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Accumulation of α-methyltyramine by the noradrenaline uptake process in the isolated rat heart

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In the perfused isolated rat heart ^{3}H-α-methyltyramine is accumulated as rapidly as noradrenaline. The accumulation of ^{3}H-α-methyltyramine is reduced by the presence of noradrenaline or other drugs known to inhibit the noradrenaline uptake mechanism. ^{3}H-α-Methyltyramine and noradrenaline probably compete for a common uptake mechanism. In these experiments less than 5% of the ^{3}H-α-methyltyramine accumulated by the heart was metabolised to α-methyloctopamine.

CATECHOLAMINE uptake in the isolated rat heart has been shown previously to be mediated by a saturable, stereochemically specific mechanism which has the kinetic properties of an active transport membrane carrier system (Iversen, 1963; 1965). This uptake process is thought to be almost entirely localised in the postganglionic sympathetic innervation of the heart, since the uptake of noradrenaline is severely reduced in hearts which lack a normal sympathetic innervation (Hertting & Schiefthaler, 1964; Potter, Cooper, Willman & Wolfe, 1965; Iversen, Glowinski & Axelrod, 1966). A wide range of sympathomimetic amines structurally related to noradrenaline can act as potent inhibitors of noradrenaline uptake in the rat heart (Burgen & Iversen, 1965). However, it is not clear whether these amines inhibit noradrenaline uptake by acting as competitive substrates for the uptake system, or whether they inhibit uptake without themselves being transported into the tissue. Recent findings suggest that at least some of the structural analogues of noradrenaline act as alternative substrates for the catecholamine uptake process. For example, α-methylnoradrenaline and adrenaline are taken up into the isolated rat heart by a process which has very similar properties to that responsible for noradrenaline uptake (Iversen, 1965; Lindmar & Muscholl, 1965; Muscholl & Weber, 1965). The histochemical studies of Hamberger, Malmfors, Norberg & Sachs (1964) and Malmfors (1965) have also demonstrated an efficient uptake of α-methylnoradrenaline into the sympathetic ground plexus of the rat iris. Tyramine uptake into rat salivary glands is mediated in part by a process which is abolished by the chronic sympathetic denervation of the glands (Carlsson & Waldeck, 1963; Fischer, Musacchio, Kopin & Axelrod, 1964). Metaraminol is also avidly accumulated in rat tissues after the administration of small doses of this compound *in vivo*; this uptake is almost completely lacking in the tissues of immunosympathectomised animals (Shore, Busfield & Alpers, 1964). The uptake of metaraminol is furthermore inhibited by drugs known to reduce the uptake of noradrenaline (Carlsson & Waldeck, 1965).

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In a previous study it was found that the accumulation of ^{3}H-α-methyltyramine in the rat heart *in vivo* was severely reduced in immunosympathectomised animals (Iversen & others, 1966). This finding, together with the results presented in the present study, suggests that α-methyltyramine is also taken up by the catecholamine transport process in the sympathetic innervation of the rat heart.

Experimental and results

^{3}H-α-Methyltyramine (generally labelled; specific activity = 3 c/mM; chromatographically pure) was generously supplied by Dr. I. J. Kopin. The uptake of α-methyltyramine in the rat heart was studied after perfusing the organ with a low concentration of the labelled amine for 5 min. This short exposure of the tissue to the labelled amine was sufficient to allow a large accumulation of the substance, but did not allow time for any appreciable conversion of the labelled amine into its β-hydroxylated derivative within the tissue.

The uptake of α-methyltyramine was measured by two methods. In the first, uptake was estimated by measuring the amount of radioactive amine disappearing from the perfusion medium during passage through the heart; this approach has previously been employed for measurements of noradrenaline uptake in this preparation (Lindmar & Muscholl, 1964). Uptake was measured in the same experiments by the alternative method of assaying the accumulated radioactive amine in the heart at the end of the perfusion.

TABLE 1. INHIBITION OF α-METHYLTYRAMINE UPTAKE BY DRUGS

Drug	Uptake of ^{3}H-α-methyltyramine-ng/g heart	Inhibition of α-methyltyramine uptake (%)
Control	9·95 ± 0·50	—
10^{-5}M Cocaine	6·85 ± 0·65	31
10^{-5}M Chlorpromazine	6·20 ± 0·25	37
10^{-5}M Phenoxybenzamine	1·55 ± 1·25	84
10^{-6}M Desipramine	5·25 ± 0·60	47
10^{-6}M (−)-Metaraminol	5·35 ± 0·10	46
2×10^{-7}M (−)-Noradrenaline	5·65 ± 0·15	43

Uptake of ^{3}H-α-methyltyramine measured as accumulation of labelled amine in the rat heart after perfusion for 5 min with a medium containing ^{3}H-α-methyltyramine (1 ng/ml). Drugs were added to this medium to yield the concentrations indicated. Results are mean values ± s.e. mean for 6 control hearts and for 4 hearts in each drug treated group.

In control hearts perfused for 5 min with a medium containing 1 ng ^{3}H-α-methyltyramine/ml, the mean uptake of labelled amine from the perfusing medium was 10·2 ± 0·95 ng/g heart (wet weight), and the amount of labelled amine accumulated in the heart during these experiments was 9·9 ± 0·50 ng/g mean ± s.e. (mean for 6 experiments). Ion-exchange chromatography on columns of Dowex-50 (Iversen & others, 1966) indicated that at least 95% of the radioactivity in the heart extracts could be accounted for as unchanged ^{3}H-α-methyltyramine; the remaining radioactivity was recovered as the β-hydroxylated derivative, ^{3}H-α-methyloctopamine. The effects of various drugs on the uptake of ^{3}H-α-methyltyramine are summarised in Table 1. The drugs were added to the

perfusion medium, together with the labelled amine. In all experiments α-methyltyramine uptake was measured by the two methods described above. The two methods always gave closely similar results, and the results quoted in Table 1 are those obtained by measuring the accumulation of labelled amine in the heart at the end of the perfusion. At concentrations of 10^{-5}M or less, chlorpromazine, cocaine, desipramine, phenoxybenzamine and metaraminol all significantly inhibited the uptake of α-methyltyramine. The presence of (−)-noradrenaline at a concentration of 2×10^{-7}M inhibited the uptake of α-methyltyramine by almost 50%. This concentration of noradrenaline was chosen as it is close to the previously determined "Km" value for the uptake of L-noradrenaline in the rat heart (Iversen, 1963). If noradrenaline and α-methyltyramine compete for uptake by a common mechanism, the presence of noradrenaline at a concentration sufficient to saturate approximately 50% of the available uptake sites should produce an inhibition of approximately 50% of the uptake of α-methyltyramine, as was indeed the case.

Discussio

The sensitivity of α-methyltyramine uptake to inhibition by a low concentration of noradrenaline and by several other drugs which are known to be inhibitors of the noradrenaline uptake process, suggests that α-methyltyramine is taken up in the rat heart by the mechanism responsible for catecholamine uptake. It seems likely that several amines which are structurally related to noradrenaline, including tyramine, α-methyltyramine, adrenaline, α-methylnoradrenaline and metaraminol can act as alternative substrates for the catecholamine uptake process. The use of amines such as α-methyltyramine may prove valuable for future studies of the detailed properties of this uptake process. The uptake of noradrenaline is a complex process, probably involving at least two phases. An initial entry of the catecholamine into the nerve terminals of sympathetic fibres is mediated by a carrier system in the axonal membrane. After this initial uptake, however, the accumulated amine undergoes a further redistribution within the nerve terminal involving an uptake or binding in intraneuronal storage particles (Potter & Axelrod, 1963). The uptake of amines such as α-methyltyramine, however, is relatively simple since this substance and other amines which lack a β-hydroxyl group are not appreciably bound in intraneuronal storage particles (Musacchio, Kopin & Weise, 1965). Furthermore, α-methyltyramine, in common with other α-methylated amines, is not a substrate for intraneuronal monoamine oxidase. The influx of this compound can therefore be estimated reliably simply by measuring the accumulation of the labelled amine in the tissue. In accordance with this prediction, the results of the present experiments showed that more than 95% of the amine removed by uptake from the perfusion medium could be accounted for as unchanged α-methyltyramine which accumulated in the tissue. During the short time of perfusion less than 5% of the accumulated amine was converted to α-methyloctopamine. Despite the short duration of the experiments the

concentration of α-methyltyramine accumulated per gram of heart was ten times higher than that present per ml in the perfusion medium, indicating that α-methyltyramine has a high affinity for the catecholamine uptake sites and is accumulated as rapidly as noradrenaline itself under similar conditions.

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The effect of butylated hydroxytoluene, butylated hydroxyanisole and octyl gallate upon liver weight and biphenyl 4-hydroxylase activity in the rat

P. J. CREAVEN, W. H. DAVIES AND R. T. WILLIAMS

When a diet containing butylated hydroxytoluene at levels of 0·01 to 0·5% is fed to growing male rats for 12 days, body weight is not affected, but liver weight is increased. The activity of liver microsomal biphenyl 4-hydroxylase is also increased by these concentrations of butylated hydroxytoluene except the lowest level of 0·01% (100 ppm). Butylated hydroxyanisole at 0·1 and 0·25% of the diet did not affect liver weight or the enzyme activity, but at 0·5% it caused some increase in enzyme activity but not in liver weight. At the level of 0·5%, butylated hydroxytoluene given for a single day caused an increase in liver weight which was observed two days later and persisted for three days after which time the liver weight did not differ from controls. Enzyme activity was not significantly altered by 0·5% butylated hydroxytoluene fed during a single day. Octyl gallate fed at 0·5% of the diet for 9 days did not affect liver weight or enzyme activity. High fat diets with or without 0·01% butylated hydroxytoluene fed for 12 days did not increase liver weight, but did increase biphenyl 4-hydroxylase activity.

IT has been shown by Gilbert & Golberg (1965) that if female rats are given butylated hydroxytoluene (BHT) daily by stomach tube for a prolonged period at a dose of 500 mg/kg, the liver becomes enlarged and there is an increase in the activity of the liver microsomal enzymes which metabolise aminopyrine, hexobarbitone and nitroanisole. Similarly, if the rats are given butylated hydroxyanisole (BHA), there is a less pronounced enlargement of the liver, but no increase in the activity of the above microsomal enzymes. In the present paper we report experiments along similar lines, except that the rats used were males and the BHT and BHA were incorporated into the diet at various concentrations.

The liver microsomal enzyme system examined was that which hydroxylates biphenyl to 4-hydroxybiphenyl and, in a few experiments, that which demethylates 4-methoxybiphenyl. These enzyme systems were chosen because 4-hydroxybiphenyl is easily estimated fluorimetrically (Creaven, Parke & Williams, 1965).

Experimental

MATERIALS

BHT (3,5-di-t-butyl-4-hydroxytoluene), m.p. 73° after recrystallisation from light petroleum, BHA (a mixture containing not less than 90% of 3-t-butyl-4-hydroxyanisole, not more than 8% of 2-t-butyl-4-hydroxyanisole and less than 1% of 4-hydroxyanisole), m.p. 52·5° and octyl gallate, m.p. 102·5° (British Drug Houses Ltd., Poole, Dorset).

ANIMALS AND DIET

Male Wistar albino rats of about 100 g weight (Porton strain; Allington Farm, Porton Down, Salisbury, Wilts) were maintained on 10 g/day of powdered 41B diet (Rank Ltd., Croydon, Surrey) and water *ad lib.* The

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antioxidants were intimately mixed with the diet when necessary. For high fat diets Walls' pure English lard or Mazola corn oil was used since these were reputed to be free of antioxidants. The diets were fully consumed each day by the rats.

ENZYME ACTIVITY

The preparation of the 10,000 *g* supernatant of rat liver homogenates, the incubation procedure and the determination of biphenyl 4-hydroxylase activity were made as described by Creaven & others (1965). In the experiments in which 4-methoxybiphenyl demethylase was determined, the method used was that of Creaven, Davies & Williams (unpublished) which is similar to that for biphenyl 4-hydroxylase except that 4-methoxybiphenyl is used as substrate instead of biphenyl.

Results

EFFECT OF CONTINUOUS FEEDING OF BHT AND BHA

Table 1 shows that if rats are allowed to eat a BHT-containing diet for 12 days, the weight of the animals is not affected compared with the controls, but the liver weight in relation to body weight is significantly

TABLE 1. THE EFFECT OF FEEDING BHT FOR 12 DAYS UPON THE WEIGHT AND THE BIPHENYL 4-HYDROXYLASE ACTIVITY OF RAT LIVER

No. of animals	Amount of BHT in diet* %	Increase in body wt. %	Liver wt g/100 g rat	Increase %	P	Biphenyl 4-hydroxylase μmole/g liver/hr	Increase %	P
10	none	36·4	4·0	—		2·33	—	
9	0·01	42·8	4·8	20	<0·001	2·58	11	N.S.
10	0·05	31·3	4·8	20	<0·001	2·86	23	0·05
10	0·10	30·7	4·6	16	<0·01	3·26	40	<0·01
10	0·25	45·4	5·4	36	<0·001	3·93	69	<0·01

* 10 g/day/rat of powdered diet 41B (Rank Ltd.).
After 12 days, the rats were killed, their livers weighed and the 10,000 *g* supernatant of the homogenised liver prepared. The biphenyl 4-hydroxylase activity of each liver was determined fluorimetrically (Creaven & others, 1965).

increased at all levels of BHT (0·01 to 0·25%) in the diet. The activity of microsomal biphenyl 4-hydroxylase is also significantly increased except at the dose level of 0·01% (100 ppm) of BHT.

Table 2 shows the results with BHA diet, and it is clear that neither liver weight nor microsomal enzyme activity, that is biphenyl 4-hydroxylase and 4-methoxybiphenyl demethylase activity, is affected by 0·1 or 0·25% (1,000 or 2,500 ppm) of BHA.

TABLE 2. THE EFFECT OF BHA ON LIVER WEIGHT AND ON CERTAIN LIVER MICROSOMAL ENZYME ACTIVITIES IN THE RAT

No. of animals	Amount of BHA in diet* %	Liver wt g/100 g rat	Biphenyl 4-hydroxylase activity μmole/g liver/hr	Increase %	P	4-methoxybiphenyl demethylase μmole/g liver/hr	Increase %	P
10	0	5·8	1·67	—		2·70	—	
6	0·1	6·0	1·33	−20	N.S.	1·98	−26	N.S
6	0·25	5·7	1·74	+4	N.S.	2·37	−12	N.S

* Diet as in Table 1.
After 12 days the animals were killed. The livers were weighed and biphenyl 4-hydroxylase and 4-methoxybiphenyl demethylase activity of the 10,000 *g* supernatant of each homogenised liver were determined fluorimetrically.

EFFECT OF BHT FEEDING FOR DIFFERENT PERIODS

BHT was incorporated in the diet at the level of 0·5% (5,000 ppm). After feeding this diet for a single day before killing the animals, there was no significant effect upon liver weight or biphenyl 4-hydroxylase activity (see Table 3). However, if this BHT-containing diet was fed for two or more days before killing, the liver weight and the microsomal enzyme activity were significantly increased.

TABLE 3. EFFECT OF FEEDING BHT FOR DIFFERENT PERIODS UP TO 6 DAYS UPON THE WEIGHT AND BIPHENYL 4-HYDROXYLASE ACTIVITY OF RAT LIVER

No. of animals	Period over which BHT 5,000 ppm (0·5%) was fed in diet* days	Liver wt g/100 g rat	Increase %	P	Biphenyl 4-hydroxylase μmole/g liver/hr	Increase %	P
5	0	5·0	—		1·14	—	
5	1	5·5	10	N.S.	1·28	11	N.S.
5	2	6·3	26	<0·001	1·59	39	<0·001
5	3	6·6	32	<0·001	1·90	67	<0·001
5	4	6·0	20	<0·001	2·53	122	<0·001
5	5	6·3	26	<0·001	2·80	146	<0·001
5	6	6·5	30	<0·001	2·45	115	<0·001

* Diet as in Table 1.
The various groups were given the BHT diet for 1–6 days so that all groups except the control received the BHT-containing diet on the 6th day and were then killed at the end of the 7th day. The livers were weighed and biphenyl 4-hydroxylase activity determined.

If, however, the BHT-containing diet was fed for one day only, and then the animals were transferred to a BHT-free diet for varying periods up to 8 days, then, as Table 4 shows, one day on the diet containing 0·5% BHT significantly affects the liver weight but not the biphenyl 4-hydroxylase activity. The effect on liver weight, however, does not appear until the second day after the day on the BHT-containing diet.

TABLE 4. THE EFFECT OF TIME AFTER A SINGLE DOSE OF BHT UPON LIVER WEIGHT AND BIPHENYL 4-HYDROXYLASE ACTIVITY IN THE RAT

No. of animals	No. of days alive after 1 day with BHT 5,000 ppm (0·5%) in diet*	Liver wt g/100 g rat	Increase %	P	Biphenyl 4-hydroxylase μmole/g liver/hr	Increase %	P
5	control, no BHT	5·4	—		1·97	—	
5	1	6·0	11	N.S.	2·21	12	N.S.
5	2	7·3	35	<0·001	2·17	10	N.S.
5	3	8·3	54	<0·001	2·25	14	N.S.
5	5	6·7	24	0·05	2·06	4	N.S.
5	6	5·6	4	N.S.	1·99	1	N.S.
5	7	6·0	11	N.S.	2·12	8	N.S.
5	8	5·5	2	N.S.	2·06	4	N.S.

* Diet as in Table 1.
Each group received this BHT diet for only 1 day but on different days (as indicated in the table) before they were killed.
All the rats were killed on the 9th day, their livers weighed and biphenyl 4-hydroxylase activity determined fluorimetrically.

Table 4 also shows that the effect of one day on 0·5% BHT (during which time each rat would have consumed 50 mg of BHT) persists for about 4 days and after that time the liver weight returns to control values.

These experiments show that the liver weight is more sensitive to BHT than is biphenyl 4-hydroxylase activity.

EFFECT OF HIGH FAT DIETS

It has been reported that BHT given with a high fat diet might produce toxic phenomena not observed with a low fat diet (Johnson & Hewgill,

1961; but see Brown, Johnson & O'Halloran, 1959). Table 5 shows that high fat diets alone for 12 days cause significant increases in biphenyl 4-hydroxylase activity and these increases are no greater when 0·01%

TABLE 5. EFFECT OF A HIGH FAT DIET WITH OR WITHOUT BHT ON THE LIVER WEIGHT AND BIPHENYL 4-HYDROXYLASE ACTIVITY IN THE RAT

No. of animals	Diet*	BHT in diet %	Increase in body wt %	Liver wt g/100 g rat	Increase %	Biphenyl 4-hydroxylase μmole/g liver/hr	Increase %	P
10	control	0	124	5·8	—	1·56	—	
5	+ 20% lard	0	92	5·3	−9	2·48	59	<0·001
5	+ 20% lard	0·01	113	5·1	−12	2·68	72	<0·001
5	+ 20% corn oil	0	107	4·8	−17	2·73	75	<0·001
5	+ 20% corn oil	0·01	104	4·8	−17	2·78	78	<0·001

* *Controls:* Diet 41B (Rank Ltd.) 15 g/day.

Experimental animals: Diet 41B (12 g) containing Walls' pure English lard (3 g) or Mazola corn oil (3 g). BHT at 100 ppm (0·01%) was added as indicated in the Table.

Each group was given the diet indicated in the table for 12 days when all the rats were killed. Body and liver weights were recorded and the biphenyl 4-hydroxylase activity of the liver determined.

BHT is incorporated into the high fat diet. None of the diets used in Table 5 increase the ratio, liver weight/body weight. This ratio, if anything, fell on the high fat diets. The fall in this ratio, however, does not account for the increase in enzyme activity.

COMPARISON OF THE ANTIOXIDANTS

Table 6 records an experiment made on five groups of rats simultaneously. It will be seen that at a level of 0·5% in the diet, the antioxidant, octyl gallate, does not affect the liver weight or the biphenyl 4-hydroxylase when fed for 9 days. Under the same conditions EHA does not affect the liver weight but it does significantly increase the

TABLE 6. THE EFFECT OF BHT, BHA, OCTYL GALLATE AND A MIXTURE OF THESE ANTIOXIDANTS UPON LIVER WEIGHT AND LIVER BIPHENYL 4-HYDROXYLASE AND 4-METHOXYBIPHENYL DEMETHYLASE OF THE RAT

No. of animals	Antioxidant added % of diet*	Liver wt g/100 g rat	Increase %	P	Biphenyl 4-hydroxylase μmole/g liver/hr	Increase %	P	4-Methoxybiphenyl demethylase μmole/g liver/hr	Increase %
8	none	5·6	—		1·93	—		2·23	—
8	BHT 0·5	7·3	30	<0·001	4·14	114	<0·001	4·18	87 P<0·001
8	BHA 0·5	6·3	12·5	N.S.	2·81	46	<0·001	—	—
6	Octyl gallate 0·5	5·1	none		2·01	4	N.S.	—	—
6	BHT 0·19 + BHA 0·19 + Octyl gallate 0·125	7·1	27	<0·01	3·33	72	<0·001	—	—

* Diet as in Table 1.

After 9 days on the diets the rats were killed, the liver wt/body wt determined. Biphenyl 4-hydroxylase activity was determined. 4-Methoxybiphenyl demethylase activity was determined in the controls and the group receiving BHT only.

enzyme activity. However, BHT affects all the parameters, the liver weight, biphenyl 4-hydroxylase and 4-methoxybiphenyl demethylase. Table 6 also shows the effect of a mixture of the antioxidants incorporated at the level of 0·5% of the diet. This mixture consisted of BHT (1,900 ppm), BHA (1,900 ppm) and octyl gallate (1,250 ppm). Liver weight

and biphenyl 4-hydroxylase activity were increased by this mixture, and the figures in Table 6 suggest the effect may have been due to its BHT content.

Discussion

The administration of certain drugs and other foreign compounds to rats and mice is known to stimulate the activity of the liver microsomal enzymes which metabolise foreign compounds (see Conney & Burns, 1962). The significance of this stimulation, as far as the well-being of the animal is concerned, is not yet fully understood, for under some circumstances it may be advantageous, whereas under others it may not. The stimulation of enzymes which destroy and detoxicate deleterious substances in the body is obviously an advantage, but the stimulation of enzymes which convert foreign compounds into toxic agents is not (see Williams, 1963).

That the administration of certain foreign compounds to animals such as rats and mice causes liver enlargement has been known for some time as a routine observation in toxicological testing (see Gilbert & Golberg, 1965), but its significance in several instances is not really understood. Gilbert & Golberg (1965) have put forward the view that this enlargement of the liver may not be a toxic manifestation but a physiological response of the liver to certain foreign compounds which is accompanied by an increase in the activity of certain liver enzymes concerned in the metabolism of these compounds. Usually relatively large doses of these compounds are needed to produce this response. Liver enlargement in rats can be obtained even with sodium benzoate if the dose is large enough, that is 8% of the diet (Deuel, Alfin-Slater, Weil & Smyth, 1954). With BHT, as shown in this paper, significant enlargement of the liver in the rat can be produced by a diet containing 100 ppm (1 mg/day/rat) of BHT (see Table 1), although this amount does not significantly increase the biphenyl 4-hydroxylase activity of the liver. Neither BHA nor octyl gallate has this effect upon liver weight even when they are fed at 5,000 ppm for 9 days, although at this level BHA does increase the enzyme activity (see Table 6). If liver enlargement has any toxicological significance then it would appear that either BHA or octyl gallate is preferable to BHT. At the present time our knowledge is insufficient to decide whether enlargement of the liver by substances like BHT is a manifestation of toxicity or not, and whether such an enlargement is peculiar to rats and mice or is a general phenomenon.

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An analysis of the action of drugs on the circular muscle strips from the caecum of the guinea-pig

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The taenia-free circular muscle strips of the caecum of the guinea-pig contracted to acetylcholine or histamine. 5-Hydroxytryptamine produced small contractile responses but high concentrations (40 μg/ml or more) induced relaxation. The contractions to 5-hydroxytryptamine were enhanced by the organophosphorus anticholinesterase drug mipafox. The responses to nicotine were always relaxations but contractions were obtained after incubating the preparation with mipafox. Hence circular muscle strips treated with mipafox were used to investigate the mechanism of contractions to all the drugs. The site of action of acetylcholine or histamine was located on the smooth muscle cells because the responses were not blocked by pentolinium, dimethylphenylpiperazinium, procaine or cocaine. Hyoscine blocked the responses to acetylcholine but left those to histamine unchanged. The contractions to nicotine were blocked by hyoscine, local anaesthetics or ganglion blocking drugs. Nicotine is thought to activate cholinergic ganglion cells. 5-Hydroxytryptamine produced contractions which were potentiated by mipafox, blocked by hyoscine and almost abolished by local anaesthetics. The responses were not modified by pentolinium but were reduced by dimethylphenylpiperazinium at a concentration which did not block the responses to acetylcholine or histamine. It is concluded that the action of 5-hydroxytryptamine was wholly indirect, on nervous tissues, part of which was located on cholinergic ganglion cells.

THE action of drugs on the circular muscle strips of the mammalian intestine has been investigated by many workers. Thus nicotine contracted the circular muscle preparation of the ileum of the cat; after treatment of this preparation with botulinum toxin, the nicotine induced contraction was replaced by an inhibition (Ambache & Lessin, 1955). Harry (1963) and Brownlee & Harry (1963) showed that the circular muscle strips of the guinea-pig ileum were insensitive to acetylcholine and contracted to histamine, nicotine or 5-hydroxytryptamine (5-HT) only after treatment with an anticholinesterase drug. Similarly, circular muscle strips from the rabbit ileum were insensitive to muscarinic drugs and did not respond at all to nicotine, histamine, 5-HT or dimethylphenylpiperazinium (Tweeddale, 1963). The circular muscle preparations of the human ileum and colon reacted with inhibitory responses only to nicotine, while 5-HT caused contraction of the preparations from the ileum but produced relaxation of those from the colon (Fishlock & Parks, 1963; 1966; Fishlock, 1964).

The caecum of the guinea-pig, like the human colon, has three bands of longitudinal muscle (the taeniae). The circular muscle is easily separated from the taeniae and thus each muscle layer can be studied in isolation. The present investigation is concerned with the mechanism of action of drugs on the caecal circular muscle strips of the guinea-pig.

Part of this work was communicated to the British Pharmacological Society in January 1965.

