca^{++} below a threshold necessary for contraction. This leads to relaxation. The action of isoprenaline is independent of membrane polarization, but requires external calcium ions. A similar hypothesis can be developed to explain the relaxing action of PGE_1. The lipid binds to the cell membrane at or very close to 5-HT D receptors. This prevents the full binding of 5-HT, and thus activates a factor leading to accumulation of calcium in the smooth muscle sarcoplasmic reticulum (Carsten, 1968), lowering free calcium ion concentration and thus producing relaxation. The link between the receptor and the calcium pump in the sarcoplasmic reticulum is related to membrane depolarization and is independent of external Ca^{++} concentration, or the sodium pump, thus differing from isoprenaline.

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Effect of glycopyrronium bromide on basal, and histamine- or gastrin-induced gastric secretion

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Acid secretory responses were obtained in rats given either intravenous histamine as dihydrochloride or gastrin subjected to partial purification. A continuous recording method for measuring gastric acid secretion was used. When the rat stomach was perfused with weak sodium hydroxide solution, glycopyrronium bromide (a powerful anticholinergic drug) blocked the acid gastric secretory effects of both histamine and gastrin. Glycopyrronium bromide in doses of 2 μg/100 g body weight of rat is well tolerated. Doses higher than 2 mg/100 g caused respiratory disturbances. The action of glycopyrronium bromide in blocking the gastric secretory effects of gastrin supports the hypothesis that gastrin acts partly by stimulating the vagus nerve.

Recent advances in the physiology of gastric secretions have shown that the vagus nerve and gastrin are the two most important factors controlling the secretion of acid gastric juice (Hollander, 1962). The secretion can be divided into basal secretion due to vagal activity and the histamine- and gastrin-induced acid secretion. The basal secretion due to vagal activity can be controlled by atropine and anticholinergic drugs. Atropine also inhibits the histamine- and gastrin-induced secretion (Gregory & Tracy, 1961; Makhlouf, McManus & Card, 1965). Atropine inhibits endogenous or exogenous gastrin in gastric pouch dogs and the secretion of acid in response to pentagastrin (Hirschowitz & Sachs, 1968).

Franco & Lunsford (1960) reported that a series of n-substituted-3-pyrrolidyl-substituted phenylacetates possessed high anticholinergic activity. In particular, glycopyrronium bromide (3-cyclopentyl mandeloyloxy-1,1-dimethylpyrrolidinium bromide) was exceptionally active in suppressing smooth muscle motility and the volume and acidity of gastric secretion. The present experiments were made to demonstrate the effects of glycopyrronium bromide on acid secretory responses to histamine and gastrin. Amure & Ginsburg (1964) showed that inhibitors of histamine metabolism also enhanced gastric acid secretory responses to exogenous gastrin. The object of the present work was to evaluate acid secretory responses to histamine and gastrin in rats pre-treated with glycopyrronium bromide.

The vagal release of gastrin is now an established mechanism (Woodward, Robertson & others, 1957), by which gastrin stimulates the gastric acid secreting glands during the phase of basal secretion due to vagal activity and functions in addition to the direct vagal action on the glands. The results show that glycopyrronium bromide inhibits this basal phase as well as exogenous histamine and gastrin-induced acid secretion; provided the present concept of the hormonal role of histamine remains valid.

EXPERIMENTAL

Male albino rats, 150 to 350 g, were anaesthetized with urethane (0·6 ml/100 g of a 25%, v/v solution, intramuscularly), and the stomach was prepared for perfusion (Ghosh & Schild, 1958) with 0·001–0·00025 N sodium hydroxide solution. The rat was chosen because it tolerates large doses of histamine without untoward effects. Another advantage is that, as the acid secretion from the stomach of the anaesthetized rat is recorded continuously in the Ghosh & Schild method, the activity of both histamine and gastrin can be measured accurately. Amure & Ginsburg (1964) described a bioassay of gastrin in rats. All drugs were given intravenously through a cannulated external jugular vein. When histamine or gastrin was being tested, glycopyrronium bromide was injected 2 min before their injection. The pH was allowed to return to normal base line before the next injection. Doses are expressed as μg of the salts, except for histamine which was expressed in μmol of histamine base. Gastrin activity was measured in units of the partially purified extract of crude gastrin powder. The response metameter chosen was the maximum fall in pH after the injection and the response metameters are plotted against log dose of gastrin.

Materials

These were histamine dihydrochloride (Light and Co.); glycopyrronium bromide (A. H. Robins Co., Inc.); hog gastrin powder was prepared according to Blair, Harper & others (1961). Fresh hog antra were collected from the abattoir and transported in ice to the laboratory. The crude gastrin powder was further subjected to partial purification by gel-filtration and acid fractionation as described by Amure & Ginsburg (1964).

RESULTS

The assay of histamine and gastrin depends on changes of pH in the effluent fluid from the lumen of the perfused rat stomach after intravenous injections of histamine or gastrin. When histamine is given, changes in the pH of the gastric effluent occur 5–7 min later and gastrin, 2–3 min later (Amure & Ginsburg, 1964).

Responses to histamine and gastrin

In all experiments, histamine and gastrin had noticeable effects on gastric acid secretions. The results showed that animals varied in their sensitivity to either of the two secretagogues. Some animals responded appreciably to low doses of either agent while other animals required large doses before any appreciable changes occurred in the pH of the gastric effluent. Table 1 shows results of typical responses to histamine (0·5 and 0·25 μmol) and to gastrin (0·4 and 0·2 units).

Table 1. *Typical effects on the gastric effluent pH of rats of histamine, gastrin and glycopyrronium bromide*

Animal I		Animal II		Animal III			
Histamine dose μmol	pH change in units	Gastrin dose in units	pH change in units	Histamine dose μmol	pH change units	Glyco-pyrronium dose μg	pH change units
0·5	−2·4	0·4	−1·5	0·5	−1·5	5	+0·2
0·5	−2·3	0·2	−0·8	0·5	−1·5	5	+0·2
0·25	−1·0	0·2	−0·8	0·2	−0·4	2	no change
0·25	−1·0	0·2	−0·8	0·1	no change	1	no change

Responses to glycopyrronium bromide

Glycopyrronium bromide in small doses (2–5 μg) given intravenously to rats of not less than 150 g, caused complete inhibition of basal acid gastric secretion and acid secretions induced by either histamine or gastrin. In some animals, glycopyrronium bromide caused an initial rise in the pH of gastric effluent. Higher doses (1–2 mg/100 g) were tolerated but above this the drug gave rise to respiratory disturbances. For these experiments, small doses (2 or 6 μg/100 g) adequately controlled acid secretory responses to histamine or gastrin. Table 1 shows the results of typical responses of acid gastric secretion to 1, 2 and 5 μg of glycopyrronium bromide.

Effect of glycopyrronium bromide and histamine

The effect on the effluent pH after histamine given before and after the intravenous injection of glycopyrronium bromide shown in Table 2, in which the typical responses are shown by results from four rats. In all experiments, gastric acid secretory responses to histamine in animals pre-treated with glycopyrronium bromide (2 μg/100 g) were less than before glycopyrronium bromide was given.

Effect of glycopyrronium bromide and gastrin

These experiments were similar to those with glycopyrronium and histamine. Partially purified gastrin was given intravenously. In each experiment, gastrin was given until at least 4 similar responses were obtained. This was followed by glycopyrronium bromide (2 μg/100 g), after the pH of the effluent had returned to control level. Two min after glycopyrronium bromide had been injected, gastrin was given in the same dose as that given before the glycopyrronium bromide. In all experiments, the results showed that glycopyrronium bromide reduced responses to gastrin. Typical results in four such experiments are in Table 2.

Table 2. *Effects of histamine (μmol) or gastrin (units) on the pH of gastric secretion in anaesthetized rats before and after intravenous injection of glycopyrronium bromide (in μg) in rats*

Substance	Dose given	pH change units	Dose given	pH change units	Dose given	pH change units	Dose given	pH change units
	Animal 1		Animal 2		Animal 3		Animal 4	
Histamine	3·0	−1·9	0·25	−0·5	0·5	−1·5	0·25	−0·8
Histamine	2·5	−1·7	0·25	−0·5	0·5	−1·5	0·5	−0·8
Histamine	2·5	−1·7	0·25	−0·5	0·2	−0·8	0·5	−0·8
Histamine	2·5	−1·7	0·25	−0·6				
Glycopyrronium ..	6		3		6		4	
Histamine	2·5	−0·9	0·25	−0·1	0·5	−0·8	0·5	−0·3
Glycopyrronium ..	6		3	No	6		4	
Histamine	2·5	−0·7	0·25	change in pH	0·5	−0·8	0·5	−0·1
	Animal 5		Animal 6		Animal 7		Animal 8	
Gastrin	0·1	−0·8	0·5	−1·2	0·4	−1·5	0·4	−0·5
Gastrin	0·1	−0·7	0·5	−1·2	0·2	−0·8	0·6	−1·2
Gastrin	0·1	−0·8	0·5	−1·2	0·2	−0·8	0·6	−1·4
Gastrin	0·1	−0·8	0·5	−1·1	0·2	−0·8	0·6	−1·4
Glycopyrronium ..	5		5		4		5	
Gastrin	0·1	−0·2	0·5	−0·8	0·2	−0·4	0·6	−0·8
Glycopyrronium ..	5		5		4		5	
Gastrin	0·1	−0·3	0·5	−0·6	0·2	−0·5	0·6	−0·9

DISCUSSION

Acid secretory responses to stimulation by histamine and gastrin as reported previously by Ghosh & Schild (1958) and Amure & Ginsburg (1964) were confirmed in all respects. The animals tolerated gastrin better than either histamine or glycopyrronium bromide. In all experiments, glycopyrronium bromide had an inhibitory effect on basal acid secretions as shown in Table 2 where the pH of the gastric effluent rose by 0·2 pH units immediately after the administration of glycopyrronium bromide, and was sustained for about 5–6 min. This effect is analogous to the inhibition of gastric acid secretion by chlorpromazine which was demonstrated in dogs (Sun & Shay, 1959) and in rats (Konturek & Radecki, 1963). The effect is probably of nervous origin and mediated by the vagus.

Glycopyrronium bromide also reduced acid secretions induced either by intravenous histamine or gastrin. This was evident in all experiments in which the animals were pre-treated with glycopyrronium bromide. The evidence adduced shows that glycopyrronium bromide is capable of reducing acid secretory responses to both exogenous histamine and gastrin.

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Comparison of intracisternally and intraperitoneally injected harmaline on body temperature and tremor in the rat

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Low doses of harmaline (1–10 mg/kg) injected intraperitoneally into rats caused hypothermia, while higher doses (10–30 mg/kg) induced tremor in addition to hypothermia. Harmaline injected intracisternally decreased body temperature without inducing tremor. To induce a maximal fall in body temperature following intraperitoneal injection of harmaline, 1000 times more harmaline was needed than after intracisternal administration. Harmaline by this route induced hypothermia much faster than by intraperitoneal injection. It is concluded that harmaline-induced hypothermia is at least partly localized in the central nervous system and is not associated with the tremor.

Harmaline injected intraperitoneally into rats induces hypothermia (Schmidt & Fähse, 1964). Recently it was shown that harmaline did not provoke hypothermia if the biosynthesis of noradrenaline was inhibited (Bruinvels & Sourkes, 1968). This suggested that the actual lowering of body temperature is mediated by noradrenaline.

That noradrenaline is involved in hypothermia is in agreement with the results of Feldberg & Lotti (1967), who showed that intraventricular administration of noradrenaline can lower body temperature in rats. Also Schmidt & Fähse (1964) demonstrated a fall in body temperature after intracerebral injection of this amine into rats. The observation that noradrenaline injected intravenously into rats results in an increase in body temperature (Jori, Paglialunga & Garattini, 1967) may suggest that the hypothermic effect of harmaline is of central origin.

Harmaline, like harmine, also causes tremor in rats (Marković & Giaja, 1951). Whether harmaline-induced tremor is associated with alterations in amine metabolism or with hypothermia is unknown. The present experiments were designed to further explore the action of harmaline on body temperature and to investigate whether or not tremor is associated with the hypothermic effect of this compound.

EXPERIMENTAL

Material and methods

Male albino rats, 100–110 g, were placed in individual cages 1·5 h before the first injection, in a room maintained at 24·5 $\pm$ 1°.

Harmaline hydrochloride (Fluka) and saline were administered intracisternally (20 μl/rat) according to Jeffers & Griffith (1962). For intraperitoneal injection a volume of 1 ml/rat was used.

Body temperature was measured with a Telethermometer (Yellow Spring Co.). A probe was inserted 3 cm into the rectum of the rats until the recorded temperature

remained constant. The change in body temperature was calculated from the area of the temperature curves of the control and the harmaline-treated animals during a constant period of time after the administation of saline or harmaline respectively.

The frequency of the tremor was measured with an electro-magnetic vibration transducer (Philips, type PR 9262), which was used in combination with a recorder.

RESULTS AND DISCUSSION

The effect of different doses of intraperitoneally injected harmaline on body temperature is shown in Fig. 1A. The deepest fall in body temperature was reached after 1 h for doses of 1–10 mg/kg, whereas higher doses (10–30 mg/kg) showed a maximum after 1·5 h. After the administration of 10–30 mg of harmaline per kg weight, a tremor with a frequency of about 10 Hz was found in addition to the hypothermia. After injection of 8 mg/kg, 50% of the rats had tremor. Lower doses of harmaline did not induce tremor.

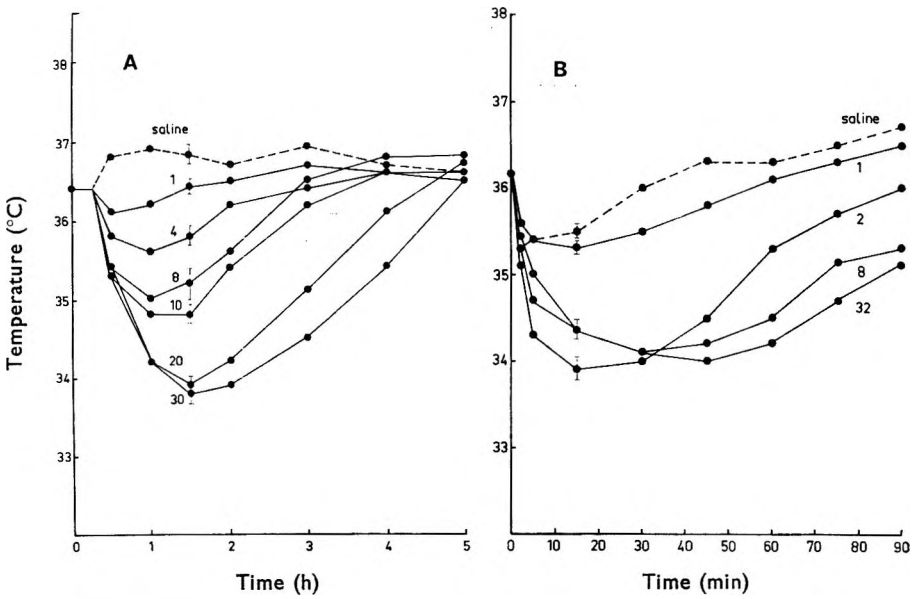

FIG. 1. Effect of (A) intraperitoneal, (B) intracisternal injection of various doses of harmaline on body temperature of the rat. Dots represent the mean of 6 experiments. Bars indicate s.e. and numbers the injected dose in, A, mg/kg, B, reg/rat.

Fig. 1B represents the fall in body temperature after intracisternal injection of different doses of harmaline. A dose of 2 μg of harmaline resulted in a maximal fall in body temperature; higher doses prolonged the duration of the hypothermia. Intracisternal injection of harmaline never caused tremor. Maximum decrease in body temperature occurred 15 min after intracisternal injection (Fig. 1B).

Fig. 2A and B represent dose-response relations after intraperitoneal and intracisternal injection of harmaline respectively. The decrease in body temperature was expressed in arbitrary units, which were proportional to the decrease in body temperature as a function of time (see methods). From Fig. 2A intraperitoneal injection of graded doses of harmaline can be seen to induce a dose-response relation which seems to be the result of two components. One elicited by doses up to 8 mg/kg, which

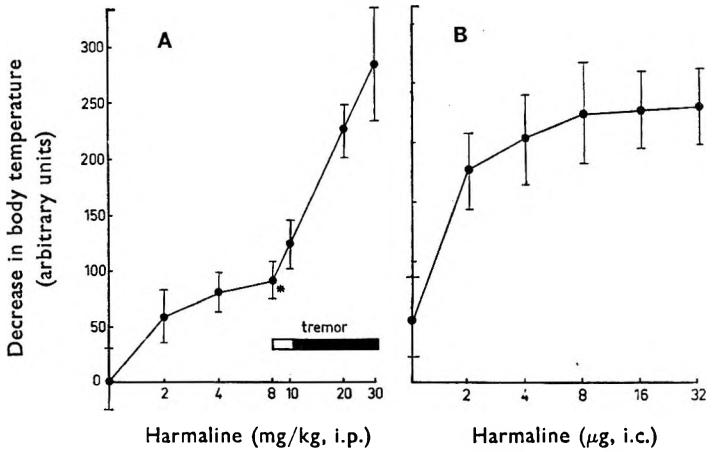

FIG. 2. Dose-response curves of the effect of, A, intraperitoneally, B, intracisternally injected harmaline on body temperature. The decrease in body temperature is expressed in arbitrary units proportional to the area beneath the temperature curves for (A) 285 min (B) 90 min after administration of harmaline or saline. Dots represent the mean of 6 experiments.* Mean of 5 experiments; one measurement was discarded according to the text of Dixon. Open bar 50% of the rats showed tremor. Solid bar 100% of the rats showed tremor.

caused a modest decrease in body temperature without tremor, and one by doses higher than 8 mg/kg which caused a marked fall in body temperature, and tremor. It is possible that the second half of the curve is caused by a facilitating effect of the tremor on the fall in body temperature. However, proof for this has yet to be provided.

Intraperitoneal injection of 32 μg of harmaline did not affect body temperature (Fig. 1A), while the same dose given intracisternally resulted in a maximal decrease of body temperature (Fig. 1B).

Thus intracisternally-injected harmaline in rats causes a fall in body temperature which occurs much more rapidly and with far lower doses than the intraperitoneally injected drug. From these results it may be concluded that the action of harmaline on body temperature is at least partly localized in the central nervous system.

The lower doses of harmaline, which caused hypothermia on intraperitoneal injection did not induce tremor. This suggests that these two phenomena are not necessarily associated. This is supported by the fact that intracisternal injection of harmaline caused hypothermia without provoking tremor. The fact that tremor did not occur in rats after intracisternal injection of harmaline, may indicate that the injected material does not reach those centres in the brain which induce tremor. Lack of association between hypothermia and tremor is also in agreement with previous results, viz. that pretreatment with a dopa-decarboxylase inhibitor or with an inhibitor of dopamine-β-hydroxylase prevents harmaline-induced hypothermia, but not the tremor (Bruinvels & Sourkes, 1968).

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The effect of two synthetic steroids on the ultrastructure of the liver of *Rattus norvegicus* L.

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Flumedroxone acetate and an analogue [17-acetoxy-3β(β-carboxypropionyloxy)-6-trifluoromethylpregn-5-ene-20-one] each produce a liver weight increase and a change in hepatic cell ultrastructure, following chronic administration in mice and rats. In all liver cells there is much proliferation of the smooth endoplasmic reticulum, which arises from the ergastoplasm, or rough membranes. An effect on esterase enzyme specificity and the evidence for the induction of an esterase isoenzyme after treatment with these steroids, is referred to. The distribution of the new smooth endoplasmic reticulum is of interest as it varies with each analogue.

Flumedroxone acetate (17-acetoxy-6α-trifluoromethylpregn-4-ene-3,20-dione; Demigran) and its analogue, 17-acetoxy-3β(β-carboxypropionyloxy)-6-trifluoromethylpregn-5-ene-20-one, have been reported to increase the liver weight of rats, *Rattus norvegicus*, L. Porton strain. These increases followed the daily intraperitoneal or oral administration of either steroid at various dose levels for a period of 3 to 14 days (Hines, 1967). Experiments on various strains of mice also produced liver weight increases; here the subcutaneous route was used and the steroids were suspended in arachis oil (Hines, unpublished observations). Pantelouris & Hines (1968) reported that these two steroid compounds cause a widening of the substrate specificity of a particular "fast running" esterolytic enzyme from the liver and serum of the adult laboratory rat, as detected by starch-gel electrophoresis, and that this wider specificity is normal in the young animal. This paper describes changes in ultrastructure of rat liver (Wistar strain) after chronic treatment with flumedroxone acetate and its analogue.

