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THE GANGLION-BLOCKING ACTIVITY OF AMINOBICYCLO-[2,2,1]HEPTANES (CONGENERS OF MECAMYLAMINE) AND BICYCLO[3,2,1]AZAOCTANES (BRIDGED CONGENERS OF PEMPIDINE)

BY

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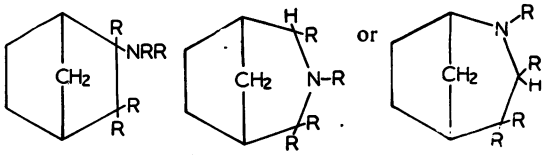
The structural requirements for strong ganglion-blocking activity and long duration of action amongst some lower homologues of mecamylamine, together with the discovery of similar activities amongst isomers with enlarged ring structures, are described. In both series of compounds it was found that the successive introduction of *C*-methyl groups surrounding the nitrogen atom resulted in a progressive increase in ganglion-blocking activity and duration of action.

The pharmacology of the potent ganglion-blocking compound pempidine, 1,2,2,6,6-pentamethylpiperidine, has recently been described (Corne and Edge, 1958; Spinks, Young, Farrington, and Dunlop, 1958).

The independent discovery of the properties of this compound by Spinks and Young (1958) originated from their observation that secondary and tertiary aliphatic amines in which the basic group is sterically hindered by attachment to a tertiary carbon atom have a modest level of ganglion-blocking activity.

By contrast, we first studied structure-activity relationships amongst certain aminobicyclo[2,2,1]-heptanes which were lower homologues of



R=H or alkyl

aminobicyclo-[2,2,1]heptanes. N atom is exocyclic

bicyclo[3,2,1]azaoctanes. N atom is endocyclic

mecamylamine. During this work we unexpectedly encountered a chemical route to bicyclo[3,2,1]azaoctanes, isomeric compounds in which the nitrogen atom was endocyclic, instead of exocyclic. Structure-activity relationships amongst these new compounds led us to discover the ganglion-blocking activity of the structurally simpler, unbridged compound pempidine. This work, which has already been very briefly outlined (Lee, Wragg, Corne, Edge, and Reading, 1958), is now reported in some detail.

METHODS

Chemical

The first four isomers of mecamylamine examined in the present work (Table I) were prepared as follows: *N*-methyl-*d*(−)-isobornylamine was prepared by the method of Wegler and Frank (1936). *N*-methyl-*d*(+)-bornylamine was prepared by the method of Forster (1899). *N*-methylfenchylamine was prepared in 20% yield by the Leuckhart reaction on fenchone. Hydrochloride sublimed >300°. (Found: C, 64.4; H, 10.8; N, 6.9. Required for $C_{11}H_{21}N$, HCl: C, 64.8; H, 10.8; N, 6.87.) *N*-methyl-camphidine was prepared by the method of Auwers (1868).

Most of the remaining compounds were prepared from nitrobicyclo[2,2,1]heptenes obtained via classical Diels-Alder reactions. Mild catalytic reduction of these intermediates gave the corresponding nitrobicyclo[2,2,1]heptanes. Catalytic reduction under more drastic conditions gave the expected aminobicyclo[2,2,1]heptanes, which could be *N*-alkylated by well-established methods to give products which undoubtedly had the structures shown as (V), (VII), and (IX) in Table II (Lee and Wragg, 1960a).

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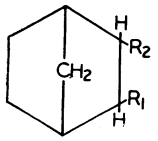
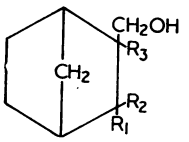
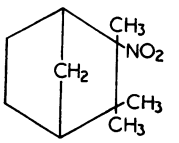


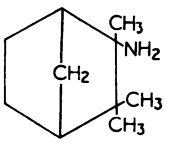
(X)



(XI)



(XII)



(XIII)

The compounds (X; $R_1 = -C_6H_5$, $R_2 = -NH.CH_3$, $-N(CH_3)_2$, or $-NH.CH_2.C_6H_5$) and (X; R_1 = cyclohexyl, $R_2 = -NH.CH_3$) were obtained similarly. The compounds (XI; $R_1 = R_2 = -CH_3$, $R_3 = -NH_2$) and (XI; $R_1 = -C_6H_5$, $R_2 = -H$, $R_3 = -NH.CH_3$) were synthesized via reactions between the appropriate nitrobicyclo[2,2,1]heptanes and formaldehyde. The compound (IV) in Table II was made by reducing the corresponding nitro-derivative chemically.

We were able to compare some of the foregoing aminobicyclo[2,2,1]heptanes (congeners of mecamylamine), in which the nitrogen atom is exocyclic, with the corresponding isomeric bicyclo[3,2,1]azaoctanes (structures (VI) and (VIII), in Table II; bridged congeners of pempidine), in which the nitrogen atom is endocyclic, as a result of a discovery made during a concurrent systematic study (Lee and Wragg, 1960b) of the aluminium lithium hydride reduction of crude 3-nitroisocamphane (XII). The basic fraction of the product mainly consisted of a single primary amine (M & B 4333) and, quite unexpectedly, two secondary amines (M & B 5561 and M & B 5562), all three of which were isomeric with the expected product (XIII).

The primary amine did not have the structure (XIII) because its *N*-methyl derivative (M & B 4334) was not identical with mecamylamine (I, Table II) (compare Stein, Sletzinger, Arnold, Reinhold, Gaines, and Pfister, 1956); moreover, both M & B 4333 and M & B 4334 were inactive when tested for ganglion-blocking activity and their actual chemical structures have not been investigated.

On the other hand, chemical evidence (Lee, Lunt, Wragg, and Barber, 1958; Lee, Wragg, Corne, Edge, and Reading, 1958) revealed that the two secondary amines had the structures (II; R=H) and (III; R=H) in Table II or geometrical isomers thereof. On the basis of chemical evidence, infra-red spectral data and the estimates of ganglion-blocking activity (see Results), we have provisionally assigned the structure (II; R=H) to M & B 5561 and the structure (III; R=H) to M & B 5562. Both compounds were *N*-methylated, giving M & B 5199 (II; $R=CH_3$) and M & B 5200 (III; $R=CH_3$) respectively, both of which are isomeric with mecamylamine. In practice, the two secondary amines formed in the aluminium lithium hydride reduction of (XII) were isolated as a mixture of 85% M & B 5561 and 15% M & B 5562, which could be separated into its components only by preparative gas chromatography. The unseparated mixture (M & B 4364) upon *N*-methylation gave M & B 4348A which consisted of 75% of the tertiary amine, M & B 5199, and 25% of its isomer, M & B 5200. M & B 4348 obtained in another experiment consisted of 55% M & B 5199 and 45% M & B 5200. These mixtures were studied as such because their separation into pure components by preparative gas chromatography was feasible only on a small scale.

We then proceeded to reduce with aluminium lithium hydride three nitrobicyclo[2,2,1]heptanes which had previously been reduced catalytically. Gas chromatography indicated that these reductions, in contrast to similar reduction of crude 3-nitroisocamphane (XII), each yielded only one product. The resulting single secondary amines were then *N*-methylated. Chemical and infra-red spectral evidence, together with the levels and duration of ganglion-blocking activity observed, led us to assign provisionally the symmetrical ring-enlarged structures (VI and VIII, analogous to II) (Table II) to the products thus obtained.

Pharmacological

The methods used were as described by Corne and Edge (1958), with the following exceptions and additions:

Most of the determinations of ganglion-blocking activity on the superior cervical ganglion-nictitating membrane preparation of the anaesthetized cat were performed using continuous (10/sec.) supramaximal pre-ganglionic stimulation. A few determinations were performed using intermittent (50/sec. for 5 sec./min.) stimulation. Several of the compounds had a short duration of action (comparable to that of hexamethonium). Greater reliance is placed on the potency estimates assigned to these compounds than to those with a long duration of action (comparable to that of mecamylamine), whose potency is difficult to estimate (Corne and Edge, 1958).

Acute oral and intravenous toxicities were studied in groups of 5 or 10 mice. Mydriatic responses in groups of 5 mice were observed after intraperitoneal, as well as oral, administration. Urinary excretion determinations were performed as described by Muggleton and Reading (1959).

The compounds were studied as the hydrohalides or acetates and all activity and toxicity determinations are expressed in terms of the cations. Other compounds used were hexamethonium bromide, acetylcholine chloride, adrenaline hydrochloride, histamine acid phosphate, nicotine hydrogen tartrate, and pilocarpine nitrate. With the exception of hexamethonium and adrenaline, all doses and concentrations of these compounds are expressed in terms of the salts.

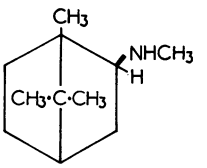
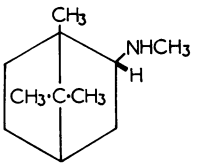
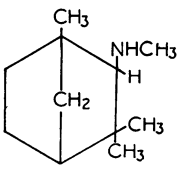
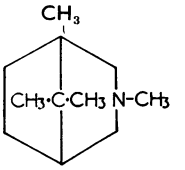
RESULTS

Four Structural Isomers of Mecamylamine

The activity of these compounds when tested on the pre-ganglionically stimulated nictitating membrane of the cat was only 4 to 7% of that of hexamethonium (Table I). None of these results was thought worth following up. A compound with an identical planar formula to *N*-methyl-*d*(−)-isobornylamine and *N*-methyl-*d*(+)-bornylamine but having an unspecified steric structure was reported by Rubinstein, Pedersen, Fakstorp, and Rønnov-Jessen (1958) to have a weak sympathetic ganglion-blocking activity and to be mildly hypertensive in the anaesthetized cat. A compound with an identical planar formula to *N*-methylfenchylamine but prepared by an unspecified route was also reported by Rubinstein *et al.* (1958) to have appreciable sympathetic ganglion-blocking activity. As our compound was short-acting and that studied by Rubinstein *et al.* was long-acting, it is unlikely that the two were identical chemically.

TABLE I

BLOCKING ACTIVITY OF 4 ISOMERS OF MECAMYLAMINE ON THE PRE-GANGLIONICALLY STIMULATED NICTITATING MEMBRANE OF THE CAT

Compound	Structure	Activity (Hexamethonium =100)
N-Methyl-*d*-(−)-isobornylamine		5
N-Methyl-*d*(+)-bornylamine		4
N-Methylfenchylamine		7
N-Methylcamphidine		6

Aminobicyclo[2,2,1]heptane Derivatives (N Atom Exocyclic, as in Mecamylamine)

All of the phenyl and cyclohexyl derivatives of structures (X) and (XI) above had a very low activity, less than 3% of that of hexamethonium.

The ganglion-blocking activity, duration of action, and oral and intravenous toxicity of the nor derivatives of mecamylamine (structures IV, V, VII, and IX) are shown in Table II. Of these compounds, only two (M & B 4086 and M & B 4620) had a prolonged duration of action on the pre-ganglionically stimulated nictitating membrane, and M & B 4086 was selected for a brief examination of its mode of action.

Properties of M & B 4086.—Mecamylamine and M & B 4086 act specifically at the ganglion since a large intravenous dose (8 mg./kg.) of either compound completely inhibited the effect of pre-ganglionic stimulation on the nictitating membrane, but had no inhibitory action on post-ganglionic stimulation or on intravenously injected adrenaline (Fig. 1).

On the isolated guinea-pig ileum, M & B 4086 in a concentration of 0.08 mg./ml. completely inhibited the effect of nicotine-induced contractions and caused a slight reduction in the response to acetylcholine but had no effect on histamine- or pilocarpine-induced contractions. The nicotine response was slow to recover after removal of the antagonist from the bath. A concentration of 0.8 mg./ml. had an initial stimulant action and blocked the effect of all four agonists.

M & B 4086 had about 50% of the activity of, but a similar duration of action to, mecamylamine when compared by the mydriatic response in mice after intraperitoneal injection.

An oral dose of 5 mg./kg. in a group of six rats showed an identical urinary excretion pattern with that shown by mecamylamine over a period of 24 hr. Thus recoveries of M & B 4086 at 2, 4, 6, and 24 hr. were 3.3, 9.6, 16.5, and 48.7% respectively. The corresponding figures for mecamylamine were 1.3, 8.8, 15.2, and 45.6%.

TABLE II

BLOCKING ACTIVITY ON THE PRE-GANGLIONICALLY STIMULATED NICTITATING MEMBRANE OF THE CAT AND TOXICITY IN MICE OF SOME AMINOBICYCLO[2,2,1]HEPTANE AND BICYCLO[3,2,1]AZAOCTANE DERIVATIVES

VS=Very short duration of action (shorter than hexamethonium). S=Short duration of action (similar to hexamethonium). M=Medium duration of action (longer than hexamethonium, shorter than pempidine). L=Long duration of action (similar to pempidine). Where the results were very variable, the range of activity is given in parentheses.