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Methods

Guinea-pigs weighing less than 500 g were killed by stunning and bleeding. The abdomen was opened and the two taeniae furthest from the mesenteric border were dissected. The caecum was cut open along its mesenteric border, washed and pinned out on a cork pad under Krebs solution. A taenia-free circular muscle strip (3 cm × 4 mm) was cut and suspended in an organ bath containing 20 ml of Krebs solution at 37° gassed with a mixture of oxygen 95% and carbon dioxide 5%. The responses, magnified 8 times, were recorded on smoked paper with an isotonic frontal-writing lever. The load on the circular muscle was about 350 mg.

The preparations were incubated with 50 μg/ml of mipafox (*NN*-diisopropylphosphorodiamidic fluoride, an organophosphorus anticholinesterase) for ½ hr, and after removing mipafox from the bath fluid a dose-response curve was made for acetylcholine, histamine, nicotine and 5-HT. This treatment with mipafox was necessary to obtain contractile responses to nicotine. Thirty min after incubating the circular muscle with an antagonist drug, and in its presence, a second dose-response curve was made.

The contact time for the agonists was 45 sec and the interval between doses was 4 min during which period the preparation was washed 6 times.

DRUGS

These were acetylcholine chloride, histamine acid phosphate, nicotine acid tartrate, 5-hydroxytryptamine creatinine phosphate, *NN*-di-isopropylphosphorodiamidic fluoride (mipafox), hyoscine hydrobromide, neostigmine methylsulphate, dimethylphenylpiperazinium iodide, cocaine hydrochloride, procaine hydrochloride and pentolinium tartrate. The concentrations of the drugs, except mipafox, are expressed as the final bath concentration in μg/ml of the base.

The composition of the Krebs solution (in g/litre of distilled water) was NaCl 6·92; KCl 0·35; $CaCl_2$ 0·28; $NaHCO_3$ 2·1; KH_2PO_4 0·16; $MgSO_4.7H_2O$ 0·29; and glucose 2·0.

Results

The caecal circular muscle strip when suspended in Krebs solution slowly went into a well-maintained contracture which reached a maximum in 1 to 2 hr. This contracture will be referred to as "tone". The tone was maintained for over 8 hr and was not abolished by hyoscine (0·1 to 1·0 μg/ml) (Fig. 1). The extent of tone exhibited by circular muscle strips varied greatly; preparations from small guinea-pigs (less than 500 g) showed less tone than those from large animals (600 g or more). The circular muscle strips were often spontaneously active.

RESPONSES TO DRUGS

Acetylcholine. The caecal circular muscle responded to acetylcholine with a contraction, the threshold dose being about 0·1 μg/ml (Fig. 1). The responses were potentiated about 25 times by mipafox and were abolished

by hyoscine (0·1 μg/ml). In the presence of this concentration of hyoscine, high doses of acetylcholine (more than 50 μg/ml) produced a biphasic effect consisting of a short-lived relaxation, followed by contraction.

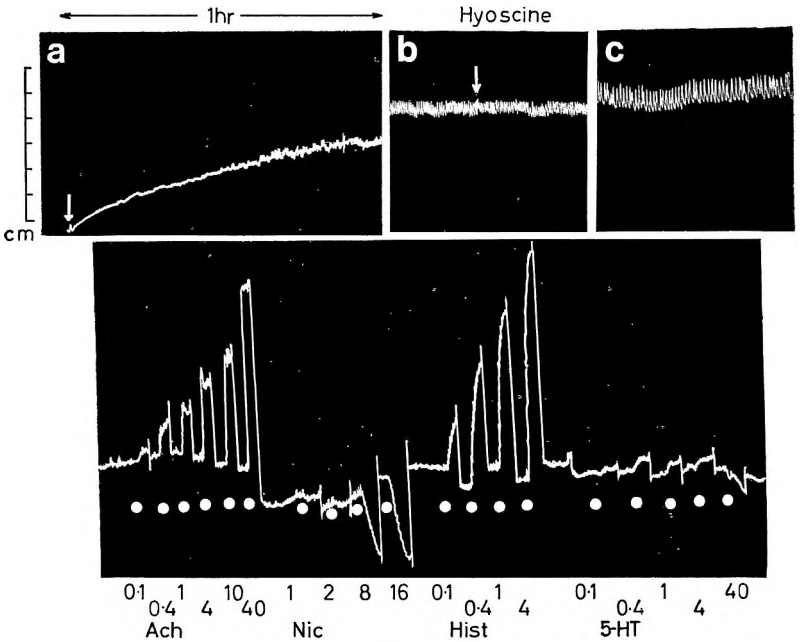

FIG. 1. The upper kymograph tracing shows the changes in tone and spontaneous activity exhibited by a caecal circular muscle strip from the guinea-pig. The preparation was set up in Krebs solution at 37°. The increase in tone (starting from the arrow) within the first hour is shown by (a). The second record (b) and the third record (c) were obtained 3 and 5 hr later respectively. Note that hyoscine (1 μg/ml) added to the bath at the arrow in b and left in bath throughout the duration of the experiment, did not reduce the tone. The lower tracing shows the typical effect of acetylcholine, nicotine, histamine and 5-HT on the circular strip. Nicotine produced only relaxation of the preparation. 5-HT caused small contractions but a high concentration (40 μg/ml) produced relaxation. The numbers represent the concentrations of the drugs in μg/ml of the bath fluid.

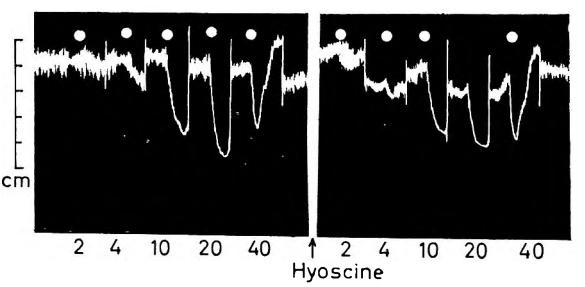

FIG. 2. The responses of the caecal circular muscle strip to nicotine in the absence and presence of hyoscine (1·0 μg/ml). Note that the high concentration of nicotine (40 μg/ml) produced a relaxation followed by a contraction. The contraction was not modified by hyoscine. The numbers refer to the concentrations of nicotine in μg/ml of bath fluid.

Histamine. The responses of the circular muscle strips to histamine were contractions. The contractions were neither potentiated by mipafox (50 μg/ml) nor depressed by hyoscine (0·1 μg/ml).

5-*Hydroxytryptamine.* It was found that 5-HT caused contraction of the caecal circular muscle and that the threshold dose was about 0·1 μg/ml.

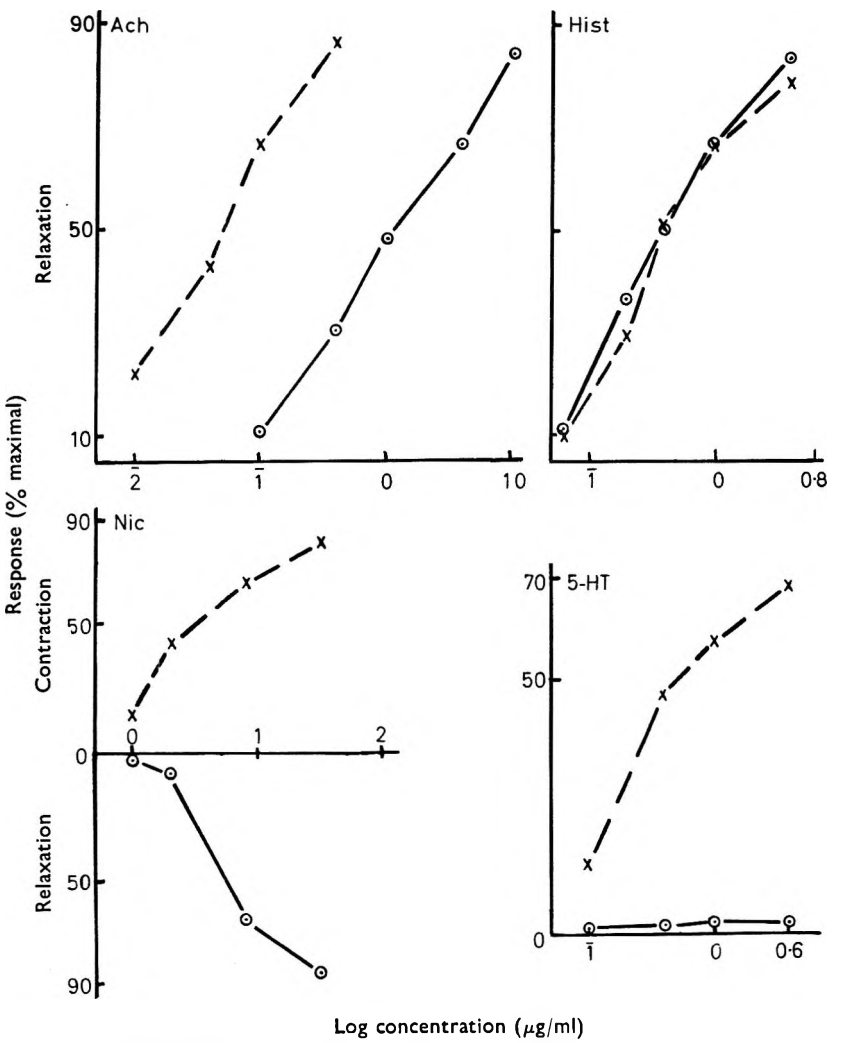

FIG. 3. The effect of treating caecal circular muscle strips of the guinea-pig with mipafox (50 μg/ml) for 1 hr on the responses to acetylcholine, histamine, nicotine and 5-HT. The results are plotted as % of maximal response (contraction or relaxation) against the log concentration in μg/ml. The circles represent the responses to the agonists and the crosses represent these responses after treating the preparations with mipafox. The responses to acetylcholine or 5-HT were enhanced but those to histamine were not. The inhibitory responses of nicotine were blocked and replaced by contractions after treatment with mipafox. Each curve represents the mean of six experiments.

These contractions were usually small and were reversed to inhibition with high doses (Fig. 1). Mipafox (50 μg/ml) enhanced the contractions and hyoscine (0·1 μg/ml) abolished or reversed them to inhibition.

Nicotine. The typical effect of nicotine is shown in Figs 1 and 2. Nicotine produced relaxation of the preparations and the response increased with dose. When a high concentration (40 μg/ml or more) of

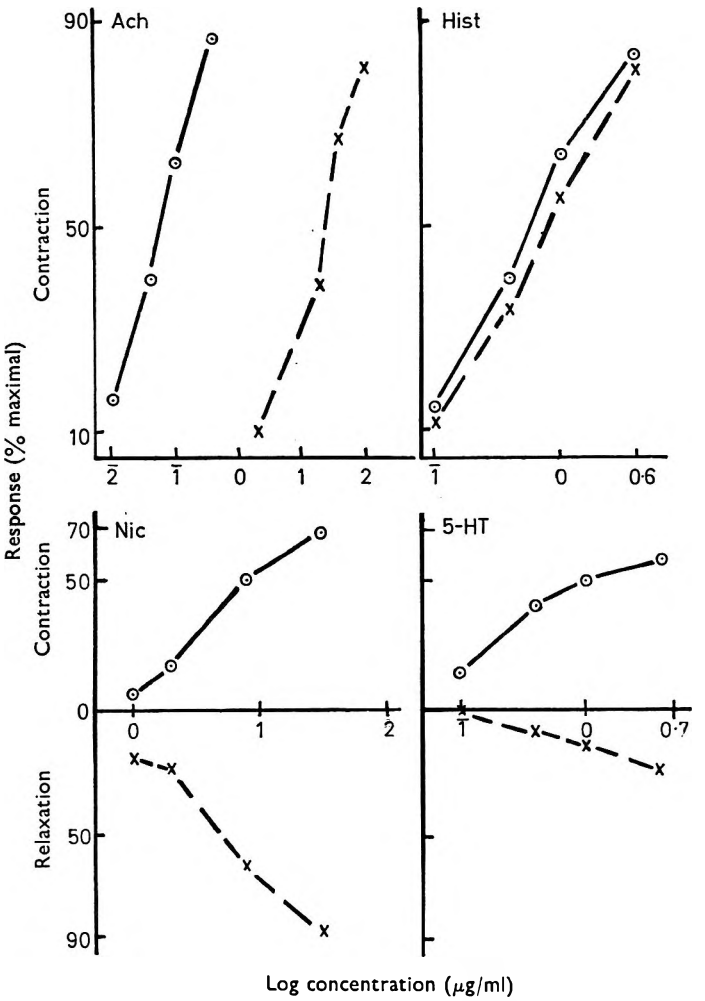

FIG. 4. The effect of hyoscine (0·1 μg/ml) on the responses of caecal circular strips of the guinea-pig to acetylcholine, histamine, nicotine and 5-HT. The control responses (circles) were obtained after the preparations had been treated with mipafox (50 μg/ml) for 1 hr. The crosses represent these responses in the presence of hyoscine (0·1 μg/ml). The results are plotted as % maximal response (contraction or relaxation) against log concentration in μg/ml. Hyoscine produced a parallel displacement to the right of the dose-response curve to acetylcholine. The responses to histamine were not modified. The contractile responses to nicotine or 5-HT were replaced by inhibitory responses. Each curve represents the mean of six experiments.

nicotine was used, a small relaxation followed by a contraction was seen. This contraction was not modified by hyoscine (1 μg/ml). After the preparations were treated with mipafox (50 μg/ml), the inhibitory responses were reversed and the contractions were then seen. For this reason the mechanism of the contractile responses to all the agonists was investigated after treating the preparations with mipafox (50 μg/ml).

The effects of anticholinesterase drugs. Fig. 3 shows the effect of an organophosphorus anticholinesterase drug, mipafox (50 μg/ml) on the dose-response curves to acetylcholine, histamine, nicotine or 5-HT. The responses to acetylcholine or 5-HT were potentiated but those of histamine were not modified. The inhibitory responses of the caecal circular muscle to nicotine were reversed and were replaced by contractions.

It was observed that the extent of potentiation to acetylcholine produced by 20 μg/ml of mipafox was the same as that produced by 50 μg/ml. Nicotine produced biphasic responses on preparations treated with mipafox (20 μg/ml) but only contractions after treatment with mipafox (50 μg/ml) and these contractions were bigger than those observed with the lower concentration of mipafox. The enhancement of the contractions to 5-HT was greater after treatment with 50 μg/ml than after 20 μg/ml of mipafox. The reason for these differences in the extent of potentiation by various concentrations of mipafox is being investigated.

Potentiation of the responses to acetylcholine or 5-HT and the reversal of the inhibitory responses to nicotine, were also produced by neostigmine (1–5 μg/ml). These concentrations of neostigmine induced a high degree of tone and the extent of the potentiation could not be determined.

Dimethylphenylpiperazinium resembled nicotine in producing relaxation of the caecal circular muscle. This inhibitory effect was replaced by contractile responses after treatment with mipafox (50 μg/ml).

The influence of hyoscine on the responses. After incubating the circular muscle strips with mipafox, the contractile responses to acetylcholine were abolished by hyoscine (0·1 μg/ml). High doses of acetylcholine produced a dose-response curve parallel to the original. The contractions to nicotine or 5-HT were reversed and replaced by inhibition but the responses to histamine were not modified (Fig. 4).

The action of local anaesthetics. Cocaine (10 μg/ml) eliminated the responses to nicotine, reduced those to 5-HT but left the responses to acetylcholine or histamine unchanged. A higher concentration of cocaine (20 μg/ml) almost abolished the responses to 5-HT but did not modify those to histamine or nicotine. A concentration of procaine (10 μg/ml) which did not antagonise the responses to histamine, greatly reduced the responses to 5-HT and almost abolished the contractions to nicotine. The dose-response curve to acetylcholine was slightly displaced to the left. The effect of cocaine (10 μg/ml) is shown in Fig. 5.

The effect of cooling on the responses. The contractile responses to all agonists were first established at 37° and then repeated after a 30 min equilibration period at any other temperature.

At temperatures lower than 37° the delay in onset of contraction was increased for all agonists but was greatest for nicotine or 5-HT. The time

taken for a response to reach maximum was also prolonged. It was found necessary to leave the drugs in the bath for 2–3 min.

At 18° the responses to 5-HT remained unchanged but those to acetylcholine, histamine or nicotine were enhanced. When the circular muscle preparation was equilibrated at 15° the contractions to histamine were potentiated or not affected and those to acetylcholine or nicotine were not modified but the responses to 5-HT were reduced. All the four agonists produced smaller contractions at 12° than at 37°. In all experiments,

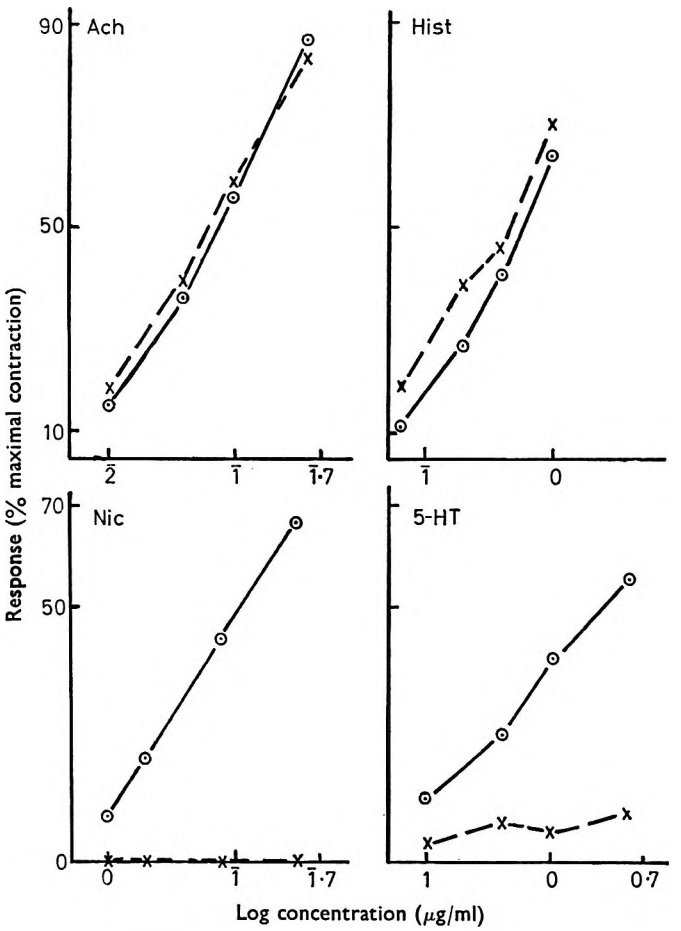

FIG. 5. The effect of cocaine (10 μg/ml) on the responses of caecal circular muscle strips to acetylcholine, histamine, nicotine and 5-HT. The preparations were treated with mipafox (50 μg/ml) for 1 hr before the control responses (circles) were established. The crosses are these responses in the presence of cocaine. The results are plotted as % of maximal contraction against log concentration in μg/ml. The contractions to nicotine were blocked, those to 5-HT were greatly reduced, but the responses to acetylcholine or histamine were not reduced. The contractions to histamine were slightly enhanced. Each curve represents the mean of eight experiments.

the contractions returned completely when the temperature was raised to 37°.

The effect of ganglion blocking drugs. The contractions to nicotine were blocked by pentolinium (5 μg/ml) but those to acetylcholine, histamine or 5-HT were not affected. Dimethylphenylpiperazinium (4 μg/ml) almost completely inhibited the responses to nicotine, greatly reduced those to 5-HT but did not modify those to acetylcholine or histamine (Fig. 6). A higher concentration of dimethylphenylpiperazinium (10 μg/ml) reduced the responses to all agonists.

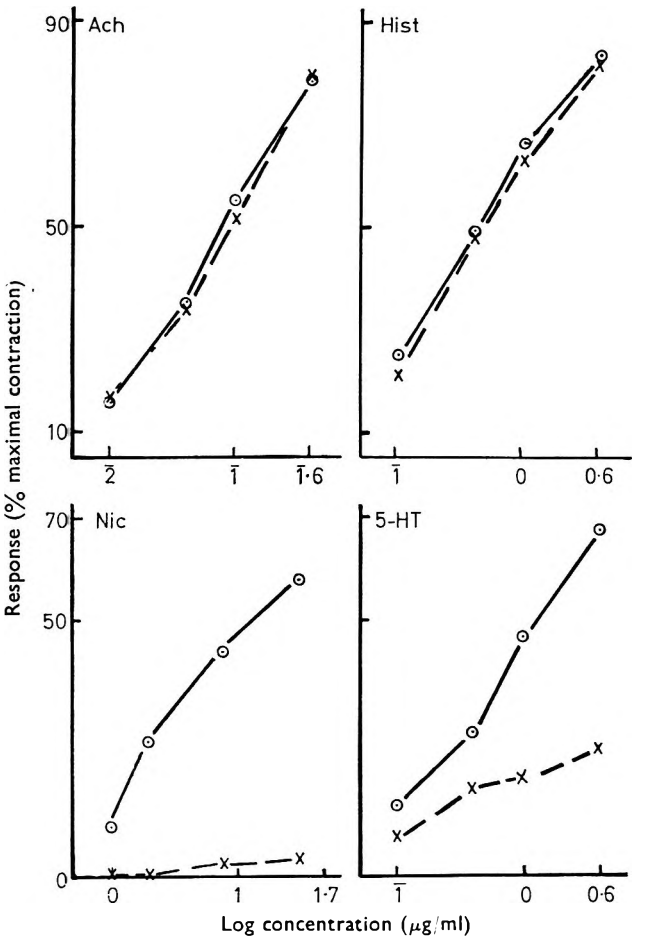

FIG. 6. The action of dimethylphenylpiperazinium (4 μg/ml) on the contractions of caecal circular muscle strips. The circles represent the control responses of the preparations after treatment with mipafox (50 μg/ml) for 1 hr. The crosses represent these responses in the presence of dimethylphenylpiperazinium. The ordinates and the abscissae are as in Fig. 5. The dose-response curves to acetylcholine and to histamine were unchanged but the responses to 5-HT were reduced and those to nicotine were almost eliminated. Each curve represents the mean of four experiments.

The effect of 5-*hydroxytryptamine in excess.* After treating the caecal circular muscle with mipafox, the dose-response curves to acetylcholine, histamine, 5-HT or nicotine were made in the absence of and also in the presence of 10 μg/ml of 5-HT. The responses to 5-HT were blocked but those to acetylcholine, histamine or nicotine were not reduced (Fig. 7).

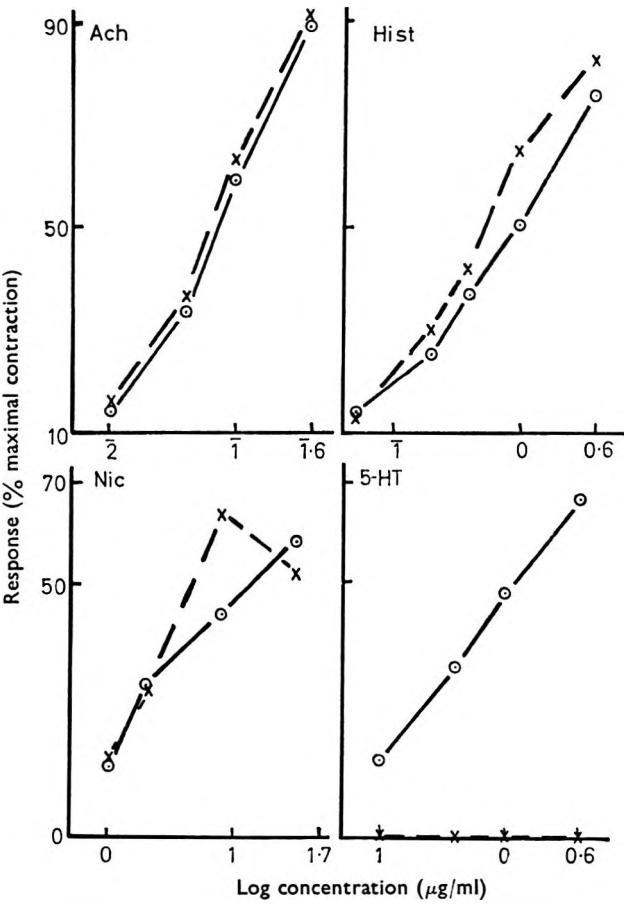

FIG. 7. The effect of 5-HT (10 μg/ml) on the contractions of the caecal circular muscle after treatment with mipafox (50 μg/ml) for 1 hr. The circles represent the control responses and the crosses represent these responses in the presence of 5-HT. The ordinates and the abscissae are the same as in Fig. 5. The responses to 5-HT were blocked but those to acetylcholine, histamine or nicotine were not reduced. Each curve represents the mean of four experiments.

Discussion

The contractions of the caecal circular muscle induced by acetylcholine were potentiated by anticholinesterase drugs, and were blocked by hyoscine but were not modified by ganglion blocking drugs or by cocaine. It seems therefore that acetylcholine stimulated muscarinic receptors sited

on the smooth muscle fibres. Procaine displaced the dose-response curve to acetylcholine slightly to the right, an action which is best explained by its known anti-acetylcholine property (Sinha, 1953; Wiedling & Tegner, 1963).

Experiments made at temperatures lower than 37° were unhelpful in producing results which could be used to explain the mechanism of action of any of these drugs. For example, at 18°, the responses to all the agonist drugs except 5-HT were enhanced. Similar potentiation of the responses to acetylcholine or histamine at temperatures lower than 37° has been reported for the guinea-pig ileum (Day & Vane, 1963) and for the guinea-pig trachealis muscle (Carlyle, 1963).

Histamine produced contractions which were neither potentiated by mipafox nor depressed by hyoscine, local anaesthetics nor by ganglion blocking drugs. Thus the action of histamine did not appear to involve the stimulation of nervous tissue. This is unlike the action of histamine on cholinergic nerves within the circular muscle strips of the guinea-pig ileum demonstrated by Harry (1963). It seems probable that such cholinergic nerves with histamine receptors are not present in the guinea-pig caecum.

Nicotine produced only a relaxation of caecal circular strips untreated with other agents. But high doses of nicotine produced a relaxation followed by a contraction. The contraction was of great interest in that it was not antagonised by hyoscine. This hyoscine-resistant action of nicotine may represent a direct effect on the smooth muscle cells. Day & Vane (1963) have suggested from their experiments on the isolated ileum of the guinea-pig, that nicotine might have a direct effect on smooth muscle fibres. An alternative consideration is that the hyoscine-resistant contraction observed in the present investigation was a 'rebound' effect resulting from the prior relaxation.