EXPERIMENTAL

Virgin female albino Wistar rats (*Rattus norvegicus*, L.) were used as they consistently produced a greater liver weight increase than male rats at similar dose levels (Hines, unpublished observations). Each rat weighed approximately 250 g, and was maintained on Lever Brothers Spittal No. 4 rat cubes with water *ad libitum*. Each steroid was suspended in water (1 ml) using compound tragacanth powder and was given by gastric intubation. Some animals were given tragacanth only by the same route, as a vehicle control; other controls received no treatment. Control and treated rats were killed by cervical fracture 24 h after the final treatment, samples were immediately taken for ultrastructural studies and the liver and the body weights were recorded.

Tissue samples for electron microscopic investigation were removed from each of the liver lobes of six rats randomly chosen from each experimental group (see Table 1).

These were fixed immediately in 1% osmic acid in veronal acetate buffer, pH 7·4, for 1 h (Palade, 1952). The samples were then rinsed in veronal acetate buffer, dehydrated through graded acetones and propylene oxide, then embedded in Araldite (Luft, 1961). Preliminary treatment for staining was by uranyl acetate incorporated in the acetone dehydrating fluids and then the sections were stained in Reynolds lead citrate (Reynolds, 1963). The sections were cut on an LKB ultra-microtome with glass knives and examined in an AEI-EM-6B electron microscope at an accelerating voltage of 60 kV.

RESULTS AND DISCUSSION

The daily administration of flumedroxone acetate or of its unsaturated analogue (17-acetoxy-3β(β-carboxypropionyloxy)-6-trifluoromethylpregn-5-ene-20-one) for 9 days produced an increase in the liver weight of Wistar rats in each case (Table 1). Flumedroxone acetate evokes a liver enlargement of 65–70% with Wistar rats compared to 85–90% with the Porton strain (see Table 1 and Hines, 1967). The stage of development and sample size, rather than strain difference probably account for the variation in overall liver weight response. Durand, Fauconneau & Penot (1965) considered that rat liver attains its "functional equilibrium" at 10 weeks but continues to grow for some weeks more; the Wistar rats that I used were at least 12 weeks old.

Table 1. *Liver weight of rats after treatment with steroid compound for 9 days*

Drug	Dose mg/kg	Mean body weight g (No. of rats) Porton rats†	Mean body weight g (No. of rats) Wistar rats	Liver weight g/100 g body weight (range, g) Porton rats†	Liver weight g/100 g body weight (range, g) Wistar rats
Flumedroxone acetate	50	138·2 (4)	225·4 (10)	7·2 (8·7–10·6)	6·2 (12·6–15·1)
17-acetoxy-3β(β-carboxypropionyl-oxy)-6-trifluoro-methylpregn-5-ene-20-one	50	136·7 (4)	248·2 (8)	7·8 (9·4–12·1)	7·0 (15·5–18·9)
Compound tragacanth powder ..	700*	137·2 (6)	—	3·9 (4·3–6·0)	—
	200	—	245·0 (14)	—	3·8 (8·3–10·6)
None	—	138·1 (8)	249·2 (10)	3·8 (4·4–7·5)	3·7 (8·1–10·7)

* Treatment continued for 14 days.
† After Hines (1967).

The fundamental ultrastructural modification after the treatment of Wistar rats with each of these synthetic steroids is classical. Comparison of liver cells from treated rats (Fig. 1B and C) with liver cells from untreated rats (Fig. 1A), shows hypertrophy of the smooth endoplasmic reticulum (ser) of the treated animals. This hypertrophy differed with each steroid. With flumedroxone acetate (Fig. 1C) a lattice work of smooth tubules (ser) grow from the nearby parallel ergastoplasm (rough membranes; rer), and occupy the whole hyaloplasm of the liver cell, a picture comparable to that following phenobarbitone treatment, 80 mg/kg i.p. daily for 5 days (Remmer, 1966). This formation of smooth (ser) from rough (rer) membranes is

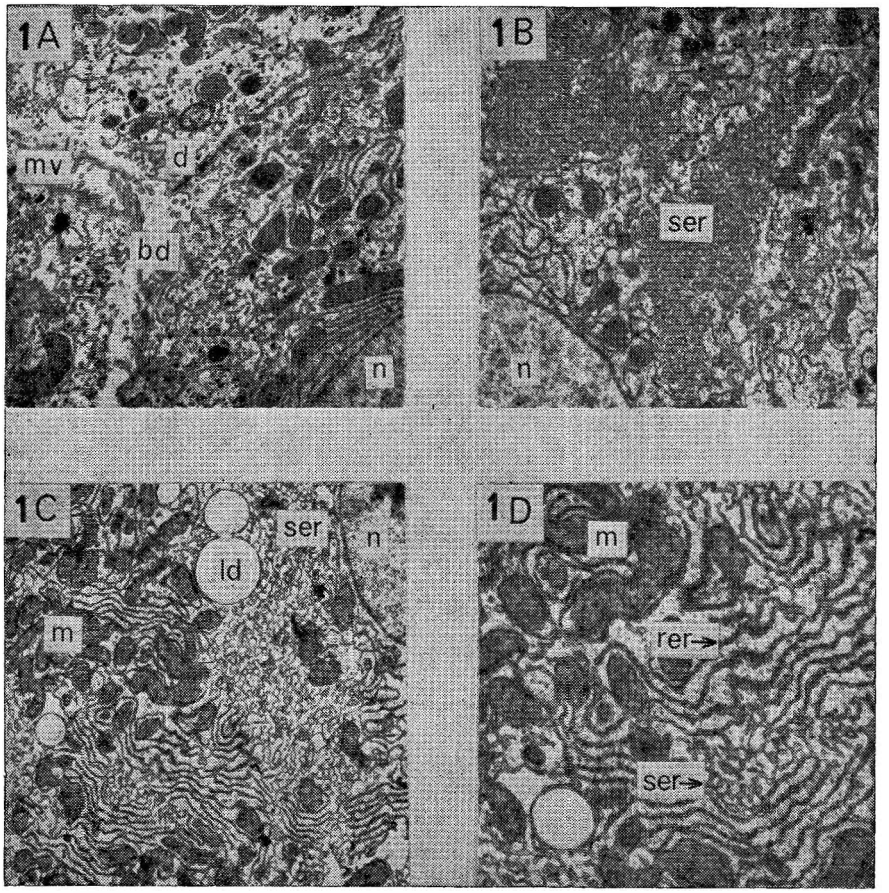

FIG. 1. Electron micrographs of rat liver cells. A. Untreated control, 1:7,400. B. 17-acetoxy-3β(β-carboxypropionyloxy)-6-trifluoromethylpregn-5-ene-20-one, 1:4,200. C. Flumedroxone acetate, 1:6,000. D. Flumedroxone acetate, 1:14,000. Abbreviations: m = mitochondrion; n = nucleus; mv = microvillus; bd = bile duct; d = desmosome; ld = lipid; ser = smooth endoplasmic reticulum; rer = rough endoplasmic reticulum.

shown more clearly in Fig. 1D. Such electron microscopic evidence indicates that smooth endoplasmic reticulum membranes are formed not through *de novo* protein synthesis, but through a process of cytoplasmic rearrangement. It has been observed that the rough endoplasmic reticulum membranes increase in liver cells even *in vitro* when respiration or oxidative phosphorylation are depressed (Loewe & Jung, 1965). For the unsaturated analogue of flumedroxone the distribution of the newly formed smooth membranes is seen to be zonal (Fig. 1B). This phenomenon was a constant feature with this steroid and has not previously been described. The origin of lipid is thought to be the hyaloplasm or the endoplasmic reticulum (du Boistesselin, 1966). In cells (e.g. sebaceous glands) engaged in the synthesis of lipids for export, it is common to find the cytoplasm filled with smooth surfaced tubules of ser (Porter, 1966). The proximity between smooth membranes and lipid (Fig. 2A), and an association between golgi, ser and lipid (Fig. 2B) was often seen following treatment with flumedroxone acetate. This suggests that the exogenous flumedroxone acetate has stimulated lipid formation through ser proliferation. On no occasion were any mitochondrial lesions observed with either steroid.

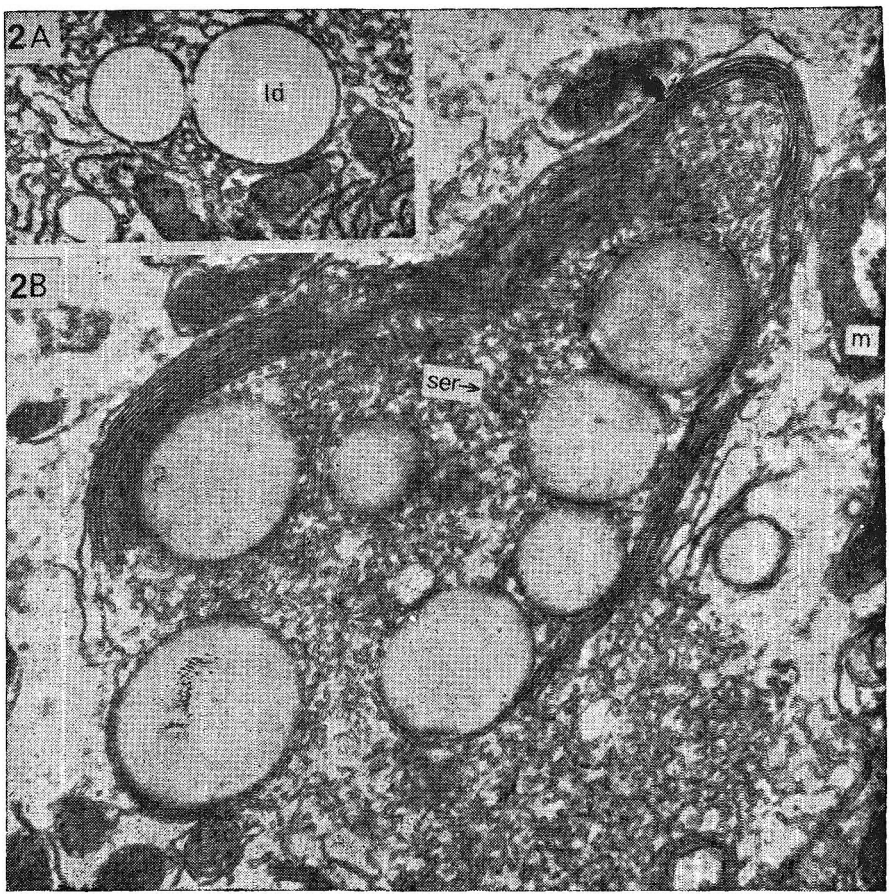

FIG. 2. Electron micrographs of rat liver cells treated with flumedroxone acetate. A. Smooth endoplasmic reticulum (ser) boundary to lipid, 1:15,500. B. Close association between ser, golgi and lipid, 1:14,000.

The chronic dosage of experimental animals with these progesterone-like synthetic steroids resulted in the following: an increase in the liver weight of various animals independent of the route of administration; a change in liver cell ultrastructure; and the appearance of two esterase isoenzymes one of which, like glucokinase (Golberg, 1966), appears in its broader reactive form as it does in the neonatal rat's liver and the second, a fast-running isoenzyme may be seen as a product of enzyme induction (Greengard, 1967; Pantelouris & Hines, 1968). Both isoenzymes were located in the microsomal fraction, a mixture of rough and smooth membranes. Chlorpromazine, phenylbutazone, SKF525-A and benzydamine can each produce in mice, after weekly treatment, a liver weight increase, a reduction in hexabarbitone sleeping time and a decrease in the retention of blood serum phosphatase (Silvestrini, Catanese & Del Basso, 1966). These drugs have been implicated in the induction of microsomal enzymes, which can activate the breakdown of the inducer itself, or their entirely different compounds (Remmer, 1964): the latter seemingly operating in this instance.

Meldolesi (1967) reviews those drugs known to bring about ser hypertrophy and suggests that 'ser hypertrophy in hepatic cells is always produced by one and the same mechanism", and further that the exogenous material which acts as the "inducer"

must itself be metabolized by enzymes which lie in the microsomal compartment of the cell. The latter fits this case, as both drugs are steroids and, as yet, are unproven inhibitors of protein synthesis. Although the overall mechanism might be the same for all ser hypertrophy, some difference must be operating in this case between the two steroid analogues. In one the ser is distributed through the cell, in the other it is zonal.

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The effect of 1,1-dimethyl-4-phenylpiperazinium on the response of mesenteric arteries to sympathetic nerve stimulation

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The effect of 1,1-dimethyl-4-phenylpiperazinium (DMPP) on the response to sympathetic nerve stimulation of rat mesenteric arteries perfused with Tyrode solution at a constant flow has been studied. DMPP (0·3 μg/ml) infused for 3 min enhanced the vasoconstriction caused by stimulation. Infusion of the same concentration for 16–40 min greatly reduced the response to nerve stimulation but did not affect the vasoconstrictor response to injected noradrenaline. The blockade of the response to nerve stimulation produced by DMPP was overcome either by adding (+)-amphetamine to the perfusion fluid or by raising the calcium concentration. Neither effect of DMPP was altered by the infusion of atropine. These effects of DMPP were similar to those seen when acetylcholine was added to the perfusion fluid except that the effects of acetylcholine were diminished or abolished by a concentration of atropine much higher than that of acetylcholine. It is concluded that the receptors at the adrenergic nerve terminals are partly muscarinic and partly nicotinic.

1,1-Dimethyl-4-phenylpiperazinium iodide (DMPP) which acts as a ganglionic stimulant, or as a ganglion blocking drug (Chen, Portman & Wickel, 1951; Page & McCubbin, 1953; Chen & Portman, 1954; Leach, 1957; Ling, 1959; Brownlee & Johnson, 1963) has also been shown to produce an increase in the rate and force of the heart by liberating catecholamines directly from the postganglionic nerve endings (Lindmar & Muscholl, 1961; Bhagat, 1966). Bentley (1962) and Wilson (1962) have shown on the other hand that DMPP inhibits the effect of stimulation of periarterial sympathetic nerves of the rabbit and guinea-pig intestine respectively. Birmingham & Wilson (1965) found that the inhibitory effect of DMPP on the intestine to sympathetic nerve stimulation had features in common with the blocking actions of guanethidine and bretylium. A similar type of blockade was observed by Rand & Wilson (1967) in the rabbit ear vessels.

Recent observations on the rat mesenteric vessels perfused with Tyrode solution have shown (Malik & Ling, 1969) that the vasoconstriction caused by stimulation of postganglionic fibres is increased when acetylcholine is infused in low concentration for a short period (15 s). The same concentration infused for a longer period (15 min) causes marked reduction or blockade of the response to sympathetic nerve stimulation. This blockade can be abolished by a concentration of atropine 20 times greater than that of acetylcholine. The blockade is also reversed by raising the calcium concentration of the perfusion fluid or by simultaneous infusion of (+)-amphetamine. In these last two respects the block by acetylcholine resembles the blockade caused by guanethidine.

The present study describes the effects of DMPP on the response of perfused mesenteric arteries of the rat to sympathetic nerve stimulation.

EXPERIMENTAL

Female albino rats, 250–300 g, were anaesthetized with ether, the abdomen opened and the superior mesenteric artery cannulated and isolated with its small resistance vessels (McGregor, 1965). A Harvard peristaltic pump (Harvard Apparatus Co., Model 1210) was used to perfuse the arteries at a constant flow of 25 ml/min with Tyrode solution of the following composition in mM: NaCl, 136; KCl, 2·7; $CaCl_2$, 1·8; $MgCl_2$, 1:1; $NaHCO_3$, 12; $NaHPO_4$, 0·42 and dextrose, 5·6. The solution was aerated with a mixture of 5% carbon dioxide in oxygen and was maintained at 22°. In some experiments the temperature of the perfusion fluid was 37°. Changes in perfusion pressure were recorded manometrically from the cannulated artery using a frontal writing lever on a kymograph. Before cannulation when the pump was operating and the flow was 25 ml/min the pressure was 60 mm Hg. During perfusion the pressure increased to 85 mm Hg. Thus the average basal pressure during an experiment was 25 mm Hg. Since the mesenteric vessels were cut along the intestine, this pressure was due to the resistance of the arterioles.

Injections of noradrenaline were made directly into the cannula leading to the superior mesenteric artery by means of a Palmer pump (F-30).

The perivascular nerves were stimulated for 20–25 s every 4 min interval with a Grass stimulator (Grass Instrument Co., Model 4C) using biphasic rectangular pulses (20 V; 1 ms; at 7/s).

The vasoconstrictor responses to both sympathetic nerve stimulation and injected noradrenaline in all experiments were submaximal.

The drugs were: 1,1-dimethyl-4-phenylpiperazinium iodide, (−)-noradrenaline bitartrate monohydrate and atropine sulphate (K & K Laboratories). Cocaine hydrochloride was generously supplied by British Drug Houses (Toronto), guanethidine sulphate by CIBA (Dorval) and (+)-amphetamine sulphate by Smith, Kline and French (Montreal).

The drugs were dissolved in normal saline just before use and added in the perfusion solution in a volume of not more than 0·5 ml/litre to obtain the final concentration. The final concentration is expressed as that of the salts.

RESULTS

The preparation of the rat mesenteric arteries was stable for periods of more than 4 h. Nerve stimulation and injected noradrenaline produced uniform responses during this time. DMPP in concentrations of less than 2 μg/ml in the perfusion fluid failed to produce any change in the basal perfusion pressure while higher concentrations produced a slight increase.

Effect of DMPP *on the response to sympathetic nerve stimulation*

When DMPP was infused in a concentration of 0·3 μg/ml for 3 min, the vasoconstriction produced by sympathetic postganglionic nerve stimulation was increased. Such an increase is seen in Fig. 1, where it lasted for 56 min. The maximum increase was 60% except in one experiment in which an increase of 170% lasting also 56 min was observed. An increase in response to nerve stimulation after the 3 min infusion of DMPP was observed at 22° and also at 37° in six preparations.

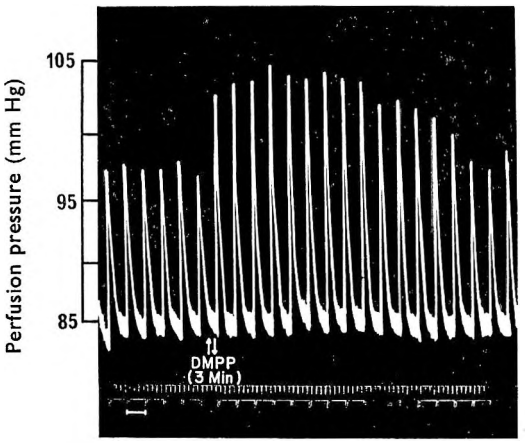

FIG. 1. The potentiating effect of DMPP on the perfusion pressure response of mesenteric arteries of rat to sympathetic nerve stimulation. The mesenteric arteries were perfused with Tyrode solution at a rate of 25 ml/min at 22°. The perivascular nerves were stimulated using biphasic pulses (20 V; 1 ms; at 7/s) every 4 min for 20 s. After 6 control responses were recorded, the infusion of DMPP (0·3 μg/ml) for 3 min markedly increased the response to nerve stimulation. The increase in response was present for about 56 min. Time Marker = 1 min interval.

However, when DMPP in the same concentration (0·3 μg/ml) was infused for 16, 20 or 40 min the initial response was increased but this was followed by inhibition which progressed until the responses to stimulation were abolished. The inhibitory effect of DMPP in 0·3 μg/ml concentration for 32 min is shown in Fig. 2, where the period of infusion was not long enough for complete inhibition. The removal of DMPP from the perfusion fluid only partially restored the responses. Preparations perfused at 37° gave similar results to those at 22° except that DMPP was more active in reducing the response at the lower temperature.

Effect of DMPP *on the response to injected noradrenaline*

To find out whether the inhibitory action of DMPP was due to a diminution in the amount of noradrenaline released or to a failure of the amine to cause vasoconstriction in the presence of DMPP, experiments were made in which the responses to submaximal nerve stimulation and then to submaximal amounts of injected noradrenaline were recorded. Care was taken to use an amount of noradrenaline (3–5 μg) which produced a similar response to that produced by stimulation. The example given in Fig. 2 shows that neither the beginning of the infusion of DMPP, nor the end of the infusion affected the response to injected noradrenaline though the infusion greatly diminished the response to nerve stimulation.