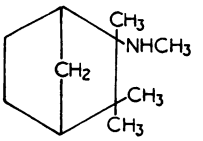
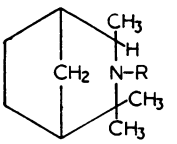
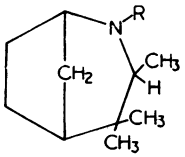
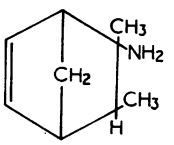
M & B No.	Structure	Activity (Hexamethonium =100)	Number of Experiments	Duration of Action	LD50 mg./kg.	
					Oral	Intravenous
	Mecamylamine ←  (I)	120 (60–190)	5	L	98	12·9
5561	→ R=H  (II)	134 (85–170)	4	L		29·9
5199	R=CH_3 ←*isomers*	100 (50–200)	5	L		28·3
	isomers → R=H  (III)					
5562	R=H	75	3	VS		30·3
5200	R=CH_3 ←	40 (25–50)	5	M		32·5
4364	Mixture of 85% M & B 5561 and 15% M & B 5562	140 (120–190)	5	L	470	35·0
4348A	Mixture of 75% M & B 5199 and 25% M & B 5200	125	2	L	214	37·0
4348	Mixture of 55% M & B 5199 and 45% M & B 5200	170 (100–210)	6		L	
4315	 (IV)	15	1	S		

TABLE II—*continued*

M & B No.	Structure	Activity (Hexamethonium = 100)	Number of Experiments	Duration of Action	LD50 mg./kg. Oral	LD50 mg./kg. Intravenous
4058	$R_1=R_2=H$	15	2	S	≏135	46·0
4086	$R_1=CH_3, R_2=H$ ←	35	2	L	138	33·8
4620	$R_1=R_2=CH_3$	50	2	L	≏165	18·1
4126	$R_1=CH_2.C_6H_5, R_2=H$	<1	1			
	(V)					
4387	←*isomers*	50	3	L	≏415	48·5
	(VI)					
4188	→ $R=CH_3$	25	2	S		68·5
4269	$R=C_2H_5$←	25	2	S		34·2
	isomers *isomers*					
	(VII)					
4443	→ $R=CH_3$	25	2	S	≏425	109
4442	$R=C_2H_5$←	20–25	2	S	≏267	54·5
	(VIII)					
4280	←	15	2	S	405	44·5
	(IX)					

Bicyclo[3,2,1]azaoctane Derivatives (N Atom Endocyclic, as in Pempidine)

The activity, duration of action, and toxicity of these compounds (structures II, III, VI, and VIII) are shown in Table II. One of the two isomers in each of the mixtures designated M & B 4364 and M & B 4348A had a higher activity and a longer duration of action than the other, and this finding considered together with the results obtained amongst mecamylamine congeners strengthened the chemical and infra-red spectral evidence in assigning the structures as shown. The effects of

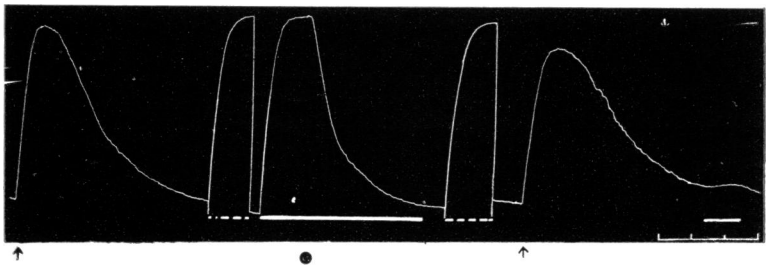


FIG. 1.—Cat, 2.4 kg., chloralose anaesthesia. Contractions of nictitating membrane. At arrows, 10 μg. adrenaline was injected intravenously; the continuous lines indicate pre-ganglionic stimulation at 10/sec.; the discontinuous lines indicate post-ganglionic stimulation at 10/sec.; at the black dot, 8 mg./kg. M & B 4086 was injected intravenously. Time, 60 sec.

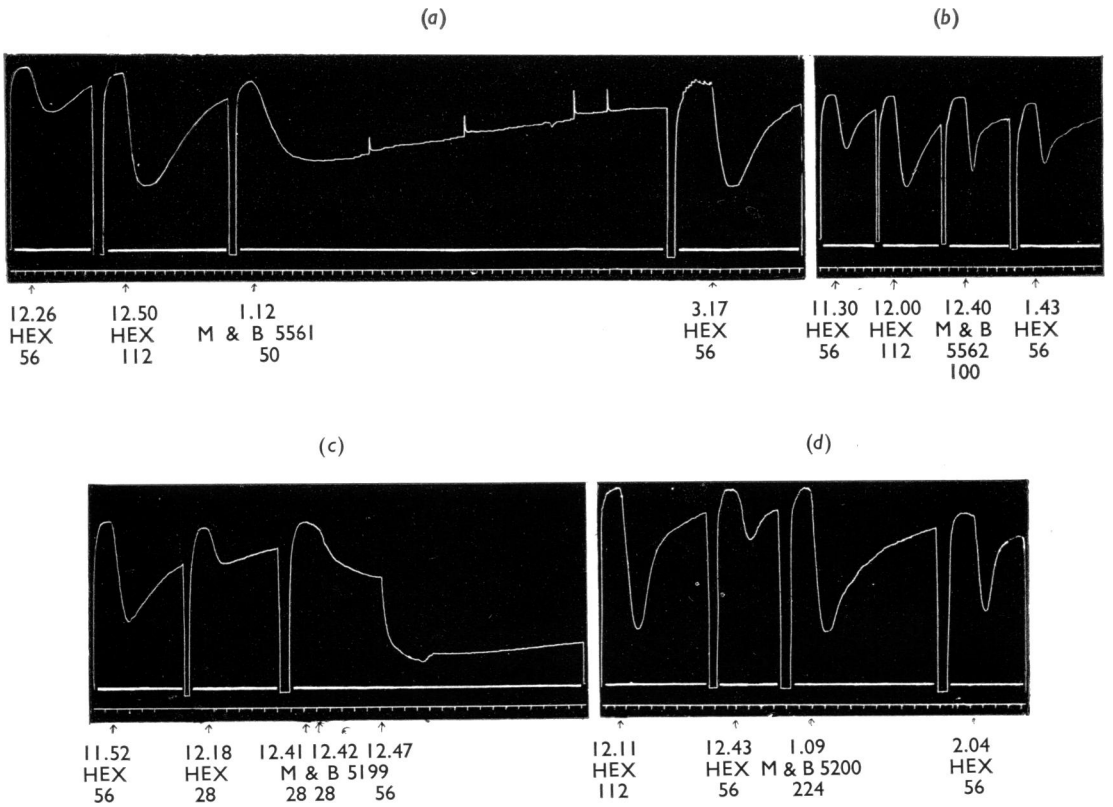


FIG. 2.—Cats, (*a*) 3.25 kg., (*b*) 2.5 kg., (*c*) 3.0 kg., (*d*) 2.3 kg., chloralose anaesthesia. Contractions of nictitating membrane. Continuous line indicates pre-ganglionic stimulation at 10/sec. Kymograph was stopped between periods of stimulation. Intravenous doses in μg./kg. at times shown. HEX = hexamethonium. Time, 60 sec.

these two pairs of isomers in comparison with hexamethonium on the pre-ganglionically stimulated nictitating membrane are shown in Fig. 2. When compared with hexamethonium on the pupillary diameter of the anaesthetized cat after removal of the superior cervical ganglion, M & B 5199 and M & B 5200 had activities of 250% and 70% respectively. When compared with mecamylamine in mice injected intraperitoneally, respective mydriatic activities of 100% and 50% were obtained. Some of the pharmacological properties of the mixture (M & B 4348A) of these two isomers were examined in more detail.

Properties of M & B 4348A.—An intravenous dose of 6.7 mg./kg. of M & B 4348A completely blocked the effect on the nictitating membrane of pre-ganglionic stimulation but had no effect on the contraction produced by post-ganglionic stimulation or by an intravenous injection of adrenaline. In the same preparation a further dose of 6.7 mg./kg. caused a small rise in blood pressure. In other experiments the pressor response to nicotine (0.1 mg./kg.) was reduced about 50% by a dose of 0.05 mg./kg., and 0.1 mg./kg. caused an almost total inhibition of the effect on the blood pressure of peripheral vagal stimulation. Doses of up to 6.7 mg./kg. had no effect on blood pressure responses to small doses of acetylcholine or histamine, and the response to adrenaline was potentiated. Respiratory arrest occurred after a total dose of 31 mg./kg. On the isolated Langendorff rabbit heart preparation doses of 2.0 to 8.0 mg. injected into the aortic cannula caused a slowing of the beat.

Oral doses of 3.3 mg./kg. and 4.0 mg./kg. of M & B 4348A and M & B 4364 respectively caused a marked mydriasis in mice within 10 min. of administration and the response was of prolonged duration.

On the isolated guinea-pig ileum a concentration of 0.067 mg./ml. of M & B 4348A completely inhibited the response to nicotine and reduced the response to pilocarpine, but had no effect on acetylcholine or histamine responses (Fig. 3). The compound was removed after being in contact with the gut for 14.5 min. and 50% recovery of the nicotine response occurred about 3 hr. later (Fig. 3).

On the isolated frog rectus abdominis muscle a concentration of 0.67 mg./ml. inhibited responses to acetylcholine without causing a contracture.

When examined for its potency on nicotine-induced convulsions in mice M & B 4348A injected intraperitoneally was found to have an ED50 of 0.065 mg./kg. The lower, less active, homologue (VI; M & B 4387) had an ED50 of 0.25 mg./kg. Consequently, these two compounds had respectively 1.66 and 0.43 times the activity of mecamylamine (ED50=0.108 mg./kg.) and 23 and 6 times the activity of hexamethonium (ED50=1.5 mg./kg.) in this test. The greater potency of amine, as compared with quaternary, ganglion-blocking compounds in this test has been described for pempidine by Corne and Edge (1958), and Stone, Meckelnburg, and Torchiana (1958) have found that peripheral ganglion-blocking activity and anticonvulsant potency are not necessarily related in structurally dissimilar compounds.

Discussion

Considering first the structure-activity relationships observed amongst mecamylamine congeners (IV, V, VII, and IX in Table II) it is apparent that the presence of a double bond in the 5,6-position had no effect on activity (compare M & B 4315 and M & B 4058). Activity was also unaffected by the substitution of a *C*-ethyl for a *C*-methyl group (compare M & B 4188 and M & B 4269). On the other hand, replacement of the *N*-methyl group in M & B 4086 by an *N*-benzyl group (M & B 4126) eliminated activity. Removal of one *C*-methyl group from the 3-position of mecamylamine (I) giving M & B 4280 (IX) resulted in a pronounced drop in activity and in a shortened duration of action. Rubinstein, Pedersen, Fakstorp, and Rønnov-Jessen (1958) have reported that a compound with this planar

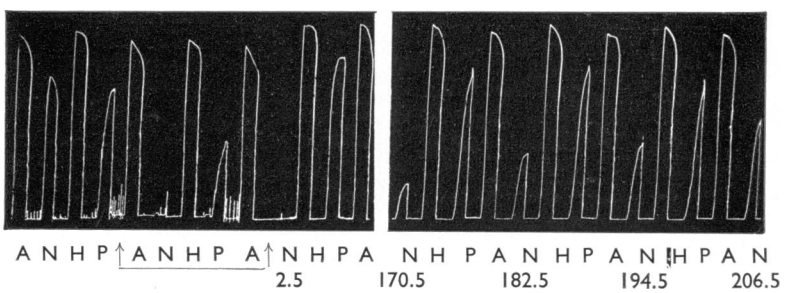


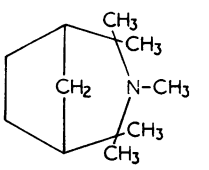
Fig. 3.—Contractions of isolated guinea-pig ileum in a 10 ml. bath. At A, 0.15 μg./ml. of acetylcholine; N, 5.0 μg./ml. of nicotine; H, 0.5 μg./ml. of histamine; P, 0.5 μg./ml. of pilocarpine. Between arrows 67 μg./ml. of M & B 4348A was present. Time in min. after removal of antagonist from the bath shown below nicotine responses.

formula, but prepared by an unspecified route, paralleled mecamylamine closely in sympathetic ganglion-blocking activity and in having a long duration of action. The pharmacological properties of our product make its chemical identity with the compound studied by Rubenstein *et al.* (1958) unlikely. By contrast, removal of a *C*-methyl group from the 2-position of (I) giving M & B 4086 (V; $R_1 = -CH_3$, $R_2 = -H$) whilst lowering activity did not shorten the duration of action. The corresponding primary amine, M & B 4058, was less active than M & B 4086, whereas the corresponding tertiary amine, M & B 4620, was similar both in activity and in duration of action to M & B 4086. A similar level of activity in related secondary and tertiary amines was later encountered again, both in the endocyclic series (for example M & B 5561 and M & B 5199), and in pempidine and the corresponding secondary amine (Corne and Edge, 1958; Spinks *et al.*, 1958).

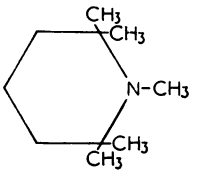
Of the two possible structures (II; $R = -CH_3$) and (III; $R = -CH_3$) for M & B 5199, the former appears more likely because an endocyclic nitrogen atom attached on each side to alkyl-substituted carbon atoms is a grouping which, in pempidine and its congeners, has since been found to be associated frequently with a high level of ganglion-blocking activity. Since infra-red spectral analysis eliminated the possibility that M & B 5200 is a geometrical isomer of M & B 5199, it probably has the structure (III; $R = CH_3$). The fact that M & B 5200 is not only less active but also has a shorter duration of action than M & B 5199 is not incompatible with the suggested assignment of structure, because subsequent work has indicated that an adjacent bridge-end α-carbon atom is less likely to confer ganglion-blocking activity on an endocyclic nitrogen atom than is a gem-dimethyl group.