The absence of hyoscine-sensitive contractile responses to nicotine in normal doses on preparations not treated with an anticholinesterase drug, seemed to suggest an absence of cholinergic ganglion cells within the circular muscle strips. However, after treatment with mipafox, nicotine induced contractions in doses which previously produced only relaxation. These contractions were abolished by hyoscine at a concentration which also blocked the responses to acetylcholine. The contractile responses to nicotine were eliminated by ganglion blocking drugs or by cocaine at concentrations which did not modify the responses to acetylcholine or histamine. Hence the responses resulted very probably from the stimulation of cholinergic ganglion cells. Thus treatment with mipafox revealed the presence of these cells within the preparation.

The reversal of the inhibitory action of nicotine to contraction was not only produced by mipafox but also by neostigmine. Also dimethylphenylpiperazinium caused relaxation of the caecal circular muscle and this response was reversed and replaced by contraction after treatment with mipafox. This is good evidence that the observations made with nicotine or mipafox did not arise from an unusual property of either compound.

It is not known why nicotine did not cause contraction of the caecal circular muscle strips before treatment with mipafox. It seems probable that a high concentration of cholinesterases may be located around cholinergic nerve endings and thus their inhibition is needed to reveal cholinergic activation by nicotine. Another possibility is that there was a large store of easily releasable inhibitory substances within the circular muscle, which masked the effect of cholinergic nerve stimulation in untreated preparations.

The contractions of the circular muscle strips induced by 5-HT like those produced by acetylcholine, were potentiated by mipafox and blocked by hyoscine. The responses were antagonised by cocaine at a concentration which blocked the effect of nicotine but not that of histamine or acetylcholine. Hence the action of 5-HT like that of nicotine, appears to be on cholinergic nerves.

The concentration of dimethylphenylpiperazinium which did not modify the responses of the caecal preparation to acetylcholine or histamine, antagonised the relaxation caused by 5-HT and by nicotine. It is therefore very likely that the action of 5-HT involved the stimulation of ganglion cells. A similar ganglionic action of 5-HT has been demonstrated on the guinea-pig ileum (Brownlee & Johnson, 1963) and on the taenia caeci (Akubue, 1966).

The contractions caused by 5-HT were attributed to activation of specific receptors because they were abolished by high concentration of 5-HT which did not modify the responses to acetylcholine, histamine or nicotine. Thus 5-HT activated specific receptors on the cholinergic nerves. One part of this action is located on ganglion cells.

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The effect of 6-azauridine on the contractile responses of the isolated ileum of the guinea-pig to drugs and to coaxial stimulation

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Small concentrations of 6-azauridine augmented the contractions of the guinea-pig isolated ileum to acetylcholine, nicotine, histamine, barium chloride and to coaxial stimulation. High concentrations partially inhibited all the responses. After prolonged contact with high concentrations of 6-azauridine, the anti-spasmogenic effect persisted long after washing out the compound. The acetylcholine output did not change under these conditions. Comparison of the actions of 6-azauridine with those of papaverine is made and the possible mechanisms of action are discussed.

THE nucleoside 6-azauridine is known for its inhibitory activity on some animal tumours (Jaffe, Handschumacher & Welch, 1957; Šorm & Keilova, 1958). This activity results from its phosphorylation *in vivo* to 6-azauridine monophosphate which in turn inhibits orotidylic acid decarboxylase (Habermann & Šorm, 1958; Handschumacher & Pasternak, 1958).

In previous papers from this laboratory (Janků, Kršiak, Volicer, Čapek, Smetana & Novotný, 1965a; Janků, Kršiak, Novotný, Volicer & Čapek, 1965b), an analysis was made of the effects of this nucleoside and its amino-analogue, 6-azacytidine, on the central nervous system. We now report the effects of 6-azauridine on the isolated ileum of the guinea-pig. Except for the work of Smith (1964), who found that the effects of some 6-azapyrimidine derivatives on smooth muscle was relaxant, these agents have not been studied.

Experimental

MATERIAL AND METHODS

Terminal isolated guinea-pig ileum bathed in modified Krebs-Henseleit solution (Eccles, 1952) at 37°, gassed with a mixture of 95% oxygen and carbon dioxide 5% was used. 6-Azauridine (Spofa) was tested on the ileum in two ways. (1) A single dose, diluted in Krebs solution with the pH adjusted when necessary with sodium bicarbonate, was added to the bath. (2) When concentrations of 6-azauridine from 2 to 8 mM were used, an equivalent amount of sodium chloride was omitted from the Krebs solution and replaced by the drug. This enabled relatively high concentrations of drug to remain in contact with the tissue for long periods without osmotic change.

The effects of adding 6-azauridine by methods (1) and (2) on the response to spasmogenic drugs were tested as follows. A dose of spasmogen

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causing approximately 40% of the maximal response was determined. This was referred to as the standard dose, the standard response being considered 100%. The size of the response in the presence of 6-azauridine was measured as a percentage of this standard response.

In other experiments, ileum taken at least 15 cm above the ileocaecal valve, was stimulated by the method of Paton (1954). In some cases the acetylcholine output from the stimulated ileum was measured in the presence of eserine salicylate, 5×10^{-6} g/ml. The bioassay was performed on guinea-pig ileum in Krebs solution containing 12·5 μg/litre eserine salicylate and 6 μg/litre morphine hydrochloride. The ileum was allowed to equilibrate with this solution for 45 min to allow for complete inactivation of the cholinesterases (Paton, 1957). The acetylcholine collections were then made in the following sequence.

(i) The ileum was allowed to rest for a period of 10 min and the acetylcholine released spontaneously into the bathing fluid was estimated.

(ii) The preparation was stimulated for 10 min by means of supermaximal rectangular pulses of 400 μsec duration and a frequency of 1 shock/10 sec and the bath fluid again analysed for its acetylcholine content.

(iii) A further 10 min spontaneous output was estimated.

(iv) The ileum was bathed in Krebs solution containing 20 mM 6-azauridine for 45 min and the ileum was stimulated continuously throughout this period. No collections were taken for assay during this time.

(v) The Krebs solution containing the nucleoside was then replaced by normal Krebs solution and the preparation was washed repeatedly for 10 min.

(vi) Estimations were then made of the spontaneous and stimulated outputs of acetylcholine for five 10 min periods: spontaneous, stimulated, spontaneous, stimulated, spontaneous.

Parallel control experiments were made which were identical with the test experiments with the exception that no 6-azauridine was present in the Krebs solution.

Results

EXPERIMENTS WITH SPASMOGENIC DRUGS

Experiments in which single doses of 6-azauridine were added to the bath revealed that 200–400 μg/ml of the drug increased the responses to nicotine, acetylcholine, histamine and barium chloride. A tenfold increase in the concentrations of 6-azauridine produced a slight increase in the tone of the preparation and the responses to the agonists were enhanced initially, but later markedly reduced.

Similarly, after 6-azauridine had been in contact with the ileum for a long time two effects were observed. The drug, 2 mM, elicited either a sustained or transient increase in the agonist responses or, as was seen in most of the experiments, reduced the contractions. Both effects were more marked after 4 mM of 6-azauridine (Fig. 1) whereas after 8 mM the inhibitory effect prevailed. Dose-response curves in the presence of 3 mM 6-azauridine are shown in Figs 2 and 3. The inhibitory effect against all

spasmogenic drugs developed gradually within the time of contact of the preparation with 6-azauridine. Furthermore, this inhibition of agonist contractions was irreversible after replacing 6-azauridine solution with Krebs solution (Figs 1, 2 and 3). In those experiments in which the drug

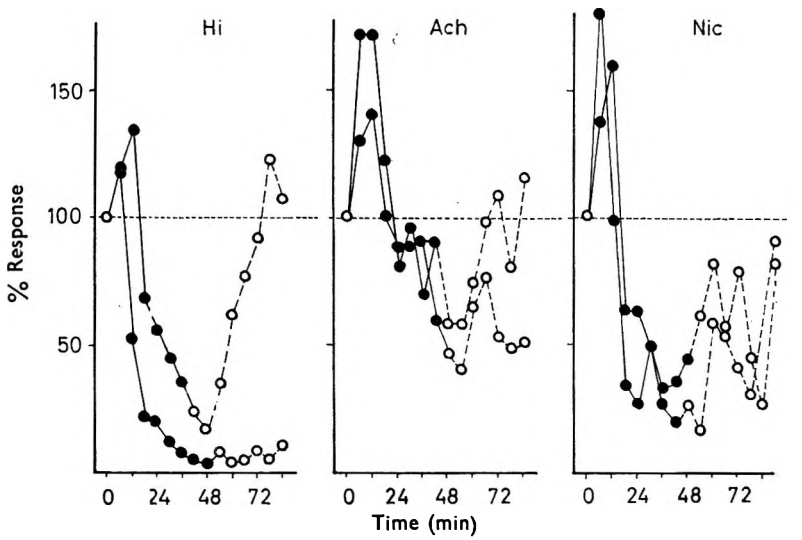

FIG. 1. The effect of 6-azauridine on the responses of the guinea-pig isolated ileum to standard doses (i.e. doses causing a contraction 40% of maximal) of histamine (Hi), acetylcholine (Ach) and nicotine (Nic) in the presence of 4 mM 6-azauridine, (●—●) and after replacing by normal Krebs solution (○ - - - ○). The responses are expressed on the ordinate as percentage of the response before adding 6-azauridine. The time of introducing the drug into the bath is indicated by zero on the abscissa.

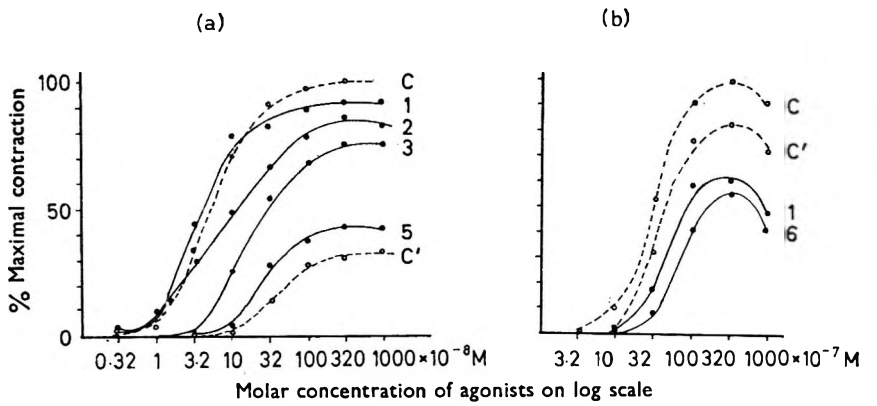

FIG. 2. Dose-response curves to (a) acetylcholine and (b) nicotine on the guinea-pig isolated ileum. The broken lines C represent the normal dose-response curves to the two agonists. The continuous lines (1) represent the responses of the agonists following treatment with 8 mM 6-azauridine. The inhibitory action of the drug became more marked with time so that subsequent dose-response curves (curves 2, 3 and 5 for acetylcholine, curve 6 for nicotine) were more depressed. On washing out the 6-azauridine the inhibition was found irreversible for acetylcholine (C′) and partly reversible for nicotine (C′).

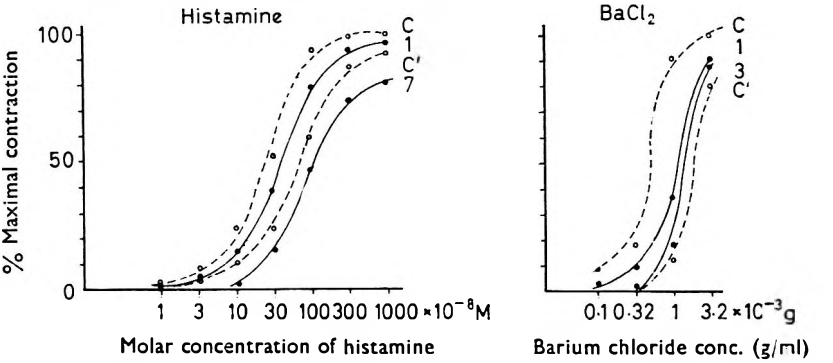

FIG. 3. Dose-response curves for histamine and barium chloride ($BaCl_2$) on the guinea-pig isolated ileum before adding 6-azauridine to the bath broken line C). The continuous lines (1) represent the response of the agonists following treatment with 8 mM 6-azauridine. The inhibitory action of the drug became more marked with time so that subsequent dose-response curves (curve 7 for histamine, curve 3 for $BaCl_2$) were more depressed. On washing out the 6-azauridine the inhibition was irreversible for $BaCl_2$ (C′) and partly reversible for histamine (C′).

(2 mM) caused a potentiation of the agonist responses, after washing it out, the responses were smaller than those obtained in the control period.

EXPERIMENTS WITH COAXIAL STIMULATION

The effect of 6-azauridine on the ileal responses to coaxial stimulation was dose dependent. Small concentrations potentiated the responses, somewhat higher concentrations caused a transient inhibition, while even higher concentrations produced sustained inhibition (Fig. 4), which never fully recovered after washing out the drug. But in control experiments prolonged electrical stimulation was not accompanied by a decrease in the height of twitches during comparable time intervals (see Fig. 5). Experiments in which part of the sodium chloride in the Krebs solution was replaced by an equivalent amount of 6-azauridine indicated that comparatively higher concentrations (20 mM) were necessary to produce marked pharmacological effects (Fig. 5). With the 20 mM dose, a gradual inhibition developed preceded occasionally by a transient increase in the

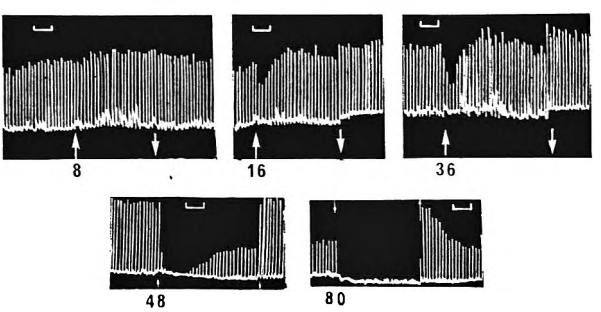

FIG. 4. The response of the isolated guinea-pig ileum to coaxial stimulation in the presence of increasing concentrations of 6-azauridine. The arrows indicate the addition (↑) and removal (↓) of the compound. The concentrations of the drug are in g/ml × 10^{-4}. Time scale: 1 min.

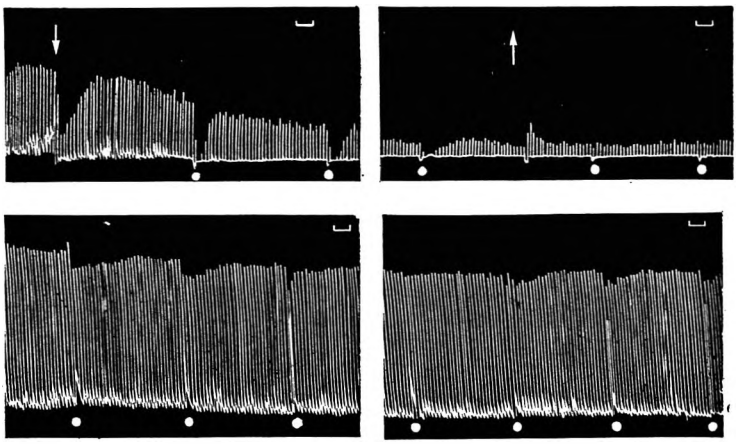

FIG. 5. The responses of the isolated ileum of the guinea-pig to coaxial stimulation during contact (45 min) with 20 mM of 6-azauridine (upper record). 6-Azauridine was present in the bath for the duration between the arrows. The lower record shows a parallel control experiment in which no 6-azauridine was added to the bath. Dots indicate points where the bath fluid was exchanged. Time scale: 1 min.

height of twitches. This inhibition was long-lasting and persisted after washing out the nucleoside. Increasing the concentration of 6-azauridine above 20 mM enhanced the inhibition.

On estimating the acetylcholine released spontaneously and by coaxial electrical stimulation, no significant difference was detected for the outputs obtained before and after a prolonged period of treatment with 20 mM of 6-azauridine. In the parallel control experiments there was a slight decrease of the acetylcholine output within the duration of experiments (Fig. 6).

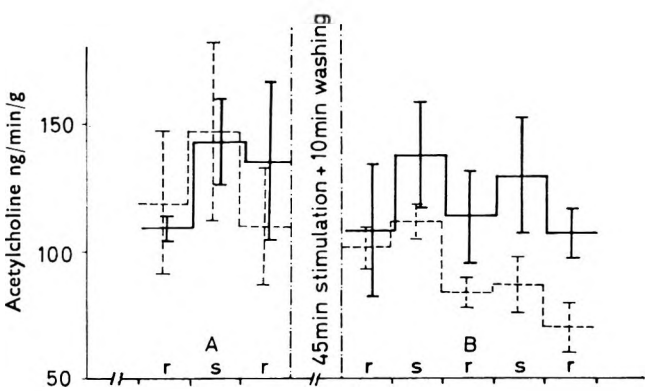

FIG. 6. The spontaneous (r) and electrically stimulated (s) output of acetylcholine from the guinea-pig isolated ileum collected over successive 10 min periods (abscissa). The continuous line indicates the mean outputs from three preparations before (A) and after (B) the 45 min contact with 20 mM 6-azauridine. The broken line indicates the mean outputs from three control preparations over comparable time intervals. The standard errors are also included. The stimulation parameters were: frequency 1 shock/10 sec; duration 400 μsec; voltage supramaximal. Time scale: 10 min.

Discussion

The effects of 6-azauridine on smooth muscle preparations have not been studied extensively. Smith (1964) demonstrated a relaxant action of 6-azauridine diphosphate and high doses of uridine and uracil on the goldfish intestine. Our experiments have confirmed the antagonistic actions of 6-azauridine to nicotine observed in previous experiments *in vivo* (Janků & others, 1965a). However, the responses to other spasmogenic drugs, acetylcholine, histamine and barium chloride, were also blocked by the same concentrations of 6-azauridine as those used to block the nicotine responses. On the other hand, it was possible to detect two distinct components of action on the isolated ileum of the guinea-pig. The first component had a rapid onset of action, and caused an immediate increase or decrease of the height of the responses observed. The second component developed slowly and remained even after removing the 6-azauridine from contact with the preparation. This component was characterised by a gradual diminution of the responses.

It is difficult to believe that the rapid changes in the reactivity of the preparation are due to the same biochemical mechanism which is also responsible for the cytostatic effects of 6-azauridine. The drug is highly ionised at physiological pH (pKa = 6·7) and therefore it hardly penetrates biological membranes, as indicated by its poor penetration of the blood-brain barrier (Habermann & Šorm, 1958; Cardoso & Symmes, 1961). We believe that the rapid component of 6-azauridine's action may perhaps be induced by a direct action on the membrane surface. The biochemical mechanism by which it exerts its action on tumour cells may be considered only in connection with the second component, which develops gradually and persists long after contact of the tissue with the nucleoside is discontinued. This effect might be dependent on the penetration of some 6-azauridine into the cells and its subsequent transformation to 6-azauridine monophosphate.

Like papaverine (Harry, 1962) 6-azauridine did not affect the acetylcholine output of the ileum, although its effects differed in that they were not readily reversible. It is concluded that the drug acts directly on the smooth muscle cells.

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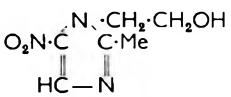
Simultaneous polarographic determination of 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole, 1-(2-hydroxyethyl)-2-methyl-4-nitroimidazole and 2-methyl-5-nitroimidazole

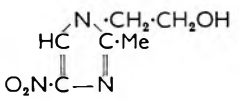
D. DUMANOVIĆ,* J. VOLKE** AND V. VAJGAND†

A method for the simultaneous quantitative polarographic determination of the three components in reaction mixtures has been proposed. The analysis is based on a separation of the half-wave potentials in strongly alkaline base solutions. The general polarographic behaviour of the three substances is also described.

THE nitroimidazole derivative 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole, (metronidazole) is used extensively as a specific agent against human trichomoniasis. Its determination, especially in reaction mixtures in the presence of the isomeric 1-(2-hydroxyethyl)-2-methyl-4-nitroimidazole and of 2-methyl-5-nitroimidazole, is most important when following the synthesis of the drug. Because of the ease of reduction of the nitro-group at the dropping mercury electrode, several methods (Danek, 1961; Kane, 1961; Cosar, Dubost, Dubost, Devoize & Pallière, 1962; Vignoli, Cristan, Gonezo & Fabre, 1963a,b) for the polarographic determination of metronidazole have been suggested, particularly for the analysis of biological materials. However, a simultaneous determination of 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole (I), 1-(2-hydroxyethyl)-2-methyl-4-nitroimidazole (II) and 2-methyl-5-nitroimidazole (III) has not been possible.



(I)



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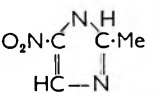

(III)

We now describe the general polarographic behaviour of these three compounds and propose the conditions for their simultaneous determination.

POLAROGRAPHIC BEHAVIOUR

The polarographic reduction of all three compounds was examined in aqueous Britton–Robinson universal buffers over the range pH 2 to pH 10·5, and in sodium hydroxide solution of various concentrations.

In acidic media metronidazole gives a 4-electron wave A deformed by a sharp maximum; wave A is followed by another wave B of about half the height of wave A. A small addition of surface-active agent, such as gelatin, suppresses the maximum on wave A (Fig. 1). With increasing pH values of the base solution, wave A increases at the expense of wave B (their sum remaining constant) until at pH 10 both waves almost coalesce

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to a single 6-electron wave. At higher pH values, a fall in the height of the combined wave A + B sets in. The time-dependence of the waves in strongly alkaline media will be dealt with later. The half-wave potentials of wave A shift to more negative voltage by −90 mV per pH unit ($E_{\frac{1}{2}} = -0{\cdot}16$ V at pH 3·10 and −0·60 V at pH 10·15) (Fig. 2). The number of electrons involved in the reduction process was determined by comparing the wave-heights with the wave-heights of substances in which the reduction mechanism is known. In addition to this, the diffusion coefficient D was calculated from the uncorrected Ilkovič equation

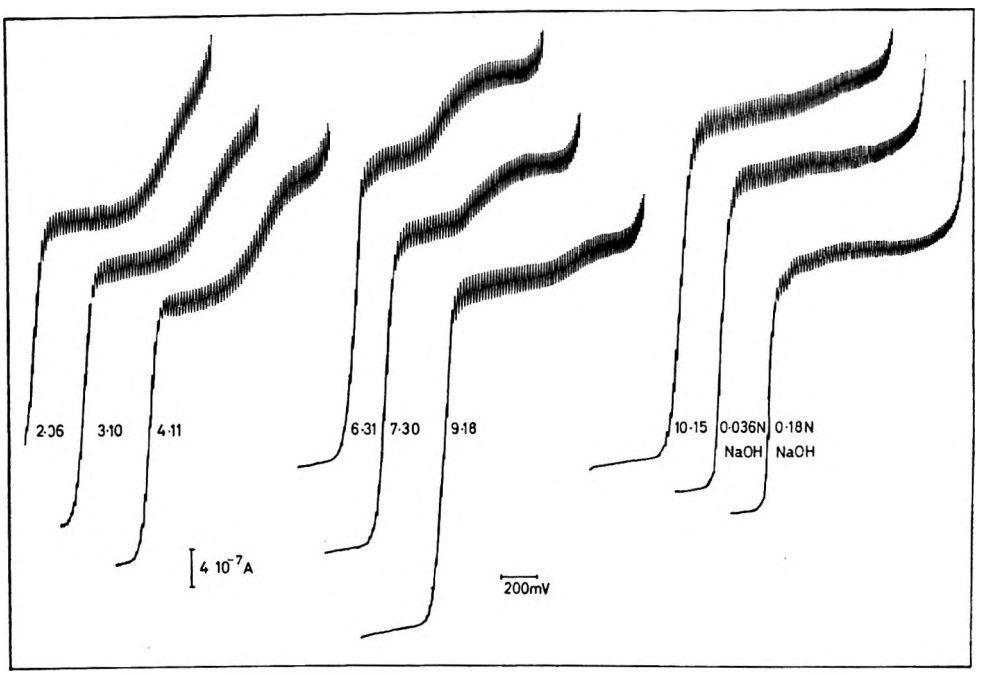

FIG. 1. Dependence of the reduction waves of 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole 2×10^{-4}M on pH; Britton–Robinson buffers, pH values are given with the curves; 0·005% gelatin; all curves except the last start at zero applied voltage; the last curve starts at −0·3 V.

at suitable pH values and the plausible value of $8{\cdot}5 \times 10^{-6}$ cm sec^{-2} was obtained for pH 4·7 and $c = 4{\cdot}95 \times 10^{-4}$M (at pH 9·3, however, the much lower value of $5{\cdot}7 \times 10^{-6}$ cm sec^{-2} was calculated; this is obviously due to the fall in height of the original 6-electron wave of this pH). The log $\bar{i}/\bar{i}_d - \bar{i}$ against E plots of the metronidazole waves show distinctly that an irreversible electrode process is operative in both waves.

The next step was the investigation of 1-(2-hydroxyethyl)-2-methyl-4-nitroimidazole behaviour as a function of pH of the supporting electrolyte. In this instance the general pattern is characterised again by the appearance of two waves A and B. Wave A is deformed by a sharp maximum, the suppression of which requires higher gelatin concentrations than that of metronidazole. In acid media the ratio of wave-heights A:B is 2:1. With increasing pH values the height of B decreases and

the wave vanishes completely at pH > 9. In strongly alkaline solutions the waves are independent of time and no colouration of the solution appears upon adding sodium hydroxide or potassium hydroxide solutions. On average, wave A is about 100 mV more negative than the same wave for metronidazole ($E_{\frac{1}{2}}$ varies from −0·15 V at pH 2·06 to −0·73 V at pH 10·15) and shifts by +97 mV/pH to more negative potentials. The wave-heights point to the fact that 4 electrons are consumed for the reduction in wave A (from the Ilkovič equation $8{\cdot}4 \times 10^{-6}$ cm sec^{-2} is obtained for D at 4·7 and $8{\cdot}5 \times 10^{-6}$ cm sec^{-2} at pH 9·3).

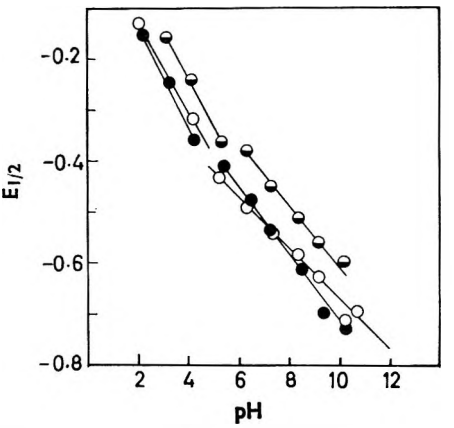

FIG. 2. $E_{\frac{1}{2}}$-pH plot for all three nitro-compounds. $E_{\frac{1}{2}}$ values are given against saturated calomel electrode. ◒ Metronidazole. ● 1-(2-Hydroxyethyl)-2-methyl-4-nitroimidazole. ○ 2-Methyl-5-nitroimidazole.