Effect of (+)*-amphetamine*

When (+)-amphetamine was added to the fluid perfusing the mesenteric arteries in a concentration of 0·2 μg/ml it caused a large increase in the response to stimulation of the sympathetic fibres; in one experiment the increase was 75%. Since (+)-amphetamine has been shown to reverse the blockade of responses to sympathetic impulses produced by guanethidine and bretylium (Day, 1962), and also blockade of responses to sympathetic impulses produced by DMPP in the rabbit intestine (Birmingham & Wilson, 1965), experiments were made to see if (+)-amphetamine

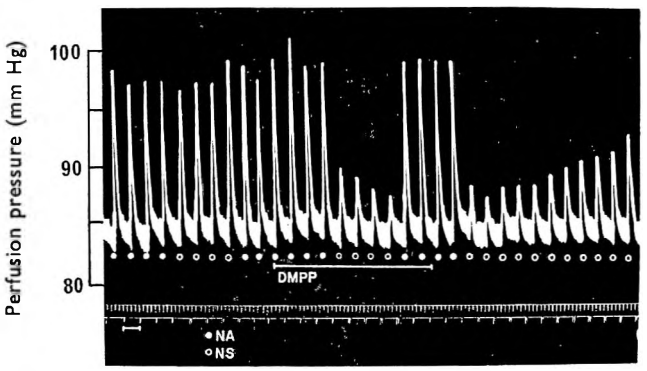

FIG. 2. Comparison of the inhibitory effect of DMPP on the responses to submaximal sympathetic nerve stimulation and to submaximal amounts of injected noradrenaline. Recording as in Fig. 1. The responses to injected noradrenaline (NA—3μg) were obtained by injecting it directly in to the cannula leading to superior mesenteric artery. Responses to sympathetic nerve stimulation (NS) were greatly inhibited, while those to injected NA remained unaffected during the infusion of DMPP (0·3 μg/ml) for 42 min. When the drug-free Tyrode solution was resumed the responses to nerve stimulation were restored partially.

would also reverse the blockade of the vasoconstrictor responses produced by DMPP in the mesenteric arteries. Fig. 3 A shows that simultaneous infusion of (+)-amphetamine (0·2μg/ml) reversed the blockade of the response to nerve stimulation produced by DMPP (2μg/ml); this effect was observed on 6 preparations.

Effect of increased calcium concentration

Since Burn & Welsh (1967) showed that increased calcium (Ca^{++}) concentration reversed the blockade of sympathetic impulses produced by guanethidine, it was of interest to see whether increased Ca^{++} concentration would also reverse the blockade of the response to nerve stimulation produced by DMPP. When Ca^{++} concentration of the perfusion fluid was raised to 4 times the normal value (i.e. from 1·8 to 7·2 mM by adding $CaCl_2$ to the perfusion fluid) the blockade produced by DMPP was partially reversed but reappeared again during the infusion of DMPP as shown in Fig. 3 B. Similar observations were made in 11 experiments.

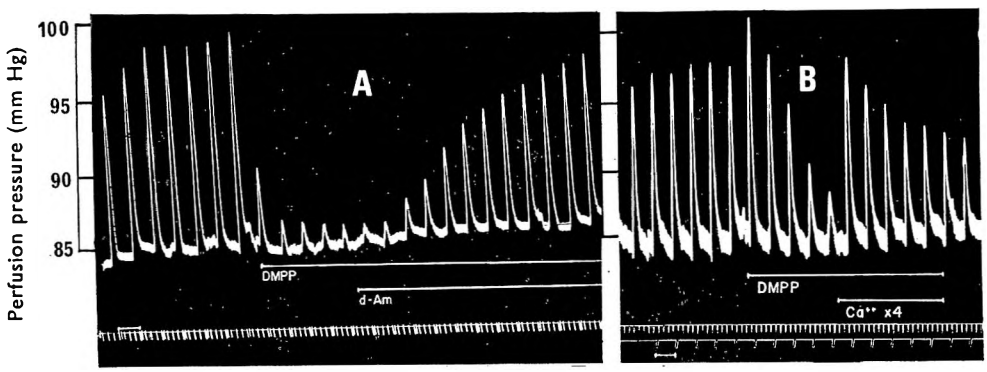

FIG. 3. A. Effect of (+)-amphetamine on the blocking action of DMPP. Recording as in Fig. 1. The responses to nerve stimulation were almost abolished when DMPP was infused in concentrations of 2 μg/ml of the perfusion fluid. (+)-Amphetamine (d-Am 0·2 μg/ml) reversed the blockade produced by DMPP.

B. Effect of increased calcium concentration on DMPP-induced blockade. Recording as in Fig. 1. Responses to nerve stimulation were inhibited by DMPP (0·3 μg/ml). Raising the calcium (Ca^{++}) concentration in the perfusion fluid to 4 times the normal value (i.e. to 7·2 mM) reversed partially the blockade produced by DMPP but it reappeared again during the infusion of DMPP.

Effect of hexamethonium

To exclude the possibility that the blocking action of DMPP on the response of mesenteric arteries to sympathetic nerve stimulation might be due to its ganglion blocking effects, experiments were made with hexamethonium. Hexamethonium (0·5 μg/ml) neither affected the response, nor reversed the blocking action of DMPP on the response to sympathetic nerve stimulation (4 experiments).

Effect of cocaine

Cocaine has been shown to increase the effect of catecholamines by impairing their uptake in adrenergic nerve terminals (see Trendelenburg, 1966). Cocaine (0·1 μg/ml) exerted a transient antagonism to partial inhibition produced by infusing the DMPP (0·3 μg/ml) for 20 min, but failed to reverse the blockade produced by a more prolonged infusion of this or higher concentrations (2 μg/ml).

Effect of bretylium and guanethidine

The effect of bretylium (0·5 μg/ml) and guanethidine (0·5 μg/ml) was investigated on the response of mesenteric arteries to sympathetic nerve stimulation and injected noradrenaline. These agents blocked the response to nerve stimulation without affecting the response to injected noradrenaline. The blockade of response to nerve stimulation produced by guanethidine and bretylium was also reversed by (+)-amphetamine and by raising the concentration of Ca^{++} to 4 times (i.e. to 7·2 mM) the normal value, but was unaffected by hexamethonium or cocaine.

DISCUSSION

The observations which have been made with DMPP follow our earlier observations on acetylcholine (Malik & Ling, 1969). When the sympathetic fibres to the perfused mesenteric arteries of rat were stimulated, the addition of acetylcholine, 2 ng/ml, to the perfusion fluid had two different effects according to the length of time for which the addition was made. When the infusion was for 15 s only, the response to stimulation increased, in some cases being doubled. However, when the addition was made for a longer time, such as 15 min, the response to stimulation was reduced and even abolished. These observations supported the view that the postganglionic fibre first releases acetylcholine and that this in turn releases noradrenaline. It appears that when acetylcholine was infused for a short period of time its effect and that of the acetylcholine released by the sympathetic fibre were additive causing an increased response. However, when it was infused for a longer time (15 min) it would occupy all the receptors on which the acetylcholine released by nerve stimulation could act, and therefore the response to stimulation would be abolished. In connection with this abolition, the surprising observation was made that the responses returned when atropine was infused in a concentration of 100 ng/ml together with the acetylcholine (5 ng/ml). This suggested that the receptors on which the prolonged infusion of acetylcholine acted to cause block were muscarinic as Lindmar, Löffelholz & Muscholl (1968) have concluded.

To see if these receptors were in fact muscarinic, experiments were made with DMPP, which is known to act on nicotinic receptors. DMPP releases noradrenaline from postganglionic terminations in the heart, and has been shown to block sympathetic terminations (Bentley, 1962; Wilson, 1962) like acetylcholine (Brücke, 1935; Burn & Rand, 1960).

The results that have been described show that DMPP, when infused for short periods, also resembles acetylcholine in causing an increased response to sympathetic stimulation. They also show that DMPP, like acetylcholine, when infused for a longer time, blocks the response to sympathetic stimulation, and that the blockade can be removed either by raising the calcium concentration of the perfusing fluid, or by the simultaneous infusion of (+)-amphetamine. The blockade produced by DMPP, 0·3 μg/ml, was however unaffected by atropine, and in this respect differed from the blockade produced by acetylcholine. The conclusion can be drawn that the receptors concerned are partly muscarinic and partly nicotinic, like receptors on sympathetic ganglia. The work of Ambache, Perry & Robertson (1956) demonstrated that muscarine itself will stimulate sympathetic ganglia, and that this action is modified by atropine.

The blocking effect of prolonged infusion with either acetylcholine or DMPP can be explained not as an effect on inhibitory receptors, but as the inhibition which follows full occupation of receptors for stimulation. It has long been known, for example, that receptors for acetylcholine at the neuromuscular junction in skeletal muscle are blocked when the motor nerve is stimulated at high rates in the presence of an anticholinesterase. In this situation the concentration of acetylcholine rises to such a level that all the receptors are occupied causing stimulation of the nerve to be ineffective.

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K. U. Malik is postdoctoral fellow of the OMHF.

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Basic dihydromorphanthridinones with anticonvulsant activity

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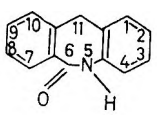
Some 11-alkylamino-5,6-dihydro-6-oxomorphanthridines and related compounds have been prepared and screened for anticonvulsant activity. One of the more active compounds, 11-dimethylamino-5,6-dihydro-6-oxomorphanthridine (ICI 45,337) was selected for further study and taken to clinical trial in epileptic patients.

There is currently considerable interest in drugs with tricylic structures as anti-depressants (Stewart, Charest & Herr, 1963; Boissier, Simon & others, 1965, among others) and as anticonvulsants (Theobald & Kunz, 1963; Davis, Winthrop & others, 1964). One such tricyclic structure (I) was chosen for investigation and a series of novel basic dihydromorphanthridinones prepared and examined for their effects on the central nervous system.

Structural requirements for anti-epileptic activity have been reviewed extensively (Spinks & Waring, 1962). Most of the currently useful drugs are weakly acidic (barbiturates, hydantoins) or chemically neutral (primidone, succinimides) and the presence of one or more –CO·NH– linkages in the molecule is considered by many writers to be associated with anticonvulsant activity. The dihydromorphanthridinones (II) described in this investigation may be regarded as cyclic amides but they are also bases and are thus unlike the drugs at present in use for the treatment of epilepsy.



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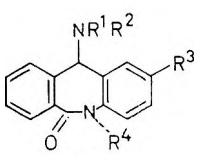


II

CHEMISTRY EXPERIMENTAL

Compounds in which the amide nitrogen atom was unsubstituted (II; R^4=H) were prepared by reaction of 11-chloro-5,6-dihydro-6-oxomorphanthridine with the appropriate amine either alone (Methods A and B) or in dimethylformamide (Method C).

Compounds in which the amide nitrogen atom was substituted (II; R^4=alkyl)

were not available by this route. 11-Chloro-5,6-dihydro-5-methyl-6-oxomorphanthridine failed to react with diethylamine at reflux temperature. The 5-alkyl compounds were, however, conveniently obtained by reaction of the sodium derivative of the 11-dialkylamino-5,6-dihydro-6-oxomorphanthridine with the appropriate alkyl halide in dimethylformamide solution (Method D).

Method A

11-Chloro-5,6-dihydro-6-oxomorphanthridine was added gradually to ten times its weight of the appropriate amine, with stirring and cooling. When the exothermic reaction was over, the mixture was heated under reflux for 30 min and then cooled and filtered. The filtrate was distilled to remove the excess of amine, the residue stirred with water, and the mixture filtered. The solid residue of crude product was then crystallized, usually from methanol or aqueous methanol.

Method B

11-Chloro-5,6-dihydro-6-oxomorphanthridine (0·01 mol) was added gradually to the amine (0·06 mol) with stirring and cooling. When the exothermic reaction was over, the mixture was heated at 90–95° for 15 min. The mixture was cooled, diluted with water and the solid collected by filtration. The crude solid product was dissolved in cold 3N hydrochloric acid, the solution filtered, and the filtrate made alkaline with ammonia solution. The precipitated solid was collected by filtration and crystallized from methanol or aqueous methanol.

Method C

11-Chloro-5,6-dihydro-6-oxomorphanthridine, dissolved in five times its weight of dimethylformamide, was mixed with three times its weight of the amine (40% aqueous solution), and the mixture heated at 90–100° for 30 min. The mixture was cooled and filtered, and the crude solid product dissolved in ice-cold 3N hydrochloric acid. The solution was filtered, and the filtrate made alkaline with ammonia solution. The precipitated solid was filtered, washed with water, and crystallized from methanol or aqueous methanol.

Method D

Sodium hydride (0·01 mol; 50% dispersion in oil) was added in portions to a stirred, cooled solution of the appropriate 11-dialkylamino-5,6-dihydro-6-oxomorphanthridine (0·01 mol) in dry dimethylformamide (20 ml), the temperature being kept between 0 and 10°. The mixture was allowed to warm to 20° and then the appropriate alkyl halide (0·01 mol) added gradually to the solution of the sodium derivative, the temperature being allowed to rise to about 40°. The mixture was finally heated at 50–60° for 1 h to complete the reaction. The mixture was cooled, poured into ice water and the precipitated solid collected by filtration and washed with water. After washing with light petroleum (b.p. 40–60°), the product was crystallized from methanol, aqueous methanol or light petroleum (b.p. 60–80°).

11-Diethylamino-5,6-dihydro-6-oxomorphanthridine hydrochloride was prepared by adding ethereal hydrogen chloride to a solution of the base in methanol. The precipitated hydrochloride was collected by filtration and crystallized from methanol; m.p. 166° (decomp.). (Found: C, 65·2; H, 7·3; N, 7·9. $C_{18}H_{20}N_2O,HCl,CH_3OH$ requires C, 65·4; H, 7·2; N, 8·0). When the hydrochloride was prepared in acetone with ethereal hydrogen chloride, and the product crystallized from methanol/ether, it

was obtained as a monohydrate, m.p. 172° (decomp.). (Found: C, 64·4; H, 6·7; N, 8·0. $C_{18}H_{20}N_2O,HCl,H_2O$ requires C, 64·6; H, 6·9; N, 8·4).

11-*Chloro*-5,6-*dihydro*-6-*oxomorphanthridine*. A mixture of 5,6-dihydro-11-hydroxy-6-oxomorphanthridine (11 g), chloroform (50 ml) and thionyl chloride (6 g) was heated under reflux for 30 min and then cooled and filtered. The solid residue was recrystallized from benzene to give 11-*chloro*-5,6-*dihydro*-6-*oxomorphanthridine*, m.p. 226° (decomp.) (Found: C, 69·3; H, 4·0; N, 5·3. $C_{14}H_{10}ClNO$ requires C, 69·0; H, 4·1; N, 5·7%).

2-*Bromo*-5,6-*dihydro*-6,11-*dioxomorphanthridine*. A mixture of 5,6-dihydro-6,11-dioxomorphanthridine (10 g), acetic acid (250 ml) and bromine (2·5 ml, 8 g) was heated under reflux on a steam bath for 4 h. The solution was cooled, and the crystals collected by filtration and recrystallized from acetic acid to give 2-*bromo*-5,6-*dihydro*-6,11-*dioxomorphanthridine* (8 g), m.p. 308–310°. (Found: C, 55·7; H, 2·7; N, 4·3. $C_{14}H_8BrNO_2$ requires C, 55·6; H, 2·7; N, 4·6%.)

2-*Bromo*-5,6-*dihydro*-11-*hydroxy*-6-*oxomorphanthridine*. Sodium borohydride (3 g) was added in portions to a stirred, cooled suspension of 2-bromo-5,6-dihydro-6,11 dioxomorphanthridine (8 g) in ethanol (200 ml), the temperature being kept at 10–15°. The mixture was stirred at room temperature for 18 h, diluted with water (200 ml), acidified with dilute hydrochloric acid and filtered. The solid 2-*bromo*-5,6-*dihydro*-11-*hydroxy*-6-*oxomorphanthridine*, m.p. 270–272°, was used directly without further purification.

2-*Bromo*-11-*dimethylamino*-5,6-*dihydro*-6-*oxomorphanthridine*. Thionyl chloride (1·5 ml) was added gradually to a solution of 2-bromo-5,6-dihydro-11-hydroxy-6-oxomorphanthridine (2 g) in dry dimethylformamide (25 ml) at 60°. When the exothermic reaction was over, the solution was cooled to 20°, and dimethylamine (12·5 ml of a 40% solution) cautiously added. The mixture was heated at 90° for 30 min, cooled, poured into water and filtered. The solid residue was suspended in water (200 ml), and the mixture acidified with 20% hydrochloric acid and filtered. The filtrate was made alkaline with ammonia solution and filtered. The solid residue was recrystallized from methanol to give 2-*bromo*-11-*dimethylamino*-5,6-*dihydro*-6-*oxomorphanthridine*, m.p. 216–217°.

2-*Chloro*-5,6-*dihydro*-11-*hydroxy*-6-*oxomorphanthridine*. Sodium borohydride (2 g) was added in portions to a stirred, cooled suspension of 2-chloro-5,6-dihydro-6,11-dioxomorphanthridine (8 g) in methanol (200 ml), the temperature being kept at 0–5°. The mixture was stirred for 1 h, diluted with water (200 ml), acidified with dilute hydrochloric acid and filtered. The solid (7 g) was recrystallized from aqueous dimethylformamide to give 2-*chloro*-5,6-*dihydro*-11-*hydroxy*-6-*oxomorphanthridine*, m.p. 268–270°. (Found: C, 64·1; H, 3·9; N, 5·5. $C_{14}H_{10}ClNO_2$ requires C, 64·6; H, 3·9; N, 5·4).

2-*Chloro*-11-*dimethylamino*-5,6-*dihydro*-6-*oxomorphanthridine*. This compound was prepared in exactly the same way as the 2-bromo-analogue, starting from 2-chloro-5,6-dihydro-11-hydroxy-6-oxomorphanthridine, but without isolation of the intermediate 11-chloro-compound.

5,6-*Dihydro*-11-*hydroxy*-5-*methyl*-6-*oxomorphanthridine*. Sodium borohydride (4 g) was added in portions to a stirred, cooled suspension of 5,6-dihydro-5-methyl-6,11-dioxomorphanthridine (Drukker & Judd, 1965) (10 g) in methanol (200 ml), the temperature being kept below 10°. The mixture was stirred for 1 h at 10–15°, diluted with water (150 ml), acidified with dilute hydrochloric acid and filtered. The solid

(9·5 g) had m.p. 198–200° and the m.p. of a sample was unchanged after crystallization from methanol. (Found: C, 75·1; H, 5·6; N, 5·8. $C_{15}H_{13}NO_2$ requires C, 75·3; H, 5·5; N, 5·85%).

11-*Chloro*-5,6-*dihydro*-5-*methyl*-6-*oxomorphanthridine*. A mixture of 5,6-dihydro-11-hydroxy-5-methyl-6-oxomorphanthridine (2·3 g), thionyl chloride (2·4 ml) and chloroform (A.R.) was heated under reflux on a steam bath for 30 min. The solvent was removed by distillation under reduced pressure, and the residue recrystallized from benzene to give 11-*chloro*-5,6-*dihydro*-5-*methyl*-6-*oxomorphanthridine*, m.p. 164–165°. (Found: C, 71·1; H, 4·4; N, 5·5. $C_{15}H_{12}ClNO$ requires C, 69·9; H, 4·7; N, 5·4%.)

5,6-*Dihydro*-6-*oxo*-11-*succinimidomorphanthridine* (cpd 31). Sodium hydride (0·8 g, 50% dispersion in oil) was added to a stirred, cooled solution of succinimide (1·7g) in dry dimethylformamide (20 ml), the temperature being kept below 15°. After stirring for 30 min, 11-chloro-5,6-dihydro-6-oxomorphanthridine (4 g) was added, and the temperature allowed to rise to 28°. The mixture was poured into ice-water (50 ml), and the precipitate collected by filtration. The solid product was crystallized from a large volume of ethanol to give 5,6-*dihydro*-6-*oxo*-11-*succinimidomorphanthridine*, m.p. 279–280°. (Found: C, 70·4; H, 4·6; N, 8·8. $C_{18}H_{14}N_2O_3$ requires C, 70·6; H, 4·6; N, 9·15%.)

5,6-*Dihydro*-6-*oxo*-11-*phthalimidomorphanthridine* (cpd 32). This compound was obtained in a similar manner from potassium phthalimide (1·8 g), suspended in dimethylformamide (20 ml), and 11-chloro-5,6-dihydro-6-oxomorphanthridine (2·4 g). The mixture was heated at 100° for 30 min and then poured into water. The product was crystallized from ethanol to give 5,6-*dihydro*-6-*oxo*-11-*phthalimidomorphanthridine*, m.p. 244–5°. (Found: C, 74·3; H, 4·0; N, 7·8. $C_{22}H_{14}N_2O_3$ requires C, 74·6; H, 4·0; N, 7·9%.)

11-N-*Ethylacetamido*-5,6-*dihydro*-6-*oxomorphanthridine* (cpd 33). Acetic anhydride (1 ml) was added to a suspension of 11-ethylamino-5,6-dihydro-6-oxomorphanthridine (0·2 g) in dry pyridine (5 ml) and the mixture heated on a steam bath for 3 min. After cooling, the mixture was diluted with water, filtered, and the solid residue washed with cold dilute hydrochloric acid. The insoluble residue was crystallized from aqueous methanol to give 11-N-*ethylacetamido*-5,6-*dihydro*-6-*oxomorphanthridine*, m.p. 221–2°. (Found: C, 73·3; H, 6·0; N, 9·7. $C_{18}H_{18}N_2O_2$ requires C, 73·45; H, 6·2; N, 9·5%.)