Perhaps the most significant implication of our results as a whole was that it had been possible to pass from the aminobicycloheptane structure, in which the nitrogen atom is exocyclic, to the isomeric bicycloazaoctane structure, in which the nitrogen atom is endocyclic, without loss in ganglion-blocking activity. Moreover, the biological results (Table II) on the pairs of isomers, M & B 4269 and M & B 4442, M & B 4188 and M & B 4443, M & B 4086 and M & B 4387, and mecamylamine and M & B 5199, conformed to a remarkably systematic pattern. In the first place, the individual compounds in each pair have both a closely similar level of ganglion-blocking activity and a similar duration of action, independent of whether the *C*-substituents are methyl or ethyl groups. Secondly, the successive introduction of *C*-methyl groups into M & B 4188 or M & B 4443 results in a stepwise increase both in activity and in duration of action in the resulting homologous series.

It thus became apparent that a high level of ganglion-blocking activity and a long duration of action would probably be exhibited by (XIV) in which the introduction of methyl groups surrounding the endocyclic nitrogen atom had been taken one step further than in M & B 5199.



(XIV)



(XV)

In practice we decided to synthesize instead the corresponding unbridged compound, pempidine (XV), which did in fact exhibit high activity and prolonged action.

We are indebted to Dr. H. J. Barber and Dr. R. Wien for their constructive interest in this work; to Mr. A. C. Rasmussen for several estimations; to Dr. H. W. Reading for carrying out the excretion experiments in rats; to Dr. D. F. Muggleton for interpretation of infra-red spectra; and to Dr. G. A. P. Tuey for separation and analysis of many samples by gas chromatography.

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CENTRAL VASOMOTOR EFFECTS OF A NEW GANGLION-BLOCKING AGENT—1 : 2 : 2 : 6 : 6-PENTAMETHYL PIPERIDINE (PEMPIDINE)

BY

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The central vasomotor effects of pempidine were studied in dogs and cats anaesthetized with pentobarbitone sodium, and in spinal cats. Pempidine lowered the threshold of electrical stimulation of the medulla oblongata to evoke a pressor response when given intravenously to cats in a dose insufficient to block autonomic ganglia. Injection of a small dose into the cerebral ventricles of dogs produced an increase in the pressor responses to occlusion of the carotid artery and to electrical stimulation of the central end of the cut vagus. In the spinal cat small doses of pempidine, given either intravenously or intrathecally, augmented the rise in blood pressure resulting from compression of the spinal cord. Since all these effects were produced by pempidine in doses insufficient to cause ganglionic block, the observed effects are attributed to central facilitation of the vasomotor responses. Thus pempidine has a stimulant action on the spinal and supraspinal centres.

It is known that piperidine derivatives block ganglionic transmission ; for example, Zamboni and Borghetti (1953) reported ganglion-blocking properties of N-ethyl piperidine. The pharmacology of 1:2:2:6:6-pentamethyl piperidine (pempidine) was independently reported by Spinks and Young (1958) and Corne and Edge (1958). This drug has a prolonged hypotensive effect attributed to strong and persistent ganglionic blockade. Harrington, Kincaid-Smith and Milne (1958) have shown that pempidine on parenteral administration passes through the blood-brain barrier and is then taken up by the brain. The present study was undertaken to determine possible central vasomotor effects of pempidine, independent of its ganglion-blocking action.

The main difficulty in the study of the central vasomotor effects of ganglion-blocking agents is that peripheral ganglionic blockade masks the central vasomotor effects of these agents (Dontas and Nickerson, 1956). To overcome this difficulty the drug was injected into the cerebral ventricles or intrathecally, or it was given intravenously in a dose too small to produce ganglionic blockade.

METHODS

The investigation was carried out in dogs and cats anaesthetized with pentobarbitone sodium (30 mg./kg. intravenously in dogs, and 35 mg./kg. intraperitoneally in cats). The animals were maintained on artificial respiration after cutting both vagi. Blood pressure was recorded from the left carotid artery. The intravenous injections were made through a polythene tube into a femoral vein, the intraventricular injections were made into the lateral ventricle as described by Bhargava and Tangri (1959), and the intrathecal injections through a needle inserted in the vertebral canal at the lumbosacral articulation.

The action of the drug on the brain stem was assessed by observing the effects on (*a*) the reflex pressor response obtained by occlusion of the right carotid artery, and by electrical stimulation of the central cut end of the right vagus nerve (by means of an electronic stimulator), and (*b*) on the pressor responses evoked by direct electrical stimulation of the medulla oblongata by means of a bipolar needle electrode using a stereotaxic technique. The electrode placement was aided by the parameters described by Wang and Ranson (1939). The site of action of the drug in the spinal cord was assessed by observing the effects on the vasomotor responses elicited by the technique of spinal compression in the spinal cat (Bhargava and Kulsreshtha, 1959). Contractions of the nictitating membrane were obtained by preganglionic stimulation of the cervical sympathetic nerve, and pressor ("nicotinic") responses by injection of acetylcholine after atropine.

Pempidine was used as the hydrochloride.

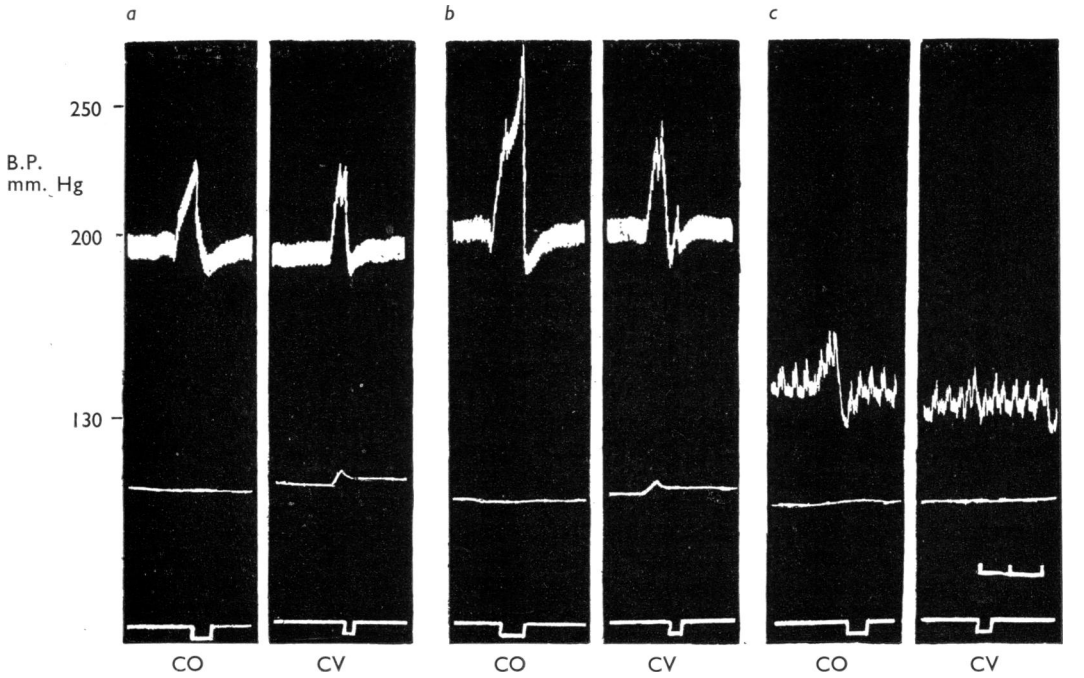

FIG. 1.—Records of arterial blood pressure (upper tracing) and of the nictitating membrane (lower tracing) of a dog, 7.2 kg., anaesthetized with pentobarbitone sodium. CO, response to 30 sec. occlusion of the carotid artery. CV, response to electrical stimulation (4 V, 10 msec., 10/sec. for 10 sec.) of cut end of the central stump of the vago-sympathetic nerve. (*a*) Control, (*b*) 10 min. after intraventricular injection of 50 μg. of pempidine, (*c*) 15 min. after intravenous injection of 0.5 mg./kg. of pempidine. Time signal in 30 sec.

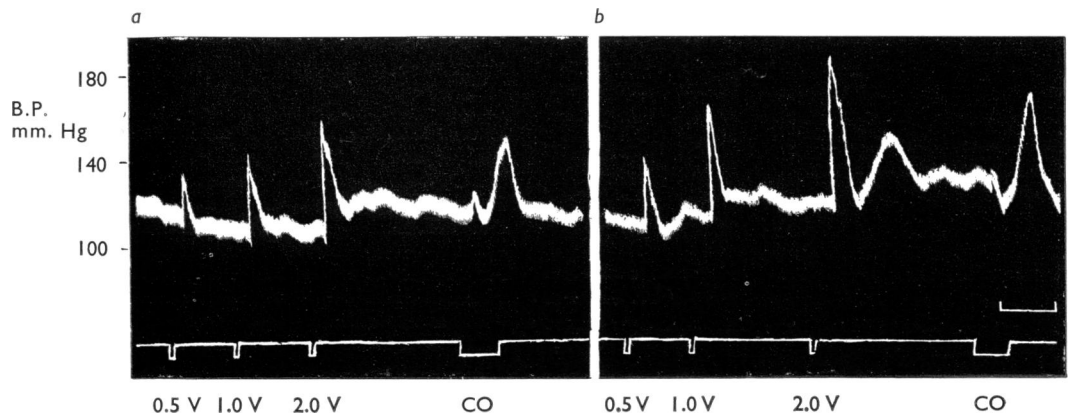

FIG. 2.—Record of arterial blood pressure of a cat, 3.0 kg., anaesthetized with pentobarbitone sodium. CO, response to 30 sec. occlusion of the carotid artery. 0.5 V, 1.0 V, 2.0 V; electrical stimulation of the medulla oblongata by means of a bipolar electrode using a stereotaxic technique. (*a*) Control, (*b*) 15 min. after intravenous injection of 25 μg. pempidine. Time signal in min. Note potentiation of all the vasomotor responses.

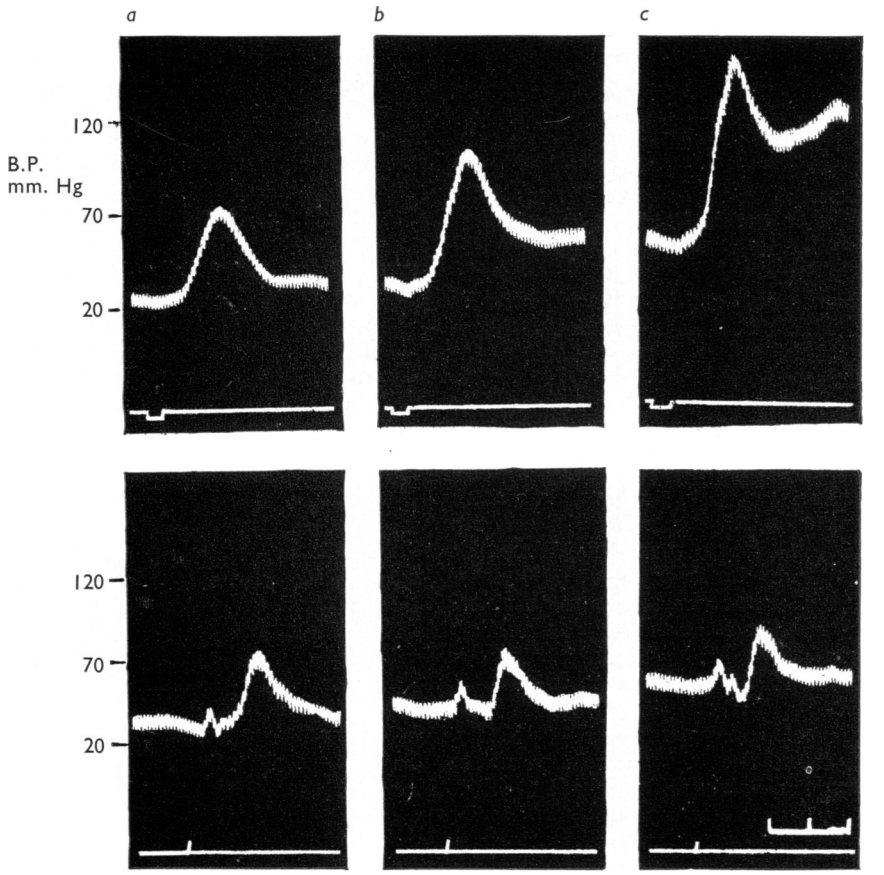

FIG. 3.—Record of arterial blood pressure of a spinal cat, 3.0 kg. Upper tracing shows vasomotor responses to compression of the spinal cord (150 mm. Hg for 10 sec.). Lower tracing shows "nicotinic" pressor responses to 150 μg. acetylcholine after atropine. Time signal in min. (*a*) Control, (*b*) 20 min. after intravenous injection of 25 μg./kg. of pempidine, (*c*) 10 min. after intrathecal injection of 5 μg./kg. of pempidine. Note the increased responses to spinal compression whereas those to acetylcholine were unaltered.