The 4-electron reduction wave of 2-methyl-5-nitroimidazole is deformed by a maximum up to pH 7·5 and, as with metronidazole, this maximum is removed by adding 0·005% gelatin. Wave A is followed by wave B which, originally, in acid media corresponds to the uptake of 2 electrons and finally disappears in sodium hydroxide solutions. Although the colour of the solution turns yellow on adding sodium hydroxide, the height of wave A is not time-dependent. The half-wave potential of wave A shifts by 90 mV per pH unit to more negative values with increasing pH (from −0·13 V at pH 2·1 to −0·72 V at pH 10·15).

The behaviour of 1-(2-hydroxyethyl)-2-methyl-4-nitroimidazole and of 2-methyl-5-nitroimidazole is in accordance with the general course of reduction for aromatic nitro-compounds (Volke, 1960). Here, e.g. in nitrobenzene, the nitro-group is reduced to phenylhydroxylamine; at more negative potentials, i.e. over the region of the second step B, a 6-electron reduction occurs leading to aniline. This mechanism is only restricted to acidic and slightly alkaline base solutions since a pre-protonation of phenylhydroxylamine is a necessary condition for the second reduction step (wave B). The mechanism for metronidazole reduction is somewhat different. Here the reduction, probably to the amine, takes place in a single 6-electron step (at higher pH values only). Similar behaviour has been found earlier, e.g. with some nitrophenols (Astle & McConnell, 1943).

In our case it could be ascribed to an influence of the vicinal $-CH_2CH_2OH$ group in the metronidazole molecule.

A strange phenomenon occurring with all three nitro-compounds should perhaps be mentioned: the waves A in the most acid buffers (pH 3) are somewhat lower than those at the other pH values. A similar discrepancy was observed earlier with 2-nitropyridine (Holubek & Volke, 1960).

It follows from the $E_{\frac{1}{2}}$-pH plot that only in strongly alkaline solutions (best in NaOH solutions of various concentrations where, e.g. in N NaOH the half-wave potentials have the following values: $-0{\cdot}57$ V for metronidazole, $-0{\cdot}68$ V for 1-(2-hydroxyethyl)-2-methyl-4-nitroimidazole, and $-0{\cdot}90$ V for 2-methyl-5-nitroimidazole) are the half-wave potentials of the three compounds sufficiently apart to enable a simultaneous quantitative determination. This is also evident from the polarograms of equimolar mixtures of all three compounds at different pH values; only above pH 11 are three independent waves obtained. There is, however, a serious drawback when working with strongly alkaline solutions, as metronidazole decomposes upon adding sodium hydroxide. The originally colourless solution turns violet and this reaction is accompanied by a decrease in the height of wave A. Finally, after about 22 hr the solution becomes colourless again and the original reduction wave completely disappears. The character of the waves of the other two compounds is not influenced in this way by the alkalinity of the supporting electrolyte. Since the wave-height of metronidazole does not change rapidly during the first 15 min after the alkalisation, quantitative analysis of mixtures in this medium is rendered possible.

To this end the concentration dependences of all three compound were investigated in sodium hydroxide solutions and the wave-height was found to be a linear function of depolariser concentration for concentrations ranging from 5×10^{-5}M to 2×10^{-3}M.

At this instant the analysis of mixtures was attempted. The examples for metronidazole and for 1-(2-hydroxyethyl)-2-methyl-4-nitroimidazole, in each case with varying concentrations of the component to be determined, and with constant concentrations of the other two components are shown in Fig. 3A and B. The concentrations of metronidazole and 1-(2-hydroxyethyl)-2-methyl-4-nitroimidazole lie between $1{\cdot}66 \times 10^{-4}$ and $1{\cdot}66 \times 10^{-3}$M. The experiments were made in 0·63N sodium hydroxide and with $7{\cdot}5 \times 10^{-4}$M solutions of the other two components.

The sensitivity of the test is adequate and the separation of the three waves is clearly visible even with $2{\cdot}5 \times 10^{-6}$M solutions. At lower concentrations a quantitative determination would be dubious and unreliable.

ANALYTICAL PROCEDURE FOR THE SIMULTANEOUS DETERMINATION

A. *The wave of the sample.* Weigh accurately about 200 mg of the sample from the reaction mixture, dissolve in distilled water and make up to 100 ml. Transfer a 2 ml portion of this solution to a dry polarographic cell, and add water (3 ml) and N sodium hydroxide (5 ml). Deaerate the solution with pure nitrogen for exactly 5 min and thereafter

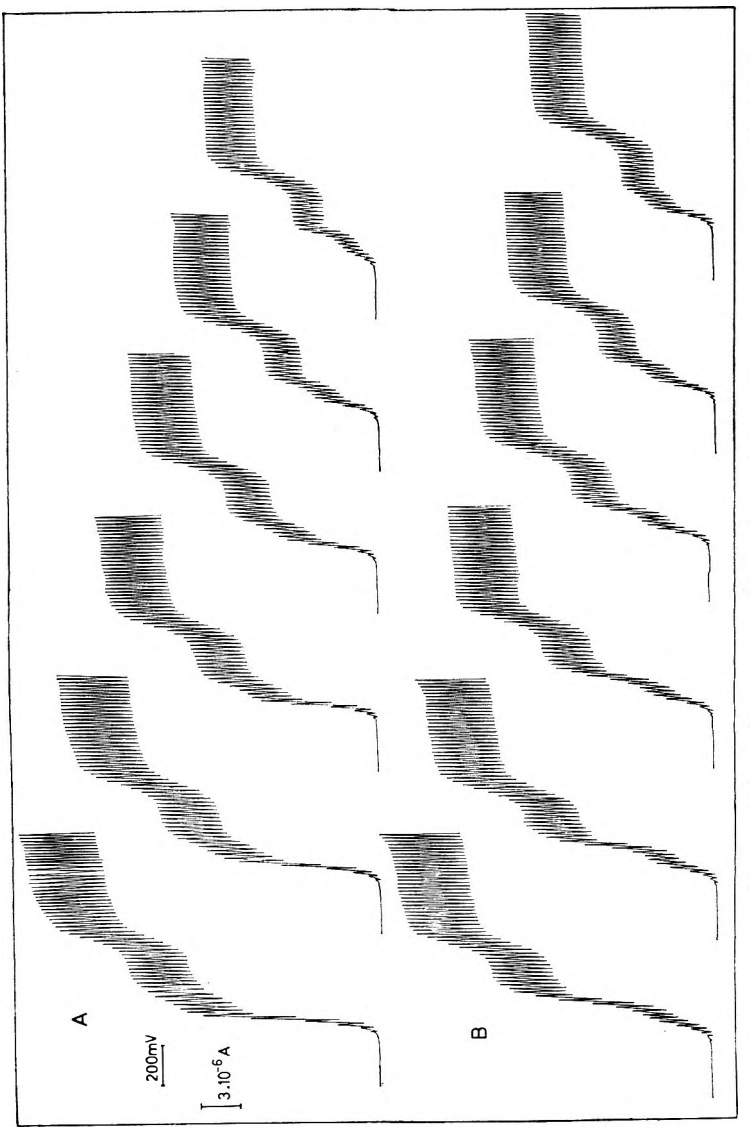

FIG. 3A. Dependence of wave-height of metronidazole on depolariser concentration in presence of 1-(2-hydroxyethyl)-2-methyl-4-nitroimidazole and 2-methyl-5-nitroimidazole. Composition of the solution: $3{\cdot}3 \times 10^{-4}$M 1-(2-hydroxyethyl)-2-methyl-4-nitroimidazole, $3{\cdot}3 \times 10^{-4}$M 2-methyl-5-nitroimidazole, 0·61N sodium hydroxide. The concentration of metronidazole varies from $1{\cdot}6 \times 10^{-4}$M to 10^{-3}M. Each curve starts at −0·3 V and ends at −1·4 V.

B. Dependence of wave-height of 1-(2-hydroxyethyl)-2-methyl-4-nitroimidazole on depolariser concentration in presence of metronidazole and 2-methyl-5-nitroimidazole. Composition of the solution: $3{\cdot}3 \times 10^{-4}$M metronidazole, $3{\cdot}3 \times 10^{-4}$M 2-methyl-5-nitroimidazole; 0·61N sodium hydroxide. The concentration of 1-(2-hydroxyethyl)-2-methyl-4-nitroimidazole varies from $1{\cdot}6 \times 10^{-4}$M to 10^{-3}M (equal additions). Each curve starts at −0·3 and ends at −1·4 V.

record the polarogram over the range from −0·3 V to −1·4 V against saturated calomel electrode.

B. *The wave of the sample with the standard addition.* Transfer a 1 ml portion of a $1{\cdot}0 \times 10^{-3}$M standard solution of metronidazole or 1-(2-hydroxyethyl)-2-methyl-4-nitroimidazole or 2-methyl-5-nitroimidazole, into a dry cell, add 2 ml of the sample solution, water (2 ml) and N sodium hydroxide (5 ml). Deaerate the solution for exactly 5 min and record the polarogram as above. To evaluate the polarograms the following formula is used:

$$\% = \frac{h \cdot c \cdot 100}{h' \cdot w}$$

% = percentage of the component to be determined in the reaction mixture,

h = wave-height for the sample,

c = concentration of the standard solution (mg in 1 ml),

w = weight of the sample (mg in 2 ml),

h′ = wave-height for the standard (i.e. wave-height for the solution with standard addition h″ minus the wave-height for the sample h).

The time-factor when working with the sodium hydroxide solution was eliminated by recording the polarograms exactly after a time interval of 5 min. The analyses were made with reaction mixtures containing about 10% of each component. In addition to this, the mixture contained the reagents necessary for converting 2-methyl-5-nitroimidazole to metronidazole. Since the reaction conditions and the composition of the solution varied for each case—the most favourable conditions for the synthesis were sought—calibration curves could not be used for evaluating the polarograms. Instead the above method of standard addition was used.

EXPERIMENTAL

All three nitro-compounds were prepared in the department of synthetic chemistry of the Galenika Laboratory and exhibited the properties described in the literature. Analytical grade reagents were used for preparing the buffer solutions and as supporting electrolytes.

The polarographic measurements were made with the Sargent Model XV Recording Polarograph and with a special thermostated cell containing a separated calomel electrode, and the dropping mercury electrode.

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The effect of frequency of coaxial electrical stimulation on the peristaltic activity of the guinea-pig isolated ileum

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Low frequency coaxial electrical stimulation (6/min to 5/sec) of the guinea-pig isolated ileum, arranged for recording peristalsis, was found either to stimulate or to maintain the existing peristaltic activity. High frequency stimulation (5/sec to 100/sec) was found to inhibit regularly the existing spontaneous peristaltic activity. The propulsive peristaltic action was also significantly diminished by high frequency stimulation. In a fatigued preparation all the applied frequencies produced the co-ordinated peristaltic contractions which were blocked by hexamethonium and by cooling. The propulsive activity of these contractions was higher after low frequency stimulation. It is suggested that block of peristalsis after high frequency stimulation might be due to activation of inhibitory elements in the gut.

IT has been established that morphine can reduce the output of acetylcholine from the guinea-pig isolated ileum both at rest and during coaxial electrical stimulation (Paton, 1957). A fundamental difference between the effects caused by stimulation at low frequencies (less than 1/sec) and those at higher frequencies was observed. For example, the output of acetycholine per shock was found to be higher at low frequencies and could be inhibited by morphine, whereas the output per shock at higher frequencies was less and morphine resistant.

The significance of the intramural nerve plexuses for the regular peristaltic activity has been repeatedly stressed (Trendelenburg, 1917; Bozler, 1949; Evans & Schild, 1953; Kosterlitz, Pirie & Robinson, 1956; Bülbring, Lin & Schofield, 1958). These nerve structures can be stimulated either coaxially (Paton, 1957) or by "field stimulation" (Härtfelder, Kuschinsky & Mosler, 1958). The present experiments were made to examine more closely the effect of various frequencies on the peristaltic propulsive activity of the guinea-pig isolated ileum and particularly to investigate the inhibitory action of high frequency stimulation on the peristaltic activity.

Experimental and results

Methods

The guinea-pig isolated ileum was immersed in a 40 ml organ bath. The bath fluid was Tyrode solution gassed with a mixture of oxygen 95% and carbon dioxide 5%. The coaxial stimulation was arranged as described by Paton (1957). The impulses were delivered by an electronic square wave stimulator. The duration of pulses was generally 1 msec, and the frequency ranged from 6/min to 100/sec.

For recording the peristaltic activity a method was used which was described in detail by Varagić & Kažić (1965). The oral end of the intestine was tied to the inflow of a U-shaped glass tube 3 mm in diameter. The aboral end of the ileum was tied to a glass tube which was connected

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to a lever with a frontal writing point used for recording the longitudinal muscle contractions. This glass tube was also connected by a soft elastic rubber tube to another U-shaped glass tube which had a side connection to a float recorder for recording the circular muscle contractions. The outflow was measured directly by collecting the propelled fluid in a cylinder. The inflow pressure ranged from 15 to 30 mm water.

Stimulation by various frequencies. When the isolated ileum was perfused by Tyrode solution at a constant small positive intraluminal pressure (about 16 to 20 mm water), a regular peristaltic propulsive activity was observed. This type of activity lasts for several hours (Varagić & Kažić, 1965). When the ileum showing this type of activity was stimulated coaxially by various frequencies, a depression of the peristaltic activity was observed after higher frequencies with either no change or stimulation after low frequencies. Hexamethonium blocked the peristaltic response to stimulation at all frequencies. An ascending order of frequencies was followed, except occasionally when the reverse order was adopted. The frequency of 6/min usually caused no change in the existing spontaneous peristaltic activity, but occasionally produced an increase in number of peristaltic waves. On the other hand, the frequency of 100/sec regularly inhibited the existing peristaltic activity. Fig. 1 shows

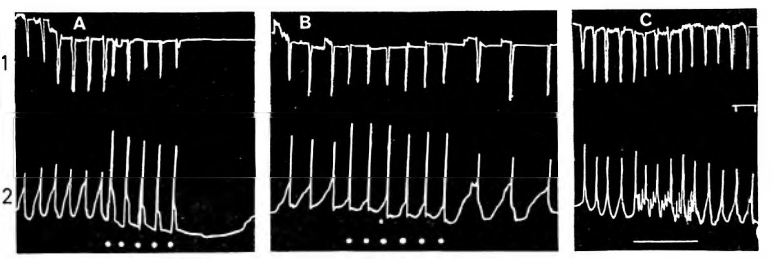

FIG. 1. The effect of electrical stimulation by various frequencies on the peristaltic activity of the guinea-pig isolated ileum. Upper record: contractions of the circular muscle. Lower record: contractions of the longitudinal muscle. At white dots in A, stimulation for 3 sec at 100/sec every 50 sec. At white dots in B, stimulation for 3 sec at 5/sec every 60 sec. At bar in C, stimulation at 6 min continuously for 3·5 min. Time: 1 min.

an experiment in which the ileum was stimulated by various frequencies in descending order. The frequency of stimulation was 100/sec in A and 5/sec in B. Arranging the period of time between stimulations to be very close to the length of time between existing spontaneous peristaltic waves caused a depression of the pre-existing activity after 5/sec frequency (B) and complete block after 100/sec (A). Stimulation with 6/min frequency caused no change in the frequency of existing peristaltic waves, as shown in C. This type of response was obtained in 10 experiments.

The propulsive activity. Generally, the stimulation with frequencies up to 5/sec was found not to affect significantly the peristaltic propulsive activity, whereas higher frequencies depressed it. Fig. 2 shows an experiment in which the propulsive activity was measured during 6 existing spontaneous peristaltic contractions and during the same number of

peristaltic contractions produced by stimulation with various frequencies. Again, the period of time between stimulations was so arranged as to be close to the length of time between the existing peristaltic contractions. It can be observed that stimulation with 5/sec (A) caused no change in the amount of propelled fluid for 6 peristaltic contractions. Stimulation with 100/sec (C) produced reductions in the amount of propelled fluid of 85% (5 experiments).

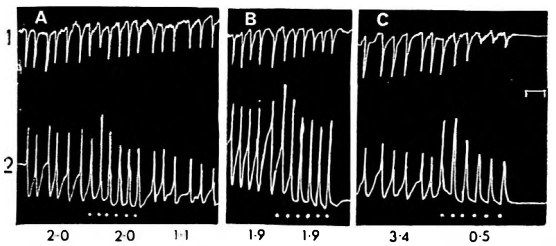

FIG. 2. The effect of frequency of electrical stimulation on the peristaltic propulsive activity of the guinea-pig isolated ileum. The numbers below the tracing indicate the amount of propelled fluid for 6 peristaltic contractions. Upper and lower records as in Fig. 1. At white dots in A, stimulation for 3 sec at 5/sec every 30 sec. At dots in B, stimulation for 3 sec at 30/sec every 30 sec. At dots in C, stimulation for 3 sec at 100/sec every 50 sec. Time: 1 min.

Stimulation during existing peristaltic activity. The depression of the existing spontaneous peristaltic activity produced by stimulation with high frequencies depends on the number of stimulations applied. Fig. 3 shows

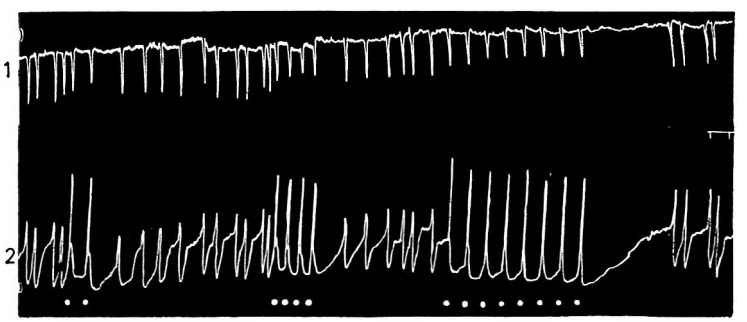

FIG. 3. The effect of high frequency stimulation on the spontaneous peristalic activity of the guinea-pig isolated ileum. Upper and lower records as in Fig. 1. At white dots, stimulation for 3 sec at 100/sec every 50 to 60 sec. Time: 1 min.

the effect of coaxial stimulation at 100/sec on the existing spontaneous peristaltic activity. Two stimulations were applied first (at white dots), then four, and finally eight and it was found that the duration of post-stimulatory inhibition was longest after 8 and shortest after 2 stimulations.

Repeating the same number of stimulations with 100/sec caused the post-stimulatory inhibition after the second stimulation to be significantly longer than after the first.

On transferring the bath fluid after high frequency stimulation of the ileum to a bath containing another segment of guinea-pig ileum, arranged for recording peristalsis, no inhibition of peristalsis could be detected.

Stimulation of the fatigued preparation. In a preparation in which a high intraluminal pressure (25–30 mm H_2O) was maintained for a long time, the existing spontaneous peristaltic activity ceased. Stimulation of this fatigued preparation coaxially with low frequencies caused peristaltic propulsive waves to be produced again. (Every single pulse need not be

FIG. 4. The effect of a low frequency stimulation on the post-stimulatory inhibition of the peristaltic activity of the guinea-pig isolated ileum. Upper and lower record, as in Fig. 1. At white dots, stimulation for 3 sec at 100/sec every 55 sec. At the arrows, stimulation for 3 sec at 2/sec every 55 sec. Time: 1 min.

followed by a complete peristaltic propulsive contraction.) Meanwhile, this type of stimulation usually initiates a series of spontaneous peristaltic contractions. High frequency stimulation (5–100/sec) for 3 sec at 30 sec intervals also produced the co-ordinated peristaltic contraction in the fatigued preparation. But the propulsive activity was smaller after high frequency than after low frequency stimulation.

The stimulatory effect could also be produced when low frequency stimulation was applied during the post-stimulatory inhibition of peristalsis caused by high frequency stimulation. In Fig. 4, the existing spontaneous peristaltic activity was inhibited by previous stimulation with 100/sec frequency (at white dots); stimulation with 2/sec frequency (at the arrows) restored the peristaltic contractions.

Stimulation at low bath temperature. Lowering the temperature of the bath fluid to 27° decreased significantly the propulsive response of the

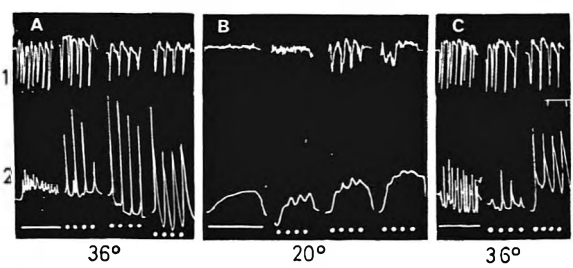

FIG. 5. The effect of cooling on the peristaltic response to various frequency stimulations of the fatigued guinea-pig ileum. Upper and lower record, as in Fig 1. In A and B, stimulation with 6/min (continuous stimulation), 2/sec, 10/sec, 100/sec. In C, 6/min, 2/sec and 100/sec. Between A and B, temperature of the bath lowered to 20°. Between B and C, temperature raised to 36°. Time: 1 min.

ileum to coaxial stimulation with various frequencies, but its peristaltic contractions were still present. Lowering the temperature of the bath to 23–20° usually blocked the effect of stimulation on the peristaltic propulsive activity. In some experiments the response to low frequency stimulation on these temperatures was more readily blocked than the effect of high frequency stimulation (Fig. 5).

Discussion

The results of the present experiments show a distinct difference between the effects of low and high frequencies of stimulation on the existing spontaneous peristaltic activity of the guinea-pig isolated ileum. The low frequencies (from 6/min to 5/sec) were found either to stimulate or to maintain the existing spontaneous peristaltic activity due to increased intraluminal pressure. Of these various low frequencies only 6/min was applied continuously, whereas higher frequencies had to be applied intermittently, at various time intervals depending on the rhythm of spontaneous peristaltic activity. Adjustment of the stimulation with low frequencies to keep pace with the rhythm of existing spontaneous peristaltic activity, caused no significant change in the co-ordinated peristaltic contractions of longitudinal and circular muscles; nor does the coaxial stimulation with various frequencies produce any change in the peristaltic activity of gut stimulated at shorter time intervals than the periods of time between the existing peristaltic contractions. This is to be expected when the intraluminal pressure is kept low. It seems therefore that the co-ordinated peristalic activity can be produced by coaxial stimulation only if time is allowed for the gut to be filled and if the intraluminal pressure is high enough. Paton (1955) has already shown that an emptying reaction can be seen in response to single shocks in an intestine distended by raising the intraluminal pressure. This type of response resembles the peristaltic reflex and is inhibited by ganglion blocking agents. Similar responses were obtained after coaxial stimulation by Harry (1962). These responses consisted of contractions both of the longitudinal and circular muscle layers and were related to the amount of acetylcholine found in the bath fluid in which the ileum was suspended.

In fatigued preparations under constant intraluminal pressure, peristaltic activity is absent, but all the applied frequencies produced the co-ordinated propulsive contractions of the longitudinal and circular muscle. However, the propulsion activity was less after high than after low frequency stimulation. The peristaltic response to all these stimulations could be blocked by hexamethonium. Sometimes, particularly after low frequencies of stimulation, the spontaneous peristaltic activity was triggered off and continued for some time even after stopping the stimulation.

The responses of the fatigued preparation to the various stimulation frequencies could be reversibly inhibited by lowering the temperature of the bath fluid to 20°. Lowering the temperature of the bath fluid to 27° only depressed the peristaltic propulsive activity, but the peristaltic

contractions were still produced by stimulation. The sensitivity of the nervous elements and synaptic transmission in the gut to cooling has already been investigated (Ambache, 1946; Kosterlitz & Robinson, 1957; Varagić & Beleslin, 1958). Paton (1957) concluded from the character of the strength-duration curve and from the pharmacological responses that during coaxial stimulation the nervous structures are stimulated. Our experiments with the cooled preparation support this conclusion.

An inhibition of the existing spontaneous peristaltic activity was regularly observed after stimulation with higher frequencies (from 5 to 100/sec). This inhibition was found to be dependent on the number of stimulations applied. Stimulation with frequencies from 10 to 25/sec produced a short-lasting inhibition, whereas stimulation with 30 to 100/sec caused an inhibition of longer duration. Our experiments do not solve the problem of the origin of this inhibition. Garry & Gillespie (1955) and Varagić (1956) have shown that the optimal frequencies for stimulation of the parasympathetic colonic nerves of the rabbit are about 5/sec, whereas the optimal frequencies for stimulation of lumbar colonic sympathetic nerves are about 50/sec. On the other hand, Rand & Ridehalgh (1965) found that guinea-pig colon responded to stimulation of either nerve at 50/sec with maximal responses. It is still possible that stimulation with higher frequencies specifically affects the adrenergic, or some other inhibitory nervous elements in the isolated ileum leading to cessation of existing spontaneous peristaltic activity. It has also been shown that in preparations where adrenaline causes relaxation and loss of tone, this effect is associated with hyperpolarisation of the smooth muscle membrane and reduction or cessation of spike activity (Bülbring, 1954; 1957). Our experiments do not exclude the possibility of an unspecific hyperpolarisation produced by high frequency stimulation. On the other hand, we were unable to show the presence of an inhibitory substance in the bath fluid after high frequency stimulation.

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A smooth muscle contracting substance in extracts of human umbilical cord

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Aqueous extracts of human umbilical arteries and vein have been shown to contain a smooth muscle contracting substance. The active principle has been distinguished from smooth muscle contracting substances which are found in mammalian tissues, namely esters of choline, histamine, 5-hydroxytryptamine, bradykinin, angiotensin, and darmstoff. Evidence indicates that the smooth muscle stimulating activity of umbilical blood vessel extracts is due to a lipid-soluble unsaturated hydroxy acid. The possible physiological function of the active substance is discussed.

THE umbilical cord is not ligated at the birth of most mammals and in veterinary obstetrics no important haemorrhage follows the division of the cord (Williams, 1931). It appears that in man, nature has also made adequate provision for the cessation of umbilical blood flow since constriction of the umbilical blood vessels follows delivery.