PHARMACOLOGY EXPERIMENTAL

Methods and materials

General. Specific pathogen-free (SPF) male and female mice of the Alderley Park strain, weighing 19–21 g and (SPF) male and female rats, 95–125 g, were used. Compounds were dissolved or suspended by ball-milling for at least 24 h in an inert dispersing agent containing per litre:—Lissapol NX 1 g, Lissapol C 1 g, Dispersol OG 1 g, and adjusted to pH 7. For parenteral injection the basic dihydromorphanthridinones were given as solutions of the hydrochlorides. Unless otherwise stated, doses of the drugs were given by stomach tube in a dose volume of 25 ml/kg body weight for mice and 5 ml/kg body weight for rats. Where comparisons are made between treated and control animals it should be understood that the control animals received an equal volume of the vehicle alone by the same route.

Mouse maximal electroshock method. Seizures were induced in mice by the application of an electrical current from a constant current stimulator through aural clip electrodes. The animals received a single shock of 0·33 s duration and an intensity of 20 mA. This current stuns the animals immediately and subsequently produces tonic extension of the hind limbs. Pretreatment with anticonvulsant drugs prevents the tonic extension following electroshock. Pretreatment times were 1 or 2 h. The number of animals in a group which fail to show tonic extension is a measure of the protection afforded by the drug. For purposes of comparison, the anticonvulsant activity of the various drugs was expressed as the dose which prevented tonic extension in 50% of a group of animals (Median Effective Dose — ED50). The confidence limits for the estimate of the ED50 were determined by logit analysis or by the method of Litchfield & Wilcoxon (1949).

Duration of action in mice. Groups of 10 mice received a dose equivalent to twice the oral ED50. The proportion of animals protected was determined at intervals of ½, 1, 2, 4, 6, 8, 12 and 24 h after dosing.

Prevention of supramaximal leptazol seizures. Groups of 10 mice received 160 mg/kg of leptazol intraperitoneally. In unprotected animals this dose of leptazol produced clonic convulsions and tonic hind limb extension, and killed most of the animals within 30 min. Drugs were given orally 1 or 2 h before injection of leptazol and ED50 values were calculated from the numbers of animals in which the hind leg tonic extension component of the normal seizure was prevented. The protective dose (PD50) was also calculated from the number of animals alive 30 min after injection of leptazol.

Rat low current electroshock test. Anticonvulsant activity was measured using the method of Bogue & Carrington (1953). In this test, the energy, in mWs, necessary to elicit hindlimb tonic extensor spasm was measured. Animals received a low current of 7·5 mA which was applied for not more than 10 s by means of aural clip-on electrodes, moistened with saline. Groups of 8–10 rats were used for the estimation of ED50 values; for less active compounds the percentage of animals protected at a particular dose is recorded.

Ataxia in mice. Three parameters measuring different aspects of drug-induced coordination were measured. These were the ability of mice to remain on a rotating 12 inch diameter roller rotating at a surface speed of 50 cm/min; the ability to remain on a 0·9 cm diameter rod rotating at 2 rev/min; and the ability to remain on, or walk to the edge of the underside of a horizontal 0·78 cm mesh. The assessment of performance was based on the length of time that the animal was able to remain on the obstacle. The neurotoxic dose was derived from the mean of the doses producing a significant degree of motor incoordination in each of the several tests.

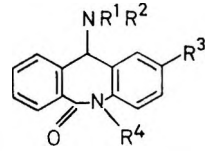
Acute toxicity. Median lethal doses were determined after administration of single oral doses to groups of 2–20 mice. The number of survivors seven days later was determined by inspection and median lethal doses (LD50) with 95% confidence limits were calculated from the proportion of animals surviving using standard methods of logit analysis.

RESULTS

Structure activity relations

Compounds are referred to by order number in Tables 2 (a), (b) and (c).

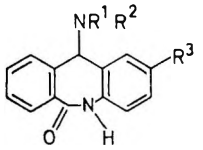
Table 1.



No.	R^1	R^2	R^3	R^4	M.p. °C	Found (%) C	H	N	Formula	Required (%) C	H	N	Method
1	Me	Me	H	H	208–9	76·4	6·6	11·0	$C_{16}H_{16}N_2O$	76·2	6·6	11·1	C
2	Et	Et	H	H	157–8	77·1	7·6	10·1	$C_{18}H_{20}N_2O$	77·1	7·2	10·0	A
3	Pr^i	Pr^i	H	H	192	77·6	7·9	9·1	$C_{20}H_{24}N_2O$	77·9	7·8	9·1	A
4	Pr^n	Pr^n	H	H	154–6	77·7	7·7	9·0	$C_{20}H_{24}N_2O$	77·9	7·8	9·1	A
5	Me	$CH_2{\cdot}CH_2OH$	H	H	167–8	71·6	6·4	9·9	$C_{17}H_{18}N_2O_2$	72·3	6·4	9·9	B
6	H	Et	H	H	178–9	76·3	6·3	10·9	$C_{16}H_{16}N_2O$	76·2	6·4	11·1	C
7	H	$CH_2{\cdot}CH_2OH$	H	H	134–5	71·3	6·1	10·4	$C_{16}H_{16}N_2O_2$	71·6	6·0	10·4	B
8	H	Pr^n	H	H	140–1	75·5	6·7	10·4	$C_{17}H_{18}N_2O$	76·7	6·8	10·5	A
9	H	$[CH_2]_2{\cdot}NMe_2$	H	H	156–7	73·7	7·5	13·3	$C_{18}H_{21}N_3O$	73·75	7·5	13·6	B
10	Me	Me	Cl	H	206–7	67·1	5·8	9·9	$C_{16}H_{15}ClN_2O$	67·0	5·2	9·8	C
11	Me	Me	Br	H	216–7	57·9	4·7	8·2	$C_{16}H_{15}BrN_2O$	58·0	4·5	8·5	C
12	Et	Et	Cl	H	174–5	68·4	6·2	8·7	$C_{18}H_{19}ClN_2O$	68·7	6·0	8·9	C
13		$-[CH_2]_4-$	H	H	193–4	77·4	6·6	10·0	$C_{18}H_{18}N_2O$	77·7	6·5	10·1	B
14		$-[CH_2]_5-$	H	H	205	78·1	6·9	9·5	$C_{19}H_{20}N_2O$	78·05	6·9	9·6	B
15		$-[CH_2]_6-$	H	H	175–6	78·6	7·3	9·3	$C_{20}H_{22}N_2O$	78·4	7·2	9·1	B
16		$-[CH_2]_2{\cdot}O{\cdot}[CH_2]_2-$	H	H	208	73·4	6·0	9·4	$C_{18}H_{18}N_2O_2$	73·45	6·2	9·5	B
17		$-[CH_2]_2{\cdot}N{\cdot}[CH_2]_2-$ CO·O·Et	H	H	223–4	69·3	6·3	11·4	$C_{21}H_{20}N_2O_3$	69·0	6·3	11·5	B
18	H	Ph	H	H	213–4	79·8	5·6	9·4	$C_{20}H_{16}N_2O$	80·0	5·4	9·3	B
19	Me	Me	H	Me	160–1	76·8	6·8	10·6	$C_{17}H_{18}N_2O$	76·7	6·8	10·5	D
20	Me	Me	H	Et	126–7	76·8	7·3	9·9	$C_{18}H_{20}N_2O$	77·1	7·2	10·0	D
21	Me	Me	H	Pr^n	107 8	77·4	7·6	9·4	$C_{19}H_{22}N_2O$	77·5	7·5	9·5	D
22	Me	Me	H	$CH_2{\cdot}CH:CH_2$	114–6	77·8	7·0	9·5	$C_{19}H_{20}N_2O$	78·05	6·9	9·6	D
23	Me	Me	H	Bu^n	107–8	78·0	7·9	9·1	$C_{21}H_{24}N_2O$	77·9	7·8	9·1	D
24	Me	Me	H	$CH_2{\cdot}Ph$	136–7	80·7	6·2	8·1	$C_{23}H_{22}N_2O$	80·7	6·5	8·2	D
25	Me	Me	H	CO·O·Et	99–100	70·2	6·4	8·7	$C_{19}H_{20}N_2O_3$	70·35	6·2	8·6	D
26	Me	Me	H	$CH_2{\cdot}CO{\cdot}NH_2$	249–50	69·6	6·4	13·7	$C_{19}H_{19}N_3O_2$	69·9	6·2	13·6	D
27		$-[CH_2]_5-$	H	Me	142–3	78·6	7·1	9·2	$C_{20}H_{22}N_2O$	78·4	7·2	9·2	D
28		$-[CH_2]_5-$	H	Et	119–20	78·4	7·5	8·9	$C_{21}H_{24}N_2O$	78·8	7·5	8·8	D
29		$-[CH_2]_5-$	H	$CH_2{\cdot}CH:CH_2$	111–2	80·0	7·0	8·5	$C_{22}H_{24}N_2O$	79·5	7·2	8·4	D
30		$-[CH_2]_5-$	H	Pr^n	116–7	79·4	7·7	8·3	$C_{22}H_{26}N_2O$	79·1	7·8	8·4	D

Compound 9 was recrystallized from ethyl acetate; cpds 29 and 30 from light petroleum (b.p. 60–80°C).

Table 2 (a). *Pharmacological activity of some substituted dihydromorphanthridinones. Doses are expressed as mg base/kg given by stomach tube*



Compound No.	Structure R¹	R²	R³	Electroshock test ED50	Leptazol test ED50	Leptazol test PD50	Acute lethal dose LD50
1 (ICI 45,337)	Me	Me	H	42	38	100	1400
2	Et	Et	H	60	25	50–100	2680
3	Pr^i	Pr^i	H	NA at 250*	25–50	200	2000
4	Pr^n	Pr^n	H	118	50–100	50–100	
5	Me	$CH_2{\cdot}CH_2OH$	H	160			
6	H	Et	H	44			
7	H	$CH_2{\cdot}CH_2OH$	H	NA at 250	>200	>200	>2000
8	H	Pr^n	H	NA at 250	25	50	
9	H	$[CH_2]_3{\cdot}NMe_2$	H	NA at 250			
10	Me	Me	Cl	34	100	100–200	
11	Me	Me	Br	49	50	50	
12	Et	Et	Cl	63	50–100	50–100	
13	$-[CH_2]_4-$		H	NA at 250			
14	$-[CH_2]_5-$		H	50†			
15	$-[CH_2]_6-$		H	NA at 250		>2000	
16	$-[CH_2]_2{\cdot}O{\cdot}[CH_2]_2-$		H	NA at 250	>200	>200	
17	$-[CH_2]_2{\cdot}N{\cdot}[CH_2]_2-$	$CO{\cdot}OEt$	H	NA at 250	200	200	
18	H	Ph	H	NA at 250	50	100	
31	$-CO{\cdot}[CH_2]_2{\cdot}CO-$		H	NA at 250	200	>200	
32	$-CO{\cdot}C_6H_4{\cdot}CO-$		H	NA at 250	200	>200	>2000
33	Et	$CO{\cdot}Me$	H	NA at 250			

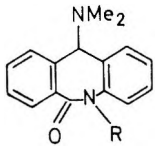
* Not active at this dose.
† Convulsions produced at this dose.

Table 2 (a) summarizes the activity of those dihydromorphanthridinones which were unsubstituted in the position 5 (II; R^4=H).

Among the most active compounds in this class were those in which the basic substituent at the position 11 was a lower dialkylamino-group (e.g. II; R^1=R^2=Me; R^3=R^4=H; ICI 45,337, cpd 1), a monoalkylamino-group (e.g. R^1=H; R^2=Et; R^3=R^4=H; cpd 6), or a piperidino-group (R^1R^2=$-[CH_2]_5-$; R^3=R^4=H; cpd 14). In the series of dialkylamino derivatives, activity decreased in the order of dimethyl, diethyl, di-n-propyl and di-isopropyl substitution. The monoethylamino-compound (cpd 6) was highly active in the electroshock test but the hydroxyethylamino-compound (cpd 7) was inactive at 250 mg/kg. Acetylation of the monoethylamino-compound (to give cpd 33) also resulted in complete loss of activity. Substitution of a halogen atom at position 2 (R^3=Hal) gave compounds with the same order of activity as cpd 1 in the electroshock test but with less activity against leptazol-induced seizures.

The effect of substitution of the amide nitrogen atom at position 5 was studied by choosing two of the most active members of the unsubstituted series, viz., the 11-dimethylamino-compound (cpd 1) and the 11-piperidino-compound (cpd 14), and

Table 2 (b). *Anticonvulsant activity of some 5-alkyl-11-dimethylamino-5,6-dihydro-6-oxomorphanthridines*

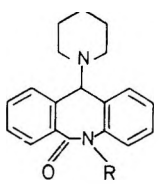


No.	R	Dose mg/kg	Electroshock test no. of mice protected
19	Me	80	10/10
		40	0/10
20	Et	100	5/5
		50	1/5
21	Pr^n	100	0/5
22	$CH_2{\cdot}CH:CH_2$	100	2/5
23	Bu^n	100	1/5
24	$CH_2{\cdot}Ph$	100	2/5
25	$CO{\cdot}O{\cdot}Et$	100	1/5
26	$CH_2{\cdot}CO{\cdot}NH_2$	100	3/5

varying the alkyl substituent at position 5 in these two structures. The effect of this substitution on anticonvulsant activity is shown in Tables 2 (b) and (c). In general, alkylation at position 5 in cpd 1 (II; $R^1=R^2=Me$; $R^3=H$) caused a diminution of activity especially with the larger alkyl groups. Alkylation at position 5 in cpd 14 ($R^1R^2=-[CH_2]_5-$; $R^3=H$) however had less effect on activity, and anticonvulsant properties in this group were in general maintained. The 5-alkyl derivatives of the 11-piperidino-dihydromorphanthridinones were generally more active than those of the analogous 11-dimethylamino-series (which includes cpd 1), but the compounds in this group all had higher acute oral toxicities. Table 2 (c) shows that in each case the ratio between the anticonvulsant and toxic doses was lower than that of cpd 1.

The dimethylamino- and diethylamino-dihydromorphanthridinones unsubstituted

Table 2 (c). *Anticonvulsant activity and toxicity of some 5-alkyl-11-piperidino-5,6-dihydro-6-oxomorphanthridines. Compounds were given orally, 1h pretreatment*



No.	R	Electroshock test, ED50 mg/kg (approx.)	Acute oral toxicity LD50 mg/kg	$\frac{LD50}{ED50}$
27	Me	8	60	7·5
28	Et	8	110	14
29	$CH_2CH:CH_2$	22	100	4·5
30	Pr^n	60	300	5

Table 3. *Central nervous system activity of compound* 1 (*ICI* 45337) *and some standard anticonvulsants in mice. Doses are expressed as mg/kg orally and* 95% *confidence limits are given in parentheses.*

Compound	Pre-treatment time (h)	Electroshock test ED50	Leptazol test ED50		Potentiation of hexobarbitone sleeping time	Neurotoxic dose (NTD)	Acute toxicity LD50	Therapeutic ratio	
			Inhibition of tonic extension	Protection from lethal effect				LD50/ED50	NTD/ED50
Cpd 1 (ICI 45,337) ..	1	25 (22–28·5)	38	110		140	1400 (1100–1700)	56	5·6
	2	47 (42–53)			250	87		30	1·9
Primidone ..	2	8 (4·9–12·9)		70	46	250	770 (560–1060)	96	31
Phenobarbitone ..	2	13·4 (12·1–14·9)	5	11	43	30	250 (230–280)	19	2·2
Phenytoin ..	2	6·2 (5·2–7·4)			100	90	270 (230–310)	44	15
Phensuximide	2	330 (276·6–393·8)	100	100		183	1513 ± 61†	8·3	1
Ethosuximide		183 ± 5·2 2070 mg/kg†	381	401 (1 h)			1530 ± 40†		

†Data from Chen, Weston & Bratton, Jr. (1963).

in the 5-position (cpds 1 and 2) were selected for extended study, and finally 11-dimethylamino-5,6-dihydro-6-oxomorphanthridine (cpd 1, ICI 45,337) was chosen on the basis of its activity and stability for chronic toxicity studies, and tested in man.

Pharmacological activity of 11-*dimethylamino*-5,6-*dihydro*-6-*oxomorphanthridine.* Table 3 summarizes the pharmacological properties of 11-dimethylamino-5,6-dihydro-6-oxomorphanthridine (cpd 1, ICI 45,337) and some anticonvulsants of clinical importance. The activity of cpd 1 is less than that of phenobarbitone, phenytoin and primidone but greater than that of phensuximide. The acute lethal dose is greater than that of any of these compounds and the calculation of a 'therapeutic ratio' shows that cpd 1 is superior to phenobarbitone in this respect (Table 3). The therapeutic ratio based on the dose producing the first signs of motor impairment and the anticonvulsant dose measured at 2 h is slightly lower than that for phenobarbitone but better than that for phenobarbitone when based on the activity at 1 h. Cpd 1 potentiated hexobarbitone sleeping time only at the relatively high dose of 250 mg/kg whereas the other compounds were active at doses in the range of 40–100 mg/kg.

The observed differences in anticonvulsant ED50 at 1 and 2 h suggested that cpd 1 was quickly absorbed and showed peak activity earlier than the other compounds.

Table 4. *Duration of action of compound* 1 (*ICI* 45,337) *and other anticonvulsants in mice. Groups of* 10 *animals received a dose equivalent to twice the oral ED*50 (*measured after* 2 *h pretreatment*). *The proportion of animals protected was determined at various intervals after dosing.*

Compound	Dose mg/kg	No. of animals protected/10 at the stated number of hours after dosing							
		½	1	2	4	6	8	12	24
Cpd 1 (ICI 45,337)	92	10	10	10	9	6	2	0	0
Primidone	16	0	3	7	8	9	8	0	0
Phenobarbitone	26·8	9	9	10	10	10	9	3	0
Phenytoin	12·4	0	4	6	8	8	7	2	0

Table 5. *Pharmacological activity of compound* 1 (*ICI* 45,337) *and some standard anticonvulsants in rats.* Effective doses are expressed as mg/kg orally fiducial limits ($P = 0{\cdot}05$) in parentheses

Compounds	Low-current electroshock test ED50	Acute oral toxicity LD50 mg/kg
Oral dosing		
Cpd 1 (ICI 45,337)	28 (25–32)	2000
Primidone	2·9 (2·5–3·4)	1000
Phenobarbitone	8·1 (6·1–11)	
Phenytoin	39·5 (29–53)	2000
Intravenous dosing		
Cpd 1 (ICI 45,337)	5·6	

The duration of action of cpd 1 and other anticonvulsants is shown in Table 4. Groups of 10 animals were given a dose equivalent to twice the ED50 and were challenged with electroshock at intervals after dosing. Cpd 1 gave maximal protection within half an hour, and maintained more than 50% protection for 6–8 h. In the same test phenobarbitone gave rapid protection and a duration of action of 8–12 h. Primidone and phenytoin gave maximum protection at 4–6 h which persisted for more than 8 h.

Anticonvulsant activity in rats. Cpd 1 was more active than phenytoin in this test but less active than phenobarbitone or primidone (Table 5). The intravenous ED50 of cpd 1 was about 1/5 of the oral ED50.

Ataxia. The mean doses of compounds producing significant incoordination are shown in Table 3. At 1 h after dosing cpd 1 produced less ataxia than at 2 h, whereas the greatest anticonvulsant effect is observed at 1 h.

Potentiation of hexobarbitone-induced and ethanol-induced narcosis. The extension of hexobarbitone sleeping time is an index of potential sedative effects of compounds which do not themselves produce hypnosis. The dose of cpd 1 required to double the sleeping time of mice treated with hexobarbitone was 250 mg/kg which is 10 times the anticonvulsant dose (Table 3). With primidone, phenobarbitone and phenytoin the effective doses in this test were 5·7, 3·2 and 16 times the anticonvulsant doses respectively.

In the ethanol potentiation test cpd 1 doubled the sleeping time of ethanol-treated mice at a dose of 250 mg/kg.

DISCUSSION

11-Dimethylamino-5,6-dihydro-6-oxomorphanthridine (cpd 1, ICI 45,337) shows anticonvulsant activity in a number of laboratory tests which detect clinically useful anticonvulsants. It is more active in antagonizing convulsions produced by electrical stimulation than chemically induced convulsions. The therapeutic ratio calculated for cpd 1 from the acute lethal and effective doses is higher than that of phenobarbitone. The ratio of neurotoxic to effective doses of cpd 1 at the time of peak activity is also greater than that of phenobarbitone.