RESULTS

The Reflex Vasomotor Responses in the Dog After Injection of Pempidine into the Cerebral Ventricles

The effects of intraventricular administration of pempidine (50 to 100 μg.) on reflex supraspinal vasomotor responses were studied in 3 dogs. The results of one experiment are shown in Fig. 1. The control records (CO) show the pressor responses to occlusion of the carotid artery for 30 sec. (CO), and the pressor response as well as the nictitating membrane contraction obtained by electrical stimulation of the central cut end of the vago-sympathetic trunk (CV). On intraventricular administration of 50 μg. pempidine the blood pressure rose about 10 mm. Hg, and both the reflex vasomotor responses increased. The pressor response to occlusion of the carotid artery increased from 30 to 80 mm. Hg, and the response to central stimulation of the vagus increased from 30 to 45 mm. Hg. The contraction of the nictitating membrane was unchanged. The subsequent intravenous injection of 0.5 mg./kg. pempidine produced a marked fall of arterial blood pressure of 50 mm. Hg, and abolished both the reflex vasomotor responses (CO and CV). The nictitating membrane response was also abolished, therefore the inhibition of the vasomotor responses can be attributed solely to ganglionic blockade.

The Vasomotor Responses in the Cat After Intravenous Injection of Pempidine

The results were essentially the same in 3 cats. Fig. 2 illustrates the graded pressor responses evoked by electrical stimulation of the medulla with increasing voltage and the pressor response to the 30 sec. occlusion of the carotid artery (CO). Fifteen min. after the intravenous injection of pempidine (25 μg./kg.) all these vasomotor responses were increased. The control response to 1.0 V was now obtained with 0.5 V, and that to 2.0 V with 1.0 V. Thus the threshold of medullary excitability was lowered after the pempidine administration.

Spinal Vasomotor Responses After Pempidine in the Spinal Cat

The effect of pempidine on the spinal vasomotor responses was studied in 8 cats after intravenous injection of 25 to 50 μg./kg. and in 3 of these also after intrathecal administration of 5 μg./kg. Fig. 3 shows the vasomotor responses to spinal compression (upper record), and the "nicotinic" pressor responses to 150 μg. of intravenous acetylcholine after atropine (lower record). After intravenous administration of pempidine (25 μg./kg.) the blood pressure rose 10 mm. Hg. Twenty min. later, the vasomotor response to spinal cord compression was increased. When 5 μg./kg. of pempidine was given intrathecally after 1 hr. and the spinal cord was then compressed 10 min. later, the vasomotor response was further increased. This augmentation of the response persisted for 1.5 hr.

After intravenous injection of 25 μg./kg. pempidine the augmented response to compression of the spinal cord appeared within 10 to 20 min. and persisted for 1.5 hr. In one cat in which 50 μg./kg. was injected intravenously the blood pressure was lowered and the response to compression of the spinal cord was depressed within 10 min. and abolished within 30 min., but this also occurred with the response to acetylcholine. In this experiment this dose of pempidine must, therefore, already have exerted its effect on ganglionic transmission.

Discussion

The results of the present investigation showed that pempidine has a central vasomotor stimulant effect at the supraspinal as well as at the spinal level. This central effect which was examined on reflex vasomotor responses is best seen when the drug is injected into the cerebral ventricles or intrathecally. On intravenous administration it can only be seen when the dose injected is too small to produce ganglion-block.

A pressor action of pempidine resulting from large intravenous doses has been reported by Corne and Edge (1958) in cats with a low blood pressure. The pressor action was not blocked by bilateral adrenalectomy or by administration of phenoxybenzamine. However, the observation that the pressor response was considerably reduced after spinal cord destruction suggests that the effect was at least partly a result of a central action. The observation that pempidine in doses insufficient to block the ganglia manifests a central vasomotor stimulation may have a bearing on its clinical use. Small doses of pempidine may raise the blood pressure by its central action, but with adequate dosage hypotension must be produced by its ganglion-blocking effect.

We would like to thank Dr. R. Wien of May and Baker Ltd., Dagenham, Essex, for the supply of pempidine.

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THE INHIBITION OF THE PERISTALTIC REFLEX BY SUBSTANCES FROM PROTEIN SOURCES

BY

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An aqueous extract of gluten, and its ultrafiltrate, depressed the peristaltic reflex of the rat jejunum. This effect was shared by similar extracts of mammalian skeletal muscle but not by such proteins as casein, gelatin, and albumin. Peptic-tryptic digestion did not abolish the effect, whereas acid hydrolysis of the active fractions destroyed the depressant substance.

Radiographic abnormalities of the small intestine have been recognized for some years as one of the features of adult patients with idiopathic steatorrhoea (Snell and Camp, 1934; Kantor, 1939), and children with coeliac disease (Golden, 1936; Flemming Møller, Nørgaard and Plum, 1948). The classical appearance of clumped masses of opaque medium invariably seen in these patients was thought to be due to alternate areas of hypotonia and hypertonia resulting from disordered motor function attributed to vitamin B deficiencies or other factors (Golden, 1945). A number of workers had observed slow transit time in these patients, but this might have been due to increased tone, as well as to decreased motility. This rather confused situation was clarified by the demonstration that the clumped appearance of the barium sulphate was not due to alternate areas of spasm and relaxation, but to flocculation of the opaque medium due to the presence of excessive quantities of mucus (Frazer, French, and Thompson, 1949). Avoidance of flocculation by the use of non-flocculating media revealed that the characteristic change in these patients was dilatation (Anderson, Astley, French, and Gerrard, 1952).

Intolerance of dietary wheat gluten has since been established as the basic cause of the enteropathy in both adults and children. Wheat gluten caused deterioration in patients with gluten-induced enteropathy if it was reintroduced into the diet of patients in remission on a gluten-free régime (Dicke, 1950; Weijers, van de Kamer, and Dicke, 1953; Anderson, Frazer, French, Hawkins, Ross, and Sammons, 1954; Ross, Frazer, French, Gerrard, Sammons, and Smellie, 1955; Frazer, Fletcher, Ross, Shaw, Sammons, and Schneider, 1959). A change from the normal feathery pattern to dilatation was observed (Anderson, Frazer, French, Gerrard, Sammons, and Smellie, 1952).

It was, therefore, of interest to study the effect of gluten, or fractions derived from it, on isolated intestinal preparations. This paper is concerned with the examination of the effect of gluten and gluten fractions, and similar preparations of other proteins, on the peristaltic reflex of rat small intestine.

METHODS

Peristaltic Reflex

All fractions were investigated for their effect on the peristaltic reflex of the isolated rat jejunum. Bülbring, Crema, and Saxby's modification (1958) of the Trendelenburg method was used. Fractions were applied to the serosal surface only.

Fractionation Procedures (Fig. 1)

Cold Aqueous Extraction.—Gluten (Energen Ltd.) from untreated wheat flour is only poorly soluble in water. An aqueous extract (A.E.) was prepared by shaking 20 g. of gluten with 200 ml. of water for 30 min. at room temperature. The suspension was centrifuged at 3,000 rev./min. for 20 min., the opalescent supernatant freeze-dried and the resultant solid used. An ultrafiltrate (A.E./U.F.) was made from the supernatant using Visking dialysis tubing (Grant, Rowe, and Stanworth, 1958).

Hot Aqueous Extraction.—This method consisted in homogenizing 20 g. of gluten in water and boiling for 20 min. to coagulate most of the protein. The mixture was then cooled and subjected to ultrafiltration. The ultrafiltrate was freeze-dried as above (A.E.H./U.F.).

Peptic-tryptic Digestion.—A peptic-tryptic digest (III) was prepared as described by Frazer, Fletcher, Ross, Shaw, Sammons, and Schneider (1959). This was further treated either by autoclaving (Frazer,

Fletcher, Ross, Shaw, Sammons, and Schneider, 1959) which denatures protein, or by ultrafiltration which removes proteins of molecular weight over 20,000. Filtrates from these processes (III A.F. and III U.F. respectively) were used in the biological investigations. The separate peptic (I) and tryptic (II) digests were not prepared.

Acid Hydrolysis.—Fractions A.E., III U.F. and III A.F. were hydrolysed by boiling 1.0 g. material with 500 ml. 6N hydrochloric acid overnight. The acid was removed by distillation *in vacuo* at 50 to 60°; water was added to the residue and the distillation repeated. Addition of water and subsequent distillation were then repeated twice. The final residue was taken up in water and the *p*H adjusted to 7.

Mammalian Skeletal Muscle.—Lean beef trimmed of excess fat was used as the source of mammalian

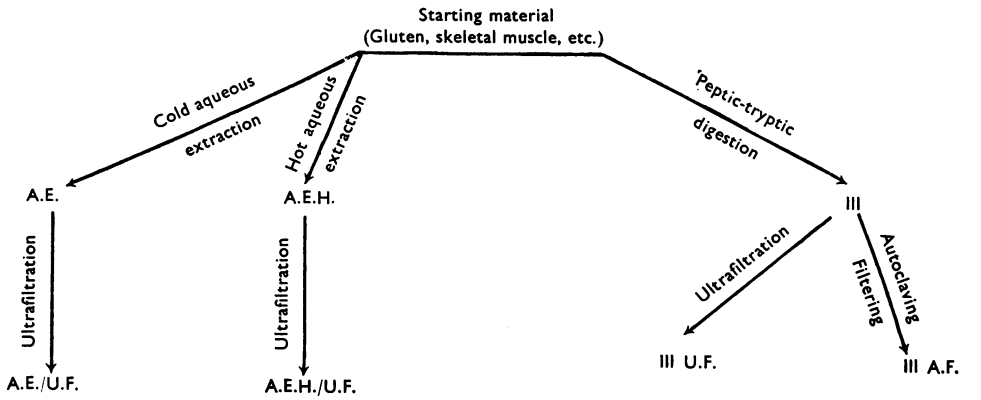

FIG. 1.—Diagram to illustrate the methods used to prepare the various fractions.

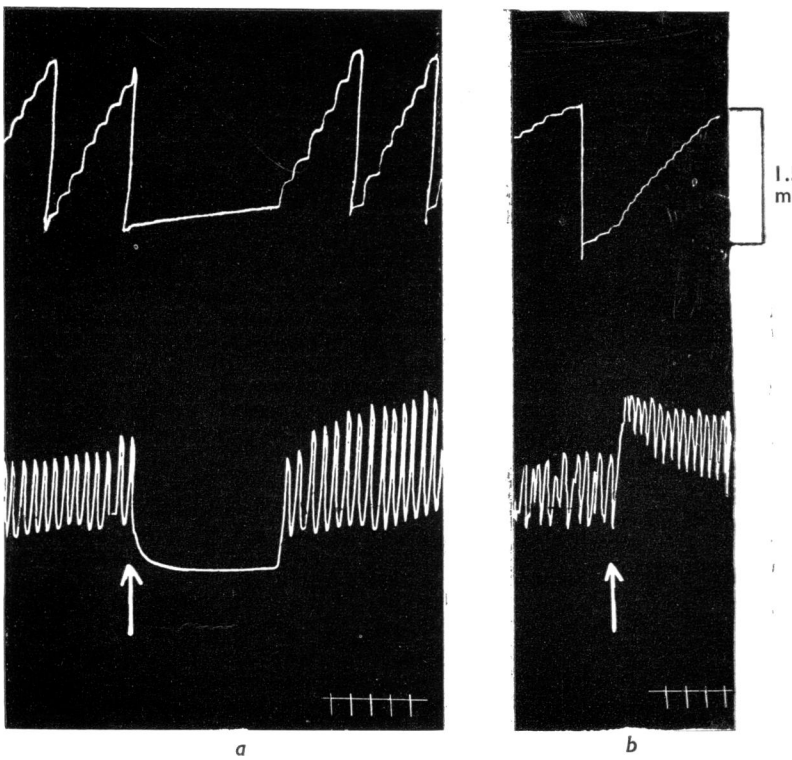

FIG. 2.—Isolated rat jejunum. Upper tracing: volume of fluid expelled during peristaltic reflex. Lower tracing: contractions of the longitudinal muscle. At (a) 2.0 mg./ml. of an autoclaved peptic-tryptic digest of gluten. At (b) 2.0 mg./ml. of an acid hydrolysate of an aqueous extract of skeletal muscle. Time, 30 sec. Doses were applied to the serosal surface.

skeletal muscle. Fractions A.E., A.E./U.F., A.E.H./U.F., III and III A.F. were prepared as described above for gluten.

Chemicals

Pure Proteins.—Casein and gelatin (British Drug Houses) and albumin (bovine plasma, fraction V, Armour laboratories) are all water soluble; samples from 10% aqueous solutions were used without further purification. In addition the following fractions were tested: casein A.E./U.F. and casein III and III A.F., and gelatin III and III A.F.

Peptides.—Glutathione, glycylglycine (British Drug Houses) and glycylglycylglycine (Nutritional Biochemicals Corporation, Cleveland, Ohio) were tested without further purification. Paper chromatography of these peptides showed no evidence of any ninhydrin-reacting impurities.

Amino Acids.—Glycine, (−)-proline, (−)-glutamic acid, (−)-tryptophan (L. Light and Co. Ltd.), (+)-glutamic acid and (−)-asparagine (British Drug Houses) were also tested.