The umbilical blood vessels have been shown to constrict in response to a variety of stimuli. Haselhorst (1929) demonstrated that the walls of cord vessels were sensitive to temperature and to irritation from handling. Schmitt (1926) showed that umbilical vessels contracted when the oxygen content of perfusing fluid was increased. Umbilical vasoconstriction may also be induced by the catecholamines, histamine, posterior pituitary extracts and 5-hydroxytryptamine (5-HT) (Panigel, 1959, 1962). These facts suggested to me the possibility of the involvement of a chemical substance which could act on the umbilical blood vessels after birth. If this speculation is correct it should be possible to recover the active substance from the umbilical blood vessels. In the present investigation, human umbilical cords have been examined for the presence of substances which might cause contraction of the umbilical blood vessels and other smooth muscles.

Experimental

Preparation of aqueous extract. The umbilical arteries and veins were dissected from fresh umbilical cords and were split open along their longitudinal axis. Blood was removed by washing with distilled water, and the vessels were dried between filter paper, weighed and then minced with 4 ml of distilled water (pH 6·5–7) for each gram of tissue. The mixture was left in the refrigerator (4°) for 12 hr before centrifugation for 15 min at 3,000 rpm. The viscous supernatant was decanted into stoppered bottles and the residue re-extracted with a further 4 ml of distilled water per gram of tissue. The two supernatants were combined and extracted with ether as described below.

Purification of aqueous extract. A slight modification of the method described by Ambache (1959) has been successfully used for the purification of the aqueous extracts of umbilical cord vessels. Aqueous extracts

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at pH 6·5–7 were shaken with equal volumes of freshly distilled diethyl ether. The separation of the two layers was assisted by centrifugation and the ether layer was discarded. The aqueous solution was next acidified to pH 3 with N hydrochloric acid and extracted with three volumes of peroxide-free ether. The two phases were separated as before and the ether layer was evaporated to dryness under reduced pressure at room temperature. The dry residue was reconstituted in 0·9% w/v sodium chloride solution just before assay.

Extract of foetal arteries. Aqueous extracts of foetal abdominal and thoracic aortae obtained at post-mortem were prepared in exactly the same way as the extracts of umbilical blood vessels. The aqueous extracts were shaken at pH 3 with 3 volumes of diethyl ether. The dry ether residue after the evaporation of the ether was taken up in 0·9% sodium chloride solution and tested for biological activity.

Extracts of Wharton's Jelly. Wharton's Jelly from fresh cords was similarly extracted.

BIOLOGICAL ACTIVITY TESTS

Rabbit ileum preparation. Segments of rabbit ileum were suspended in a 5 ml organ bath of Tyrode solution. The bath solution at 32° was bubbled with air. Records were made with a frontal writing lever of 3 : 1 magnification with a 3 g load on the muscle.

Guinea-pig ileum. From animals weighing 200–250 g, a piece of terminal ileum was removed and suspended in a 5 ml organ bath of Tyrode solution at 32°. The tension on the tissue was approximately 2 g. The frontal writing lever had a magnification of 10 : 1.

Rat uterus. This was set up as described by Amin, Crawford & Gaddum (1954). The bath fluid was gassed with oxygen at 28°.

Guinea-pig proximal colon. This was set up in a 10 ml organ bath as described by Botting (1965).

Rat colon. The proximal 4–5 cm of colon was suspended in a 5 ml organ bath at 28°. The aerated bath fluid corresponded to that used by Gaddum, Peart & Vogt (1949).

Umbilical artery preparations. Fresh umbilical cords were used. Approximately 4–5 cm of the spirally cut arteries were suspended in a 5 ml organ bath of Krebs bicarbonate solution gassed with oxygen.

Pithed rat blood pressure. Rats weighing 250–300 g were anaesthetised with ether and pithed by passing a strong wire through the orbit down the cerebrospinal axis. Artificial respiration was maintained by a Palmer small animal pump. Blood pressure was recorded from a carotid artery by means of a condon manometer.

Rabbit blood pressure. Rabbits (1·8–2·5 kg) were anaesthetised with urethane (8 ml of a 20% solution i.v.) and injected with heparin (500 units/kg) as an anticoagulant. Arterial blood pressure was recorded from the carotid artery by a mercury manometer and drugs were injected in the external jugular vein.

Cat blood pressure. Cats weighing between 2·3–4 kg were anaesthetised with ether followed by chloralose (80 mg/kg i.v.). The blood pressure was

recorded from a cannulated femoral artery by a mercury manometer. Drugs were injected into a femoral vein.

Paper chromatography. Ascending chromatograms of the umbilical vessels extracts were run on Whatman No. 1 filter paper. The following solvent systems were used. (1) Organic, upper phase of the following mixture: ethyl methyl ketone-water-diethylamine (60:20:3) (Vogt 1955, 1957c). (2) Butanol-acetic acid-water (4:1:5) (Partridge, 1948). (3) Phenol saturated with 0·01N hydrochloric acid. The chromatograms were developed at room temperature (18–20°) for 22–24 hr in an atmosphere of nitrogen. The localisation of the active substance was detected by biological assay. Strips of paper were cut at variable intervals from the starting line to the solvent front, and eluted by descending chromatography overnight in 10 ml graduated cylinders with distilled water (pH 6·5–7). The eluates were acidified to pH 3 with 0·1N hydrochloric acid and extracted with three volumes of freshly distilled diethyl ether. The ether extracts were evaporated to dryness at room temperature under reduced pressure. The dry extracts were dissolved in Tyrode solution just before assay on guinea-pig proximal colon.

Paper electrophoresis. Paper electrophoresis was on Whatman No. 1 filter paper at pH between 5 and 9 in phosphate buffer as described by Ambache (1957). The position of the active substance on the dry paper was ascertained by assay on the guinea-pig proximal colon.

Enzyme Incubation. (i) *Chymotrypsin.* To 1 ml of ether-purified umbilical blood vessels extract (≡100 μg dry weight of extract) was added 0·3 mg of crystalline chymotrypsin. The solution at pH 8·0 was placed in a water-bath at 37° for 1 hr. A control tube containing an equiactive concentration of bradykinin and 0·3 mg of chymotrypsin was similarly incubated. Another control tube containing 100 μg of umbilical blood vessels extract at pH 8 was also incubated. At the end of 60 min the three solutions were assayed for biological activity on guinea-pig proximal colon.

(ii) *Trypsin.* To each of two test-tubes containing 100 μg/ml umbilical blood vessels extract and 1 μg/ml angiotensin was added 10 mg of trypsin. The tubes were incubated at 37° for 60 min. At the end of this period the two solutions were tested for activity on the guinea-pig proximal colon.

CHEMICAL TESTS

Chemical tests on umbilical vessels were made in solutions or on chromatograms.

Amino-acid test. Papers were sprayed with 0·1% ninhydrin in water-saturated butanol and left in an oven at 100° for 5–10 min.

Test for -NH- group. The modified method of Reindel & Hoppe (1953) as described by Ambache (1959) was used. Filter paper containing the active extracts was exposed to chlorine dioxide followed by spraying the paper with 1% benzidine solution in 10% acetic acid.

Colour test for higher fatty acids. To 0·1 ml of a saturated solution of Rhodamine B in benzene were added 0·1 ml of 1% aqueous solution of uranyl acetate and 0·2 ml of a benzene solution of ether purified umbilical

blood vessels extract (≡50 μg). A control tube containing the reagents and 0·2 ml benzene without extracts was prepared simultaneously and both tubes were shaken. With this procedure the higher fatty acids form benzene-soluble red additive compounds which fluoresce (Feigl, 1956) under ultraviolet light.

Colour test for hydroxy-fatty acids. 0·1 ml of a freshly prepared 0·5% solution of sym-diphenylcarbazide in tetrachloroethane was added to 50 μg/ml solution of umbilical blood vessels extract. A control tube with same volumes of reagents and without the umbilical extracts was also prepared. The tubes were shaken intermittently and the colour in the lower tetrachloroethane was noted.

Phenyl isocyanate. This test was made as described by Ambache (1959). To 1 ml of umbilical vessels extracts (≡100 μg) was added 0·02 ml of phenyl isocyanate in a test-tube which was then left at room temperature for 1 hr; during this time the tube was shaken intermittently. The solution was centrifuged to remove the precipitated diphenyl urea and the supernatant after decanting was tested for biological activity.

*NN'-carbo-di-*p*-tolyl imide.* To 100 μg of umbilical vessels extracts in diethyl ether was added 1 mg of the reagent. A second sample of the cord extract was used as an untreated control. The two samples were left at room temperature for 30 min and were then evaporated to dryness and taken up in Tyrode solution and tested for activity on the guinea-pig proximal colon.

Potassium permanganate. To 1 ml of aqueous solution of umbilical vessels extract (≡100 μg dry weight) was added 0·025 ml of a 2% solution of potassium permanganate. The pH of the mixture was 7·5. After 5 min at room temperature the solution was acidified to pH 3 and extracted with 3 volumes of diethyl ether. The ether phase after evaporation was reconstituted in 0·9% sodium chloride solution and assayed on guinea-pig proximal colon.

Iodine bromide. 0·2 ml of a saturated solution of iodine bromide was added to 1 ml of umbilical cord extract (100 μg). The solution was left standing for 15 min at room temperature and the excess of iodine bromide was inactivated by adding excess of sodium thiosulphate. The extract was acidified to pH 3 and extracted with 3 volumes of ether. The control was a tube with the same amount of iodine bromide inactivated with sodium thiosulphate before the addition of the active extract. It was partitioned under identical conditions.

DRUGS USED

Acetylcholine chloride, histamine acid phosphate, 5-hydroxytryptamine creatinine sulphate, bradykinin, angiotensin, diphenhydramine, atropine sulphate, methysergide bimaleate, trypsin and chymotrypsin.

Results

Extraction. After extraction of the minced umbilical blood vessels twice with 4 ml of distilled water per gram of the tissue, less than 20% of

the biological activity was left. For this reason the residue was not further re-extracted.

Purification by partition with ether. Fig. 1 shows the recovery of the active substance from the aqueous extracts of the umbilical blood vessels

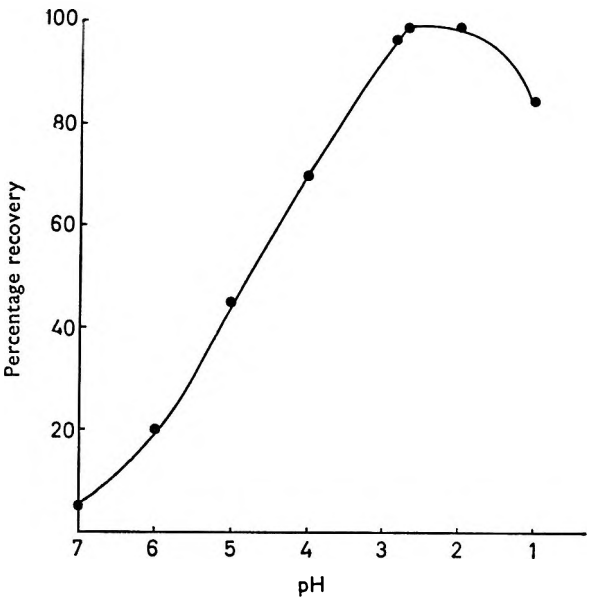

FIG. 1. Recovery of the active substance after partitioning the aqueous extracts of umbilical blood vessels with three volumes of diethyl ether at various pH values, expressed as percentage of the original activity found per ml of ether phase.

after partition with three volumes of diethyl ether at various pH values. The yield of the active material was almost 100% between pH 2 and 3 (Fig. 1). The low recovery at pH 1 was found to be due to the active substance being partly destroyed. The yields of the active substance after purification are given in Table 1.

TABLE 1. YIELDS OF ACTIVE SUBSTANCE PURIFIED BY PARTITION FROM 100 G OF TISSUE

pH of the partition	Weight of active residue	Purification—from original weight of tissue
2·5	10 mg	10,000 ×
2·3	12 mg	8,333 ×
3·0	8 mg	12,500 ×
3·0	9 mg	11,111 ×

Activity test. The ether-purified umbilical vessels extracts contracted smooth muscles, namely guinea-pig ileum and colon, rat colon and uterus, rabbit ileum and isolated umbilical artery preparations (Fig. 2). Guinea-pig proximal colon (Botting, 1965) was found to be the most sensitive

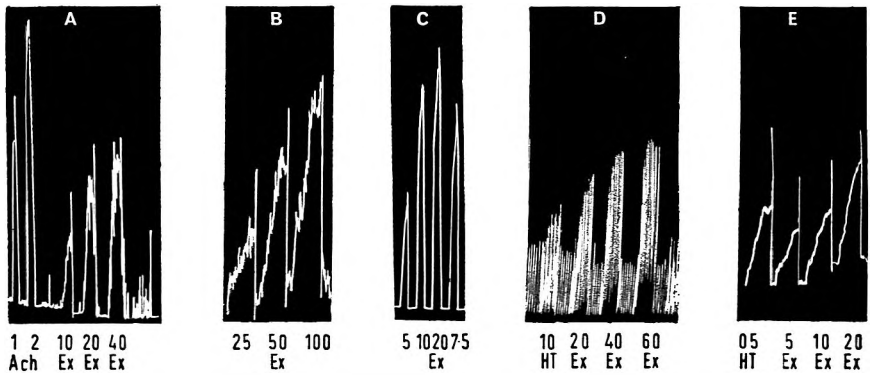

FIG. 2. Effect of umbilical cord vessels extracts on various smooth muscle preparations suspended in 5 ml isolated organ baths. A. Guinea-pig terminal ileum. B. Rat colon. C. Rat uterus. D. Rabbit ileum. E. Umbilical artery preparation. Ex. Doses of ether purified extracts in μg. HT. 5-Hydroxytryptamine. Ach. Acetylcholine. All doses in μg.

preparation and has been used for the routine biological assay of the active substance. Extracts of foetal aortae and of Wharton's Jelly in a concentration of 200 μg/ml had no smooth muscle stimulating activity.

Other pharmacological actions. The active substance in the umbilical blood vessels produced vasodepression in the cat, rat and the rabbit (Fig. 3). This property has been used to distinguish the active principle from other known substances with similar properties.

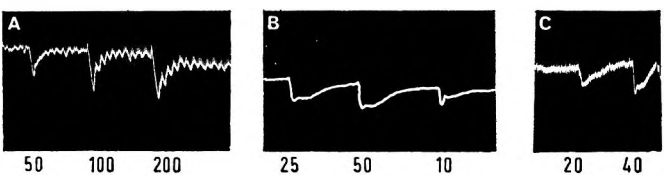

FIG. 3. Effect of intravenous injections of umbilical cord vessels extract on the blood pressure responses of (A) anaesthetised cat (2·2 kg), dose interval 5 min; (B) anaesthetised rabbit (1·9 kg), dose interval 20 min; (C) pithed rat (300 g), dose interval 10 min. All doses in μg/kg.

Dialysis. The active substance in the umbilical blood vessels slowly dialysed through a cellophane membrane. In dialysis experiments of extracts against distilled water, equilibrium was reached slowly. After 6 hr the activity in the water was almost nil and there was no loss of activity in the extract. After 14 hr the activity was equally distributed on both sides.

Stability. The stability of the active substance in the umbilical blood vessels depended on pH and temperature. After 7 days at room temperature at pH 7 there was no loss of activity, whereas at pH 1 or 2 more than 50% of the activity was destroyed after standing at room temperature for 8 hr. The effect of boiling the aqueous extracts in sealed containers at various pH is shown in Fig. 4. The active substance was not destroyed

by boiling for 30 min at pH 3–9. At pH 7, one sample was left boiling for 2 hr without loss of activity.

Solubility. The active substance is soluble in water, ethanol and acetone. It is also soluble in ether and chloroform at acid pH but almost insoluble in light petroleum.

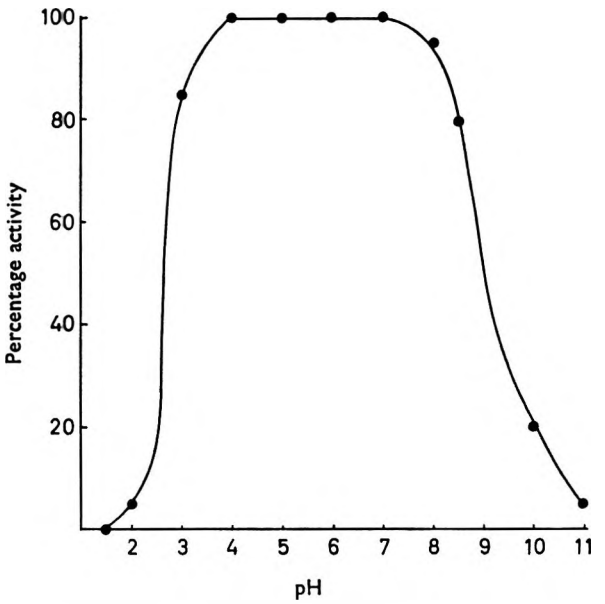

FIG. 4. Percentage smooth muscle stimulating activity left after standing umbilical cord vessels extracts in a boiling water-bath for 30 min at different pH values.

Electrophoresis. The active substance tended to move towards the anode; this tendency was increased at alkaline pH. Thus at pH 6·3 under a potential gradient of 12 V/cm the active substance had moved 0·5 cm towards the anode after 4 hr. At pH 8·5 under a similar potential gradient the activity was found 4·0 cm from the origin towards the anode after 4 hr. Endosmosis was in the direction of the cathode as shown by glucose and the above values were arrived at after correction in the migration values had been made for this effect.

Paper chromatography. Chromatograms of ether-purified extracts of umbilical blood vessels were developed in three solvent systems. The active substance was detected by biological activity as described under Methods. The results of these experiments are summarised in Table 2.

TABLE 2. Rf VALUES OF ACTIVE SUBSTANCE IN CORD VESSELS

Solvent system	Rf values	Recoveries
		%
Phenol-hydrochloric acid (saturated)	0·8 –0·92	80
Butanol-acetic acid-water (4 : 1 : 5)	0·85–0·95	70
Ethyl methyl ketone-diethylamine-water (60 : 3 : 20)	0·78–0·96	76

DISTINCTION OF THE ACTIVE MATERIAL FROM PHARMACOLOGICALLY ACTIVE SUBSTANCES OF NATURAL OCCURRENCE

The contractions of various smooth muscle preparations produced by umbilical blood vessels extract could be distinguished from those produced by acetylcholine, 5-HT and histamine.

Acetylcholine. The contractions of the guinea-pig proximal colon produced by the umbilical cord extracts were resistant to atropine. Concentrations of atropine (10^{-7}–10^{-6} g/ml), while abolishing the responses of the guinea-pig colon to acetylcholine, did not modify responses to the umbilical cord extracts (Fig. 5).

Histamine. In a similar manner the active substance could be distinguished from histamine. Diphenhydramine (10^{-7} g/ml) while abolishing the contractions of the guinea-pig proximal colon produced by histamine did not affect responses to the umbilical vessels extract (Fig. 5).

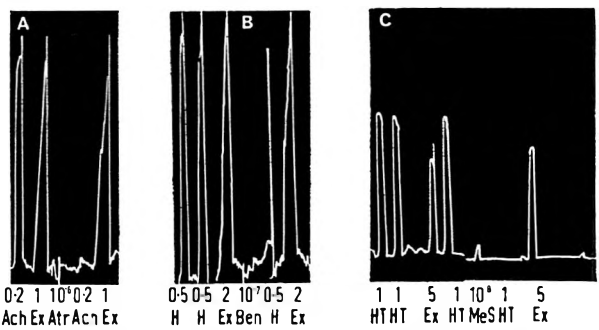

FIG. 5. Comparison of the effects of acetylcholine (Ach) and umbilical vessels extract (Ex) before and after (A) atropine (Atr) on guinea-pig proximal colon; contact time 90 sec, dose interval 10 min. (B) Histamine (H) and Ex on a similar preparation before and after diphenhydramine (Ben) and (C) 5-hydroxytryptamine (HT) and Ex before and after methysergide (MeS) on non-pregnant rat uterus; dose interval 5 min, contact time 2 min.

In all experiments the antagonists were injected 1 min before the doses of the stimulant drugs. All doses in μg.

Apart from this the active substance in the cord vessels could be distinguished from histamine for the following reasons. (1) Histamine is a base: the active substance in the cord vessel behaves as a weak acid. (2) Whereas the umbilical cord extracts caused the rat colon and uterus preparations to contract, histamine even in very large doses (100 μg) failed to contract these preparations.

5-Hydroxytryptamine. Antagonists of 5-HT do not effectively abolish the contractions of the guinea-pig ileum and colon produced by this amine. For this reason the isolated rat uterus preparation was used to demonstrate that the smooth muscle stimulating activity of umbilical cord vessels extract was not due to 5-HT.

Methysergide (10^{-8} g/ml) abolished the contractions of the rat uterus produced by 5-HT whereas contractions of this preparation produced by umbilical vessels extract were unaffected by even larger doses (10^{-8}–10^{-5} g/ml) of methysergide (Fig. 5).

Effect of incubation with chymotrypsin and trypsin. There was no loss of activity on incubating the extracts of umbilical blood vessels at pH 8·0 with trypsin, or with crystalline chymotrypsin for 60 min at 37°. Controls consisting of chymotrypsin and bradykinin or angiotensin and trypsin when similarly incubated were inactive after 60 min (Fig. 6).

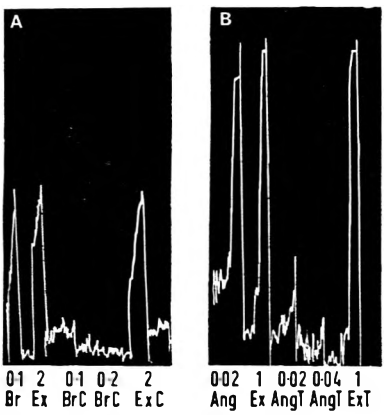

FIG. 6. Guinea-pig proximal colon. Effect of incubation with chymotrypsin and trypsin on the smooth muscle stimulating activity of umbilical cord vessels extract and of bradykinin and angiotensin. A. Br and Ex control responses to bradykinin and cord extracts. BrC and ExC responses after incubation of bradykinin and cord extract with chymotrypsin for 60 min. B. Ang and Ex control responses to angiotensin and cord extract. AngT and ExT responses after 60 min incubation with trypsin. Doses in μg.

This together with the negative ninhydrin and -NH- group test appears to show that the active substance is unlikely to be a polypeptide.

CHEMICAL NATURE OF THE ACTIVE SUBSTANCE IN THE UMBILICAL CORD VESSELS

The solubility of the active substance in the umbilical blood vessels in various organic solvents, its behaviour on partition with ether and its migration towards the anode at alkaline pH, all suggest that it is a lipid soluble acid. The following evidence based on chemical tests and colour reactions is produced to suggest that the active substance is an unsaturated hydroxy acid and that the integrity of hydroxyl and carboxyl groups and at least one double bond are essential for the smooth muscle contracting action.

Inactivation of the active substance by NN'-*carbo-di*-p-*tolyl imide.* The carboxyl binding reagent *NN'*-carbo-di-*p*-tolyl imide converts carboxylic acids into mono-acylated di-ureides (Zetsche, Lüsher & Meyer, 1938). This test for the presence of the carboxyl group was made on three different samples of ether-purified umbilical cord extracts. When the samples treated with reagents were assayed on the guinea-pig proximal colon it was found that the reagent had inactivated the treated samples to the extent of 80–90%.

Colour test for carboxylic acid. This test was made on several batches of ether-purified umbilical vessel extracts. With 100 μg of the active substance dissolved in benzene, in presence of the reagents uranyl acetate and Rhodamine B a pink colour appeared in the benzene layer.

Inactivation of the active material by phenyl isocyanate. (i) *Presence of OH group.* Phenyl isocyanate reacts with free hydroxyl groups to form phenyl urethane. Umbilical cord extracts treated with this reagent were inactivated after standing at room temperature for 1 hr.

(ii) *Evidence of unsaturation.* Inactivation by potassium permanganate. The active substance in the umbilical cord vessels was inactivated by treating ether purified extracts with neutral potassium permanganate. A control sample without permanganate retained its activity.

Inactivation by iodine bromide. Samples of extracts treated with iodine bromide showed a loss of almost 100% activity. Controls showed no loss in activity.

The results of these experiments suggest that the active substance is a lipid soluble unsaturated hydroxy acid. The active substance in the umbilical blood vessels, however, can be distinguished from various lipid soluble acids of natural occurrence.

SLOW REACTING SUBSTANCE A

The smooth muscle stimulant (SRS-A) released from the lungs of perfused sensitised guinea-pigs has been examined by Brocklehurst (1953, 1955). Like the active substance in the umbilical vessel extract it is soluble in water and behaves similarly on electrophoresis. However SRS-A can be distinguished from the umbilical cord extracts because unlike the cord extract it causes no fall in arterial blood pressure and has no stimulant effect on the isolated rat uterus preparation (Vogt, 1957a).

The unsaturated fatty acid SRS which is split off from lecithin by cobra venom and which stimulates smooth muscles (Vogt, 1957b) can also be excluded because its action on guinea-pig ileum decreases with successive applications and finally disappears; no such tachyphylaxis is seen with the umbilical vessels extracts.

Irin. Extracts of rabbit iris contains a smooth muscle stimulating substance to which the name irin has been given (Ambache, 1957). Ambache (1959) has shown that irin is a long chain unsaturated hydroxy fatty acid. The active substance in the umbilical cord extracts can be distinguished from irin because unlike the cord extracts irin does not stimulate the rat uterus preparation. Also irin is thermolabile and at pH 9 it is inactivated even at room temperature. The active substance in the umbilical cord is not inactivated by boiling for 30 min at pH 3–9.

Darmstoff. The lipid soluble darmstoff (Vogt, 1957a, b) consists of acetal or semi-acetal phosphatidic acid. It can be distinguished from umbilical blood vessels extracts because unlike the cord extracts it stains with fuchsin-sulphurous acid. It can also be distinguished from darmstoff by paper chromatography in ethyl methyl ketone-diethylamine-water (60 : 3 : 20). In this solvent system darmstoff has an Rf of 0·4–0·45 on Whatman No. 1 paper (Vogt, 1957b) whereas the active substance in the

umbilical vessels run in this solvent system has an Rf of 0·8–0·9. Unlike the umbilical cord extracts, Darmstoff is not vasodepressor in rabbits and cats (Vogt, 1958).