In view of its potentially useful therapeutic ratio and low acute and chronic toxicity (Baker, 1966: personal communication), cpd 1 was taken to clinical trial (Grant, 1966: personal communication).

The patients used in this study were refractory cases of mixed epilepsies, and were

already receiving phenobarbitone, phenytoin or primidone and in most cases one or two other anticonvulsants. Under these conditions cpd 1 did not produce any anticonvulsant effect over and above that produced by the existing treatment. The early appearance of drug-induced rashes in a high proportion of patients precluded the possibility of investigating the anticonvulsant activity of cpd 1 alone. No symptoms of sedation or nausea were observed at doses up to 2 g/day.

It is known that some anticonvulsants, particularly phenobarbitone, induce hepatic microsomal metabolizing enzymes (see review by Conney & Burns, 1963). Metabolic studies (Platt, 1966: personal communication) showed that cpd 1 also stimulated the formation of hepatic microsomal metabolizing enzymes producing a similar pattern of enzyme induction to that caused by phenobarbitone. It is therefore probable that cpd 1 was being given to patients in whom metabolizing enzymes had been induced and who were capable of inactivating it more rapidly than patients who had not previously received phenobarbitone.

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A note on the colorimetric determination of butyrophenones with 3,5-dinitrobenzoic acid

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Butyrophenones react with 3,5-dinitrobenzoic acid in an alkaline medium with the formation of a red coloured complex. This reaction can be used for the quantitative determination of these drugs in pharmaceutical preparations. The influence of the ethanol concentration, the alkalinity and the reagent concentration was investigated separately. The results show that the proposed method is suitable for the assay of solutions and tablets. It requires less time than existing methods.

Few publications have described methods for the quantitative determination of butyrophenones in pharmaceutical preparations. In 1961, Demoen published an ultraviolet assay method for haloperidol which, though sensitive, is not specific. He also described a titration in acetic acid; but a large amount of drug is required; and a colorimetric method for the determination of haloperidol in urine based on the reaction of haloperidol with methyl orange. Demoen's ultraviolet method has also been used by Janicki, Brenner & Schwartz (1968) to determine droperidol in combination with fentanyl. In our laboratory, we (Van den Bossche, Haemers & De Moerloose, 1969) developed a method for the quantitative determination of fluorinated drugs in pharmaceutical preparations based on the colorimetric determination of the fluoride ion, formed after combustion in a Schöniger flask, with alizarine fluorine-blue. This method has also been used to assay butyrophenones. Soep (1961) determined butyrophenones in biological fluids by fluorine determination; he found it impossible to determine butryrophenones with the usual ketone reagents and we can confirm this. Also, the colour reaction of haloperidol with 1,3-dinitrobenzene, described by Thomas & Dryon (1967) cannot be used for the direct determination of butyrophenones in aqueous solution.

Tattje (1958) described an assay method for α-β unsaturated ketones (thujone, pulegone, piperitone) in volatile oils with 3,5-dinitrobenzoic acid in alkaline medium. As all butyrophenones react in the same way, we sought the most favourable reaction conditions using a solution of the butyrophenone pipamperone as hydrochloride.

EXPERIMENTAL AND RESULTS

In the examination of the reaction conditions, the influence of ethanol concentration (20–70%), strength of sodium hydroxide (0·2–0·9N) and concentration of 3,5-dinitrobenzoic acid (0·5–3·0%) was assessed. Whatever the concentration of 3,5-dinitrobenzoic acid and the alkalinity, maximum extinction occurred with an ethanol concentration of 30% v/v. Although the sensitivity of the reaction is optimal at that concentration, the colour is stable for too short a time and because of this an ethanol concentration of 40% was chosen since the colour stability is much increased while the sensitivity diminishes only slightly. Increasing the alkalinity increases sensitivity and reaction velocity, the colour stability however diminishes so a final concentration of 0·6 N was chosen. Colour stability is also affected by the reagent and as concentration increases stability diminishes. A final concentration of 1,5% 3,5-dinitrobenzoic acid was found to be most suitable.

Other butyrophenones, required the same optimal reaction conditions.

The absorption spectrum of the red coloured complex shows a maximum at 525 nm. All the butyrophenones examined had maximum absorption at the same wavelength. Furthermore, all butyrophenone-3,5-dinitrobenzoate complexes obey Beer's law in a concentration range from 0 to 1 mg.

Method

Reagents. Ethanol 96% (aldehyde-free: U.S.P. XVII); 3,5-dinitrobenzoic acid (7·5% in ethanol 96%); 3 N sodium hydroxide in water.

Aqueous solutions. Dilute the sample of butyrophenone with water to give a 0·05 to 0·25 mg/ml solution. Pipette 4·0 ml into a test-tube and add consecutively aldehyde-free ethanol (2·0 ml) colour reagent (2·0 ml) and 3N sodium hydroxide solution (2·0 ml). Mix, and measure the extinction after 8 to 12 min in 1 cm cells at 525 nm. Calculate the concentration of butyrophenone by comparison with the extinction of a similarly-treated standard solution or by means of a calibration curve. In this way, solutions for oral or parenteral administration and tablets containing water-soluble butyrophenones, such as pipamperone HCl, can be determined.

Ethanolic solutions. Dilute the sample of the butyrophenone with the aldehyde-free ethanol to obtain a 0·1 to 0·5 mg/ml solution. Pipette 2·0 ml into a test-tube and add consecutively water (4·0 ml) colour reagent (2·0 ml) and 3N sodium hydroxide solution (2·0 ml). Measure extinction as under aqueous solution above. In this way, tablets containing ethanol-soluble butyrophenones, such as haloperidol, can be assayed.

Using the proposed method, we have examined pharmaceutical preparations containing butyrophenones. Table 1 contains the results.

Table 1. *Results obtained by the proposed method*

Sample	Found (%)
Azaperone injection 40 mg/ml	98·1
Benperidol drops 1 mg/ml	99·3
Droperidol injection 2·5 mg/ml	98·6
Fluoanisone drops 25 mg/ml	100·3
Haloperidol drops 2 mg/ml	99·8
Haloperidol tablets 0·5 mg	100·7
Pipamperone 2 HCl tablets 40 mg	98·5
Trifluperidol HCl drops 1 mg/ml	99·3

The results prove that the proposed method is suitable for the determination of butyrophenones in pharmaceutical preparations. It can be used for aqueous as well as for alcoholic solutions. Preliminary extraction of preservatives, such as methyl- or propylparaben, is unnecessary. The method is accurate (standard deviation: 0·9%) and can be executed in minimum time.

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The control of oil-in-water emulsion consistency using mixed emulsifiers

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Mixtures of emulsifiers of the surfactant-fatty alcohol type are widely used to stabilize oil-in-water emulsions and to alter their consistency from fluid to semi-solid. A mechanism, involving the formation of a viscoelastic network in the continuous phase, is proposed to explain this self-bodying action. Evidence has been adduced from published data on a variety of systems. Support for this concept is also provided by the results obtained from a concentric cylinder study of the rheology of liquid paraffin in water emulsions containing a fixed concentration of cetostearyl alcohol and varying amounts of polyoxyethylene sorbitan monooleate, cetomacrogol 1000, sodium dodecyl sulphate or cetrimide.

Formulation of oil-in-water emulsions having the mechanical properties of a semi-solid pose both theoretical and practical problems. Wide use is made of combinations of an anionic or cationic or non-ionic surfactant with a fatty alcohol, as in emulsifying wax B.P., cetrimide emulsifying wax B.P.C. and cetomacrogol emulsifying wax B.P.C. This control of rheological behaviour is effected by varying the amount of "mixed emulsifier" in the formulation according to its "self-bodying" action. In the present paper this is defined as the ability of a mixed emulsifier to produce a mobile emulsion when present at low concentrations and a semi-solid preparation when present in moderate amounts (about 10% or less of the total weight of the emulsion). This criterion is used because increasing the proportion of most emulsifiers in an emulsion formulation increases the apparent viscosity (see, for example, Sherman 1964, 1968). The essential feature of the self-bodying action is the introduction of a large elastic component into the rheological behaviour.

This paper attempts to present a coherent explanation of the mechanism of the self-bodying action of a variety of mixed emulsifiers using published data, and results from a re-examination of emulsion systems for which the proposed theory does not, at first, appear to apply. The data are derived from work using the surfactants sodium dodecyl sulphate, potassium laurate, cetrimide, cetomacrogol 1000, polyoxyethylene sorbitan monolaurate and monooleate, the alcohols oleyl, lauryl, cetyl and cetostearyl and the oils isopropyl myristate, liquid paraffin, light liquid paraffin, arachis and castor and thus the data cover a representative range of ingredients. The systems discussed range in consistency from fluid to solid, many showing both liquid and solid properties. Ideally, therefore, the data should be presented in the form of viscoelastic parameters, such as moduli of rigidity, compliances, coefficients of viscosity and retardation and relaxation times. These may be derived from, for example, creep or oscillatory testing. In general, however, continuous shear methods have been used and although having limitations (see Davis, Shotton & Warburton, 1968; Barry & Warburton, 1968) they often provide information from which useful conclusions may be drawn.

A SUGGESTED MECHANISM OF THE SELF-BODYING ACTION OF MIXED EMULSIFIERS

The proposed explanation of the mechanism by which the consistency of the emulsions to be discussed is controlled is based on the explanation offered by Barry (1968a) to account for the self-bodying action of the mixed emulsifier sodium dodecyl sulphate-cetyl alcohol in liquid paraffin emulsions. This mechanism is therefore considered in detail, using the above formulation as a model system.

In the preparation of the emulsions, a mixture of cetyl alcohol in liquid paraffin at 65–70° is poured into an aqueous sodium dodecyl sulphate solution maintained at the same temperature, the resulting mixture being agitated until cold. An emulsion is formed and stabilized by complexing of the amphiphile and surfactant at the oil-water interface. Usually the concentrations of the emulsifiers are greater than those required to provide a complex condensed film at the interface, and excess material modifies the rheological properties of the emulsions. The excess surfactant remains in the aqueous phase, either monodispersed or as micelles, depending on its concentration and the temperature. Below the critical micelle concentration (CMC), mobile, somewhat unstable emulsions are formed. Above the CMC, a micellar pseudophase is present during the cooling process, and some of the amphiphile will diffuse from the oil into the aqueous phase. More will diffuse as the amphiphile concentration in the oil phase increases, due simply to a raised concentration gradient from oil to water. However, the amount of water-insoluble amphiphile which diffuses into a non-micellar aqueous environment is small. When the surfactant concentration is greater than the CMC, significant quantities of amphiphile may be solubilized within the micelles alongside the surfactant molecules. A small proportion of the oil phase may also be solubilized. The mixed micelle formed may have a typically spherical shape or alternatively it may exist as a lamellar smectic liquid crystal. This liquid crystal phase can form at low concentrations because of the closer molecular packing allowed when repulsion between the ionized head groups of the surfactant is reduced by interposition of the amphiphile, by the strong interaction of the alkyl chains and by the necessity of separating water away from these chains (Lawrence 1958, Boffey, Collison & Lawrence, 1959). Thus, as the temperature is decreased the amphiphile becomes progressively less soluble in the oil, diffuses from the disperse phase and is solubilized by the micellar phase. However, when the temperature falls below the melting point of the amphiphile (minus any depression caused by the other ingredients), solubilization is no longer possible (Hyde, Langbridge & Lawrence, 1954) and the amphiphile precipitates. This precipitate which may consist of finely divided amphiphile, or possibly an intimate mixture of amphiphile, surfactant and water, may maintain its structure for some time, forming metastable frozen micelles or frozen liquid crystals. This results in the formation of a viscoelastic gel which entraps oil globules and is responsible for the marked increase in consistency. I consider that this method of aggregation is more important than the theory of polymolecular adsorption of the surfactant (Cockbain, 1952; Riegelman, 1962) or the electrical theory (Higuchi, Okada & Lemberger, 1962) when dealing with the rheological properties of semi-solid emulsions of high alcohol content.

The water-soluble component of the mixed emulsifier

In considering the ability of a mixed emulsifier to form semi-solid emulsions, the part played by the surfactant needs examination. The surfactant should be capable of

combining with the amphiphile to form a complex, condensed film at the oil-water interface, to stabilize the emulsion. It should also be present in an amount sufficient to form micelles in the aqueous phase.

Using the mixed emulsifier sodium dodecyl sulphate-cetyl alcohol, Barry (1968a) has shown by continuous shear rheometry that when the surfactant is in excess of its CMC considerable structural order exists in the emulsion in the form of a gel-like network. During shearing in a Ferranti-Shirley viscometer, aggregates of globules were disrupted, releasing entrapped continuous phase, and thus the flow curves were in the form of hysteresis loops. The areas of these loops were a measure of the amount of breakdown which had taken place during the shearing cycle, and this in turn was related to the extent of gel formation in the continuous phase. Both of these increased rapidly with emulsifier concentration above the CMC. The fact that aggregates had been broken down during shearing was confirmed microscopically and by particle size analysis. Stronger evidence for the presence of a viscoelastic network was derived from creep experiments (Barry 1968a). The creep compliances fell rapidly as the surfactant concentration increased up to the CMC and there was a less rapid change upon further increase. Similarly, there was a steeply ascending portion to each viscosity curve, followed by a region where the slope decreased as emulsifier concentration increased. The initial fall in compliance and rise in viscosity with increase in emulsifier concentration were considered to reflect the build-up in structure in the continuous phase. Once the emulsion globules were well linked together by a gel structure of sufficient size, further addition to this network had a relatively minor effect on the viscoelastic parameters and the curves flattened out.

An increase of both the yield value and the plastic viscosity with surfactant concentration for autoclaved liquid paraffin emulsions containing sodium lauryl sulphate and a fixed concentration of cetyl alcohol were shown by Axon (1956). The findings of Talman, Davies & Rowan (1968) do not agree. These authors used a Ferranti-Shirley cone and plate viscometer in automatic mode to examine the effect of two ionic surfactants—cetrimide and sodium lauryl sulphate—and two non-ionic surfactants—Sorbester Q12 (polyoxyethylene sorbitan monolaurate) and cetomacrogol 1000—on the rheology of some liquid paraffin emulsions. At low shear rates the rheograms showed either a spur or an abrupt change of slope. The shear stress at this point was reported as a static yield value and their results are reproduced in Fig. 1 a, b, p. 539. The values from this type of experiment are probably dependent in part on the instrument and the mode of operation and, although they are not a measure of fundamental parameters, they may be used to show a trend when comparing members of a closely related series of materials. But the material must neither fracture in, nor be expelled from the cone-plate gap. Talman & others (1968) found that for a fixed concentration of cetostearyl alcohol the static yield value went through a maximum as the surfactant concentration increased. This finding does not support the suggested mechanism of self-bodying action. A re-examination of their systems using concentric cylinder geometry (Rotovisko) instead of a cone and plate (see experimental section) showed emulsion consistency to increase steadily with surfactant concentration to the extent that the torque reading went offscale at 5% concentration of the ionic surfactants. Only the maximum shear stress readings obtained at the lowest shear rate available were determined and they are plotted in Fig. 1 a and b. As with the Ferranti-Shirely viscometer results, the Rotovisko results as determined are only valid in as far as they establish a trend in a series of materials, as they will depend, amongst other

things, on the inertia of the torque sensing apparatus. But the increase in consistency agreed with the behaviour of the emulsions when they were loaded into the viscometer; the resistance to the insertion of the bob into the cup due to the presence of the sample in the gap increased steadily as the concentration of surfactant increased, which does *not* indicate a maximum in the relevant rheological properties. It would seem, therefore, that provided the surfactant concentration is not so high as to promote instability, the consistency of the systems increases as surfactant concentration increases.

In the official B.P. and B.P.C. oil-in-water creams, the surfactant concentrations are above the CMC values, and thus these preparations should show evidence of structure in the continuous phase. This has been confirmed by Davis (1969) who examined them by a continuous shear method and obtained rheograms of the hysteresis loop type with yield stress. On creep testing, viscoelastic curves were obtained, which also indicates a network structure linking globules of disperse phase.

The oil-soluble component of the mixed emulsifier

The oil-soluble component should be an amphiphilic compound which by itself promotes water-in-oil emulsions, and is capable of complexing with the hydrophilic component to form a complex film at the oil-water interface. It should be present at least in sufficient concentration to form this close-packed mixed monolayer, when a mobile emulsion will be formed; to form a semi-solid preparation at the test temperature it should be near or above its saturation concentration in the oil. In the systems examined by Barry (1968a) this was so. Similarly, Axon (1956) has shown that the plastic viscosity and the yield value of liquid paraffin emulsions containing sodium lauryl sulphate and cetyl alcohol increase exponentially with cetyl alcohol concentration. Talman, Davies & Rowan (1967) examined emulsions formulated with different oils, several surfactants and with the oil-soluble components oleyl, lauryl and cetostearyl alcohols. The concentration of oleyl alcohol was *below* its saturation point in the oil phase and all emulsions prepared with this alcohol were fluids of low apparent viscosity and zero static yield value. On increasing the concentration of this alcohol, but still remaining within the limits of its solubility, only a slight rise in apparent viscosity was recorded and this the authors attributed simply to an increase in disperse phase content. This is in agreement with the self-bodying postulate. Fluid emulsions were also produced by Talman & others using low concentrations of cetostearyl alcohol. On increasing the alcohol concentration, a position was reached at which the apparent viscosity versus concentration curve diverged markedly from that given by the oleyl alcohol emulsions. This divergence concentration was identical with the saturation concentration of the alcohol in the oil. Above the divergence concentration, static yield values were reported, which increased with further increase in the alcohol concentration. This is precisely what would be expected if the theory holds. The third alcohol tested by Talman & others was lauryl alcohol. It was miscible in all proportions with liquid paraffin and the rheological behaviour of emulsions containing it was found to be dependent on the type of water-soluble component. Fluid products of low apparent viscosity were obtained with non-ionic components, but cetrimide and sodium lauryl sulphate produced more viscous emulsions, some of which exhibited static yield values. Now a small amount of the alcohol would be expected to partition with the micellar phase, but this should not precipitate on cooling to 25°, as the penetration temperature

(see, e.g. Barry & Shotton, 1967b), and hence the minimum temperature for solubilization, should be less than the melting point of the alcohol. Thus the emulsions should not exhibit semi-solid properties such as significant static yield values. Samples of emulsions containing the largest proportion of lauryl alcohol (which exhibited the highest yield values in the series) have been prepared (see experimental) and they were viscous pourable preparations and not semi-solid. Their static yield values and apparent viscosities, as measured in the Rotovisko viscometer, were only about one tenth that of similar emulsions containing cetostearyl alcohol. Thus, overall, the data of Talman & others (1967) agree well with the requirement that the amphiphile should diffuse from the disperse phase and precipitate on cooling, and these authors explained their results on the basis of a diffusion process.

There is a limit to the amount of, for example, cetyl alcohol which can diffuse out of the oil phase to be solubilized in the hot aqueous micellar phase. In many formulations, the concentration of the alcohol remaining in the oil phase may exceed its maximum solubility in the oil when the emulsion is cooled, and this should lead to precipitation within the disperse phase. Although such precipitation within disperse phase globules would have no direct significant effect on the bulk rheology of the emulsion, it is important as it indicates that the solubility of the alcohol in the oil has been exceeded. Barry (1968a) has published photomicrographs of such crystals which were sufficiently well formed to distort the emulsion globules and similar crystals have been detected by Talman & Rowan (1968).

Viscoelastic structure

The presence of a viscoelastic structure entrapping disperse phase globules is crucial to the suggested mechanism of the self-bodying action of mixed emulsifiers as defined. For preparations in which the elastic properties (both instantaneous and retarded) predominate i.e. semi-solid creams, the disperse phase in the *cooled* emulsion may be considered to approximate simply to a geometric diluent which serves as loci for the establishment of a gel network. Barry & Shotton (1967a, c) have photographed gels prepared from mixtures of cetyl alcohol, sodium dodecyl sulphate and water, in which solid alcohol provided loci and frozen liquid crystal the gel matrix. Both continuous shear data and creep analysis supported the hypothesis of a network, and the apparent relative viscosity versus temperature curve went through a maximum as the frozen smectic phase melted to liquid crystal. This result has been confirmed by Davis (1969), using the identical system, when he found that the compliance in a creep test (which is qualitatively equivalent to the reciprocal of the apparent viscosity) went through a minimum at the same temperature. When oil is introduced into the ternary system so as to form semi-solid creams, the rheological behaviour still strongly suggests a viscoelastic network (Barry 1968a). Davis (1969) has investigated aqueous cream B.P. by creep analysis at different temperatures and has found that the compliance had a minimum value at a similar temperature as the ternary system dealt with above, which again suggests melting of a gel network. However, it is difficult to demonstrate unequivocably the presence of a gel matrix existing in the bulk of an emulsion. It is not readily apparent microscopically in an undiluted emulsion, as fine resolution is difficult, and on diluting the system the labile gel structure readily breaks down. In such a diluted preparation, remnants of the network can be detected, but to photograph them clearly it is easier to increase the proportion of the alcohol. Barry (1968b) has published photomicrographs of diluted emulsions in which this has been done, and in

ordinary light a network enclosing disperse phase droplets was visible. Both precipitated cetyl alcohol and any frozen pseudomorphic mixture of surfactant, amphiphile and water will be optically anisotropic and show up between crossed polarizers. As this was the situation in these photomicrographs, further evidence of the nature of the gel network was provided. Photomicrographs under phase contrast of a filamentous structure enveloping globules or dispersed in the continuous phase have also been published by Talman & Rowan (1968).