RESULTS

Effect of Peptic-tryptic Digests of Gluten.—As the primary interest of this investigation was the possible inhibitory effect of digests of gluten on the peristaltic reflex, the following procedure was adopted. The isolated intestine was first tested for its response to 2.0 mg./ml. of the autoclaved filtered peptic-tryptic digest of gluten (III A.F.).

TABLE I

EFFECT OF FRACTIONS OF GLUTEN AND OTHER PROTEIN SOURCES ON THE PERISTALTIC REFLEX OF THE RAT JEJUNUM

Doses in mg./ml. Number of experiments shown in parentheses.

Material	Aqueous Extract		Ultrafiltrate of Aqueous Extract		Peptic-tryptic Digest	Ultrafiltrate of Peptic-tryptic Digest		Filtrate of Autoclaved Peptic-tryptic Digest	
	A.E.		A.E./U.F.	A.E.H./U.F.	III	III U.F.		III A.F.	
	Untreated	Acid Hydrolysate	Untreated		Untreated	Untreated	Acid Hydrolysate	Untreated	Acid Hydrolysate
Gluten	Inhibition 0·2–2·0 (3)	No effect 2·0 (1)	Inhibition 0·02–0·4 (33) No effect 0·02 (1)	Inhibition 0·2–0·4 (5)	Inhibition 2·0 (1)	Inhibition 0·2–2·0 (5) Doubtful 0·4 (1)	No effect 2·0 (2) Doubtful 3·2 (1)	Inhibition 1·5–2·0 (7)	No effect 2·0 (3)
Skeletal muscle	No effect 0·4–1·0 (2) Inhibition 2·0 (4) Doubtful 2·0 (1)	Stimulation 2·0 (1)	No effect 0·2–1·0 (5) Inhibition 2·0 (1)	Inhibition 0·2–1·0 (7)	Inhibition 2·0 (2) Doubtful 0·4 (1)			Inhibition 2·0 (2)	
Casein	No effect 2·0 (1)	Stimulation 2·0 (1)	No effect 2·0 (1)		Inhibition 2·0 (2)			Inhibition 2·0 (1) No effect 2·0 (1)	
Gelatin	No effect 2·0 (1)	Stimulation 2·0 (1)			No effect 1·9–2·0 (4)			No effect 2·0 (2)	
Albumin	No effect 2·0 (1)	Stimulation 2·0 (1)							

Inhibition usually occurred (Fig. 2a). Occasionally there was no response ; the intestine was then discarded. Frequently a slight increase of sensitivity to this inhibitory effect was observed during the course of the experiment. Inhibition occurred immediately on injecting the material into the bath. The preparatory and the emptying phase of the reflex were affected simultaneously, movements ceased and usually the baseline of the longitudinal muscle trace dropped. The duration of the effect varied with the sensitivity of the jejunum. With less sensitive preparations movements returned while the material was still in the bath ; with more sensitive preparations the bath fluid was changed, after the effect had lasted for 2 to 3 min. After washing, the preparation was rested for approximately 10 min., when movements invariably returned.

Effect of Aqueous Extracts of Gluten.—In order to obtain further information regarding the nature of the active material in gluten an aqueous extract of the unmodified protein was tested as well as various further modifications. The results are summarized in Table I. The aqueous extract of the whole protein (A.E.) was about 10 times more active than both the original peptic-tryptic digest (III A.F.), and the peptic-tryptic digest without treatment by autoclaving (III). Ultrafiltration of the aqueous extract and of the peptic-tryptic digest did not alter their activity.

Effect of Acid Hydrolysis on the Activity.—Acid hydrolysis completely abolished the activity of the whole protein, as well as of the peptic-tryptic digest after autoclaving and filtration, and of its ultrafiltrate.

Effect of Other Proteins on the Peristaltic Reflex.—A comparison with other proteins or protein-containing material was made in order to determine whether the inhibitory effect of gluten on the peristaltic reflex is specific for this substance. The results are shown in Table I. An aqueous extract of muscle (A.E.) as well as its ultrafiltrate (A.E./U.F.) depressed the peristaltic reflex slightly, but the activity was less than one-fifth of the corresponding gluten fraction. However, the ultrafiltrate of muscle extracts obtained by boiling (A.E.H./U.F.), as well as the peptic-tryptic digest, were as potent as the corresponding gluten fractions. After acid hydrolysis extracts of skeletal muscle did not inhibit but stimulated the intestine. This stimulation consisted of an increase in the rate of peristalsis and a rise of the baseline of the longitudinal muscle trace (Fig. 2b).

Solutions of the three pure proteins, casein, gelatin and albumin, showed no depressant activity in concentrations of 2 mg./ml. After peptic-tryptic digestion casein but not gelatin inhibited the peristaltic reflex. After acid hydrolysis the three pure proteins, like the skeletal muscle extracts, produced a stimulating effect.

Effect of Peptides and Amino-acids.—Six amino-acids, glycine, (−)-proline, glutamic acid ((+)- and (−)-), (−)-asparagine and (−)-tryptophan, as well as three peptides, glutathione, glycylglycine and glycylglycylglycine, were tested in concentrations of 1.0 to 2.0 mg./ml. In no experiment was there any depression of peristalsis. Occasionally a slight stimulating effect was observed. Tryptophan, the only amino acid that is destroyed by acid hydrolysis, showed such a stimulating effect.

Discussion

The results of this investigation show that some protein sources such as gluten and mammalian skeletal muscle contain water-soluble material that will inhibit the peristaltic reflex in the isolated rat intestine, when applied to the serosal surface. No inhibition was observed with casein, gelatin and albumin under the same conditions. The nature of the active material is as yet undetermined. It is not a large protein, as ultrafiltration, which removes molecules of a weight of over 20,000, did not reduce this effect. Digestion with pepsin and trypsin did not affect activity. This is further evidence that the material is not likely to be a whole protein. Moreover, in one experiment, peptic-tryptic digestion of casein led to the appearance of activity in previously inert material. This was possibly due to the preparation of smaller molecules. Furthermore, denaturation of the protein of such digests by autoclaving did not reduce their activity. The active principle is not a free amino acid, as acid hydrolysis completely abolished the depressant effect on the peristaltic reflex, and in some instances even led to the appearance of a stimulating action which was also exhibited by most of the pure amino acids. Attempts at further purification and identification are being carried out.

These studies are part of a collaborative programme of work on the pathogenesis of gluten-induced enteropathy which is being undertaken in this department under the general direction of Professor A. C. Frazer. We wish to thank Mrs. B. Matthews for technical assistance and the Energen Foods Co. Ltd. for supplies of gluten.

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A GANGLION-STIMULATING PRINCIPLE PRESENT IN PEPTONE

BY

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(RECEIVED FEBRUARY 16, 1960)

One of us (W. T. B.) has previously described a pressor effect of peptone in the rat. This effect was considered secondary to a release of sympathin by the stimulation of sympathetic ganglia. The present experiments show that peptone causes contraction of guinea-pig ileum and inferior eyelid of the rat which is antagonized by hexamethonium. Atropine inhibits equiactive doses of peptone and acetylcholine on the guinea-pig ileum and eserine potentiates them. Boiling peptone in concentrated hydrochloric acid or N sodium hydroxide destroys its activity; incubation with proteolytic enzymes (trypsin, pepsin, and chymotrypsin) has no effect on its activity. It is concluded that peptone acts upon the intramural ganglia of the guinea-pig ileum and on sympathetic ganglia which supply the inferior eyelid of the rat.

In a previous paper one of us described the pressor effect of peptone in the rat (Beraldo, 1958). The peptone-induced pressor effect was not abolished by adrenalectomy, but it was abolished by anti-adrenaline drugs and by paralysing doses of nicotine and hexamethonium. These experiments suggested that peptone stimulated sympathetic ganglia with the consequent release of sympathin.

In pursuing this investigation we have studied the effect of peptone on the guinea-pig ileum, the inferior eyelid of the rat and on the superior cervical ganglion of the cat. The results of these experiments are presented in this paper.

METHODS

Guinea-pig Ileum.—The preparation was suspended in 10 ml. aerated Tyrode solution at 35°. The contractions were recorded by means of a frontal writing lever. The substances employed to make the intestine contract were always used in submaximal dose. Doses were applied for 1 min., and then washed out; they were given at intervals of 2 to 3 min. Hexamethonium and cocaine were allowed to act for periods of up to 4 min.

Inferior Eyelid of the Rat.—This is a modification of the method described by Gertner (1956) for studying the effects of drugs acting on smooth muscle and sympathetic ganglia. Wistar rats weighing 350 to 450 g. were used. The animals were anaesthetized with ether followed by intravenous chloralose (12 mg./100 g.). A cannula was inserted into the trachea. The femoral vein was cannulated with a hypodermic needle mounted in a polythene tube. Heparin (50 to 100 units/100 g. body weight) was injected through the venous cannula. The lingual artery was similarly cannulated. The solutions, if not otherwise stated, were injected through the lingual artery by means of a 1 ml. tuberculin syringe and were washed in with 0.1 ml. of normal saline. The eyelid movements were recorded by means of a thread tied through the lower eyelid and fixed to an isotonic lever with a frontal writing point; the magnification was approximately 17 fold.

Nictitating Membrane of the Cat.—Cats were anaesthetized with ether followed by intravenous chloralose (80 mg./kg.). The lingual artery was cannulated with a hypodermic needle mounted in a polythene tube. All solutions were injected through the lingual artery. The nictitating membrane was connected to an isotonic lever which gave a magnification of about 10 fold.

Drugs Used.—Chloralose (Merck) 120 mg./10 ml. solution in saline. Heparin (Vitrum) 5% solution (5,000 units/ml.). Witte's peptone (Friedr. Witte, Rostock) 10 to 20% fresh solution in distilled water. The peptone solution was acidified with concentrated hydrochloric acid to *p*H 2 to 3, shaken with permutit (1 g. of permutit/g. of peptone), filtered and neutralized before use. This treatment removed histamine from the solution. Most of the experiments were performed with Witte's peptone, but other peptone preparations were also used (Pfanstiehl Peptone and Bacteriologic Peptone, Parke, Davis).

The quantities of hexamethonium bromide (Bistrium, Squibb), atropine sulphate (Poulenc Freres),

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1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) (Parke, Davis), cocaine hydrochloride (Enila), acetylcholine hydrochloride (Roche) and eserine sulphate (Evans Medical Supplies Ltd.) used are expressed as weights of the salts. Nicotine (Schering-Kahlbaum) is given as the weight of the base. Chymotrypsin, crystallized salt free; and trypsin, 50% magnesium sulphate (Worthington Biochemical Sales Co.). Pepsin, 2 x crystallized (Nutritional Biochemical Corporation). Darco (activated carbon, Atlas Powder Co.) and permutit, Folin (Anachemia).

RESULTS

Experiments on the Guinea-pig Intestine

When peptone in a dose of 20 to 40 mg. (0.1 to 0.2 ml. of a 20% solution) was added to the guinea-pig ileum, the muscle responded by a contraction in about 40% of the preparations. In these same preparations 0.01 to 0.02 μg. of acetylcholine usually produced a contraction of 3 to 5 cm. In the remaining 60%, peptone commonly failed to produce a response and the sensitivity to acetylcholine was correspondingly low; the effective dose of acetylcholine lay between 0.1 and 0.5 μg. When 0.5 to 1 μg. of eserine was added to the bath the sensitivity of the preparation to acetylcholine and to peptone increased by about 5 to 15 times.

Fig. 1 illustrates an experiment in which pronounced potentiation of the peptone response had occurred.

When atropine was added in a concentration which inhibited equiactive doses of peptone and acetylcholine, the recovery of the response to subsequent doses of the drugs followed a parallel course. This fact suggested that acetylcholine or some other ester of choline was a constituent of the peptone solution. Fig. 1 (B) shows the results obtained in one such experiment.

The possible presence of a choline ester in the peptone was investigated. 5 ml. of 10% peptone solution was adjusted with N sodium hydroxide to *p*H 8.5. Control experiments were performed with 5 ml. of acetylcholine solution (10 μg./ml.) at the same *p*H. Both solutions were left at room temperature (20°). After 1 hr. the *p*H of the solutions was readjusted to 7.4 and the activity of each solution was tested on the guinea-pig ileum. The activity of the acetylcholine solution had disappeared completely, but that of the peptone remained unaltered.

Eserinized guinea-pig ileum did not become desensitized or show tachyphylaxis on repeated additions of peptone to the bath over a period of 7 to 8 hr.

Mode of Action of Peptone.—The question remained whether or not peptone acted directly on the smooth muscle or on the nervous structures in the intestine.

Cocaine.—If peptone acted by stimulating nervous structures in the intestine, cocaine would be expected to abolish its effect. Feldberg (1951) has shown that cocaine in weak concentrations paralyses the nervous tissue of the gut, thereby abolishing the contractions to nicotine but not to histamine, the effect of which is actually increased. The fact that cocaine paralyses the nervous structures without reducing the response to histamine may be taken as evidence against a nervous component in the action of histamine on the intestine.