Prostaglandin. The lipid-soluble acidic material with smooth muscle stimulating and vasodepressor activity present in the seminal plasma and in accessory genital glands (prostaglandin) has been shown to include several closely related unsaturated fatty acids (Euler, 1936; Goldblatt, 1935; Green & Samuelsson, 1964). Like the active principle in the cord vessels the prostaglandins are lipid soluble acids and migrate towards the anode on paper electrophoresis. The prostaglandin and the active substance under study produce vasodepression in the cat, rat and rabbit (Fig. 3). However they differ because: (1) the active material in the human seminal plasma has an Rf value of 0·45 on Whatman No. 1 paper when run in ethyl methyl ketone-diethylamine-water (60 : 3 : 20). The Rf value of the active substance in the cord vessels under identical conditions is 0·8–0·9 (Table 2). (2) According to Euler (1936) the active material in the seminal plasma is stable to boiling at pH 1–7 for 20 min. Umbilical cord extracts have been shown to be inactivated by boiling at pH 1 and 2.

Discussion

The solubility of the active substance present in the umbilical vessels in various organic solvents, its extraction by partition with ether at acid pH and its migration towards the anode at alkaline pH, all suggest that it is a lipid soluble acid. The inactivation of the active material by chemical reagents which bind with specific functional groups has revealed the presence of hydroxy and carboxyl groups and at least one pair of double bonds in the molecule. The activity of the extract is dependent upon the integrity of these functional groups and double bonds.

The active substance in the cord vessels can be distinguished from various lipid soluble acids of natural occurrence which stimulate smooth muscles including irin, darmstoff, SRS-A and prostaglandins.

Widely diverse functions have been suggested for the various lipid soluble acids extracted from tissues. Prostaglandins could facilitate impregnation by motor effects on the male and female genital tract or accessory glands. Darmstoff is assumed to be involved in the physiological movements of the gut (Vogt, 1958). Irin possibly plays a role in the function of the iris (Ambache, 1957). Thus the functions attributed to the lipid soluble acids are those of local hormones. The physiological significance of the active material in the umbilical cord vessels is a matter for speculation but it provides a possible explanation of the contractions of the cord vessels at birth by acting as a local hormone. It is hoped to show whether this is so. Because of the absence of innervation in the vessels of the umbilical cord the question of the active substance acting as a neurohormone does not arise.

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A note on the diffusion of drugs through artificial phospholipid membranes

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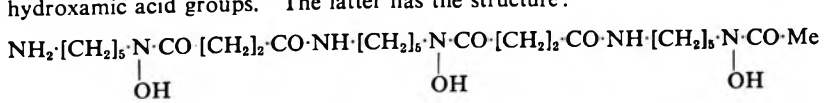
A relatively simple method for the assessment of the passive diffusion of drugs through artificial phospholipid membranes composed of lecithin and collodion yields data on the permeability of some pharmaceutical substances through lipid barriers. Although there are inherent limitations in using artificial phospholipid membranes as substitutes for more complex biological membranes, this model system can furnish information on permeability kinetics and on the effect of structural groups on biological activity.

MANY efforts have been made in the last three decades to devise simpler models of biological membranes for the examination of transport phenomena. Membrane permeability and equilibria have been discussed by Meyer & Sievers (1936), Adair (1937), Meyer (1937), Teorell (1937) and Wilbrandt (1959); the kinetics of diffusion (Laidler & Shuler, 1949; Zwolinski, Eyring & Reese, 1949); penetration (Davson & Danielli, 1952) and thermodynamics of irreversible processes (Kedem & Katchalsky, 1958; Katchalsky, 1960) have been reported. After we had completed our work the review of Lakshminarayanaiah (1965) on transport phenomena in artificial membranes appeared. But there have been comparatively few direct studies on the permeability of drugs through artificial phospholipid membranes. A qualitative study on the permeability of collodion-beef brain lipid membranes by Weatherby (1943, 1949) showed that these membranes displayed asymmetry potentials which varied according to the pH of the fluid to which they were exposed and that they possessed a high degree of permeability to the lipid-soluble undissociated molecules of nicotine and salicyclic acid. Changes in degree of ionisation by structural modifications also changed the rate of permeation through these membranes. Brodie (1964), and Schanker, Tocco, Brodie & Hogben (1961), observed that gastrointestinal epithelium, renal tubular epithelium, blood brain barrier, and boundaries of various tissue cells, acted as lipid-like barriers preferentially permeable to lipid-soluble undissociated drug molecules with rates of transfer related to lipid-water partition coefficients and dissociation constants of drugs. It therefore appeared of interest to obtain data on the permeability characteristics through collodion-lecithin membranes of a series of biologically interesting compounds available in our laboratories. These were: ferrioxamine-B hydrochloride* (Bickel, Hall, Keller-Schierlein, Prelog, Vischer & Wettstein, 1960), glutethimide (Tagmann, Sury & Hoffman, 1952), thalidomide, salicylic acid, morphine, nalorphine and

From CIBA Ltd., Basle, Switzerland.

* Ferrioxamine-B is the Fe^{3+} complex of deferrioxamine, an amphoteric compound with one strongly basic aliphatic amino-group and three weakly acidic hydroxamic acid groups. The latter has the structure:



2,4-di(diethylamino)-6-(2-phenylacetylhydrazino)-1,3,5-triazine, a potent blocker of polysynaptic reflexes and a depressant of muscle spindle activity (Bein & Fehr, 1962).

Experimental

Purification of lecithin. The lecithin was purified egg lecithin (Merck) which was freed of cephalin by passing it in a methanolic solution through a column of Dowex 1 × 4 (50–100 mesh) resin in bicarbonate form according to the method of Perrin & Saunders (1960). The lecithin passed through and the cephalin was held on the resin column. The product was further purified using an ethyl methyl ketone-acetone mixture and lecithin was obtained as a waxy mass having an N:P ratio of 1·01. Weatherby (1943, 1949) used an ill-defined mixture of soybean and brain phosphatides.

Preparation of collodion-lecithin membranes. The method of preparation of membranes was similar to that used by Weatherby (1943). A 10 ml aliquot of a solution of collodion (1%) and lecithin (1%) in absolute ether-absolute ethanol (9:1) was pipetted over clean mercury contained in a petri dish of 110 mm diameter, the solvent was allowed to evaporate under controlled conditions for about 9 hr. The membrane was then cut out and immersed overnight in distilled water until ready for use. All membranes were prepared under similar controlled conditions. Their average thickness was 20 $\pm$ 2 μ using a Mikrotest magnetic thickness measuring instrument.

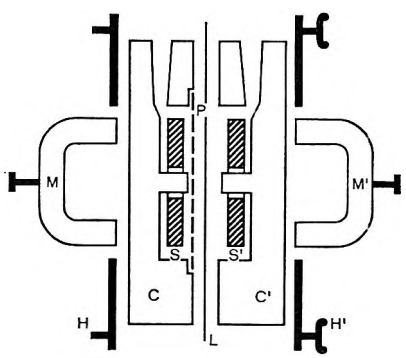


FIG. 1. Diffusion cell assembly. CC', circular glass cells with ground edges. SS', stirrers rotating on vertical axes. MM', magnets (synchronised). L, collodion-lecithin membrane. P, supporting round metal plate with circular holes. HH', aluminium circular holders with tightening screws.

Method of measuring diffusion characteristics. The diffusion experiments were run in the apparatus shown in Fig. 1 (the design of apparatus kindly supplied by Dr. Majer of CIBA Ltd., Basle). It consists of two circular ground glass cells CC' of capacity approximately 20 ml each. The cells are provided with magnetic stirrers SS' for continuous stirring of solutions in the compartments by synchronised motor. The membrane

is supported in place by a thin stainless steel round plate P with circular holes. After setting the membrane in place, cells are screwed together by two round aluminium gaskets on either side and mounted on a support assembly. A known concentration of drug, in buffers of known pH, was used on the right side and an equal volume of same buffer on the left side. In some experiments, an equal volume of distilled water was used in the left compartment. After suitable intervals, an aliquot of fluid was analysed. The diffusion of most compounds was studied in 0·1 m phosphate buffer pH 7·4, that of salicylic acid at two other pH values 2·5 and 8·5.

Estimations. Phenol red and salicylic acid were estimated by the method of Schanker & others (1958), aniline by Bratton & Marshall's method (1939), ferrioxamine-B by Tripod & Keberle's method (1962), glutethimide-^{14}C and thalidomide-^{14}C by liquid scintillation counting, Val^5-angiotensin-Asp-β-amide by paper chromatography, morphine and nalorphine by ultraviolet spectrophotometry in N hydrochloric acid solutions of appropriate dilutions.

Results and discussion

The compounds that did not diffuse through a collodion-lecithin membrane from a solution in 0·1M phosphate buffer at pH 7·4 buffer to water were (conc. μg/ml): phenol red (100), ferrioxamine-B hydrochloride (1600), *N*-[2-(β-ethoxyphenyl)acetyl]ferrioxamine-B hydrochloride (1600) and Val^5-angiotensin-Asp-β-amide (1000). This last compound showed marked degradation to peptides and amino-acids. These compounds also do not pass through *in vitro* rat intestinal preparations in significant concentrations (Misra, Hunger & Keberle, 1966).

The diffusion of all substances in Table 1, except thalidomide which has limited solubility in phosphate buffer pH 7·4, was studied at 1 mmolar concentration. The permeability constants for these compounds were obtained by using the equation derived by Lueck, Wurster, Higuchi, Lemberger & Busse (1957) for describing a diffusion process under quasi-steady state conditions in which two well-stirred liquids, either or both containing a solute, are separated by a barrier permeable to the solute:

$$\log (C_o - 2\,C_b) = -\frac{2\,K}{2{\cdot}303}\,t + \log C_o$$

where C_0 is the initial concentration of the penetrant in the solution on the side of origin, C_b the concentration of penetrant in receiving chamber, t the time of sampling. K is the permeability constant of the membrane, which, according to Lueck & others (1957), is defined as $K = A.D.(DC)/V.L$, where A is the cross-sectional area of the membrane; L is the thickness of the membrane; V is the volume of each of the two chambers; D is the diffusion coefficient; (DC) is the distribution coefficient between solution and membrane.

A plot of log ($C_o - 2\,C_b$) against time of sampling gave a straight line with a slope equal to $-2K/2{\cdot}303$ for at least 4 hr with the exception of salicyclic acid in acid medium.

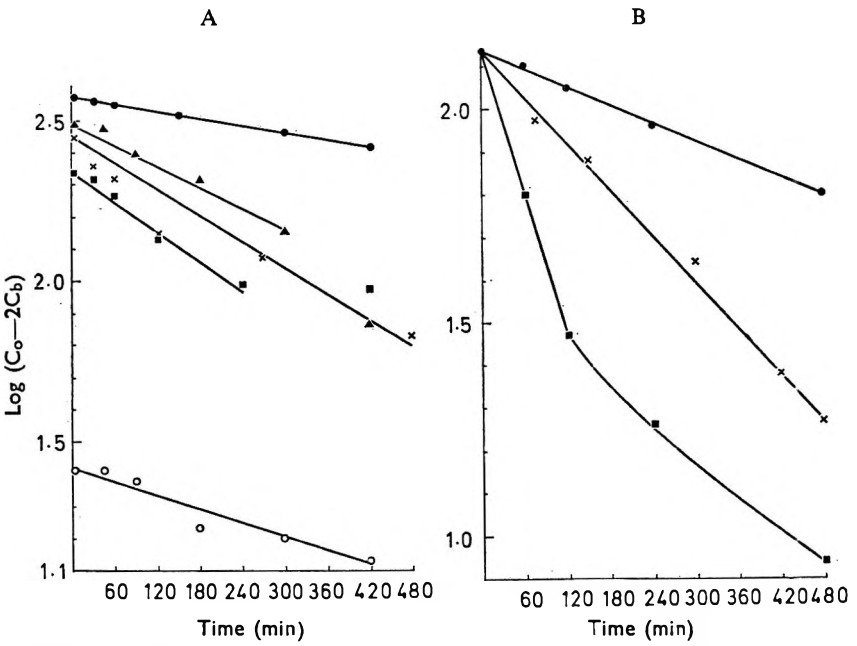


FIG. 2A. Diffusion drugs from solutions through a collodion-lecithin membrane. ●——● 2,4-Di(diethylamino)-6-(2-phenylacetylhydrazino)1,3,5-triazine 1 mmolar solution in 0·01 N hydrochloric acid; ▲——▲ nalorphine; ×——× morphine hydrochloride; ■——■ glutethimide-^{14}C; ○——○ thalidomide-^{14}C all in 0·1 mmolar solution in 0·1 M phosphate buffer pH 7·4.

B. Diffusion of salicylic acid from 1 mmolar solutions of different pH values through collodion-lecithin membrane. ●——● 0·1 M Borate buffer pH 8·5; ×——× 0·1 M phosphate buffer pH 7·4; ■——■ 0·1 M citrate buffer pH 2·5.

These plots are given in Fig. 2A. With salicylic acid (Fig. 2B) at pH 2·5 a straight line relationship is obtained only for the first 2 hr probably due to adsorption of acid on membrane.

Salicylic acid diffused two to three times faster at pH 7·4 and about eight times faster at pH 2·5 than at pH 8·5 (Table 1). Nalorphine diffused through collodion-lecithin membrane at pH 7·4 at a rate comparable to morphine. The triazine derivative diffused approximately 15 times slower at pH 2·0 than did salicylic acid at pH 2·5. The limited solubility at physiological pH of the triazine derivative provided indirect though insufficient evidence that the stomach could be an important site for its absorption. Glutethimide-^{14}C diffused more slowly than salicyclic acid at pH 7·4 and twice as fast as thalidomide-^{14}C. Glutethimide-^{14}C also permeated faster through *in vitro* rat intestinal preparations than did thalidomide-^{14}C.

The limited solubility of many pharmacologically potent lipid-soluble drugs in buffers and the affinity with which some drugs are adsorbed and held on the phospholipid membrane impose some limitations on the present method.

TABLE 1. PERMEABILITY CONSTANTS OF DRUGS THROUGH COLLODION-LECITHIN MEMBRANES

Compound	Conc. used (μg/ml)	pH of buffer used	Permeability constant ($K \times 10^4$) (min^{-1})
Salicylic acid	133	8·5	82
	138	7·4	205
	133	2·5	643
2,4-Di(diethylamino)-6-(2-phenyl-acetylhydrazino)-1,3,5-triazine	371·5	2·0	41
Nalorphine	311	7·4	120
Morphine hydrochloride	285	7·4	159
Glutethimice-^{14}C	217	7·4	177
Thalidomide-^{14}C	25·8	7·4	84

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Anticonvulsant and some neuropharmacological properties of 2-methyl-3-*o*-tolyl-6-chloro-4(3*H*)-quinazolone and related compounds

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Three 2-methyl-3-*o*-tolyl-4(3*H*)-quinazolone derivatives and five 3-*p*-bromophenyl-4(3*H*)-quinazolone derivatives were screened for oral anticonvulsant, analgesic and hypothermic properties. The anticonvulsant properties were assessed using a modified maximal electroshock seizure test and the leptazol threshold test. 2-Methyl-3-*o*-tolyl-6-chloro-4(3*H*)-quinazolone, being the compound with the greatest anticonvulsant action, was examined in more detail. It is about 1½ times more potent than phenytoin sodium against electroshock induced convulsions, and its potency ratio with phenobarbitone is 1·2 to 1. Against leptazol-induced convulsions, it is 10 times more potent than troxidone. Its sedative-hypnotic properties are much less than those of phenobarbitone sodium and it also has a much weaker potentiating action on phenobarbitone-induced hypnosis. There is no reduction in the spontaneous motor activity of normal and amphetamine-stimulated rats at its ED50 dose level. None of the compounds possesses any analgesic or hypothermic properties.

SOME quinazol-4-one derivatives possess potent hypnotic and other neuropharmacological properties (Gujral, Saxena & Tiwari, 1955; Gujral, Kohli & Saxena, 1955). As some hypnotic drugs and their chemical congeners possess anticonvulsant activity, Gujral, Saxena & Tiwari (1957) screened some of the quinazol-4-one compounds for anticonvulsant action and found 2-methyl-3-*o*-tolyl-quinazol-4-one (QZ-2) quite potent against electroshock and leptazol-induced convulsions. Bianchi & David (1960) screened a series of 2,3-disubstituted quinazolones and found 2-methyl-3-*p*-bromophenyl quinazolone (BDH-1880) to be the most potent as an anticonvulsant in the same tests.

We have synthesised some 4(3*H*)-quinazolones and examined them for their anticonvulsant, hypothermic and analgesic actions. The chemical structure of the compounds is given in Table 1. As the compound 2-methyl-3-*o*-tolyl-6-chloro-4(3*H*)-quinazolone (compound No. 8) possessed marked anticonvulsant action, it was examined in greater detail for its anticonvulsant action, sedative hypnotic properties, hypnotic potentiation action and its effects on spontaneous motor activity. Its ED50, LD50 and therapeutic indices for anticonvulsant action were also calculated. Troxidone, phenytoin sodium and phenobarbitone sodium were reference standards. The compound No. 8 was synthesised by Salimath, Patel & Shah (1956). It crystallises from dilute ethanol in colourless granules, m.p. 167°, is soluble in ethanol but insoluble in water M 284·5; the molecular formula is $C_{16}H_{13}ClN_2O$.

Experimental

The compounds were administered in 5% gum acacia such that the dose/g body weight was contained in 1 ml; control groups received 5% gum acacia only. Drugs were given orally to rats of 80–120 g weight, and injected intraperitoneally in mice of 15–25 g. Animals were allowed

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free access to food and water, except during experiments, because starvation modifies the supramaximal-electroshock seizure pattern test by prolonging the tonic-extensor component, and reduces the threshold for minimal electroshock seizure (Davenport & Davenport, 1948).

Anticonvulsant activity. The anticonvulsant activity of the quinazolones was assayed by a modificd maximal electroshock seizure test in normal albino rats, using corneal electrodes with current strength of 150 mA for 0·3 sec (Harned & others, 1953) and also by protection of albino rats against leptazol-induced convulsions (Swinyard, Brown & Goodman, 1952).

The compounds were administered orally in doses of 200 and 300 mg/kg, except compound No. 8 which was given in doses ranging from 5–300 mg/kg. In the electroshock test, convulsions were induced by convulsometer before the drug, to assess the positive response, and also 2 hr after the drug when the abolition of the hind leg extensor component was taken as the end-point. Leptazol convulsions were induced in rats by injecting 80 mg/kg in the loose subcutaneous tissue of the back 1 hr 50 min after giving the test drug.

Analgesic effect. The rat tail radiant heat method of Davies, Raventos & Walpole (1946) was used and the effect of drugs was noted at 30 min intervals for 2 hr. after the compounds in 200 and 300 mg/kg doses.

Hypothermic effect. Any change in body temperature in rats was observed by noting the rectal temperature 30 min before, and at 30 min intervals for 2 hr after the compounds in 200 and 300 mg/kg doses.

Neurological toxicity. This was studied before and after administration of the drug by neurotoxicity tests outlined by Swinyard & others (1952).

The preliminary screening showed compound No. 8 to be the most potent of the compounds examined; it therefore received further attention.

Sedative-hypnotic properties. The hypnotic activity of compound No. 8 was assessed in 10 albino mice. Three doses, 5, 10 and 20 mg/kg, were given intraperitoneally. Two control groups of 10 animals were also used; one was given phenobarbitone 5, 10 and 20 mg/kg and the other 5% gum acacia alone. The mice were observed for loss of righting reflex for 8 hr. Results were calculated in mice hr units.

Hypnotic potentiation activity. Compound No. 8 and phenobarbitone were given in doses of 5, 10 and 20 mg/kg intraperitoneally to groups of animals and another group was given 5% gum acacia. Pentobarbitone sodium was injected 30 mg/kg intraperitoneally 30 min after drug administration and the sleeping time of compound No. 8-treated group was compared with that of the phenobarbitone- and gum acacia-treated groups.

Spontaneous motor activity. The effect on spontaneous motor activity of compound No. 8 and phenobarbitone was examined in groups of albino rats in doses of 20, 40, 80 and 160 mg/kg. Each dose was given to five animals and the effect was assessed before and 2 hr after the drug.

The effect of drugs on amphetamine-stimulated rats was similarly studied. Amphetamine sulphate was given, 2 mg/kg intraperitoneally, 2 hr 45 min after drug administration.

Acute toxicity studies. Acute toxicity studies of compound No. 8 were made in rats, eight doses being used. The drug was administered orally and the animals were observed for 48 hr. The LD50 value was calculated by the method of Litchfield & Wilcoxon (1949).

Results

ANTICONVULSANT ACTIVITY

Modification of the supramaximal seizure pattern test. The results of assay of the eight compounds are summarised in Table 1. Of these, compound No. 8 was the most potent. According to the Litchfield & Wilcoxon method, the oral ED50 of compound No. 8 was 18 mg/kg (27·9–11·6); that of phenytoin sodium was 28 and phenobarbitone 22 mg/kg.

TABLE 1. ANTICONVULSANT ACTIVITY OF 4(3H)-QUINAZOLONES AGAINST ELECTRO-SHOCK AND LEPTAZOL INDUCED CONVULSIONS

Compound No.	Protection against maximal electro-shock seizure pattern test				Protection against leptazol-induced convulsion			
	Dose 200 mg/kg		Dose 300 mg/kg		Dose 200 mg/kg		Dose 300 mg/kg	
	No. of rats used	Protection %	No. of rats used	Protection %	No. of rats used	Protection %	No. of rats used	Protection %
2-Methyl-3-*p*-bromophenyl-6-bromo-4(3*H*)-quinazolone (1)	20	10	20	10	20	20	20	25
2-Methyl-3-*p*-bromophenyl-6,8-dibromo-4(3*H*)-quinazolone (2)	10	Nil	10	30	10	Nil	10	20
2,6-Dimethyl-3-*p*-bromophenyl-4(3*H*)-quinazolone (3)	10	Nil	10	20	10	Nil	10	40
2-Methyl-3-*p*-bromophenyl-6-chloro-4(3*H*)-quinazolone (4)	10	10	20	40	10	20	10	40
2-Methyl-3-*p*-bromophenyl-6-chloro-8-bromo-4(3*H*)-quinazolone (5)	10	Nil	10	20	10	Nil	10	30
2-Methyl-3-*o*-tolyl-6,8-dibromo-4(3*H*)-quinazolone (6)	10	Nil	10	20	10	Nil	10	20
2-Methyl-3-*o*-tolyl-6-chloro-8-bromo-4(3*H*)-quinazolone (7)	20	65	20	75	30	70	30	70
2-Methyl-3-*o*-tolyl-6-chloro-4(3*H*)-quinazolone (8)	30	100	30	100	40	80	40	90
Dilantin sodium 80 mg/kg	30	90	—	—	—	—	—	—
Troxidone 500 mg/kg	—	—	—	—	30	90	—	—

Leptazol threshold test. Results are given in Table 1. Compound No. 8 was found to be the most potent; its oral ED50 was 23 mg/kg (11–48) while that of troxidone was 200 mg/kg.

Analgesic and hypothermic activity. Of the eight compounds tested, none was found to have any analgesic or hypothermic activity.

Sedative-hypnotic properties. The mean sleeping time in the untreated control groups (gum acacia treated rats) was 21 mice-hr units and with

the approximate ED50 dose of compound No. 8 and phenobarbitone (about 20 mg/kg in both instances) it was 34 mice-hr units and 50·8 mice-hr units respectively.

Hypnotic potentiation. The mean onset of hypnosis with phenobarbitone treated rats was 1 hr 15 min, and with compound No. 8 it was 14 min. Other data are in Table 2.

TABLE 2. HYPNOTIC POTENTIATING EFFECT OF COMPOUND NO. 8 AND PHENOBARBITONE ON PENTOBARBITONE-INDUCED HYPNOSIS. ROOM TEMPERATURE, 28°

Drug administered intraperitoneally mg/kg	Pentobarbitone sodium intraperitoneally mg/kg	Mean sleeping time in min	Potentiation %	P value compared with phenobarbitone
Compound No. 8				
5	30	38 ± 3·5	58	
10	30	45·5 ± 5·4	90	<0·05>0·001
20	30	53 ± 7·0	121	<0·05>0·001
Phenobarbitone				
5	30	48·5 ± 5·2	102	
10	30	62·5 ± 6·8	160	
20	30	68·5 ± 9·2	185	
Gum acacia 5%	30	24 ± 2·2	—	

Spontaneous motor activity. There was no reduction in spontaneous motor activity of normal and amphetamine-stimulated rats at doses of 20 and 40 mg/kg orally with compound No. 8. A reduction produced by this compound at a dose of 160 mg/kg was about the same as that produced by 40 mg/kg of phenobarbitone orally, both in normal and amphetamine stimulated rats.

The experimental results indicate that compound No. 8 is a compound with some promise, being more active than phenobarbitone without the drawback of sedative-hypnotic effect. Its LD50 is 6 g/kg. The therapeutic index is above 250.

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A note on perfusion with labelled compounds to determine intestinal absorption

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An *in vivo* perfusion technique has been adapted for the measurement of intestinal absorption of labelled compounds. Salicylic acid, labelled with ^{14}C, in solution with or without phenylephrine or caffeine, was perfused through the ligated and cannulated small intestine *in situ* of the rat. Absorption was estimated from the difference in count of the solutions entering the gut and the outflow which was sampled at 10 min intervals. Some 40–45% of the acid was found to be absorbed at each of three consecutive 10 min samplings. This was lower than, but consistent with, reported results derived by chemical assay. In the presence of the other two drugs the absorption of salicylic acid increased to 70%.

SEVERAL *in vivo* procedures for determining intestinal absorption have been described (e.g. Cori, 1925; Sheff & Smyth, 1955; Nissem & Smith, 1965). Shanker, Tocco, Brodie & Hogben (1958) described a procedure for determining intestinal absorption in anaesthetised rats. Buffer solutions containing the drugs or compounds are perfused, at a rate which can be varied, through the intestine from the duodenal to ileal end by means of a perfusion pump. The ileal outflow is collected and the degree of absorption determined from the difference of concentrations of the compound entering and leaving the intestine as determined by chemical analysis. We have used this procedure, with suitable modifications, to measure the uptake of salicylic acid. This drug was chosen because of its wide use both as a single component and in combination with other drugs. The final analysis was made by radioisotope activity measurement.

Experimental

Preparation. Female Wistar rats, 80–100 g, were fasted for 24 hr and anaesthetised with pentobarbitone, 35 mg/kg, administered intraperitoneally. A midline incision was made in the abdominal region, the intestine exposed and cannulated at the duodenal and ileal ends with tygon cannulae having an inside diameter of 2·5 mm and outside diameter of 3·5 mm. The stomach and caecum were closed off by ligatures, taking care not to occlude major blood vessels. The intestine was replaced in the abdomen, the incision clamped and perfusion through the intact small intestine initiated immediately.