The viscoelastic network is also important as an aid to emulsion stability as it hinders close approach of disperse phase droplets and thus retards coalescence and ultimate cracking of the emulsion.

CONCLUSION

It would appear that the suggested mechanism of the self-bodying action of mixed emulsifiers discussed is sufficient to explain the ability of a number of mixed emulsifiers to produce semi-solid emulsions, particularly when the surfactant is ionic. When this is non-ionic, the surfactant-alcohol complex is probably weaker than that formed by ionic surfactants as the non-ionic molecule is bulky (Rowe 1965). This explains the results of Talman & others (1968) and my own findings which show that emulsions formulated with ionic surfactants and cetostearyl alcohol have a greater consistency than those containing non-ionic surfactants. Davies (1969) considers that this difference also explains why chlorhexidine cream B.P.C. contains more surfactant (cetomacrogol) and cetostearyl alcohol than do those official oil-in-water creams based on ionic surfactants combined with cetostearyl alcohol. However, I consider that part of the increased quantity of mixed emulsifier in this cream is probably required so as to form a more extensive network as the liquid paraffin concentration is low (10 w/w) and thus the globules are more widely separated.

On the basis of the self-bodying mechanism, the requirements for the mixed emulsifiers may be listed:

Lipophilic component

1. This should be an amphiphilic compound which by itself promotes water-in-oil emulsions and is capable of complexing with the hydrophilic component at the oil-water interface.

2. Its concentration should at least be sufficient to form a close-packed mixed monolayer with the hydrophilic component. To promote semi-solid emulsions at room temperature it should be near or above the saturation concentration in the oil.

3. Excess material should diffuse readily from the warm oil phase into the warm aqueous micellar phase, and there be solubilized.

4. The melting point should be sufficiently high to precipitate solubilized material at moderate temperatures.

Hydrophilic component

1. This should be a surface-active agent which by itself promotes oil-in-water emulsions and is capable of complexing with the lipophilic component at the oil–water interface.

2. Its concentration should at least be sufficient to form a close-packed mixed

monolayer with the lipophilic component. To promote semi-solid emulsions it should be in excess of its critical micelle concentration in the aqueous phase.

3. It should be capable of solubilizing the lipophilic component whilst warm.

EXPERIMENTAL AND RESULTS

Materials. Water and sodium dodecyl sulphate were as used by Barry & Shotton (1967a), liquid paraffin as used by Barry (1968a), polyoxyethylene sorbitan monolaurate (Sorbester Q12, from Howards Ltd., Ilford, Essex) and cetrimide, cetostearyl alcohol and cetomacrogol 1000 were commercial grades used without further purification.

Preparation of emulsions. Emulsions were prepared according to the general formula liquid paraffin, 50·0, cetostearyl alcohol, 7·0, water-soluble component, 0·125 to 5·0, distilled water to 100·0 g. The alcohol, dissolved in liquid paraffin at 65–70°, was added to an aqueous solution of the water-soluble component at the same temperature and mixed with a Silverson Multi-Purpose High Speed Laboratory Mixer fitted with an axial flow head and emulsor mesh. The resulting emulsions were cooled rapidly and each was distributed into several separate ointment jars, which were then stored for not less than 10 days at 25 ± 1° before testing. The contents of a separate jar were used for each test.

Rheological examination. Emulsions were examined with a Haake Rotovisko viscometer using measuring cup MV and rotary bob MVI (concentric cylinders) at 25 ± 0·1°, and all measurements were made at least twice and results averaged. In general, systems showed complex rheological behaviour, including shear thinning and hysteresis. At high shear rates, the high consistency creams tended to be ejected

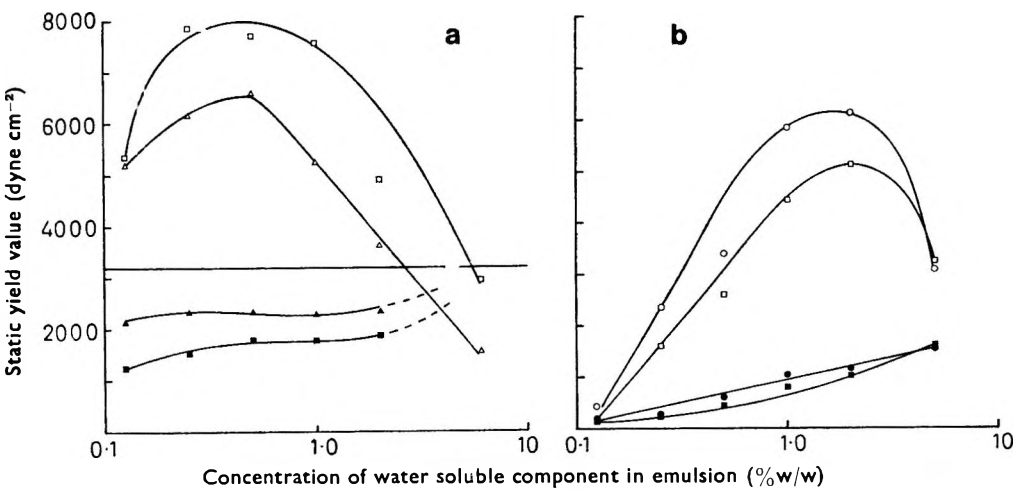

FIG. 1. Effect of water soluble component concentration (% w/w) on the static yield value of emulsions containing 7% w/w cetostearyl alcohol. (a), -△-, sodium dodecyl sulphate, -□-, cetrimide (static yield values determined by Talman & others (1968), using a Ferranti-Shirley cone and plate viscometer). -▲-, sodium dodecyl sulphate, -■-, cetrimide (static yield values determined with a Rotovisko concentric cylinder viscometer at 7·05 s^{-1}). Horizontal line represents shear stress scale limit of Rotovisko viscometer. (b), -○-, cetomacrogol 1000, -□-, Sorbester Q12 (static yield values determined by Talman & others (1968), using a Ferranti-Shirley cone and plate viscometer). -●-, cetomacrogol 1000, -■-, Sorbester Q12 (static yield values determined with a Rotovisko concentric cylinder viscometer at 7·05 s^{-1}).

from the cylinder gap. When smaller diameter inner cylinders were used (so as to extend the range of measurable shear stress) these creams fractured in the gap. As the work was performed to compare results with the static yield values derived from a Ferranti-Shirley cone and plate viscometer (Talman, Davies & Rowan, 1968) reported results are limited to the maximum shear stress reading obtained at the lowest available shear rate (7·05 s^{-1}) starting from rest. These are plotted in Fig. 1 a and b, together with the results of Talman & others (1968).

Acknowledgement

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LETTERS TO THE EDITOR

Effect of a single dose of phentolamine and MJ 1999 on aspirin-induced gastric ulceration in rats

The preventive effect of α-methyldopa in two types of stress-induced gastric ulcer in rats has previously been demonstrated (Djahanguiri, Hemmati & others, 1967). These authors have also shown that the production of stress-induced ulcers is prevented by the pretreatment of rats by the α-blocking agent, phentolamine, and aggravated by the β-blocking agent, MJ 1999 {4-[1-hydroxy-2'-(isopropylamino)ethyl]methane sulphonanilide HCl} (Djahanguiri, Sadeghi & Hemmati, 1968).

I have now examined the effect of phentolamine and MJ 1999 on aspirin-induced gastric ulceration in rats.

Rats of either sex, weighing 90–110 g were given intraperitoneally 2 ml/100 g weight of normal saline to prevent dehydration. They were housed in individual cages and food was withheld for 24 h. The animals were divided into four groups. The first two groups (each of 20 rats) were injected intraperitoneally with 2 mg/kg of phentolamine and 10 mg/kg of MJ 1999 respectively. Thirty min later these two groups and a third group (20 rats) were injected with acetylsalicylic acid (150 mg/kg, i.p.) suspended in olive oil. The fourth group (10 rats) were given 1 ml/100 g weight of olive oil by the same route. Gastric damage was measured 5 h after the last injection. The rats were killed by a blow on the head and the stomachs were immediately removed, opened along the greater curvature, washed with water and examined by direct lighting (by an observer to whom the treatments were not known) for the presence of focal haemorrhagic erosions. Any haemorrhagic area 2 mm or greater in its largest dimension was considered as a positive. The frequency of these spots found in the glandular part of the stomach ranged from 2 to 12. Microscopic examination showed the presence of ulceration in the mucosal layer, rarely reaching the muscularis mucosa and always accompanied by oedema, necrosis and haemorrhage.

The results are summarized in Table 1. The percentage of gastric lesions after

Table 1. *The incidence of gastric ulceration in rats treated with adrenergic blocking agents and aspirin*

Drugs and doses (mg/kg body weight)	Number of rats	Number with ulcers	Percentage
Phentolamine 2 + aspirin 150	20	2	10*
MJ 1999 10 + aspirin 150	20	17	85
0 + aspirin 150	20	16	80
Olive oil (1 ml/100 g body weight) ..	10	0	0

* $P < 0.001$ when compared with the aspirin group value.

the administration of aspirin in the control group is similar to that obtained by Brodie & Chase (1967). The results also show that phentolamine, at a dose of 2 mg/kg, significantly prevented the occurrence of gastric ulceration in the rats. MJ 1999 did not affect the incidence of aspirin-induced gastric ulceration. The statistical significance was calculated by the χ^2 method.

The role of the sympathetic nervous system in the pathogenesis of acute gastric ulceration is supported by much experimental evidence. The role of sympathetic excitation has been demonstrated by the ulcerogenic action of noradrenaline injected into the left gastric artery of the dog (Nicoloff, Peter & others, 1965). Lynch, Highley

& Worton (1964) observed the ulcerogenic action of phenylephrine. Sun & Shay (1960) demonstrated that the late phase of gastric secretion produced by insulin hypoglycaemia could be blocked by an adrenergic-blocking agent and Emas (1964) found a decrease of gastric acid secretion after administration of guanethidine in the cat. By time-study experiments it has been claimed that the primary change, in the course of the development of stress-induced acute gastric ulcer, in which there is a high level of blood catecholamine (Euler, 1964), is a trophic disturbance which is produced under the influence of sympathetic excitation (Anichkov & Zavodskaya, 1965).

In the pathogenesis of aspirin-induced acute erosive lesions in the stomach, one of the more attractive mechanisms recently proposed is the loss of the mucus "barrier" of the stomach (Menguy, 1966). Kent & Allen (1966) found aspirin to produce deleterious effects on the protective mechanisms of the stomach by reducing the rate of synthesis of mucus. They observed that the biosynthesis of macromolecules in cells of the gastric mucosa was inhibited by a selective action of salicylate. Menguy (1966) has suggested that this loss of "barrier" permits the back diffusion of hydrochloric acid [the theory proposed by Davenport (1966)] into the aspirin-damaged gastric mucosa.

Whatever the pathogenic mechanisms of aspirin-induced gastric ulceration may be, the results of the present study would lend support to the theory that the sympathetic nervous system is implicated in the production of such lesions and that α-adrenergic receptors may be involved.

I wish to express my indebtedness to Dr. R. H. Salter in the preparation of this manuscript. The generous supply of phentolamine (Regitine) by Dr. Nik-Eteghad from Ciba Pharmaceutical Co. and of MJ 1999 by Mead Johnson Pharmaceutical Co. are acknowledged.

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The non-specificity of diethyldithiocarbamate

When an inhibitor of tyrosine hydroxylase, 3-iodo-L-tyrosine (3-IT) (200 mg/kg, s.c.), is injected into rats, there is a reduction in the concentration of noradrenaline in the brain. Sodium diethyldithiocarbamate (DDC) (500 mg/kg, s.c.) causes a greater reduction in brain noradrenaline than does 3-IT. If these same doses of 3-IT and DDC are administered simultaneously, the depletion of noradrenaline is significantly less than that obtained with DDC alone (Goodchild, 1969). The interpretation of these results depended on the fact that the decrease in noradrenaline after DDC has previously been attributed to inhibition of dopamine-β-hydroxylase (Collins, 1965; Carlsson, Lindqvist & others, 1966). I have now measured the level of tyrosine in the brains of rats treated with DDC with the object of challenging the utilization of tyrosine in the brain.

Adult, male white rats (150–200 g) were injected with DDC (500 mg/kg, s.c.) killed at various times after injection and the brain tyrosine levels estimated (Waalkes & Udenfriend, 1957). The results are shown in Table 1.

Table 1. *The effects of DDC on rat brain tyrosine levels.* Tyrosine levels are expressed as the percentage of uninjected controls. The figures in parentheses are the standard errors of the means (each value represents the mean of 5 determinations). The absolute value for the tyrosine level in control brains was 11·49 μg/g.

Time after injection of DDC	% Tyrosine	Significance value
20 min	102 (±4)	N.S.
30 min	120 (±2)	$P < 0{\cdot}02$
1 h	135 (±5)	$P < 0{\cdot}01$
2 h	134 (±6)	$P < 0{\cdot}01$
3 h	123 (±8)	N.S.

There was a significant increase in brain tyrosine levels 30 min after injection and this persisted for a further 90 min. This increase could arise from an increased availability of tyrosine, or from an inhibition of its enzymic degradation. Taylor, Stubbs & Ellenbogen (1969) have recently reported that DDC can inhibit tyrosine hydroxylase *in vitro*. Thus it appears that in addition to its inhibitory action on the conversion of dopamine to noradrenaline, DDC may also inhibit tyrosine hydroxylase as shown by the increased tyrosine levels reported here. The decrease in noradrenaline content of rat brain after treatment with DDC may therefore be attributed to the inhibition of tyrosine hydroxylase in addition to dopamine-β-hydroxylase.

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An antihistamine compound with potent antibradykinin activity

Bradykinin is considered to play an important role in numerous pathological processes, and an effective antagonist *in vivo* may be of value in controlling pathological reactions induced by endogenous bradykinin. Certain antihistamine compounds, particularly phenothiazine derivatives, possess antibradykinin activity *in vitro* when tested on the isolated ileum of the guinea-pig (Mariani, 1961; Rocha e Silva & Leme, 1963; Horowitz & Mashford, 1969). Some thioxanthene and dibenzocycloheptene derivatives also exert powerful antibradykinin activity *in vitro* (Leme & Rocha e Silva, 1965). Nevertheless, inhibition *in vivo* has been difficult to demonstrate and, even then, antagonists such as the salicylates and the fenamates are often effective only in one or two preparations, as, for example, against the bronchospasm induced by bradykinin in guinea-pigs (Collier & Shorley, 1963).

The present report relates to the effectiveness of a potent antihistamine compound (halopyramine) both in reducing the action of bradykinin on the rat isolated uterus and on capillaries in rat skin, and in reducing thermic oedema in rat paws which results from the formation and release of endogenous bradykinin (Starr & West, 1967). Halopyramine hydrochloride (Synopen, Geigy), a chlorobenzyldimethylpyridyl derivative of ethylenediamine, is a white powder, readily soluble in water, and in all of the following experiments a stock solution was made by dissolving it in normal saline to provide a 0·1% (w/v) solution.

On the isolated uterus of a rat in oestrus, halopyramine was found to be an effective antagonist of bradykinin, a concentration of 1×10^{-7} in six experiments reducing the contractions produced with 1 ng bradykinin (in a 5 ml bath) by about 35% and with 0·5 ng by about 49%. The antagonist was quickly removed from the preparation by washing (Fig. 1a). Concentrations of 2×10^{-7} g did not alter the 5-hydroxytryptamine responses and histamine is inactive. Doses of bradykinin (1·25 and 2·5 μg) were next injected intradermally into depilated skin of rats with azovan blue dye (10 mg/kg) in their circulation. Groups of 15 adult albino animals were used, some of which had been injected 5 min before the bradykinin with halopyramine intraperitoneally (10 mg/kg). Twenty min after the bradykinin injection, the rats were killed and their dorsal skin was removed, cleaned of fat and dried at 56°. The blue dye exuded into the injection sites was extracted by the method of Judah & Willoughby (1962) and assayed using a Beckman spectrophotometer. Halopyramine significantly reduced the amount of dye released from the capillaries by the bradykinin (see Fig. 1b). Intraperitoneal doses of 10 mg/kg did not alter the blueing of rat skin induced by 5-hydroxytryptamine or histamine.

In other experiments, the hindpaws of groups of 15 adult albino rats under light ether anaesthesia were heated at 46·5° for 30 min (Rocha e Silva & Antonio, 1960), some of the groups being injected 5 min before the heating with either 5 or 10 mg/kg halopyramine intraperitoneally. Thirty min later, the rats were killed and the size of the oedema was expressed as a percentage increase in limb weight compared with the weight of the opposite unheated limb. This method of assessing oedema gave results which were comparable with those obtained using plethysmography. The higher dose of halopyramine significantly reduced the swelling resulting from the heat application (Fig. 1c). Starr & West (1967) have previously reported that large doses of more specific antihistamine compounds (as, for example, mepyramine, 50 mg/kg) had no effect on the thermic oedema and did not reduce the local capillary effect of bradykinin although several non-steroidal anti-inflammatory compounds were potent inhibitors.

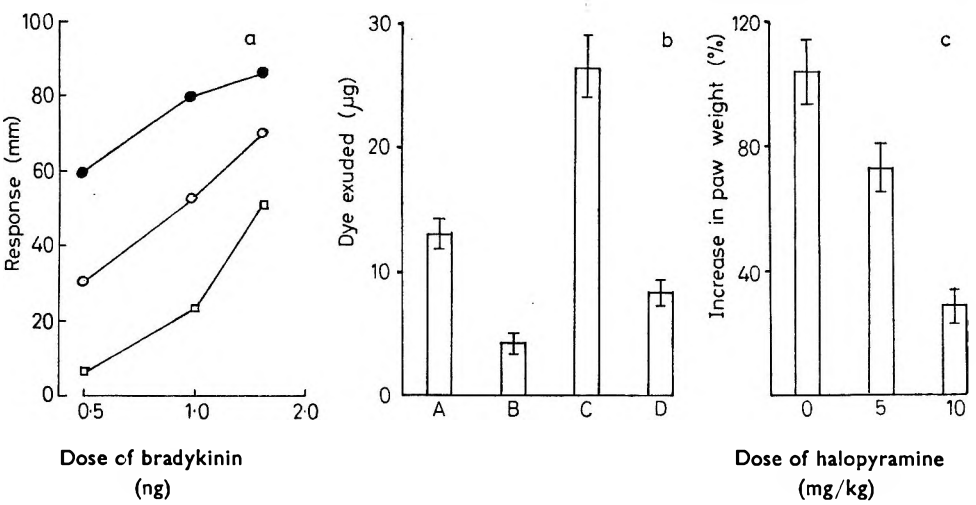

FIG. 1a. Dose-response curves of rat uterus to bradykinin. Effect of halopyramine. Mean of 6 experiments. ●—● control. ○—○ 0·1 μg/ml of drug. □—□ 0·2 μg/ml of drug.

b. Effect of intraperitoneal doses of 10 mg/kg halopyramine (B and D) on the dye exudate (μg) in rat skin produced by the intradermal injection of bradykinin (A and B, 1·25 μg: C and D, 2·5 μg). Results are the means (± s.e.) of 15 experiments.

c. Effect of intraperitoneal doses of halopyramine (mg/kg) on the % increase in paw weight of rats after heating at 46·5° for 30 min. Results are the means (± s.e.) of 15 experiments.

The present study shows that halopyramine possesses antibradykinin activity both *in vitro* and *in vivo*, and significantly reduces the thermic oedema resulting from the release of endogenous bradykinin. This compound may well lessen inflammation in clinical conditions.

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Inhibition by morphine of the release of [^{14}C]acetylcholine from rat brain cortex slices

Morphine elevates levels of total brain acetylcholine in rats and mice (Giarman & Pepeu, 1962; Hano, Kaneto & others, 1964). In the rat brain, "bound" acetylcholine was increased while "free" acetylcholine was decreased (Crossland & Slater, 1968). Previously morphine had been shown to decrease the release of acetylcholine from guinea-pig ileum (Schaumann, 1957; Paton, 1957; Cox & Weinstock, 1966) and from brains of anaesthetized cats (Beleslin & Polak, 1965; Beleslin, Polak & Sproull, 1965).