When in the present experiments the nervous structures were paralysed by cocaine, the ileum did not respond to nicotine and peptone, but

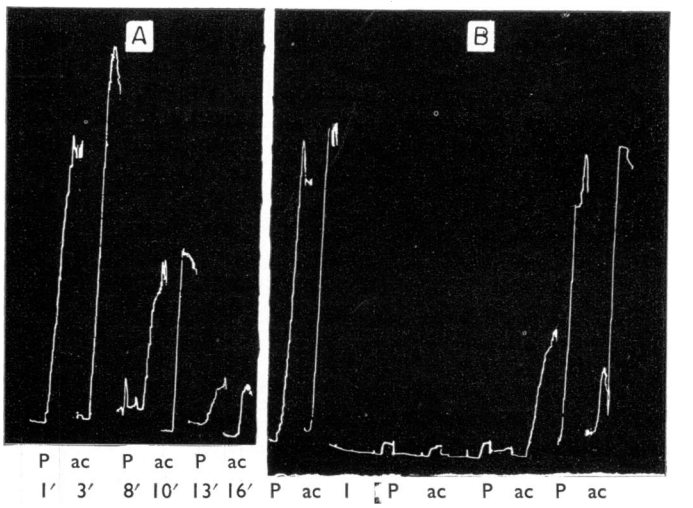

FIG. 1.—Guinea-pig ileum preparation in 10 ml. bath. A, Effect of 1 μg. eserine, added previously to the bath, on contractions caused by 40 mg. peptone (at P) and by 0.02 μg. acetylcholine (at ac). Time in min. shows the gradual reduction both of the acetylcholine and peptone response as the eserine potentiation disappears. B, Effect of 1 μg. atropine (1), allowed to remain in the bath for 1 min., on contractions caused by 40 mg. peptone (at P) and 0.02 μg. acetylcholine (at ac). 1 μg. eserine was maintained in the bath throughout the experiment. A period of 3 min. elapsed between (A) and (B). Acetylcholine and peptone were added to the bath at 2 min. intervals.

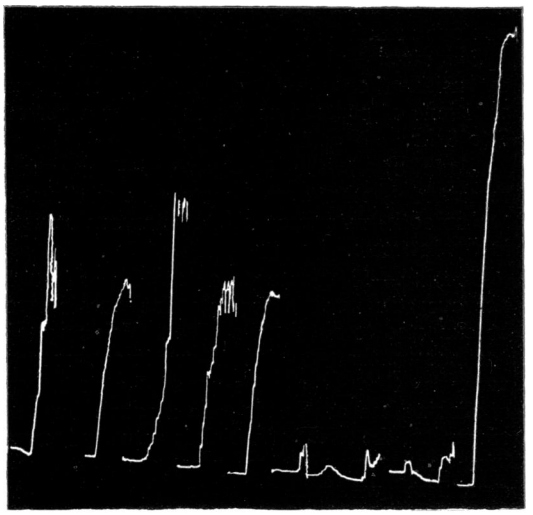

FIG. 2.—Guinea-pig ileum preparation in 10 ml. bath. Effect of 300 μg. cocaine (C) (allowed to remain in the bath for 2 min.) on the response to 20 mg. peptone (at P), 3 μg. nicotine (at Ni) and 0.1 μg. histamine (at H). The substances were added to the bath at 2 min. intervals.

histamine retained its full effect and at times produced a stronger contraction. The results of these experiments suggest that peptone acts on the guinea-pig intestine by stimulating nervous structures in the intestinal wall. This is illustrated in Fig. 2.

Hexamethonium.—Since equally strong contractions to peptone and nicotine were abolished by cocaine, the further question arose whether peptone acted by stimulating nerve cells or nerve fibres in the intestinal wall. An answer was sought by paralysing the ganglion cells of the myenteric plexus with ganglion-blocking agents such as hexamethonium. The effect of hexamethonium (100 μg. to 10 mg.) on the response to doses of peptone (10 to 20 mg.) was tested 22 times on 11 different preparations. The response was reduced in 17 of the tests, unchanged in 3 and augmented in 2. In 7 preparations the reduction was between 80 and 100%, and in 10 between 26 and 64%. The percentage reduction in the response to peptone was obtained by measuring the height of the contractions immediately before and during the action of hexamethonium. In order to test the effectiveness of ganglionic blockade after paralysing doses of hexamethonium, either 1,1-dimethyl-4-phenylpiperazinium iodide, a potent ganglion-stimulating drug studied by Chen, Portman, and Wickel (1951), or nicotine was used. When the responses of the intestine to equiactive doses of nicotine and peptone were blocked with hexamethonium, the recovery of sensitivity to subsequent doses of these drugs followed a parallel course, as shown in Fig. 3. Similar results were obtained when 1,1-dimethyl-4-phenylpiperazinium iodide was used instead of nicotine. Since the action of peptone under these conditions was similar to that of nicotine and DMPP, it is suggested that the effect of peptone on the guinea-pig ileum was due, at least in part, to stimulation of parasympathetic ganglia in the intestinal wall. The gradual return of the response to peptone, 1,1-dimethyl-4-phenylpiperazinum iodide or nicotine after blockade induced by hexamethonium can be explained on the basis of the gradual recovery of the sensitivity of the ganglia following the removal of the antagonist.

The Action of Peptone on the Inferior Eyelid of the Rat

It has been shown by Gertner (1956) that the contraction of the inferior eyelid of the anaesthetized rat provides a sensitive method for studying the effects of drugs acting on smooth muscle and sympathetic ganglia.

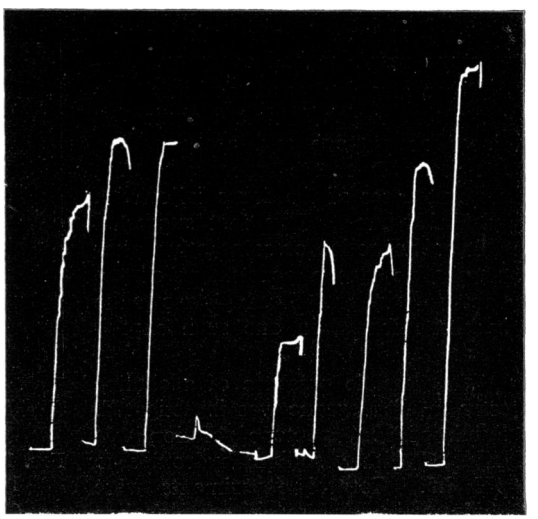

FIG. 3.—Guinea-pig ileum preparation in 10 ml. bath. Effect of 200 μg. hexamethonium (He) (allowed to remain in the bath for 4 min.) on the response to 20 mg. peptone (at P) and 5 μg. nicotine (at Ni). 1 μg. eserine was maintained in the bath throughout the experiment. The substances were added to the bath at 2 min. intervals.

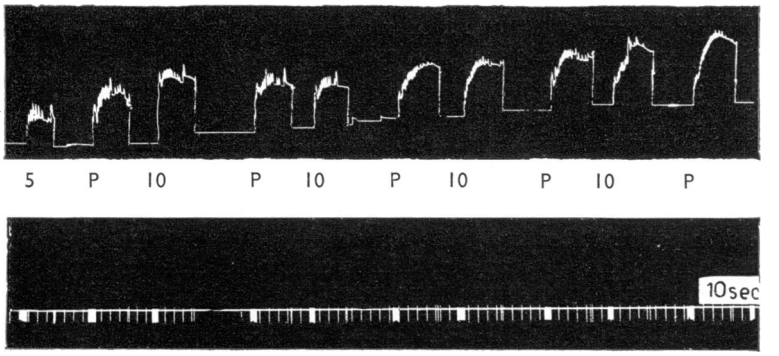

FIG. 4.—Inferior eyelid of rat (400 g.). Chloralose anaesthesia. 5, 10=5 and 10 μg. 1,1-dimethyl-4-phenylpiperazinium iodide. P=5 mg. Witte's peptone. The injections were made into the lingual artery at 10 min. intervals.

Since peptone stimulates sympathetic ganglia in the rat (Beraldo, 1958), it was decided to investigate the effect of peptone on the response of the inferior eyelid of the rat.

When peptone (1.25 to 2.5 mg. or 0.025 to 0.05 ml. of 5% solution/100 g. of body weight) was injected into the lingual artery the inferior eyelid contracted within a few seconds. Repetition of the same dose of peptone at 10 min. intervals produced a constant effect; desensitization or tachyphylaxis did not occur. The effect of peptone was compared with that of 1,1-dimethyl-4-phenylpiperazinium iodide. Thus, the contraction of the inferior eyelid produced by 5 to 10 mg. of peptone in a group of 12 rats was roughly equivalent to that produced by 5 to 50 μg. of 1,1-dimethyl-4-phenylpiperazinium iodide. The result is shown in Fig. 4.

Peptone Response After Hexamethonium.—In order to investigate whether peptone acted directly on the smooth muscle fibre of the inferior eyelid of the rat or through stimulation of sympathetic ganglia, paralysing doses of hexamethonium were used. The effectiveness of ganglionic blockade was tested with 1,1-dimethyl-4-phenylpiperazinium iodide. The response to peptone (2 to 5 mg./100 g. body weight) was followed in a series of 10 rats which had been previously treated with hexamethonium (0.5 to 5 mg.). In 6 of the rats the responses were reduced by 100%; in 2 the reduction lay between 50 and 60%, and in 2 it remained unchanged as compared with the height of the tracing of the contractions of the inferior eyelid immediately before the injection of the hexamethonium. Fig. 5 shows a partial blockade of the effect of peptone and 1,1-dimethyl-4-phenylpiperazinium iodide on the inferior eyelid of the rat as produced by 2.5 mg. hexamethonium. The blockade and recovery of the response to peptone and 1,1-dimethyl-4-phenylpiperazinum iodide followed, as on the guinea-pig ileum, a parallel course suggesting a similar mechanism of action for the two substances.

Effect of Peptone on the Nictitating Membrane of the Cat

The nictitating membrane of the cat did not contract when a dose of 10 to 50 mg. peptone was injected into the lingual artery (3 experiments). However, the injection of 5 to 10 μg. 1,1-dimethyl-4-phenylpiperazinum iodide or nicotine in the same preparation caused strong contractions. Thus, peptone failed to stimulate the superior cervical ganglion of the cat.

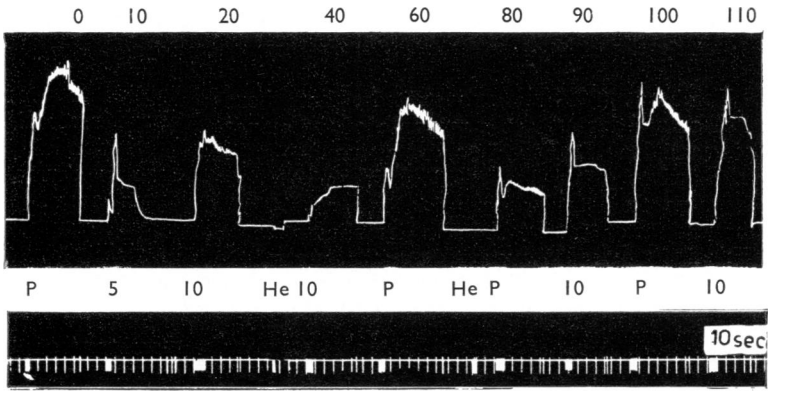

FIG. 5.—Inferior eyelid of rat (370 g.). Chloralose anaesthesia. P=20 mg. Witte's peptone. 5, 10=5 and 10 μg. 1,1-dimethyl-4-phenylpiperazinium iodide. He=intravenous injection of 2.5 mg. hexamethonium. A period of 10 min. elapsed between the injection of hexamethonium and those of 1,1-dimethyl-4-phenylpiperazinium iodide and peptone which were injected into the lingual artery according to time intervals given in min. at the upper part of the tracing.

Some Properties of the Active Principle Present in Peptone

The active principle was dialysable. It was destroyed by boiling in concentrated hydrochloric acid as in the method for extraction of histamine (Code, 1937). However, it withstood boiling for 1 hr. in 0.5 N hydrochloric acid; boiling for 30 min. in sodium hydroxide destroyed it. The treatment of peptone solution with permutit, which removed histamine, did not remove the active principle from the solution. The active principle was partially retained on charcoal, but it was not possible to elute it with 0.05N or 0.1N hydrochloric acid.

The incubation of peptone for 2 hr. with proteolytic enzymes such as chymotrypsin, pepsin and trypsin had no effect on the activity of peptone when tested on guinea-pig gut or inferior eyelid of the rat. Parallel tests were performed with bradykinin and peptone incubated with chymotrypsin. While the activity of the bradykinin was destroyed in 30 min. by chymotrypsin, the activity due to peptone remained unaltered.

Discussion

The results of the experiments described above provide evidence of a ganglion-stimulating principle in peptone. The smooth muscle stimulating effect on the gut was not abolished or diminished when peptone was maintained at *p*H 8.5 for 1 hr. at 20°, whereas equiactive doses of acetylcholine, when submitted to similar treatment, were inactivated. These results indicate that the ganglion-stimulating principle in peptone is unlikely to be acetylcholine or some other ester of choline.