Perfusion procedure. Drug solution (300 ml) in a 400 ml Erlenmeyer flask was placed into a constant temperature water bath and maintained at 37°. The solutions were perfused through the intestine at a rate of 1·5 ml/min by means of a Sigmamotor Peristaltic pump (Sigmamotor, Inc., Model A–L–4–F, Middleport, N.Y.) The level of the solutions was such that flow through the preparation was not influenced by gravity or hydrostatic pressure.

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The perfusion rate was adjusted before each perfusion. The small intestine was cleared of particulate matter by perfusing for 30 min with drug-free solution and then for 30 min with drug solution to displace the first wash. Perfusion was continued for a further 30 min with collections of the ileal outflow in separate 10 min intervals. Three 0·1 ml samples of each 10 min collection were taken for counting.

The procedure was repeated with 5 animals for each drug solution. The drug solutions employed were as follows (mM/litre water). Solution 1. Salicylic acid, 1·0; NaCl, 145·0; KCl, 4·56; $CaCl_2$, 1·26; Na_2HPO_4, 1·33; NaH_2PO_4, 0·33. Solution 2. Salicylic acid, 1·0; phenylephrine HCl, 0·03; NaCl, 145·0; KCl, 4·56; $CaCl_2$, 1·25; Na_2HPO_4, 1·33; NaH_2PO_4, 0·33. Solution 3. Salicylic acid, 1·0; caffeine, 0·09; NaCl, 145·0; KCl, 4·56; $CaCl_2$, 1·25; Na_2HPO_4, 1·33; NaH_2PO_4, 0·33.

The ratio of phenylephrine and caffeine to salicylic acid was similar to that in commercial preparations. For the study, ^{14}C-carboxyl labelled salicylic acid was added to each solution, 5 μc/300 ml, and each solution thoroughly agitated to give a homogeneous mixture. The radiochemical purity of the labelled salicylic acid was established by paper partition chromatography and autoradiography.

Counting procedure. The 0·1 ml volumes of perfusate obtained from each animal at the various time intervals were added to 10 ml of a liquid scintillation solvent system in glass counting vials [diphenyloxazole 0·4%, naphthalene 5·0%, cellosolve 300 ml, dioxane 300 ml and toluene to 1000 ml: Baxter, Fanning & Swartz (1964)]. The vials were sealed with screw caps and agitated to ensure a homogeneous mixture.

The initial activity was determined by taking 0·1 ml samples of the solutions before perfusion and adding these to 10 ml amounts of the liquid scintillation solvent system. All samples were stored for 24 hr at −20° in the detector deep freeze for dark and temperature adaptation.

The activity of each sample was determined at the balance point for ^{14}C by means of a liquid scintillation detector and associate beta spectrometer (Ekco Model N664A and Scaler Model N610A). A blank sample was used to measure the background. The true net cpm for each sample

TABLE 1. INTESTINAL ABSORPTION OF SALICYLIC ACID[1]

Time interval (min)	Total absorption %		Absorption %/g bodyweight	
	$\bar{X}$	Sx	$\bar{X}$	Sx
Salicylic acid alone				
10	40·7	8·2	0·49	0·12
20	43·2	9·3	0·51	0·14
30	44·9	9·8	0·51	0·06
Salicylic acid + phenylephrine				
10	67·7	15·0	0·61	0·16
20	68·6	16·4	0·62	0·17
30	67·3	10·4	0·60	0·12
Salicylic acid + caffeine				
10	71·5	15·1	0·63	0·14
20	72·9	13·7	0·64	0·15
30	73·3	13·6	0·65	0·14

[1] Values are arithmetic means of 5 animals.

was determined and corrected for quenching by means of an internal standard of hexadecane-1-^{14}C.

The percentage absorption at each time interval for each sample was calculated from the loss of activity from the initial solution. These values are in Table 1.

Preliminary perfusion through the tygon tubing and cannula with labelled salicylic acid drug solution established that there was no loss of initial activity due to absorption or adsorption in the tubing.

Results and discussion

The intestinal absorption of salicylic acid was observed to be 40·7, 43·2 and 44·9% at time intervals of 10, 20 and 30 min. These values are lower but consistent with those reported by Shanker and his associates (1958), using chemical analysis. The radioisotope approach does appear to offer several distinct advantages. The assay procedure is simple particularly when compared to the complexity of chemical analytical methods, and offers an unlimited sensitivity and specificity. In this study millimoles of salicylic acid were evaluated with the use of low microcurie levels of activity. The specificity is illustrated by the use of the identical counting procedure to determine the absorption of salicylic acid in the presence of caffeine and phenylephrine. The latter is of particular interest as chemical analytical methods could not distinguish between phenylephrine and salicylic acid. This problem would undoubtedly be true for many drug combinations. The presence of phenylephrine or caffeine was observed to increase the intestinal absorption of salicylic acid.

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Inhibition of amine uptake in tubero-infundibular dopamine neurones and in catecholamine cell bodies of the area postrema

SIR,—Previous studies using the histochemical fluorescence method (*cf.* Hillarp, Fuxe & Dahlström, 1966) have demonstrated the existence of tubero-infundibular dopamine neurones, the terminals of which converge on the primary capillary plexus of the hypophysial portal system (Fuxe, 1963; 1964; Fuxe & Hökfelt, 1966; Lichtensteiger & Langemann, 1966). Since these dopamine nerve terminals and their non-terminal axons lie outside the blood-brain barrier as do the catecholamine cell bodies of the area postrema (Fuxe & Hillarp, 1964) it has proved possible to examine the uptake of catecholamines in these neurones. It was found that these neurones, like the peripheral adrenergic neurones (Hamberger, Malmfors, Norberg & Sachs, 1964; Malmfors, 1965), have an amine uptake-concentration mechanism which is unaffected by reserpine treatment (Fuxe & Hillarp, 1964). *In vitro* experiments by Hamberger & Masuoka (1965) on the uptake of amines in brain slices showed that most of the central dopamine and noradrenaline neurones appear to have this mechanism. Experiments with the peripheral and central noradrenergic neurones (Malmfors, 1965; Carlsson, Fuxe, Hamberger & Lindqvist, 1966) showed that this uptake-concentration mechanism (the "membrane pump"), which in all probability is localised at the level of the nerve cell membrane, can be blocked by desipramine and (+)-amphetamine but not by reserpine. The present investigation aimed at examining the effects of these drugs on the uptake mechanism of the tubero-infundibular dopamine neurones and that of the catecholamine cell bodies of the area postrema and of the superior cervical ganglia in reserpine-treated animals.

α-Methylnoradrenaline (0·2–1 mg/kg) and dopamine (1–5 mg/kg) were given slowly in the sublingual vein of male Sprague-Dawley (200–250 g) rats, reserpine-pretreated (10 mg/kg, i.p., 4–12 hr before killing) to block the uptake in the amine granules and deplete the endogenous monoamines (Carlsson, 1965). In the experiments with dopamine, nialamide (100 mg/kg, i.p.) was given 3 hr before death. The rats were killed 15 min after the amine injection by decapitation under light ether anaesthesia. To certain groups of rats, desipramine (25 mg/kg, i.p.) and (+)-amphetamine (10–25 mg/kg i.p.) respectively were administered 30 min before the amine injection. The animals were killed and the medulla oblongata, diencephalon and the superior cervical ganglia were dissected, freeze-dried and treated with formaldehyde gas for 1 hr (Dahlström & Fuxe, 1964; Hamberger, Malmfors & Sachs, 1965). From all animals iris stretch preparations were made (Malmfors, 1965). The catecholamine cell bodies of the area postrema and of the superior cervical ganglia were studied only after α-methylnoradrenaline injection.

In the rats treated with reserpine or reserpine-nialamide alone, no fluorescent dopamine nerve terminals could be seen in the external layer of the median eminence, nor could any catecholamine cell bodies be distinguished in the area postrema. After α-methylnoradrenaline or dopamine injection, green fluorescent nerve terminals and non-terminal axons appeared in the median eminence, in the rat iris and around the basal arteries with low to strong intensity depending on the dose used. The catecholamine nerve cell-bodies of the area postrema and the superior cervical ganglia appeared with a green fluorescence of medium to strong intensity. If desipramine was given before the amine injection, no fluorescence appeared in the area postrema catecholamine cells, whereas the appearance of fluorescence in the dopamine nerve terminals and non-terminal axons of the median eminence could not be prevented. However, in the same

animal the uptake of amine into the noradrenaline nerve terminals of the iris and of the basal arteries was completely blocked. This was true also for the noradrenaline cell bodies of the superior cervical ganglion. If, on the other hand, (+)-amphetamine was given, the amine uptake was completely blocked both in the dopamine nerve terminals and non-terminal axons of the median eminence and in the catecholamine cell bodies of the area postrema. The uptake of amine was blocked also in peripheral noradrenaline cell bodies and terminals.

The present paper gives further evidence for the view that the "membrane-pump" is distributed along the entire catecholamine neurone (cell body, axon, terminal) since the uptake is blocked in all parts of the neurone. The findings strongly suggest that desipramine is active only on noradrenaline neurones, and not on dopamine neurones, whereas amphetamine is able to block the uptake of dopamine or α-methylnoradrenaline in both noradrenaline and dopamine neurones. Thus, fundamental differences probably exist between the dopamine and noradrenaline neurones. Experiments with brain slices and on the dopa-induced amine accumulation in the brain have given similar results (*cf.* Carlsson & others, 1966).

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Central origin of the lenticular opacities induced in mice by opiates

SIR,—Weinstock (1961) showed that opiates injected subcutaneously or intraperitoneally induced a transient clouding of the lens of the mouse. This activity of the opiate was shown to parallel its analgetic activity and suggested a similarity of the receptors for each response. The lenticular response was thought to originate within the eye particularly because the uptake of tritiated levorphanol in the lens and in the brain was found to be virtually equal after intraperitoneal injection of labelled levorphanol (Smith, Karmin & Gavitt, 1966a). Furthermore, the lenticular effect of parenteral levorphanol was potentiated when concentrated adrenaline solutions were instilled into the eye. However, the fact that tolerance to the lenticular effect of opiates could be blocked by actinomycin or puromycin (Smith, Karmin & Gavitt, 1966b) suggested a more complex etiology for the opacity phenomenon.

We have now given levorphanol intracerebrally in a dose (22 μmol/kg) too low to produce a significant incidence of opacities when given intraperitoneally, and one third of the mice developed opacities (Table 1).

TABLE 1. THE INCIDENCE OF LENTICULAR OPACITIES PRODUCED BY INTRACEREBRAL INJECTION OF LEVORPHANOL COMPARED WITH INTRAPERITONEAL INJECTION IN FEMALE SWISS-WEBSTER MICE (20–25 G)

Treatment	No. Mice	No. Opacities	% Opacities	Deaths
Levorphanol, 22 μmol/kg (i.c.) (given in 30 μl at pH 7)	45	15	33	11
Levorphanol, 22 μmol/kg (i.p.)	25	1	4	0
0·9% Saline, 30 μl (i.c.)	29	0	0	0

The ED50 for intraperitoneal levorphanol was 118 μmol/kg.

As further evidence for a central origin of the lenticular effect, levorphanol-^{3}H, with a specific activity of 5 μc/μmol and diluted with carrier to provide a dose of 22 μmol/kg in a volume of 30 μl, was injected intracerebrally into the right cerebral hemispheres of mice. Thirty min after injection the mice were killed by decapitation and the brains, lenses, hearts and plasma separated. The tissues except for the lenses were washed in saline, homogenised in 0·1N hydrochloric acid and the clear supernatant counted in a liquid scintillation spectrometer (Smith & others, 1966a). The results were as follows: [tissue (no. of animals) μc/g or ml ± s.e.]: cerebrum (right) (5) 256 ± 46; cerebrum (left) (5) 186 ± 76; brain stem (4) 183 ± 81; plasma (5) 23·2 ± 6·5; heart (5) 23·4 ± 5·1; lens (4) 13·3 ± 5·0. Tissues of 2 mice were pooled for each independent value. Scans of the radiochromatographs prepared from brain stem or cortical extracts revealed only single radioactive areas. These areas corresponded to the carrier levorphanol. It appears that the labelled substance is concentrated in the region of the injection site but it is also present in high concentrations in stem and left cerebrum. The levorphanol-^{3}H seems to be transported from the injection site to other brain areas by cerebral fluid rather than by the blood because the blood has a relatively low concentration of radioactivity. The fact that the lens shows even less tritium than blood is further evidence in favour of a central origin.

It is therefore concluded that levorphanol induces lenticular opacities by a process initiated in the brain. Because of the previously demonstrated interaction between opiates and catecholamines to produce opacities, adrenergic nerves may be involved in this process.

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Effect of probenecid on the level of homovanillic acid in the corpus striatum

SIR,—Active transport mechanisms seem to be involved in the removal of organic acids from the cerebrospinal fluid to the blood, as evidenced by perfusion and clearance experiments with the ventriculocisternal system *in vivo* (Pappenheimer, Heisey & Jordan, 1961; Prockop, Schanker & Brodie, 1962). The different substances tested, for example, *p*-aminohippuric acid and diodrast, compete for the same saturable transfer processes, which seem to be similar to those found in the renal tubules.

Probenecid reduces the renal excretion of a variety of organic acids, which includes acid monoamine metabolites such as 5-hydroxyindoleacetic acid (Despopoulos & Weissbach, 1957) and homovanillic acid, derived from the amines 5-hydroxytryptamine and dopamine, respectively. These amine metabolites also occur in the brain, with about the same distribution as the corresponding amines (Roos, 1962; Andén, Roos & Werdinius, 1963; Bernheimer, 1964). The present experiments investigated whether probenecid, given alone and in combination with reserpine or haloperidol, would interfere with the levels of homovanillic acid in the brain.

Adult hooded rats of either sex, five animals in each experiment, were treated with probenecid (50, 100 or 200 mg/kg i.p.), followed after 30 min by reserpine (10 mg/kg i.p.) or haloperidol (2 mg/kg i.p.). After another 3 hr the homovanillic acid was measured fluorimetrically in the pooled corpora striata (Andén & others, 1963). Control groups were run with none, or only one, of the drugs for the corresponding time intervals. The results are given in Table 1. In a few experiments (data not shown) dopamine was assayed fluorimetrically (Carlsson & Waldeck, 1958; Carlsson & Lindqvist, 1962).

TABLE 1. LEVELS OF HOMOVANILLIC ACID IN THE CORPUS STRIATUM OF RATS, 3·5 HR AFTER VARIOUS DOSES OF PROBENECID

Probenecid mg/kg i.p.	Homovanillic acid µg/g		
	Controls	Reserpine treated	Haloperidol treated
0	0·2 ± 0·06 (3)	0·6 ± 0·15 (3)	1·5; 1·4 (2)
50		0·6; 0·9 (2)	
100		1·2; 1·1 (2)	
200	0·5 ± 0·07 (3)	1·9 ± 0·15 (3)	2·7 ± 0·35 (3)

Reserpine (10 mg/kg i.p.) or haloperidol (2 mg/kg i.p.) was given 30 min after probenecid. The values are means ± s.e. of the means. Figures in brackets indicate number of experiments. Each experiment was performed on five pooled organs.

Normally, the concentration of homovanillic acid in the corpus striatum is rather low in rats, about 0·2 µg/g tissue (Juorio & Vogt, 1965; Juorio, Sharman & Trajkov, 1966), compared to that of dopamine (3–4 µg/g). Reserpine

produced a threefold increase in homovanillic acid in 3 hr, probably due mainly to release and breakdown of the stored dopamine, which had disappeared almost completely. The increase in homovanillic acid corresponded to only about 10% of the released dopamine, indicating a rapid removal of homovanillic acid, or the existence of alternative metabolic pathways for dopamine (see Juorio & others, 1966).

Pretreatment with probenecid, 200 mg/kg, given 30 min before reserpine increased the homovanillic acid level to about 1·9 μg/g in 3 hr, i.e. tenfold. Smaller doses of probenecid in combination with reserpine caused less pronounced increases. With probenecid alone, 200 mg/kg, 3·5 hr, there was a more moderate, two- to threefold increase in homovanillic acid, comparable with that produced by reserpine alone, while the dopamine level was not significantly changed. Reserpine and probenecid thus seem to increase the homovanillic acid concentration in the corpus striatum by different and independent mechanisms.

Haloperidol also produced a clear increase in homovanillic acid in 3 hr, without affecting the dopamine level. The increase has been attributed to an impaired elimination of the metabolite or, probably more important, to an increased amine turnover secondary to receptor blockage (Andén, Roos & Werdinius, 1964; Roos, 1965). However, since probenecid pretreatment nearly doubled the homovanillic acid level, the mechanisms which increase the acid are probably not identical for haloperidol and probenecid.

The results could be explained by an active transfer mechanism, which removed homovanillic acid from the brain tissue and which was inhibited by probenecid.

Acknowledgements. This study was supported by a grant from the Faculty of Medicine, University of Göteborg, Sweden. For generous supplies of drugs I am indebted to Astra Ltd., Södertälje (probenecid), Swedish Ciba Ltd., Vällingby (reserpine), and Leo Ltd., Hälsingborg (haloperidol). For technical assistance I thank Miss Gun Alfredsson and Mrs. Ilona Olofson.

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Potentiation of the noradrenaline-releasing action of tyramine by monoamine oxidase inhibitors

SIR,—Severe hypertension has been reported in patients treated with monoamine oxidase inhibitors after eating cheese, beans, or certain yeast extracts (Blackwell, 1963; Blackwell, Marley & Mabbitt, 1965). The tyramine content of these foods is considered to be the principal pressor substance (Blackwell & Mabbit, 1965; Horwitz, Lovenberg, Engelman & Sjoerdsma, 1964). I now report the influence of monoamine oxidase inhibition on the noradrenaline-releasing effect of tyramine in rats.

Adult Wistar male albino rats, 200 to 300 g, were given intraperitoneally pargyline 80 mg/kg. Twelve hr after this treatment the rats were deprived of food and 24 hr after the drug they were given different doses of tyramine by mouth. Four hr after the last treatment the rats were killed and their hearts were analysed for noradrenaline fluorometrically (Brodie, Dablac & Costa, 1966). Control animals fasting for 12 hr received tyramine only. The results (Table 1) demonstrate that the monoamine oxidase inhibitor potentiated the effect of tyramine in depleting noradrenaline and indicate that the enhancement of the pressor effects of tyramine by mouth (Tedeschi & Fellows, 1964) is caused by an increased release of peripheral noradrenaline.

TABLE 1. AUGMENTATION BY PARGYLINE OF THE EFFECT OF TYRAMINE IN DEPLETING NORADRENALINE IN RATS

Treatment (drugs in mg/kg)	Heart noradrenaline, µg/g (mean and range), 4 hr after the depleting agent
Controls	0·83 (0·80–0·85)
•Tyramine 12·5	0·77 (0·75–0·78)
" 25·0	0·65 (0·61–0·67)
" 50·0	0·48 (0·46–0·50)
Pargyline (80 mg)	0·94 (0·93–0·95)
Pargyline (80 mg) + tyramine (12·5)	0·92 (0·86–0·95)
" + " (25·0)	0·50 (0·40–0·52)
" + " (50·0)	0·21 (0·18–0·25)
" + " (25·0) + ••(desipramine (10)	0·80 (0·75–0·87)

• Tyramine hydrochloride diluted in saline was given by mouth, 1 ml/100 g, 24 hr after pargyline.
•• Desipramine was given intraperitoneally 20 min before the tyramine.

Both the hypertensive and noradrenaline-depleting actions of tyramine are known to be blocked by desipramine (Gessa, Vargiu & Crabai, 1966) and it is interesting that the experiments show an analogous effect in monoamine oxidase inhibited animals.

Acknowledgement. Abbott Laboratories, North Chicago, Ill., kindly supplied the pargyline.

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Differences in the response to insulin of pathogen-free mice and mice bred conventionally

SIR,—In 1961 comparisons were made of the responses to insulin of mice from a number of strains at room temperature (21°). Since 1961 these strains have been transferred into pathogen-free buildings by caesarian derivation and one or two of the strains were re-tested. Alterations in response cannot be attributed solely to the lack of pathogen burden, since in the five intervening years a number of generations have been produced; and also considerable genetic drift may have ensued particularly in the random bred LAC grey mice.

TABLE 1. THE REACTION TO INSULIN OF STARVED CONVENTIONALLY BRED MICE COMPARED WITH THAT OF STARVED PATHOGEN-FREE MICE

Strain	No. of tests	Approx. ED50 milliunits/kg mouse	Mean slopes of regression
	Conventionally bred mice at 21° (1961)		
LAC grey	2	1,845	1·28
DBA/1	2	875	2·84
	Pathogen-free mice at 21° (1966)		
LAC grey	2	12,050	1·87
DBA/1fCFWLac ..	2	3,250	4·10
	Pathogen-free mice at 33°		
LAC grey	2	2,450	4·54
DBA/1fCFWLac ..	2	1,700	3·31

Table 1 gives the relevant comparisons of the ED50 values and of the mean slopes of the regression lines. Both strains of mice are now much less sensitive to insulin at 21° (six and three times less sensitive). At 33° the mice are still not as sensitive as the original mice at 21°. With both strains of mice the precision of the reaction is increased but the difference is less than significant ($P = 0·7$ $P = 0·2$). These results are in agreement with the statement made by Davey (1962) that pathogen-free mice are less sensitive than conventionally bred mice to toxic substances.

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Fluorimetric detection of tranylcypromine in urine

SIR,—Trans-2-phenylcyclopropylamine (tranylcypromine) is a potent inhibitor of monoamine oxidase (Maas & Nimmo, 1959) and is widely used as an antidepressant agent. At present there is no specific chemical method for its detection in the amounts in which it occurs in biological tissues. An investigation was made to determine whether the compound possessed fluorescent properties.

Fluorescence was measured by an Aminco-Bowman spectrophotofluorimeter (Cat. No. 4–8202B) fitted with Aminco thermoelectric cooler and X-Y recorder.

At an emission of 290 mμ the compound possessed two excitation peaks, at 220 and 270 mμ. A small peak observable at 290 mμ was due to Rayleigh and Tyndall scatter (Udenfriend, 1962). When excited at 220 mμ, there was a main peak at 290 mμ and a smaller second-order emission peak at 565 mμ (uncorrected). A similar spectrum was obtained with excitation at 265 mμ.

When solutions of tranylcypromine sulphate were excited at 220 mμ, the intensity of fluorescence measured at 290 mμ, was proportional to concentration from 0·1–4 μg/ml. Above 4 μg/ml the curve deviated from linearity due to self-absorption. The intensity of fluorescence was increased by decreasing the temperature of the solution from 15–5°. In an investigation of the effect of pH on the intensity of fluorescence of a 10 μg/ml solution, it was maximal below pH 7, and at greater pH values fell away rapidly, no fluorescence being observable above pH 11. It was found that the pH at which the intensity had fallen to that equivalent to a 5 μg/ml solution was 8·4, which agrees well with the value for the pH of 8·55 determined by potentiometric titration. This suggests that it is the ionised species ($R \pm NH_3$) and not the free base ($R - NH_2$) which is the fluorescent form.

Detection in urine. Preliminary investigations showed that benzene was a suitable solvent for this extraction procedure.

Urine (15 ml) to which 75 μg of tranylcypromine had been added was placed in a 100 ml glass-stoppered centrifuge tube and adjusted to pH 11–14 with 5N NaOH. Benzene (30 ml) was added and the tube was shaken mechanically for 20 min. It was then centrifuged and the benzene layer removed by means of a dropping pipette and transferred to a tube containing 0·1N NaOH (5 ml). After shaking for 1 min to wash the benzene layer, the tube was centrifuged and a 25 ml aliquot of the benzene layer added to 0·1N H_2SO_4 (1 ml) in a 100 ml glass-stoppered centrifuge tube. This was shaken mechanically for 15 min and centrifuged. The acid layer was removed by means of a dropping pipette and transferred to a test tube. A further 1 ml of 0·1N H_2SO_4 was added to the benzene layer in the centrifuge tube which was shaken for another 15 min. It was then centrifuged and the acid layer transferred to the test tube containing the first acid extract. The bulked acid extracts were well mixed and a 0·5 ml aliquot removed and transferred to a test tube containing 0·1N H_2SO_4 (2 ml). After mixing this solution was cooled in a refrigerator for 30 min to 5°. Its fluorescence was then read at 220 mμ excitation and 290 mμ emission (uncorrected), and compared with that of a control blank urine which had been taken through the extraction procedure.

In 3 patients given single doses of tranylcypromine sulphate 20 mg, the drug appeared in the urine within 2 hr and disappeared after 12–16 hr. It was detected in all specimens from 8 patients receiving tranylcypromine sulphate, 10–20 mg daily, alone or in combination with trifluoperazine hydrochloride.

Quantitative estimations of tranylcypromine sulphate in urine are made difficult by interference in fluorescent activity by tyramine and tryptamine. Attempts to overcome this are being made.

Acknowledgements. Paul Turner is in receipt of a Wellcome Senior Research Fellowship in Clinical Science. We thank the Board of Governors of St. Bartholomew's Hospital for a grant to J. H. Young. Professor Taylor and Drs. Linford Rees and C. M. B. Pare kindly allowed us access to their patients. Smith Kline and French Laboratories Ltd., provided tranylcypromine sulphate (Parnate). Dr. D. M. G. Armstrong, Mr. C. J. Thompson and Miss E. P. Saunders gave valuable assistance.

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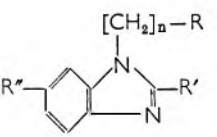
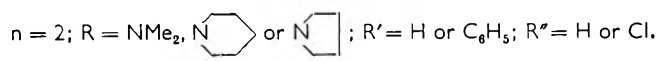
Inhibitory effect of 1-alkylbenzimidazoles on gastric secretion in the rat

SIR,—While examining the pharmacological effects of a new series of benzimidazole derivatives of general structure (I) we observed that they exerted an inhibitory effect on secretion of gastric acid and gastric juice.



(I)

The most active compounds are those in which



With the most active derivatives, the dose causing a 50% inhibition of secretion of gastric acid and gastric juice in the rat is 5 to 10 mg/kg intramuscularly, while the intramuscular LD50 values are about 200 mg/kg.

Some effects of one of the compounds lying in the middle of the range of activity are now described. The compound is 1-(2-piperidinoethyl)benzimidazole (H-635). The inhibitory effect of this compound on gastric secretion in the rat is seen in Table 1.