KCl accelerates the release of acetylcholine; for example, from rat isolated diaphragm (Mitchell & Silver, 1963), rat cerebral cortex slices (Polak & Meeuws, 1966) and the superior cervical ganglion of the cat (Brown & Feldberg, 1936).

The effect of morphine on the accelerated release of [^{14}C]acetylcholine by KCl from rat brain cortex slices has now been examined.

Slices of rat cerebral cortex were prepared as described by McIlwain & Rodnight (1962). Two incubation media were used: the first contained (M) 0·13 NaCl, 0·004 KCl, 0·002 $CaCl_2$, 0·025 $NaHCO_3$, 2×10^{-4} eserine sulphate (Mann, Tennenbaum & Quastel, 1939) and will be referred to as "4 mM KCl medium"; the second contained (M) 0·103 NaCl, 0·031 KCl, 0·002 $CaCl_2$, 0·025 $NaHCO_3$, 2×10^{-4} eserine sulphate (Mann & others, 1939) and is designated "31 mM KCl medium". Both media contained 0·005 M [^{14}C]glucose (uniformly labelled) except when employed for reincubation or for extraction of [^{14}C]acetylcholine from the slices. Media were adjusted to pH 7·4 with HCl before incubation. Slices (200 ± 10 mg wet weight) were placed in 50 ml beakers containing 3 ml of incubation medium. Vessels were incubated at 37° for varying periods of time in an atmosphere of 95% oxygen and 5% carbon dioxide.

In the first set of experiments, slices were incubated in 31 mM KCl medium with and without 10^{-3} M morphine for 75 min. In the second experiments slices were first incubated in 4 mM KCl medium for 60 min to accumulate [^{14}C]acetylcholine in the slices; one aliquot of these slices was reincubated in 31 mM KCl medium for 15 min, while a second portion was reincubated in 31 mM KCl containing morphine 10^{-3} M for the same period.

At the end of the incubations, the vessels were chilled to 0° in crushed ice. Slices were separated from media by centrifugation at 2200 *g* for 20 min at 0°. Slices were then homogenized in 3 ml of fresh incubation medium. Both slices and media were analysed for their [^{14}C]acetylcholine content as described by Browning & Schulman (1968).

Results in Table 1 show that when morphine (10^{-3} M) was present in the incubation

Table 1. *Influence of morphine on the formation or release of* [^{14}C]*acetylcholine by slices of rat cerebral cortex*

	^{14}C-Acetylcholine μg/g				
	Formation			Release	
Addition	Total	Slices	Medium	Slices	Medium
None	18·10 ± 0·74	2·30 ± 0·16	15·70 ± 0·73	1·08 ± 0·05	1·12 ± 0·15
Morphine 10^{-3} M ..	12·25 ± 0·90*	1·99 ± 0·07	10·30 ± 0·69*	1·23 ± 0·04†	0·75 ± 0·04†

* $P < 0·01$ † $P < 0·05$.

medium there was a decrease in the amount of [^{14}C]acetylcholine formed but this occurred largely in the medium. Since the [^{14}C]acetylcholine in the medium was synthesized in the slices and transferred to the medium as a result of the releasing action of KCl, it may be presumed that morphine retarded this action of KCl. That this may be so is seen from the effect of morphine on the release of preformed [^{14}C]acetylcholine from slices. The presence of morphine in the incubation medium was associated with a significant decrease in the amount of [^{14}C]acetylcholine released from the slices into the medium. And, conversely, the amount of [^{14}C]acetylcholine that remained in slices was greater in the incubation medium containing morphine.

Morphine inhibits the release of acetylcholine in other systems, for example, from guinea-pig ileum (Schauman, 1957; Paton, 1957; Cox & Weinstock, 1966) and from cat brain (Beleslin & Polak, 1965; Beleslin & others, 1965). Our experiments substantiate these reports and indicate that morphine had a similar effect on rat cerebral cortex. The increased acetylcholine content of rat brain after administration of certain drugs has been attributed to inhibition of acetylcholine release by these drugs rather than to stimulation of acetylcholine synthesis or to inhibition of cholinesterase (Crossland & Merrick, 1954). Our finding that morphine inhibited the release of acetylcholine from rat cerebral cortext suggests that the observed increase in total and "bound" acetylcholine content of rat brain associated with morphine administration (Giarman & Pepeu, 1962; Crossland & Slater, 1968) may well be due to inhibition of acetylcholine release at cholinergic synapses in the brain. This effect would also explain the decrease of "free" acetylcholine observed by Crossland & Slater (1968) in brain after morphine administration to rats.

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The effect of narcotics and narcotic antagonists on the tail-flick response in spinal mice

Irwin, Houde & others (1951) reported that the tail-flick response of rats to radiant heat has the characters of a simple reflex. The ability of spinal rats to respond to this stimulus has been confirmed and extended (Winter & Flataker, 1951; Bonnycastle, Cook & Ipsen, 1953). Morphine inhibited the reflex in spinal rats but a quantitative difference observed between its effect in spinal rats and intact animals indicated that a second action might exist in its capacity to increase supraspinal inhibitory mechanisms. We have used the tail-flick test in mice to elucidate the mechanism of action of morphine and the narcotic-antagonist analgesics (Harris & Pierson, 1964; Dewey, Harris & others, 1969; Harris, Dewey & others, 1969) and have found a high correlation of activity with a number of narcotic analgesics to exist between species.

We have confirmed and extended the observation that cholinergic agents such as oxotremorine and physostigmine also reduced this response (Harris & others, 1969; Howes, Harris & others, 1969). In addition, we have shown that an increase in central adrenergic or 5-hydroxytryptamine tone will increase the activity of morphine in intact mice in the tail-flick test (Dewey, Harris & others, 1968). We have now attempted to increase our knowledge about this testing procedure by studying the narcotics, the narcotic antagonists, and some of the neurochemicals discussed above in spinal mice.

Male albino mice of the Swiss-Webster strain (18–25 g) had transections made under ether anaesthesia. A dorsal midline incision was made and the spinal cord was exposed between the fifth and sixth thoracic vertebrae. The cord was cauterized and the wound was closed with silk sutures. Attempts to transect the cord at a higher level resulted in death from respiratory paralysis or uncontrolled bleeding. There were few deaths from the surgical procedure. Water and food were presented *ad libitum*. Within 3 to 4 h all mice were quite active. Simple physiological stimuli showed that the cord section was positive. Most of the mice responded to the radiant stimulus of the tail-flick apparatus within 4 s, the variability among the reaction times being less than is usually observed in normal mice. Animals not responding within 4 s were not used. The results obtained were averaged with a second reading taken 30 min later, after which the drug was given subcutaneously in the flank; readings were made 20 min later. Mice not responding within 10 s were removed from the apparatus and considered to be 100% affected. The % maximal possible inhibition was calculated using the following formula:

$$\frac{\text{test}-\text{control}}{10-\text{control}} \times 100 = \% \text{ maximal possible inhibition}$$

The ED50 for morphine in intact mice is 6·25 mg/kg in this procedure, in our hands. In intact animals, 10 mg/kg of morphine sulphate usually gives near 100% maximum possible inhibition as do all the higher doses used in these experiments.

The results in Table 1 show the effect of morphine on spinal mice. It is much less active than in intact mice. Irwin & others (1951) found a similar decrease in its potency in spinal rats. They concluded that it had an inhibitory effect on the spinal reflex and a stimulatory effect on supraspinal inhibitory processes. These supraspinal processes were removed in the spinal animals explaining the observed decrease in potency of morphine. Our mouse evidence differs from the findings with spinal rats in that increasing the dose of morphine to a high level did not increase its activity in mice. In spinal mice as opposed to spinal rats there appears to be a limit to the ability of morphine to inhibit the reflex.

Table 1. *The effects of morphine and naloxone on the tail-flick test in spinal mice*

Drug	Dose mg/kg	N	Control time (s)	Test time (s)	% maximal possible inhibition
Morphine	10	19	2·6	3·5	18
Morphine	20	23	2·8	4·6	32
Morphine + naloxone	20 1	13	2·2	2·5	3
Morphine	40	30	2·4	3·6	16
Morphine + naloxone	40 1	19	2·1	2·1	0
Morphine	62·5	17	2·3	3·6	18
Morphine + naloxone	62·5 1	10	2·2	2·0	0
Morphine	120	8	2·7	3·2	7

Naloxone, a nearly pure antagonist (Blumberg, Dayton & Wolf, 1966), antagonized the activity of morphine (Table 1). This indicates that, in this procedure, the antagonist's activity is not limited to an effect on supraspinal mechanisms. The narcotic-antagonist analgesics, pentazocine and cyclazocine, at normal doses were almost without activity in the tail-flick test in intact mice. In spinal mice, at very high doses where some activity is seen for cyclazocine in normal animals (Harris & Pierson, 1964), pentazocine (60 mg/kg) and cyclazocine (100 mg/kg) caused a 19 and 27% maximum inhibition in spinal mice. There was complete inhibition in a few of the animals but no effect on most. Morphine on the other hand delayed the reaction time for most mice.

As there are cholinergic inhibitory cells in the spinal cord (Eccles, 1964) it might be expected that the activity of physostigmine and oxotremorine in normal mice would be largely due to spinal inhibition. If this were so these drugs should be relatively equally active in spinal mice. Physostigmine (0·4 mg/kg) and oxotremorine (0·1 mg/kg), both of which cause greater than 80% inhibition in intact mice, gave 13 and 10% maximum possible inhibition in spinal mice, indicating that other central cholinergic mechanisms may play a role in the action of compounds, like the narcotic analgesics, active in this test.

This evidence from the analgesic and the cholinergic drugs indicates that although a spinal reflex is involved in this procedure, the supraspinal influences might be more important than effects at the lower level of the cord. Additional evidence for this point of view is provided by the very high correlation shown to exist between activity in the tail-flick test and addiction potential in man (Archer, Harris & others, 1964).

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The effects of reserpine on the distribution of [^{14}C]5-hydroxytryptamine in the rat

It has been stated that in the rat, as in many other species, reserpine facilitates the oxidative deamination of both endogenously bound and parenterally administered 5-hydroxytryptamine (5-HT) (Erspamer, 1956a; Garattini, Lamesta & others, 1961; Airaksinen, 1963; Axelrod & Inscoe, 1963; Snyder, Wurtman & others, 1964). The results to be presented in this letter are not entirely consistent with these observations.

Male Wistar rats of 230 ± 10 g were treated intraperitoneally with reserpine (5 mg/kg) or the vehicle solution (20% ascorbic acid). Eighteen h later they were anaesthetized with pentobarbitone, pithed (Shipley & Tilden, 1947) and given a 1 min infusion of 4·08 μg (4 μCi) of [^{14}C]5-HT creatinine sulphate monohydrate into the femoral vein. At the end of the infusion, a plasma sample and heart and kidney tissues were processed for their total radioactivity and unchanged [^{14}C]5-HT content by the methods previously described (Fozard, 1969). The results are shown in Fig. 1.

There was no significant difference as a result of reserpine pretreatment in the total radioactivity levels of plasma, heart or kidney, or in the unmetabolized [^{14}C]5-HT content of heart and kidney. The small sample of plasma obtainable during the collection period did not allow routine determination of the unmetabolized [^{14}C]5-HT content of the plasma total radioactivity. The tissue to plasma ratios of total radioactivity (Weiner & Trendelenburg, 1962) for the ascorbic acid and reserpine pretreated groups respectively were 0·9 ± 0·10 and 0·92 ± 0·13 for hearts and 1·74 ± 0·24 and 2·14 ± 0·41 for kidneys.

The rapid metabolism of [^{14}C]5-HT accumulated by hearts and kidneys of reserpine pretreated rats has been shown to be the result of oxidative deamination by monoamine oxidase (Fozard, 1969), and was predictable from the earlier observations of Erspamer (1956a), Airaksinen (1963) and Axelrod & Inscoe (1963). However, in the present work an unexpected finding was that the results obtained in animals given reserpine were not significantly different from those obtained in the vehicle-pretreated controls. The explanation may be related to the dose of 5-HT used and its mode of administration.

Both Erspamer (1956b) and Airaksinen (1963) demonstrated that in normal rats the proportion of a small dose of amine excreted as 5-hydroxyindoleacetic acid was greater than that excreted from a larger dose. Airaksinen (1963) found the proportion of deaminated metabolites when 5-HT was given subcutaneously or by slow intravenous injection was greater than when given by rapid intravenous injection. In his experiments, pretreatment with reserpine increased the amount of 5-hydroxyindoleacetic acid after intravenous injection of 5-HT such that the difference usually observed

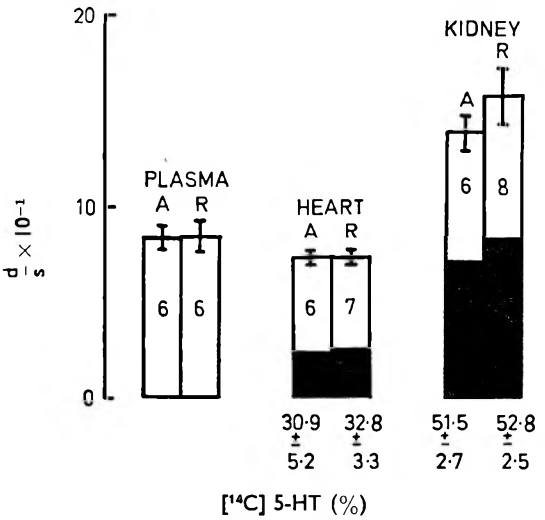

FIG. 1. The distribution of radioactivity after infusion of 4·08 μg (4 μCi) of [14C]5-HT creatinine sulphate monohydrate into reserpinized pithed rats (R) and pithed rats treated with the vehicle solution of 20% ascorbic acid (A). The histograms show the mean total radioactivity in disintegrations per 0·1 s per ml of plasma or per g of heart or kidney tissue with standard errors. The proportion of the total radioactivity which is present as the unchanged [14C]5-HT is represented by the solid portion of the histogram. The actual percentages with standard errors are given below each histogram.

between subcutaneous and intravenous administration with respect to 5-hydroxyindoleacetic acid excretion was annulled. In the present experiments the effect of slowly infusing a small dose of [14C]5-HT by the intravenous route can be equated with the concept of a slow leaching of a larger dose from a subcutaneous depot. Therefore one might expect not only the rapid extensive metabolism of [14C]5-HT by deamination, but also a lack of difference between normal and reserpinized animals in this respect. Such a suggestion would also explain the inconsistency between the present results and those reported by Axelrod & Inscoe (1963), where pretreatment of rats with reserpine (5 mg/kg) increased the metabolism and reduced the tissue concentrations of a larger dose (250 μg) of [14C]5-HT after rapid intraveous injection.

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Monoamine oxidase inhibition and bretylium on adrenergic neuronal transmission

Bretylium is known to exert a monoamine oxidase inhibitory action *in vivo* (Malmfors, 1968; Clarke & Leach, 1968); I have now investigated this effect of bretylium upon adrenergic neuronal transmission.

Female Wistar rats were anaesthetized with either sodium pentobarbitone (60 mg/kg) or urethane (1·5 g/kg) both given intraperitoneally. The systemic blood pressure was recorded from the right common carotid artery and drugs were administered into a femoral vein. Intravenous eserine (25–40 μg) was used to stimulate the sympathetic nervous system.

Pretreatment of rats with nialamide (100 mg/kg, i.p.), for 2 or 18 h did not affect the pressor response to eserine, nor did it prevent the ability of bretylium (5 or 10 mg/kg, i.v.) to antagonize the eserine response (Fig. 1a). Under these conditions dexamphetamine (100 μg, i.v.) produced a reversal of the bretylium-induced blockade of eserine.

It seems that the acute adrenergic neuronal blocking activity of bretylium is not linked with any monoamine oxidase inhibitory property of this drug. Under these experimental conditions bretylium exerted a pronounced hypotensive effect showing that it can block not only the excessive and possibly, therefore, atypical sympathetic discharge attributed to eserine, but also the far less intense resting sympathetic discharge contributed by the anaesthesia. This correlates well with the effects of

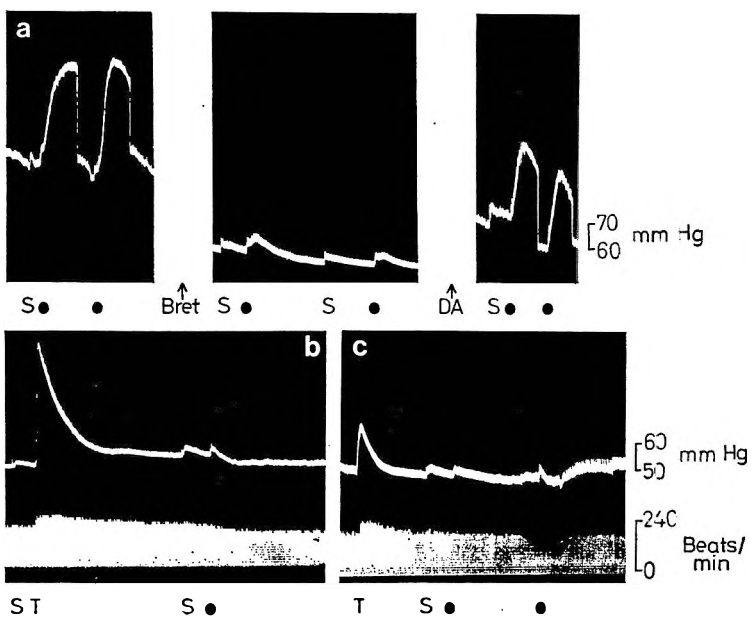

FIG. 1. (a) Rat blood pressure, sodium pentobarbitone anaesthesia, pretreated with nialamide, 100 mg/kg for 2 h. Effect of bretylium (BRET), 5 mg/kg, on the response to eserine (●), 30 μg. Dexamphetamine, 100 μg, was given at (DA). (b) Rat blood pressure (upper record) and heart rate (lower record), sodium pentobarbitone anaesthesia, reserpine-treated, 5 mg/kg for 18 h + nialamide 100 mg/kg for 6 h. Effect of nialamide treatment on the response to tyramine (T), 50 μg and eserine (●), 40 μg. (c) As in (b) except bretylium 5 mg/kg was given for 6 h. S = 0·3 ml normal saline.

bretylium seen under normal conditions in the rat (Cass & Spriggs, 1961), as does the reversal of adrenergic neuronal blockade with dexamphetamine (Spriggs, 1966), and implies that the normal pharmacological mechanism of action of bretylium in sympathetic nerve blockade was in no way altered by monoamine oxidase inhibition.

In rats anaesthetized with sodium pentobarbitone and pretreated with reserpine (5 or 10 mg/kg) for 18 h, the cardiovascular response to tyramine (50 μg) was abolished and repeated doses of eserine failed to give rise to a pressor response. The pretreatment of reserpinized rats with nialamide (100 mg/kg) or bretylium (5 mg/kg) given subcutaneously 6 h before use, restored the cardiovascular response to tyramine but caused little or no restoration of the response to eserine (Fig 1, b and c). In non-reserpinized rats there is an almost complete recovery of the hypertensive effect of eserine, 5 to 6 h after an intravenous injection of bretylium (5 mg/kg). Finally, neither the bretylium or nialamide pretreatment described above, caused a rise in the resting level of the systemic blood pressure or heart rate of reserpinized rats beyond that normally encountered in these preparations.

The restoration of the cardiovascular response to tyramine is consistent with the monoamine oxidase inhibitory actions attributable to bretylium and nialamide under reserpinized conditions and is probably mediated through the accumulation of endogenously formed catecholamines within the postganglionic adrenergic nerves. The specific intraneuronal location of this amine fraction might well account for its inability to be released by sympathetic nerve impulses. However, with bretylium, the assumption has to be made that its noted duration of adrenergic neuronal blockade in non-reserpinized rats is still applicable under reserpinized conditions.

The restoration of adrenergic transmission obtained by Häggendal & Malmfors (1969) with nialamide in reserpinized rats given a subsequent injection of dopamine, appeared to be mediated through the release of newly synthesized noradrenaline. In the experiments here reported, it is highly probable that insufficient endogenously formed amine occurred under the nialamide treatment to overcome the competitive inhibitory effect of reserpine on the intraneuronal granular binding mechanism (Stjärne, 1966) at the site held to be responsible for adrenergic nerve function (Häggendal & Malmfors, 1969). In this respect, Stjärne (1966) has provided evidence that the biosynthesis of noradrenaline is much less sensitive to inhibition by reserpine than is the granular storage mechanism.