Feldberg (1951) demonstrated that hexamethonium depressed the response of the guinea-pig and rabbit ileum to potassium chloride, and Ambache and Lessin (1955) showed that the response to potassium chloride was consistently reduced after treatment with botulinum toxin. This finding was taken as evidence that part of the action of potassium on the intestine was by stimulation of nervous structures. Both of these authors used relatively large doses of potassium chloride (10 to 15 and 8 to 20 mg., respectively) to evoke contraction of the guinea-pig ileum. Since in our experiments peptone was used in doses ranging from 20 to 40 mg., the action of peptone on the intestine could not have been caused by potassium in the peptone. Further, the effect of peptone disappeared after boiling the solution for 30 min. with N sodium hydroxide or with concentrated hydrochloric acid.

It has been shown that histamine stimulates the superior cervical ganglion of the cat (Konzett, 1952; Trendelenburg, 1954). It was therefore possible that the response of the inferior eyelid to peptone was mediated through the release of histamine. The effect of histamine on the eyelid response was tested in 3 experiments by injecting doses of 0.1 to 1 mg. into the lingual artery. In none of the experiments did the inferior eyelid respond to doses in that range, although a typical response was obtained with peptone. It is interesting to contrast these results with those obtained on the guinea-pig ileum: treatment of this preparation with cocaine abolished the effect of peptone and nicotine, but not that of histamine.

Both the spasmogenic effect of peptone on the guinea-pig ileum and its action on the inferior eyelid of the rat might have been caused by the release of 5-hydroxytryptamine. Robertson (1954a, 1954b) found that 5-hydroxytryptamine stimulated the perfused superior cervical ganglion of the cat and that this action was not blocked by hexamethonium. Rocha e Silva, Valle and Picarelli (1953) obtained evidence that 5-hydroxytryptamine acted on the postganglionic cholinergic fibres of the intramural nervous system of the guinea-pig ileum, which explains why hexamethonium failed to inhibit the action of 5-hydroxytryptamine on this preparation. The spasmogenic action of peptone on the guinea-pig ileum, as well as its action on the inferior eyelid of the rat, however, were diminished or completely inhibited by hexamethonium.

The possible presence in peptone of an active peptide or peptides which might account for its ganglion-stimulating action was investigated. The incubation of peptone with proteolytic enzymes such as chymotrypsin, pepsin and trypsin, however, had no effect on the activity of peptone. These results, however, do not exclude the possibility that the activity is due to a peptide, or peptides, present in peptone.

The negative results obtained with the nictitating membrane of the cat are at variance with those obtained with the inferior eyelid of the rat. The failure of peptone to stimulate the superior cervical ganglion of the cat remains unexplained.

It is possible that peptone formed during protein digestion may be concerned with motility of the gastro-intestinal tract in some animal species.

The authors would like to thank Dr. M. Rocha e Silva (Faculdade de Medicina, Ribeirao Preto) and Eline S. Prado (Escola Paulista de Medicina) for kindly supplying bradykinin and 1,1-dimethyl-4-phenylpiperazinium iodide respectively). This work was supported in part by funds received from the Conselho Nacional de Pesquisas.

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THE ABSORPTION OF PROTHIDIUM BY *TRYPANOSOMA RHODESIENSE*

BY

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(RECEIVED AUGUST 19, 1959)

When rats, heavily infected with *Trypanosoma rhodesiense*, were injected with Prothidium and killed 1 to 5 hours later, measurable amounts of the drug could be extracted from the parasites: a million trypanosomes have been shown to absorb 0.01 to 0.06 μg. of Prothidium *in vivo*. When viewed with the fluorescence microscope, treated trypanosomes appeared to concentrate Prothidium particularly in the blepharoplast and other cytoplasmic granules. Prothidium was absorbed by trypanosomes *in vitro* in less than 30 min. When equilibrium had been reached the concentration of the drug inside the trypanosome was approximately 400 times the concentration outside, in the range of concentrations studied.

Prothidium (7-amino-2-(2-amino-6-methylpyrimidin-4-yl)amino-9-*p*-aminophenylphenanthridine 10,1'-dimethobromide) was introduced by Watkins and Woolfe (1956) as a powerful prophylactic agent against cattle trypanosomiasis. When exposed to ultra-violet light, dilute solutions of the compound in distilled water show a brilliant orange fluorescence with a peak absorption of 315 mμ, which is detectable down to dilutions of 1 μg./ml. This property of fluorescence has been used to study the absorption of Prothidium by *Trypanosoma rhodesiense*.

METHODS

Trypanosomes.—The strain of *T. rhodesiense* used throughout these experiments was the Maun strain which was isolated in December 1955. The parasite, which is polymorphic, produces a fatal infection in hooded Norwegian rats bred in this Institute. It is sensitive to Prothidium; the minimum effective dose in infected mice is 3.75 mg./kg.

Appearance of Trypanosomes after Treatment with Prothidium.—A fresh blood film was taken from the tail of a heavily infected rat 1 hr. after an intraperitoneal dose of 7.5 mg./kg. of Prothidium. The coverslip was sealed to the slide with a Vaseline-paraffin wax mixture and the preparation was examined with the fluorescence microscope (Fig. 1). The fluorescence microscope was a simple arrangement of components; no costly apparatus was used.

Extraction and Estimation of Prothidium.—Intraperitoneal doses of 7.5 mg./kg. of Prothidium were given to 8 rats, all of which had 40 or more trypanosomes in each low-power field of a fresh blood preparation. The animals were killed 4.5 to 5 hr. later and the blood collected and heparinized.

The trypanosomes were separated by differential centrifugation, washed three times in saline and then counted; they were still actively motile at this stage. The trypanosomes were spun down, the saline was removed and replaced by 4 ml. of 5N HCl in order to extract the drug from the parasites. Five min. later, the debris was removed by centrifugation and the supernatant examined under ultra-violet light. Untreated trypanosomes were used as a control and were similarly extracted. The acid extract from treated trypanosomes fluoresced yellow in the ultra-violet whereas the controls did not fluoresce at all. Prothidium fluoresces yellow in acid solution and orange at *p*H 7.0 to 8.5. The fluorescence is quenched at *p*H 9.0 and over; the reactions are reversible. The Prothidium was estimated quantitatively by determining the optical density of the acid extract in a Beckman spectrophotometer (the control extract serving as a blank), using the wavelength 315 mμ. The U.V. absorption curve for Prothidium in 5N HCl is shown in Fig. 2. A standard curve prepared at 315 mμ is shown in Fig. 3.

Absorption of Prothidium by the Trypanosomes in Vitro.—Solutions of Prothidium in saline were prepared so that 0.5 ml. samples contained 20 to 90 μg. of Prothidium and these samples were placed in a series of small glass tubes of 0.5 cm. bore. A concentrated suspension of trypanosomes was obtained by centrifuging the blood of a heavily infected rat. These were washed in nutrient medium (1 part of horse serum with 3 parts of Ringer solution containing 0.2% of glucose) and then suspended in this medium so that there were 140 to 280 million trypanosomes/

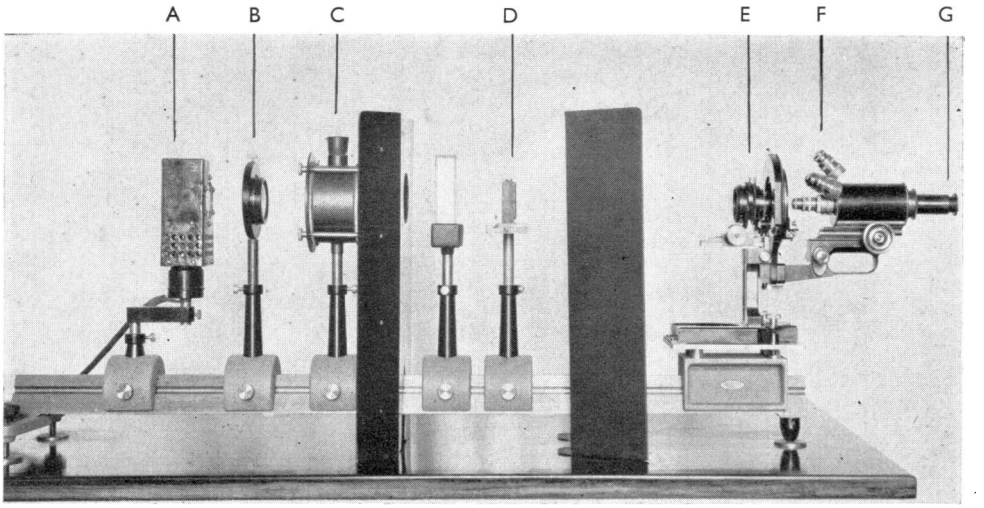

FIG. 1.—The fluorescence microscope. **A**, Activating light source (box type mercury vapour lamp). **B**, Collecting lens. **C**, Heat absorber. **D**, Wratten 18A primary filter. **E**, Cardioid condenser. **F**, Apochromatic objectives (2 mm. 1.4 NA used in this work). **G**, Ocular × 6 (no secondary filter).

ml. Samples of this suspension measuring 0.5 ml. were then placed in each of the small tubes containing Prothidium and incubated for 30 min. at 35°. Control tubes containing Prothidium and nutrient medium alone were also set up. After incubation the trypanosomes were spun down by centrifugation (800 *g*) and the supernatant of each tube was removed into 3 ml. of 5N HCl and the Prothidium content determined. Thus the amount of the drug absorbed by the trypanosomes could be calculated. The partition ratio (concentration inside trypanosomes/concentration outside) was calculated by the method of Hawking (1944). The amount of Prothidium in the trypanosomes was given by the amount which had disappeared from the supernatant; the volume of 100 million trypanosomes was taken to be 2.4 mm.^{3}

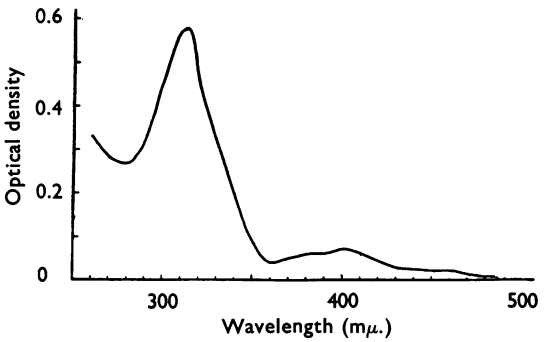

FIG. 2.—The ultra-violet absorption curve of Prothidium in 5N HCl.

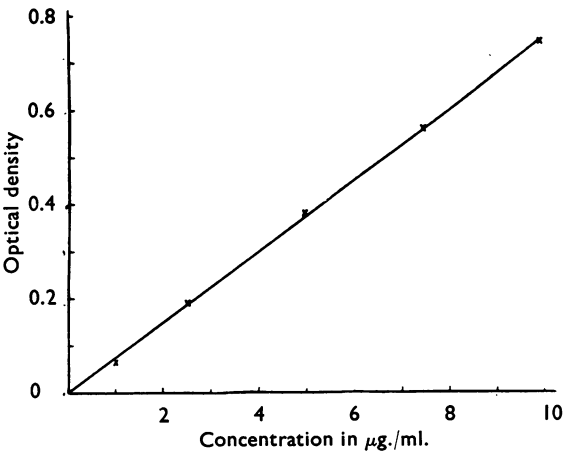

FIG. 3.—Standard curve showing the ultra-violet absorption of Prothidium in 5N HCl at various concentrations, using the wavelength 315 mμ.

RESULTS

Trypanosomes from rats treated with Prothidium exhibited a brilliant orange fluorescence (Fig. 4). The intensity of the fluorescence varied with the type of trypanosome. In the stumpy forms the blepharoplast, the nucleus and the cytoplasmic granules around it shone brilliantly. Some granules in the anterior region of the trypanosome also fluoresced brightly; they

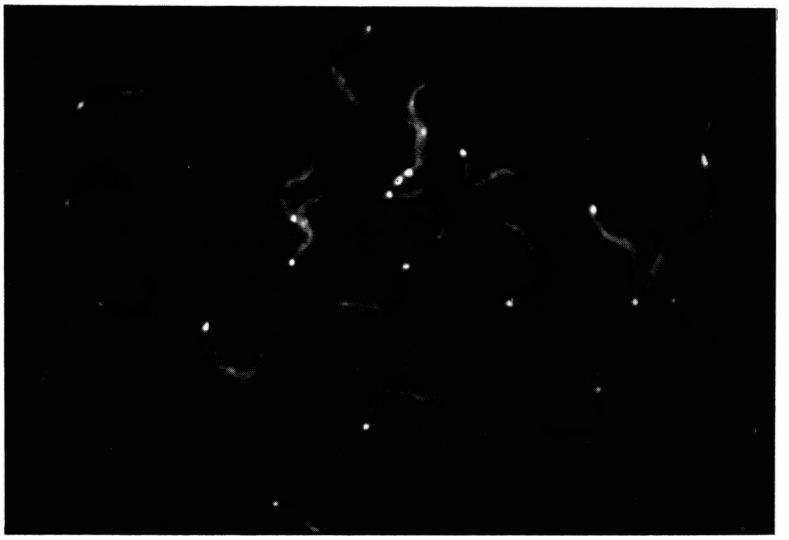

FIG. 4.—*Trypanosoma rhodesiense* after treatment with Prothidium *in vivo*. × 2,750.

were probably volutin granules. The cytoplasm also fluoresced but the flagellum was invisible. The red cells and plasma were non-fluorescent. Long thin trypanosomes did not show much fluorescence in the cytoplasm or nucleus, but their cytoplasmic granules and blepharoplasts shone brilliantly. Thus both types of trypanosome had a strong affinity for Prothidium, which was absorbed and deposited in the blepharoplast and other cytoplasmic granules.