TABLE 1. THE EFFECT OF 1-(2-PIPERIDINOETHYL)BENZIMIDAZOLE ON GASTRIC SECRETION IN THE RAT

No. of animals	Dose mg/kg i.m.	Inhibition (%) of secretion of:		
		Free acid	Total acid	Gastric juice
12	9·0	38·9	42·2	26·4
25	12·0	74·2	60·6	51·6
27	25·0	78·8	61·6	66·3
	ED50 mg/kg i.m.	10·0	11·2	16·4

The compound also increases gastric emptying time and inhibits intestinal motility when given intramuscularly or orally. These actions are shown in Table 2.

TABLE 2. THE EFFECT OF 1-(2-PIPERIDINOETHYL)BENZIMIDAZOLE ON GASTRIC EMPTYING TIME AND INTESTINAL MOTILITY IN THE RAT

No. of animals	Dose mg/kg	Increase (%) in gastric emptying time	Inhibition (%) of intestinal motility *in vivo*
12	12·5 i.m.	66	25
12	25·0 i.m.	166	not investigated
12	50·0 oral	131	not investigated
12	100·0 oral	322	45·0

This compound has no effect on either the acetylcholine-induced contraction of excised rat duodenum *in vitro* or acetylcholine-induced vasodepression in cats and dogs *in vivo*. We consider the compound to selectively block parasympathetic but not sympathetic transmission for the following reasons.

1. The vasodepression caused by cervical vagal stimulation in the cat is abolished in doses which do not influence the contraction of nictitating membrane induced by preganglionic electrical stimulation of the superior cervical ganglion. At the same time, the compound fails to alter the vasodepressive reaction to intravenously injected acetylcholine.

2. The bradycardia and vasodepression due to thoracic vagal stimulation are abolished by doses which do not affect the tachycardia and vasopressor effect induced by preganglionic electrical stimulation of the stellate ganglion.

3. The rise in blood pressure after intravenous injection of tetramethylammonium bromide is not affected by pretreatment with H-635; this rise is, however, completely blocked by tetraethylammonium bromide, a ganglion blocking drug also inhibiting sympathetic ganglia.

The effect of H-635 in blocking parasympathetic transmission seems also to occur in the central nervous system. The compound inhibits the rage reaction evoked in conscious cats by intrahypothalamic injection of carbachol, a reaction with a pathway which involves cholinergic transmission sites and which is not affected by either reserpine, chlorpromazine or phenobarbitone.

In addition, the compound exhibits a general sedative action and antagonises or reduces the increased motility of mice seen after desoxyephedrine. Similar effects were observed with other members of this chemical series.

The selective blockade of parasympathetic transmission and the sedative effect exerted on the central nervous system in general, and on the hypothalamus in particular both seem to be responsible for the inhibitory action of these compounds on gastric secretion.

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Alkaloids from *Mitragyna javanica*, Koord. and Valeton and *Mitragyna hirsuta*, Havil

SIR,—We have isolated a number of alkaloids from ethyl acetate extracts of the leaves of *Mitragyna javanica*, Koord. and Valeton and *Mitragyna hirsuta*, Havil. The chemical constitution of two and possibly three new alkaloids is now presented (Table 1).

The extracts of *Mitragyna javanica* yielded ajmalicine, mitraphylline, isomitraphylline, Pa 7 [which may be identical with vineridine (Kasymov, Yuldashev & Yunusov, 1965a,b)] and *mitrajavine*, together with a yellow alkaloid, m.p. 238–240°.*

The extracts of *Mitragyna hirsuta* yielded mitraphylline, isomitraphylline, rhynchophylline, isorhynchophylline and *hirsutine*.

Mitraphylline, isomitraphylline, rhynchophylline, isorhynchophylline and ajmalicine were identified by comparison of the m.p., mixed m.p., $[\alpha]_D$, Rf values (thin layer chromatography), ultraviolet and infrared spectra of the isolated and authentic samples of these alkaloids.

The data obtained indicate that Pa 7 is an oxindole alkaloid of a methoxy-mitraphylline type (I; R = OMe). The nmr spectrum of Pa 7 (Table 2) shows a splitting pattern of the aromatic protons (ca. 3·4 τ, 2H; ca. 2·9 τ, 1H) which is similar in chemical shift and integral to the aromatic splitting pattern (ca. 3·4τ, 2H; ca. 2·8 τ, 1H) seen in the nmr spectrum of 6-methoxy-*N*-methyl oxindole but not in the nmr spectra of the 4,5 or 7-methoxy analogues. This indicates that the aromatic methoxy group of Pa 7 is in the 11 position of I. The 5–10 τ region of the nmr spectrum of Pa 7 differs from the corresponding region of speciophylline (Beckett, Shellard, Phillipson & Lee, 1965) but shows similarities to that of mitraphylline and isomitraphylline, suggesting that the C/D/E ring junctions are the same in Pa 7, mitraphylline and isomitraphylline.

Since the isolation and characterisation of Pa 7, Kasymov & others (1965a,b) have reported the constitution of vineridine and vinerine as being of the 11-methoxy-mitraphylline type. The m.p. of Pa 7 is similar to that of vineridine (179–180°) and although the reported optical rotations differ, vineridine +22·7° (*c*, 2·32, pyridine), it is possible that these two alkaloids are identical.†

The alkaloids mitrajavine and hirsutine were shown to be indoles by colour tests, and by ultraviolet, infrared and nmr spectra. The physical data, along with elemental analysis and equivalent weight determinations indicate the structure of mitrajavine to be that of a methoxy-ajmalicine type (II; R = OMe) with a molecule of water of hydration indicated by a two-proton singlet in the nmr spectrum at about 7·2 τ which disappears upon deuteration. The closed E ring is indicated by the presence of a three-proton doublet for the C19-Me (9·10 τ)‡ and the C19-H one-proton multiplet at about 5·6 τ. A *cis* C_3H orientation is indicated by the absence of any *trans* CH bands (below 2800 cm^{-1}, KCl disc) in the infrared spectrum (Wenkert & Roychaudhuri, 1956; Bohlmann, 1957; Rosen, 1961) and the presence of a one-proton multiplet in the nmr spectrum at 5·55 τ (Wenkert, Wickberg & Leicht, 1961a,b; Uskokovic, Bruderer, Planta, Williams & Brossi, 1964). The aromatic splitting pattern of mitrajavine (3·47 τ, 1H; 2·91 τ, 2H) is similar to that of mitragynine (Beckett,

* Found: C, 73·3; H, 4·7; N, 10·7; OMe, 0%; *M*, 313 (by mass spectroscopy); λmax (abs. ethanol) 256, log ϵ = 4·24; 285, log ϵ = 4·03; 294, log ϵ = 4·10; 305, log ϵ = 4·15; 380, log ϵ = 4·51; 398, log ϵ = 4·62. ν (KCl), 3450, 3280, 1642, 1605 cm^{-1}, base $HClO_4$, m.p., 254°. Found: C, 55·4; H, 4·1; N, 9·7%.

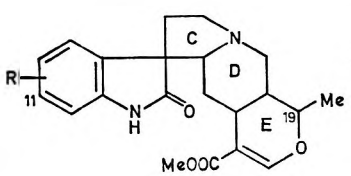
† Work is in progress to determine the identity or non-identity of the two alkaloids.

‡ This upfield position suggests that the methyl group is shielded by the indole ring.

Shellard & Tackie, 1965) (3·52 τ, 1H; 3·08 τ, 2H) showing that the aromatic methoxy group (6·10 τ) is in the 9 position of II. This evidence indicates that mitrajavine is a 9-OMe compound of the ajmalicine type (II, R = 9-OMe) with the C_3H *cis* to the nitrogen lone pair.

The physical data, along with elemental analysis and equivalent weight determinations indicate the structure of hirsutine to be of the corynantheidine-type (III). The methyl of the ethyl group is indicated by the three-proton triplet at 9·21 τ in the nmr spectrum. The ester and methoxy groups appear at 6·29 and

TABLE 1. PROPERTIES AND SPECTRAL DATA OF SOME *Mitragyna* ALKALOIDS



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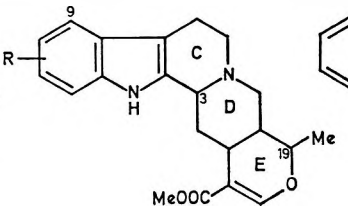

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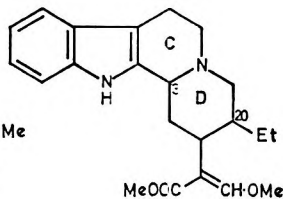

III

Analyses	C	H	N	OMe	Equiv. wt.
Structure I (R = OMe) requires for $C_{22}H_{26}N_2O_6$	66·3	6·5	7·0	15·5	398
Pa 7 found	66·0	6·45	7·45	11·5§	404†
Structure II (R = OMe) requires for $C_{22}H_{26}N_2O_4{\cdot}H_2O$ ..	66·0	7·0	7·0	15·5	400
Mitrajavine found	65·7	6·9	7·1	15·4	385†
Structure III requires for $C_{22}H_{28}N_2O_3$	71·7	7·6	7·6	16·85	368
Hirsutine found	70·4	8·3	7·7	17·8	370†

	Pa 7 I R = 11-OMe		Mitrajavine II R = 9-OMe		Hirsutine III	
Melting point (Recryst. from dry ether)	180°		117°		101°	
$\alpha]_{23}^{D} \pm 0{\cdot}5°$ ($CHCl_3$)	+77·4° (*c*, 0·195)		−37·6° (*c*, 0·26)		+68·6° (*c*, 0·32)	
Approx. Rf* (a) alumina/$CHCl_3$	0·50		0·80		0·70	
(b) silica gel/ether	0·10		0·05		0·0	
Ultraviolet spectra (abs. ethanol)	λ mμ	log ε	λ mμ	log ε	λ mμ	log ε
	220	4·97	227	4·47	226	4·64
	240	4·76	265	3·89	282	3·84
	282	4·15	292	3·73	290	3·79
	291	4·11				
Infrared spectra (Nujol) cm^{-1}						
–NH	3500		3500		3400	
Ester >C = O	1700		1685		1695	
>C = C<	1620		1615		1620	
Oxindole >C = O	1705		—		—	
C/D *trans* bands (KCl)	—		No bands between 2800 and 2500 cm^{-1}			
Derivative m.p. (from abs. ethanol)	methyl iodide 205°		hydrobromide 230°		methyl iodide 254°	
Analyses of derivatives	$C_{22}H_{28}N_2O_6{\cdot}CH_3I$		$C_{22}H_{28}N_2O_4{\cdot}HBr{\cdot}2H_2O$		$C_{22}H_{28}N_2O_3{\cdot}CH_3I$	
	Req.	Found	Req.	Found	Req.	Found
	C 51·1	51·6%	C 52·6	51·5%	C 53·9	53·4%
	H 5·4	5·8	H 6·2	6·1	H 6·1	6·1
	N 5·2	5·3	N 5·6	5·4	N 5·5	5·0

* Thin layer chromatography. Reference values: Mitraphylline, (a) 0·17, (b) 0·03. Ajmalicine, (a) 0·83, (b) 0·45. Corynantheidine, (a) 0·83, (b) 0·72.

† Titration in non aqueous media.

§ The presence of two methoxy groups is indicated in the nmr spectrum (Table 2).

TABLE 2. NMR SPECTRA OF SOME *Mitragyna* ALKALOIDS IN $CDCl_3$, 60 MC
(τ values, ppm from tetramethylsilane)

Protons	Pa 7 I	Mitrajavine II	Hirsutine-III
–C–*Me*	—	—	9·21 triplet
19 Me / –O R	8·88 doublet (J = 6·5 cps)	9·10 doublet (J = 6·5 cps)	—
–CO–OMe	6·41 singlet	6·29 singlet	6·29 singlet
–OMe	—	—	6·22 singlet
Ar–OMe	6·18 singlet	6·10 singlet	—
19 R / –O H	ca. 5·6 multiplet	ca. 5·6 multiplet	—
C_3H *cis*	—	5·55 multiplet	5·5 multiplet
aromatic	ca. 3·4 (2H) ca. 2·9 (1H) multiplets	3·47 (1H) 2·91 (2H) multiplets	ca. 2·7 (4H) multiplet
olefinic H	2·53 singlet	2·42 singlet	2·65 singlet
–N–	1·20* singlet	1·30* singlet	1·82* singlet

* Disappears on deuteration.

6·22 τ. A *cis* C_3H is demonstrated by the absence of *trans* CH bands (below 2800 cm^{-1}, KCl disc) in the infrared spectrum and a one-proton *cis* C_3H multiplet in the nmr spectrum at 5·55 τ. The absence of an aromatic methoxy group is shown by the lack of an aromatic three-proton singlet at about 6·15 τ and the presence of four aromatic protons (unsplit signal at about 2·7 τ). This evidence suggests that hirsutine is an alkaloid of the corynantheidine-type with the C_3H *cis* to the nitrogen lone pair.

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The effects of barbiturates on the activity of the catecholamine neurones in the rat brain

SIR,—Previous biochemical and histochemical studies have demonstrated the importance of neuronal impulse flow for the disappearance of catecholamines and 5-hydroxytryptamine after inhibition of their biosynthesis (Fuxe & Gunne, 1964; Dahlström, Fuxe, Kernell & Sedvall, 1965; Andén, Corrodi, Dahlström, Fuxe & Hökfelt, 1966; Corrodi & Malmfors, 1966). The methyl ester of α-methyltyrosine (H 44/68) has been found by ourselves to be a potent inhibitor of catecholamine synthesis without affecting the uptake-storage mechanism of the intraneuronal granules (Andén & others, 1966; Corrodi, Fuxe & Hökfelt, 1966; Corrodi & Hanson, 1966). This substance could therefore be used to reveal any changes that may occur in the activity of the brain catecholamine neurones under the influence of pharmacological, physiological or experimental conditions. This approach was originally suggested to us by Hillarp a few years ago. A decrease in impulse flow would thus have the effect of a decreased rate of disappearance of the catecholamines from the axon terminals after treatment with H 44/68, while an increased impulse flow would increase the rate of disappearance of the catecholamines after inhibition of synthesis. In the present study the effects of barbiturates on the activity of the catecholamine neurones were studied in two experimental situations. In the first experiment a single intraperitoneal injection of pentobarbitone sodium, 40 mg/kg, was given to male Sprague-Dawley rats (150–250 g). The animals at an environmental temperature of 25° were kept heavily sedated, without righting reflex, by repeated injections of small amounts (10–15 mg/kg). Fifteen min after the first injection the animals were treated with H 44/68 (250 mg/kg. i.p.) and were killed by decapitation 4 or 8 hr later. Control animals received either pentobarbitone or H 44/68 in the same way as the experimental animals.

In another experiment phenobarbitone sodium was used. The rats which were killed 4 hr after H 44/68 received a large dose of phenobarbitone sodium (100 mg/kg i.v.), 15 min before H 44/68 administration (250 mg/kg i.p.) and a small dose (40 mg/kg. i.p.) 2 hr before killing. This treatment made the rats sleep deeply. The rats which were killed 8 hr after the H 44/68 injection received two large doses of phenobarbitone sodium (100 mg/kg i.v., 8 hr 15 min and 4 hr before killing) and two small doses of the drug (40 mg/kg i.p. 6 and 2 hr before killing). Control groups were treated in the same way as in the previous experiment. The rectal temperature was recorded frequently and found to be normal in all animals.

For the histochemical investigation defined parts of the brains were dissected, freeze-dried, treated with formaldehyde gas, embedded and sectioned using the histochemical fluorescence method developed by Hillarp (Falck, Hillarp, Thieme & Torp, 1962; see review by Hillarp, Fuxe and Dahlström, 1966). Whole brains were removed and analysed for dopamine and noradrenaline (Bertler, Carlsson & Rosengren, 1958; Carlsson & Waldeck, 1958; Carlsson & Lindqvist, 1962).

The biochemical determinations (see Tables 1 and 2) demonstrated that the brains of rats treated with barbiturates showed a significant decrease in the rate of disappearance of dopamine both 4 and 8 hr after H 44/68 treatment compared to rats treated with H 44/68 alone. The rate of disappearance of brain noradrenaline, however, was not much affected by the barbiturate administration. No certain changes were obtained in the amine levels of the catecholamine neurones after treatment with barbiturates alone.

With the aid of the histochemical fluorescence method it was found that the

dopamine nerve terminals of the nucleus caudatus putamen, nucleus accumbens, the tuberculum olfactorium and the median eminence of rats treated with barbiturate and H 44/68 had a greater fluorescence intensity than those of animals treated with H 44/68 alone. The difference in intensity was more evident 8 hr than 4 hr after H 44/68 treatment. The central noradrenaline nerve terminals of various areas, for example, the hypothalamus, and the neocortex, of animals treated with barbiturates and H 44/68 or with H 44/68 alone did not show any clear difference in the degree of depletion obtained 4 and 8 hr after administration of the drugs. The same was also true for the central dopamine and noradrenaline cell bodies. No obvious change in the appearance of the central catecholamine and 5-hydroxytryptamine neurones was obtained after treatment with barbiturates only.

TABLE 1. THE CONTENT OF NORADRENALINE AND DOPAMINE IN RAT BRAIN AFTER PENTOBARBITONE AND H 44/68 EXPRESSED AS A PERCENTAGE OF NORMAL VALUES ± S.E.M. OF 4 EXPERIMENTS
(Dosage and treatment see text)

Treatment		Noradrenaline (%)	Dopamine (%)
Nontreated		100·0 ± 2·5	100·0 ± 3·8
Pentobarbitone	4 hr	93·8 ± 6·7	98·6 ± 4·4
H 44/68	4 hr	45·9 ± 0·8	*26·3 ± 1·4
Pentobarbitone + H 44/68	4 hr	55·5 ± 1·5	*45·0 ± 0·6 (*P = 0·001)
Pentobarbitone	8 hr	104·1 ± 3·4	98·1 ± 4·5
H 44/68	8 hr	32·9 ± 1·3	*12·9 ± 0·2
Pentobarbitone + H 44/68	8 hr	34·6 ± 0·5	*30·8 ± 1·9 (*P = 0·001)

TABLE 2. THE CONTENT OF NORADRENALINE AND DOPAMINE IN RAT BRAIN AFTER PHENOBAREITONE AND H 44/68 EXPRESSED AS A PERCENTAGE OF NORMAL VALUES ± S.E.M. OF 4 EXPERIMENTS
(Dosage and treatment see text)

Treatment		Noradrenaline (%)	Dopamine (%)
Nontreated		100·0 ± 2·5	100·0 ± 3·8
Phenobarbitone	4 hr	98·3 ± 3·7	104·1 ± 3·1
H 44/68	4 hr	44·5 ± 3·3	*24·2 ± 0·8
Phenobarbitone + H 44/68	4 hr	50·0 ± 1·0	*44·7 ± 4·2 (*P < 0·005)

Thus, both the histochemical and biochemical experiments support the view that pentobarbitone and phenobarbitone induce a markedly decreased impulse flow in the various dopamine neurones, with a subsequent decreased rate of synthesis in these systems. This has the effect of slowing up of the depletion obtained by an inhibition of dopamine synthesis. In other words, the dopamine neurones are in a lower state of activity than normal during barbiturate sleep. It is not known whether this effect is indirect or direct. The noradrenaline neurone systems, however, were hardly affected by the barbiturates.

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Concerning the regulation of some diverse biochemical reactions underlying the inflammatory response by salicylic acid, phenylbutazone and other acidic antirheumatic drugs

Sir,—Under this title, we proposed the hypothesis that lysyl ϵ-amino-groups of certain proteins are important binding sites for acidic anti-inflammatory drugs, and that when so bound these drugs may inhibit enzymic reactions which depend upon the availability of these ϵ-amino-groups—either for binding an essential cofactor (for example pyridoxal phosphate) or for directing the enzymic reaction (for example tryptic-like proteolysis or mitochondrial phosphorylation) (Whitehouse & Skidmore, 1965). We now have evidence that these particular drugs may also influence other biochemical reactions which might be involved in the inflammatory response, by acting as pseudo-antimetabolites and inhibiting the metabolism of certain derivatives of aromatic amino-acids.

Enzymes resembling chymotrypsin (E.C. no. 3.4.4.5) in their substrate specificity have been found in the granules of rat mast cells (Lagunoff & Benditt, 1963) and have been implicated in the swelling of the paw of the rat after the local injection of an inflammatory agent (Hladovec & Rybák, 1963) and also in the anaphylactic release of histamine either from sensitised lung of the guinea-pig (Austen & Brockelhurst, 1961a) or from rat peritoneal mast cells (Keller, 1963). Chymotrypsin hydrolyses esters and amides of aromatic amino-acids, leucine, methionine and even histidine (Kloss & Schröder, 1964) as well as proteins, and releases histamine from mast cells with concomitant degranulation (Uvnas & Antonsson, 1963; Saeki, 1964). Sodium salicylate inhibits a guinea-pig lung protease (Ungar, Yamura, Isola & Kobrin, 1961) and the anaphylactic release of histamine in guinea-pigs (Mongar & Schild, 1957) and rabbits (Haining, 1956). Mörsdorf, Donner & Cornellisson (1966) found that the *N*-acetyltyrosine esterase present in the inflamed rat paw was powerfully inhibited by several acidic anti-inflammatory drugs.

We have made kinetic studies on the inhibition of crystalline beef α-chymotrypsin (British Drug Houses Ltd.) by some of these acidic drugs and have found that, with the exception of indomethacin (*N*-*p*-chlorbenzoyl-5-methoxy-2-methylindole-3-acetic acid) and its indol-3-yl-α-propionic analogue, they all compete with the enzyme substrate when this is *N*-acetyltyrosine ethyl ester (ATEE).

An *N*-methylene analogue of indomethacin (*N*-*p*-chlorbenzyl-5-methoxy-2-methylindol-3-yl-α-propionic acid) did, however, act as a competitive inhibitor of ATEE hydrolysis by chymotrypsin. Indomethacin itself (an *N*-acyl compound) was slowly hydrolysed by chymotrypsin to yield *p*-chlorbenzoic acid and 5-methoxy-2-methylindole-3-acetic acid.

TABLE 1. EFFECTIVENESS OF SOME ANTI-INFLAMMATORY ACIDS IN INHIBITING CHYMOTRYPTIC HYDROLYSIS OF *N*-ACETYLTYROSINE ETHYL ESTER, AND 5-HT FORMATION BY DOPA DECARBOXYLASE (BEEF ADRENAL MEDULLA)

Drug	K_I for	
	Chymotrypsin	Dopa decarboxylase
Salicylic acid	9×10^{-3}M	$1{\cdot}5 \times 10^{-2}$M
Ibufenac	5×10^{-3}M	6×10^{-4}M
Phenylbutazone	3×10^{-4}M	2×10^{-4}M
Indomethacin	No inhibition	1×10^{-4}M
Flufenamic acid	$1{\cdot}4 \times 10^{-4}$M	5×10^{-5}M
Mefenamic acid	9×10^{-6}M	4×10^{-5}M

K_I = dissociation constant of the (inhibited) enzyme-drug complex; determined graphically (Dixon, 1953).

Experimental conditions.

Chymotrypsin. Substrate concentrations 1×10^{-3}M and 6×10^{-4}M ATEE, 15 μg crystalline α-chymotrypsin all in 10 ml 10 mM sodium phosphate pH 7·50, ionic strength 0·15 (sodium chloride). Drugs and substrate added in the same buffer, enzyme added in water (30 μl 0·5 mg/ml). Reaction followed titrimetrically with a pH-stat (Radiometer, Copenhagen), using 10 mM sodium hydroxide (ionic strength 0·15).

Dopa decarboxylase. 6-fold purified enzyme from adrenal medulla. Incubation volume 0·6 ml containing 0·083 M sodium phosphate pH 6·8, 6 mM β-mercaptoethanol and 1·67 mM EDTA. Substrate concentrations 3·8 and $7{\cdot}6 \times 10^{-5}$M 5-hydroxytryptophan. 2·5 mg enzyme protein added. ^{14}C-5-HT measured after paper chromatography (Somerville, 1964).

5-Hydroxytryptamine (5-HT) has been implicated as an inflammatory mediator, at least in the rat (Spector & Willoughby, 1965). We have found that these aromatic acidic drugs also inhibit 5-HT formation by the aromatic L-amino-acid decarboxylase (dopa decarboxylase) present in bovine adrenal medulla. This decarboxylase contains strongly-bound pyridoxal phosphate and even after ammonium sulphate fractionation and gel-filtration of the enzyme, the activity of the preparation is increased only by 30% by adding excess pyridoxal phosphate (15 μM). Kinetic analysis has shown that the drugs compete with the substrate, 5-hydroxytryptophan; not with the coenzyme. [This is exactly the converse of the action of these drugs in inhibiting histamine formation by substrate-specific mammalian histidine-decarboxylases (Skidmore & Whitehouse, 1966).]

Table 1 shows the relative potencies of some acidic anti-inflammatory acids, currently used as clinical antirheumatic drugs, as competitive inhibitors of chymotrypsin and dopa decarboxylase. Glycyrrhetic acid-3-hemisuccinate (carbenoxolone 0·9 mM) and chloroquine phosphate (1·8 mM), which are examples of a non-aromatic and a non-acidic anti-inflammatory drug respectively, did not affect the chymotryptic hydrolysis of *N*-acetyl tyrosine ester or formation of 5-HT. Lauric acid (1·0 mM), which inhibits the anaphylactic release of histamine (Austen & Brocklehurst, 1961b) did not inhibit the chymotryptic hydrolysis of ATEE.

These drugs did not inhibit rat liver homogentisate oxidase (with 2 mM substrate and drugs in saturated solution, pH 7·2 or 25 mM sodium salicylate), indicating that they do not inhibit the metabolism of all phenolic derivatives of aromatic amino-acids. Unlike dopa decarboxylase and chymotrypsin, homogentisate oxidase will act only on one substrate (2,5-dihydroxyphenylacetate). It would thus seem that only enzymes with a fairly broad (binding) specificity for aromatic substrates may suffer competitive inhibition by aromatic anti-inflammatory acids. Even this viewpoint is not an exclusive one, for the action of these drugs in inhibiting histamine formation (Skidmore & Whitehouse, 1966) may be considered *either* as competitive inhibition of the binding of the aromatic cofactor (pyridoxal phosphate) *or* non-competitive inhibition of the enzyme due to drug-binding at a site not involved in binding the substrate (L-histidine).

In summary, we believe these drugs may influence several enzyme systems underlying the inflammatory response by at least two modes of action at the molecular level, which include the neutralisation of active (lysine) ϵ-amino groups as previously discussed (Whitehouse & Skidmore, 1965), and by behaving as antimetabolites to competitively inhibit enzymes such as dopa decarboxylase and (non-enteric) chymotryptic hydrolases.

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