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Absence of central nervous system effects of practalol [ICI 50,172; 4-(2-hydroxy-3-isopropylaminopropoxy)-acetanilide], a new adrenergic β-receptor blocking drug

Practalol [ICI 50,172; 4-(2-hydroxy-3-isopropylaminopropoxy)-acetanilide] is a potent adrenergic β-receptor blocking agent which, in contrast to some older drugs from this group {propranolol, Kö 592 [1-(isopropylamino)-3-(*m*-tolyloxy)-2-propanol HCl]}, is almost free from local anaesthetic properties (Dunlop & Shanks, 1968). Since it has been shown that several β-receptor blocking drugs, like propranolol, Kö 592, and 2-isopropylamino-1-(p-nitrophenyl) ethanol (INPEA), exhibit marked effects on the central nervous system, ranging from central depression to central stimulation (Ammon & Estler, 1968; Leszkovszky & Tardos, 1965; Murmann, Almirante & Saccani-Guelfi, 1966), we examined the central nervous system effects of practalol in white mice. (±)-Propranolol was used for comparison in some of the tests.

Male NMRI-mice, which had free access to standard diet (Altromin R, obtained from Altromin GmbH, Lage/Lippe, Germany) and tap water, were used.

Spontaneous motor activity of single mice. The animals were placed into circular activity cages of 14 cm diameter. In each cage two photo-cells and light beams were installed. When the animals moved along the circular path they interrupted the light beams, and the number of interruptions per time unit was measured. Recordings were made for 2 h after the animals had received 1, 5, or 20 μg/g of practalol or the same doses of (±)-propranolol subcutaneously. Activity was measured in counts/30 min.

Spontaneous orientational hypermotility of grouped mice. Groups of 5 mice were placed into a Basile activity cage. The floor of this cage is made up of steel bars insulated from each other and charged with a low current. When moving around the animals close the electric circuits and the contacts per time unit are counted. The activity was measured for 15 min after the animals had received 1, 5, or 20 μg/g practalol subcutaneously. Activity was measured as contacts/15 min.

Traction test. This test (Julou, 1956) measured sedation and muscular relaxation in mice treated with 1, 5, or 20 μg/g practalol or propranolol subcutaneously 45 min beforehand.

Effect on hexobarbitone anaesthesia. Mice were pretreated subcutaneously with 1, 5, or 20 μg/g practalol or (±)-propranolol. 30 min later, 120 μg/g of hexobarbitone was injected intraperitoneally, and the sleeping time measured. Sleeping time was equated with absence of righting reflexes.

Effect on propanidid anaesthesia. Mice were pretreated subcutaneously with 1, 5, or 20 μg/g practalol or (±)-propranolol. 30 min later they were injected with 40 μg/g propanidid intraperitoneally, and the sleeping time was measured as before.

Effect on leptazol seizures. Mice were pretreated subcutaneously with 20 μg/g practalol or the same dose of (±)-propranolol. 30 min later leptazol was given intravenously in increasing doses from 50–120 μg/g. The number of animals that exhibited tonic extensor spasms and died within 30 min was recorded. From these data the LD50 for leptazol was calculated (Litchfield & Wilcoxon, 1949).

It is obvious (Table 1) that the effects of practalol differ in most respects from those of propranolol. Like Leszkovszky & Tardos (1965), we found propranolol to increase the seizure threshold and the LD50 of leptazol and to prolong hexobarbitone anaesthesia. In the same way, the sleeping time after propanidid was prolonged by 20 μg/g (±)-propranolol. Practalol, on the other hand, did not significantly affect the hexobarbitone and propanidid anaesthesia and the LD50 of leptazol. Both

Table 1. *Central nervous system effects of practalol and (±)-propranolol*

Test	Time after injection of drug (min)	Saline	Practalol (μg/g s.c.) 1	Practalol (μg/g s.c.) 5	Practalol (μg/g s.c.) 20	(±)-Propranolol (μg/g s.c.) 1	(±)-Propranolol (μg/g s.c.) 5	(±)-Propranolol (μg/g s.c.) 20
Spontaneous motility of single mice (counts/30 min) ..	0–30	120 ± 12 (28)	155 ± 14* (28)	195 ± 17* (28)	167 ± 13* (28)	138 ± 23 (20)	134 ± 14 (24)	119 ± 12 (24)
	30–60	38 ± 6 (28)	56 ± 12 (28)	114 ± 22* (28)	45 ± 11 (28)	68 ± 23 (20)	60 ± 11 (24)	51 ± 9 (24)
	60–90	23 ± 5 (28)	37 ± 11 (28)	78 ± 20* (28)	43 ± 10 (28)	18 ± 4 (20)	37 ± 7 (24)	29 ± 6 (24)
	90–120	23 ± 7 (28)	30 ± 11 (28)	63 ± 21 (28)	14 ± 6 (28)	14 ± 3 (20)	32 ± 9 (24)	27 ± 8 (24)
Orientational hypermotility of grouped mice (counts/15 min)	0–15	615 ± 61 (20)	813 ± 46* (20)	809 ± 52* (20)	795 ± 53* (20)	—	—	—
Sleeping time after 120 μg/g hexobarbitone i.p. (min)	30	51 ± 5 (16)	47 ± 5 (16)	45 ± 5 (14)	51 ± 4 (14)	53 ± 9 (9)	53 ± 12 (8)	75 ± 6* (21)
Sleeping time after 40 μg/g propanidid i.p. (min)	30	1·8 ± 0·2 (10)	2·1 ± 0·2 (10)	1·8 ± 0·2 (9)	2·1 ± 0·2 (13)	2·2 ± 0·2 (9)	2·2 ± 0·2 (10)	2·6 ± 0·2* (15)
LD50 of leptazol and confidence limits (μg/g i.p.) ..	45	75 68–86	—	—	68 56–83	—	—	92* 79–105

Results are expressed as mean values ± s.e. The number of animals is given in parentheses.
* Values significantly different from control, $P \leq 0.05$.

drugs had no effect on the behaviour of animals in the traction test. The only significant effect of practalol was on spontaneous motility. All doses tested increased the orientational hypermotility of grouped mice. In single animals motility was increased for 90 min after a dose of 5 μg/g practalol and for only 30 min by 1 and 20 μg/g, whereas propranolol did not markedly influence the spontaneous activity of single mice.

Thus, (±)-propranolol shows central depressant properties (Leszkovszky & Tardos, 1965; Murmann & others, 1966), whereas practalol exerts only weak, if any, central stimulant effects.

We wish to thank Dr. H. P. Kuemmerle, Rhein-Pharma GmbH, Heidelberg, Germany for kindly supplying practalol. Hexobarbitone (Evipan-Natrium) and propanidid (Epontol) were gifts from Farbenfabriken Bayer A.G., Leverkusen, Germany.

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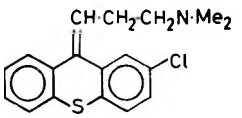
Oxidation of chlorprothixene with ceric sulphate

Recently, Agarwal & Blake (1969) described a photometric titration procedure for the analysis of a series of therapeutically useful phenothiazine derivatives and their dosage forms. The procedure involved the titration of an acidic solution of the drug with ceric sulphate solution. The equivalence point was determined by following the change in absorbance of the solution at 420 nm. Chlorprothixene (I), *trans*-isomer of 2-chloro-9-(3-dimethylaminopropylidene) thioxanthene which is chemically and pharmacologically related to chlorpromazine, was one compound for which the analysis proved unsuccessful because of the formation of a precipitate during the titration. The composition of the precipitate and the chemical reaction involved in its formation have now been examined.



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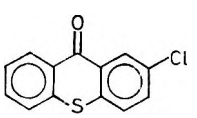

II

Chlorprothixene (500 mg) was suspended in 10% H_2SO_4 (3 ml). Sufficient ethanol was added to effect solution. Ceric sulphate solution (0·1N) was added slowly until the formation of a heavy yellow precipitate was complete. The reaction mixture was left in an ice bath for 30 min, after which the precipitate was separated by filtration washed with distilled water, dried, and crystallized from ethanol-chloroform, m.p. 51–152°. Found: C, 63·0; H, 3·1. $C_{13}H_7ClOS$ requires C, 63·3; H, 2·9.

The infrared spectrum indicated strong absorption, characteristic of the carbonyl group, at 1642 cm^{-1}. The spectrum for chlorprothixene did not show this absorption peak. The nmr spectrum of the reaction product showed peaks in the region of 2·3–2·7δ, due to phenyl protons; whereas the spectrum for chlorprothixene, showed peaks at 2·5–2·85δ (phenyl protons), 4·1δ (=CH–), 7·4–7·6δ (–CH_2–CH_2–), and 78·2δ (–NMe_2). These data indicate that the dimethylaminopropylidene group was removed from the chlorprothixene molecule as a result of the oxidation process producing 2-chloro-10-thiaxanthenone (II). The identity of this product was confirmed by synthesis from chlorobenzene and *o*-mercaptobenzoic acid in a concentrated sulphuric acid medium as described by Gilman & Diehl (1959); m.p. 152–153°; mixed melting point with the reaction product obtained by oxidation of chlorprothixene gave no depression. In addition, their infrared and nmr spectra were identical.

We are grateful to Dr. C. S. Mahajanshetti for obtaining the nmr spectral data.

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Antidotes for phosgene-induced pulmonary oedema

It may be of interest to record the results of certain experiments on antidotes for phosgene-induced pulmonary oedema in laboratory animals, made in this department some years ago.

Death from phosgene poisoning in frogs could be prevented simply by placing the frogs in a bath of normal saline immediately after exposure to the gas (Boyd & Stewart, 1953). The saline solution apparently prevented diffusion of body fluids through the skin, the permeability of which had been apparently increased by phosgene.

Boyd & Perry (1960) found that pulmonary oedema in rabbits, after exposure to phosgene, was accompanied by the outpouring into respiratory tract fluid of a fluid similar in composition to blood plasma. Similar results were obtained in cats and dogs by Boyd & Perry (1963) who noted that postural pulmonary drainage did not clear this fluid from the lungs and did not prevent death. We have tried unsuccessfully to prevent or cure phosgene-induced pulmonary oedema by many means including the use of antihistamine agents, Janus green, goitrogenic agents and inhalation of ammonia gas. We did find, however, that death rates from phosgene were reduced in rabbits, cats and albino rats if the animals were allowed to inhale the phosgene through the nose rather than directly into the trachea. Since phosgene is fairly labile, this was due presumably to destruction of the gas in the naso-bucco-pharynx. We could not induce nasal breathing in dogs as they persisted in panting and drawing air directly into the trachea. Presumably as a result of this, we were unable to lower death rates to phosgene in dogs by this means.

The experiments on nasal breathing were made upon 18 adult rabbits, 14 cats, 80 albino rats and 21 dogs. The animals were anaesthetized with thiopentone sodium and a glass cannula was ligated into the trachea of half of the animals of each species. When the anaesthesia wore off, the animals were exposed to phosgene after the technique of Boyd & Perry (1960). After exposure, the tracheotomy tube was removed, the wound closed by sutures and the animals observed until recovery or death. The mortality rate in the tracheal-cannulated rabbits was 88% versus 25% in rabbits inhaling phosgene through the nose. Corresponding mortality rates in cats were 100% and 50% and in albino rats 90% and 40%. The combined mortality rate in these three species to inhaling phosgene directly into the trachea was 91% and to inhaling the gas through the nose 39%. The probability (P) that these percentage death rates were the same was less than 0·001. There were 3 deaths in 24 controls, which were tracheal-cannulated but not exposed to phosgene.

Boyd, MacLachlan & Perry (1944) reported similar results from inhalation of ammonia gas by rabbits and cats. Necrosis of the tracheo-bronchial mucosa was greater in animals which inhaled ammonia gas directly into the trachea than in animals which inhaled it through the nose. Boyd, MacLachlan & Perry (1944) referred to the phenomenon as the "naso-bucco-pharyngeal filter". It would appear that this filter may be of great significance in reducing the toxicity of inhalants.

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Fenfluramine and body temperature

Fenfluramine is an anorectic drug structurally similar to amphetamine but devoid of central and cardiovascular stimulant properties in experimental animals and in men (Colmore & Moore, 1966; Le Douarec, Schmitt & Laubie, 1966; Ziance & Kinnard, 1968). The experiments here reported suggest, however, that fenfluramine may display stimulant properties in certain experimental conditions, such as the increased availability of catecholamines produced by dopa in monoamine oxidase inhibitor pretreated mice. (+)-Amphetamine sulphate, (±)-fenfluramine hydrochloride and S992 [trifluoromethylphenyl(benzoyloxy)ethylamino-2-propane], were tested in different experimental conditions according to the following schedule: Group 1: saline; group 2: pheniprazine (10 mg/kg i.p.); Group 3: L-dopa (25 mg./kg i.p.); Group 4: pheniprazine + L-dopa.

(+)-Amphetamine sulphate, 2 mg/kg, i.p., given to control mice did not affect the body temperature (Fig. 1B), while it was hyperthermic on increasing the dose to 5 mg/kg (Fig. 1C). Fenfluramine and S992 were never hyperthermic when given to control mice up to a dose of 30 mg/kg, i.p.

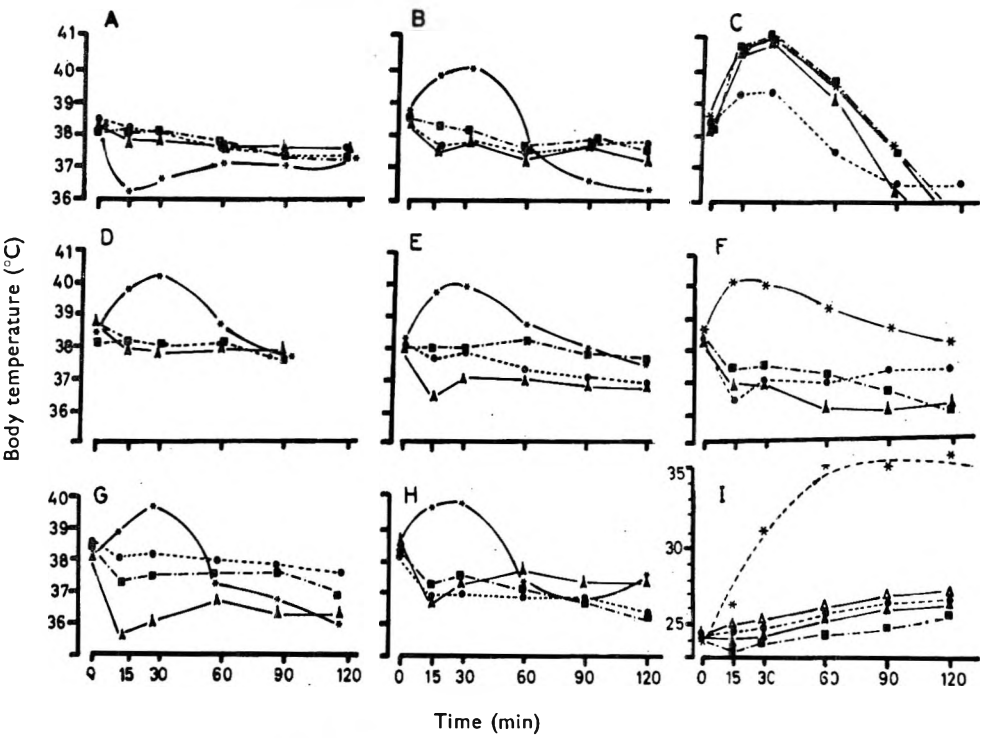

FIG. 1. A to H: Body temperature of groups of 5 mice receiving i.p. injections of: ●—● saline, ▲—▲ dopa, 25 mg/kg, ■-·-·-■ pheniprazine, 10 mg/kg, ★—★ pheniprazine + dopa. Except group A, in all other figures, beside this treatment, mice received i.p. amphetamine, fenfluramine or S992 at the doses listed below. Pheniprazine was given 16 h before the test. Fifteen min after the tested drugs, L-dopa was given and body temperature recorded 15, 30, 60, 90 and 120 min later. A. Saline. B. Amphetamine 2 mg/kg. C. Amphetamine 5 mg/kg. D. Fenfluramine 7·5 mg/kg. E. Fenfluramine 15 mg/kg. F. Fenfluramine 30 mg/kg. G. S992 15 mg/kg. H. S992 30 mg/kg.

I: Indicates the body temperature of mice injected i.p. with 5 mg/kg of reserpine 16 h before the test. △—△ saline, ★- - - - -★ amphetamine, 5 mg/kg, ▲—▲ fenfluramine 15 mg/kg, ●- - - - -● fenfluramine 30 mg/kg, ■- · - · -■ S992 15 mg/kg.

From Fig. 1A it can be seen that L-dopa displays a mild hypothermic action in monoamine oxidase-blocked mice. This hypothermia is reversed to a clear hyperthermia not only by amphetamine but also by fenfluramine and S992 at any dose studied. The hyperthermia was accompanied by clear signs of excitation. From a more detailed examination it appears that amphetamine (2 mg/kg) and fenfluramine (7·5 mg/kg) act similarly in all the four experimental conditions (Fig. 1B and 1D). At higher doses (Fig. 1C, E, F) the picture is different. Amphetamine itself elicits hyperthermia, which is further increased by L-dopa or pheniprazine. On the contrary, fenfluramine alone produces hypothermia which is unaffected by L-dopa or pheniprazine.

In comparison with the above conditions, where catecholamine levels are increased, the effect of fenfluramine on body temperature was tested on mice made hypothermic by reserpine. After reserpine, amphetamine increases the body temperature while fenfluramine and S992 fail to modify it (Fig. 1 I).

These studies show that the effect of fenfluramine and its derivative may differ from or be similar to amphetamine according to the availability of catecholamines. Also Le Douarec & others (1966) concluded, on the basis of different experiments, that fenfluramine may show both sedative and stimulant effects at the same time.

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BOOK REVIEW

ISOLATION AND IDENTIFICATION OF DRUGS in pharmaceuticals, body fluids and post-mortem material. Edited by E. G. C. Clarke assisted by Judith Berle. Pp. xxii + 870 (including index). The Pharmaceutical Press, London, 1969. £14.

Isolation and Identification of Drugs is, as is claimed in the descriptive brochures, an entirely new book which should fill a gap which has long been apparent in the literature of human toxicology. This book has been produced as a companion volume to the Extra Pharmacopoeia and indeed there is a marked resemblance in size and style. It is offered as a practical manual and data book for forensic scientists, toxicologists, analytical chemists, pharmacists, biochemists, pathologists and police surgeons; whilst of undoubted value to the former groups the value to police surgeons must be limited. Since it is a companion volume to the Extra Pharmacopoeia, it is perhaps unfortunate that the habit encountered in the toxicological literature of intermixing data for mice (rats, guinea-pigs etc.) and men has been allowed to invade the present volume. However, Professor Clarke and his team of collaborators and assistants have succeeded admirably in their task and are to be congratulated on the production of a volume which has been in almost constant use (in at least one laboratory) since its publication.

The concept of this work is ambitious in that an attempt has been made to provide details of methods and techniques for the identification of many drugs under condi-

tions ranging from the primitive to the more sophisticated. The book is divided into four parts and a little time spent in studying the layout and mastering the coding systems used to refer, for example, to chromatographic systems, will speedily repay the effort involved. Part 1 contains ten chapters describing the theory and practice of the relevant experimental methods, for example, extraction methods, the various chromatographic techniques, ultraviolet and infrared absorption spectrophotometry, colour tests and microcrystal tests. Also included is a useful summary of the pathways of biotransformation which drugs undergo in biological systems. The remaining chapter in Part 1 describes some rapid screening methods suitable for the hospital biochemist working against the clock; it was disappointing to find that the comparatively inexpensive and rapid technique of thin-layer chromatography was not adopted in this section in preference to traditional tests, many of which have outlasted their usefulness. The data obtained for a very wide range of drugs, by following the methods and using the chromatographic systems described in Part 1, are given in most instances in the text of Part 1, the drugs being listed in alphabetical order. The same data are classified, for example in ascending order of Rf value, and tabulated in Part 3 of the book which also includes reproductions of some 450 infrared spectra with the major peaks labelled.

Part 2 contains monographs for over 1000 compounds. Each monograph includes synonyms, structural formula, molecular weight, melting point and solubilities. In addition, some or all of the following are given: notes on suitable extraction methods of recovery of the drug from biological systems, references to appropriate chromatographic systems, ultraviolet and infrared absorption data, methods for quantitative analysis, details of metabolism, dose, toxicity (mostly animal data) and summaries of relevant published cases.

Part 4 comprises appendices giving details of reagents and tests referred to in Parts 2 and 3 and a bibliography of the references cited in the text. Finally there is an excellent index.

Isolation and Identification of Drugs is undoubtedly destined to become one of the classic books of toxicological literature: it contains well-tried and properly evaluated techniques and data relating to an extensive range of drugs. It is to be hoped that this book will enjoy the wide circulation that it deserves and that prospective purchasers will not be deterred by its price, since the extensive content of the book and its overall usefulness will be found to amply repay this.

ANN E. ROBINSON

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