Absorption of Prothidium by Trypanosomes.—Table I shows a million trypanosomes absorbed between 0.01 and 0.06 μg. of Prothidium from the blood of a rat 4.5 to 5 hr. after intraperitoneal injection. When trypanosomes were exposed to the drug *in vitro*, equilibrium was reached in 15 to 30 min. After 30 min. the organisms were still actively mobile, but had absorbed a considerable proportion of the Prothidium originally present (Table II). Fig. 5 shows the partition of Prothidium between trypanosomes and medium. The partition ratio diminished with increasing concentrations of Prothidium. In Fig. 5, the mean concentrations of drug have been calculated from the data in Table II. In these experiments there was a tendency for the curve to flatten off at the higher concentrations; even at the lower concentrations the slope did not reach 45°. Over the range of concentrations of drug used (10 to 45 μg./ml.) the partition ratio ranged from 140 to 590. For the purpose of comparing Prothidium with other drugs a mean figure for the partition ratio may be taken as 400.

TABLE I

THE AMOUNTS OF PROTHIDIUM RECOVERED FROM THE TRYPANOSOMES OF RATS HEAVILY INFECTED WITH *T. RHODESIENSE*

7.5 mg./kg. Prothidium was given intraperitoneally.

Hr. after Dose	Number of Trypanosomes Extracted × 10^6	Total Drug Extracted in μg.	Drug Absorbed by 10^6 Trypanosomes in μg.
4·5	323	20·4	0·063
4·75	307	12·52	0·041
	280	17·2	0·062
	247	6·4	0·025
	272	32·0	0·012
	142	6·4	0·044
	102	31·0	0·031
5·0	178	10·0	0·056

DISCUSSION

The above experiments have demonstrated that "fixation" of Prothidium by *T. rhodesiense* occurs in less than 1 hr. both *in vivo* and *in vitro*.

It has also been shown that there is a quantitative relation at equilibrium between the concentration of Prothidium inside the trypanosomes and that outside; the partition ratio over the range of concentrations studied was approximately 400. The partition ratio for stilbamidine

TABLE II

THE ABSORPTION OF PROTHIDIUM BY *T. RHODESIENSE* AFTER EQUILIBRIUM HAS BEEN REACHED *IN VITRO*

Temperature 35°; duration of exposure 30 min.

Initial Concentration of Drug: μg./ml.	Trypanosomes/ml. × 10^6	Final Concentration of Drug: μg./ml.		Partition Ratio	Drug Absorbed by Trypanosomes: μg. (Mean in Brackets)
		Supernatant Fluid	Trypanosomes		
43·6	71·3	30·8	7,500	200	12·8
		28·4	10,100	355	16·4
		34·8	4,900	140	8·85
		31·6	7,000	220	12·1
					(12·3)
28·8	71·3	17·6	6,600	375	11·3
		16·6	7,200	435	12·3
		18·7	6,000	410	10·4
		17·6	6,600	375	11·3
					(11·3)
12·4	71·3	5·4	3,150	580	6·4
		10·0	5,900	590	2·4
		8·4	4,950	590	4·1
		7·2	4,200	585	5·3
					(4·5)
42·8	140	33·6	3,600	107	9·1
		32·4	3,100	96	10·25
					(9·7)
25·8	140	18·7	2,120	114	7·25
		20·0	1,940	97	5·85
					(6·6)
10·2	140	9·2	2,950	320	0·8
		8·4	3,200	380	1·8
					(1·3)

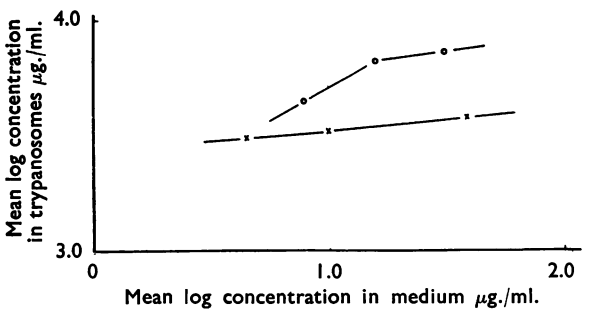

FIG. 5.—The relationship between the concentration of Prothidium in the medium and the concentration in the trypanosomes after equilibrium has been reached at 35° (30 min. exposure).

is about 1,100, for reduced tryparsamide about 5,000 and for acriflavine is about 8,000 (Hawking, 1938 and 1944) so that Prothidium was absorbed less avidly than these three compounds. Examination, by the fluorescence microscope, of trypanosomes which have absorbed the drug shows that it is not evenly distributed over the surface of the cell, but that it is concentrated into the blepharoplast and certain other granules. In this respect, the absorption of Prothidium by trypanosomes is similar to the absorption of stilbamidine (Hawking and Smiles, 1941; Hawking, 1944). The behaviour of stilbamidine is closely similar to that of trivalent arsenicals and acriflavine (Yorke, Murgatroyd and Hawking, 1931; Hawking, 1938) although studies of drug resistance indicate that stilbamidine is absorbed by a different receptor (Lourie and Yorke, 1938; Fulton and Grant, 1955).

Histochemical studies have led Ormerod (1951) to suggest that phenanthridinium compounds act by splitting cytoplasmic ribonucleoprotein into its constituent nucleic acid and protein. Newton (1957) has shown that Promidium (Ethidium bromide) rapidly inhibits deoxyribonucleic acid synthesis in *Strigomonas oncopelti* whilst ribonucleic acid and protein synthesis is apparently unaffected. Since Prothidium is also a phenanthridinium compound, it may be that it exerts a similar action on *T. rhodesiense*.

My grateful thanks are due to Dr. F. Hawking for his advice and encouragement and to Messrs. Boots Pure Drug Company for supplying the Prothidium. I would also like to thank Miss B. C. Staehelin for her willing and skilful technical assistance and Mr. M. Young for taking the photographs.

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THE ABSORPTION, DISTRIBUTION AND EXCRETION OF PROTHIDIUM IN RATS, RABBITS AND CATTLE

BY

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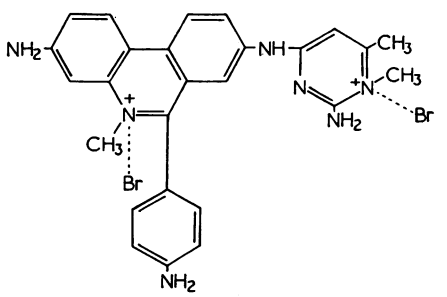
(RECEIVED AUGUST 19, 1959)

2 - Amino-7 - (2-amino-6-methylpyrimidin-4-ylamino)-9-*p*-aminophenylphenanthridine 10,1′ - dimethobromide (Prothidium), a prophylactic drug against cattle trypanosomiasis, was concentrated in the liver and kidneys of rats and rabbits after intraperitoneal or intracardial injection; it was detectable in these organs for 7 days in rats and 10 days in rabbits. The drug protected adult rats against *Trypanosoma vivax* for 8 weeks. Histological examination of the organs of rats treated with Prothidium indicated that no damage had been incurred from the treatment. When cattle were treated subcutaneously, elongated swellings appeared at the site of injection which disappeared within 6 weeks. Excretion of unchanged Prothidium occurred *via* the bile in rats and the drug was detectable in the bile for 9 days, but no Prothidium could be detected in the faeces or urine of rats or rabbits. No metabolic products of the Prothidium were found in the tissues or plasma of rats, rabbits, or cattle. In rat liver perfused for 6 hours with Prothidium only the unchanged drug was recovered. A depot of Prothidium was formed at the site of subcutaneous injection in cattle and this remained for at least 3 months. The prolonged prophylactic action was probably due to the formation of this depot since Prothidium injected intraperitoneally into a calf was excreted at a similar rate to that observed in rats and rabbits.

Prothidium (2 - amino - 7 - (2 - amino - 6 - methyl - pyrimidin - 4 - ylamino) - 9- *p* - aminophenylphen - anthridine 10,1′-dimethobromide) was introduced by Watkins and Woolfe (1956) as a powerful prophylactic agent against cattle trypanosomiasis.



An earlier paper (Taylor, 1960) has described the absorption of Prothidium by *Trypanosoma rhodesiense*. The present studies are concerned with the absorption, distribution and excretion of Prothidium when administered to rats, rabbits and cattle. The effectiveness of the drug as a prophylactic agent against *T. vivax* in rats was also investigated.

METHODS

Prothidium is an orange powder, readily soluble in water or acid. It is less soluble in alcohol and other organic solvents. Solutions are strongly fluorescent, the colour depending on *p*H: acid solutions fluoresce yellow, neutral solutions fluoresce orange, and the fluorescence is quenched by strong alkali. A specially purified sample of Prothidium (kindly supplied by Dr. G. Woolfe of Messrs. Boots Pure Drug Company, Nottingham) was used in all experiments with the exception of those in cattle, where commercial Prothidium was used.

The free base may be extracted into butanol from alkaline solution and quantitatively back-extracted into N sulphuric acid. The solution in sulphuric acid has a characteristic ultra-violet absorption spectrum (maximum at 315 mμ, Fig. 1) and this has been used throughout to identify the drug in tissue extracts. For the estimation of Prothidium at low concentrations it was more convenient to use the more sensitive fluorimetric method described below.

Chemical Methods

Extraction of Prothidium from Tissues.— Preliminary evidence suggested that Prothidium was strongly bound to proteins; dialysis of solutions of Prothidium in plasma or in serum at 4° against

normal saline extracted only 50% of the drug. When a solution of Prothidium in water was dialysed in a similar manner, almost all the drug passed into the dialysate.

Good recovery of Prothidium from either tissue or from plasma was obtained when the material (5 g.) was homogenized with 2N sulphuric acid (30 ml.) in an all-glass Potter homogenizer. The resulting suspension was centrifuged at 16,000 *g* at 4° for 40 min., and the solids were discarded. The supernatant fluid was extracted with ether (10 ml.) to remove fats and subsequently made alkaline by the addition of sufficient 40% sodium hydroxide (7 ml.) to neutralize the sulphuric acid. The Prothidium base was extracted into butanol (10 ml.) and centrifuged to facilitate separation.

The loss on extraction was small and was accepted as a necessary defect of a simple routine method for use on large numbers of samples. For liver, the loss, estimated by recovery of Prothidium (100 μg.) added to a liver homogenate (5 g.) and extracted under the standard conditions described above, was 25 μg. (25%). Recovery from plasma (5 ml.) was 95%. No correction factor has been applied in the estimation of recovery in actual experiments since the percentage loss varies somewhat with different tissues and fluids. The distribution in different organs is unaffected by these losses.

Estimation of Prothidium by Fluorescence.—The butanol extract was washed once with an equal volume of distilled water and dried over anhydrous potassium carbonate (2.5 g.) for 30 min. Fluorescence of the dried butanol extract was measured using a Farrand fluorimeter with an orange secondary filter (Wratten No. 25). A blank was obtained by extracting normal tissue in a similar manner. The concentration of Prothidium present was estimated by comparison with a standard curve (Fig. 2). The performance of the fluorimeter was standardized using aqueous eosin solution at a final concentration of

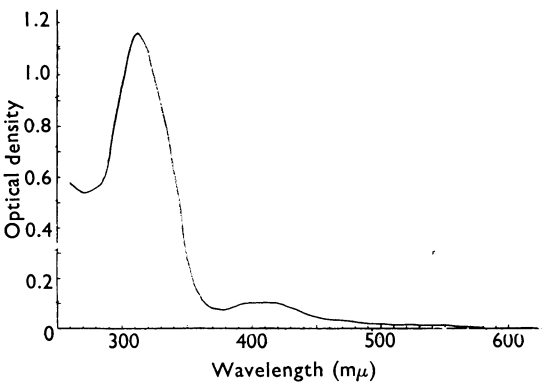


FIG. 1.—The ultra-violet absorption curve of Prothidium in N sulphuric acid.

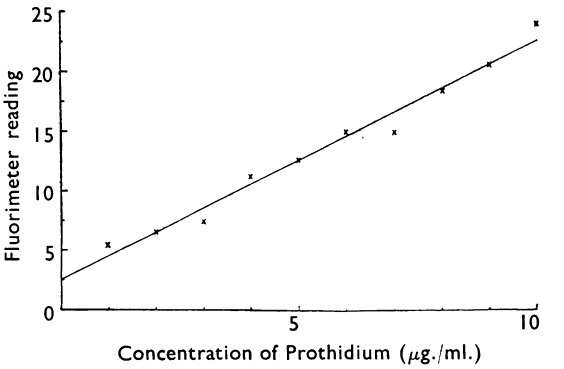


FIG. 2.—The calibration curve of Prothidium for the Farrand fluorimeter.

50 μg./ml. As a check that the fluorescence was due to Prothidium, a sample of the butanol extract was mixed with N sulphuric acid and separated by centrifugation. The ultra-violet spectrum of the sulphuric acid extract was measured in a Beckman spectrophotometer; the peak of the absorption spectrum curve (260 mμ–500 mμ) served to characterize the drug (Fig. 1).

Biological Methods

Tissue Concentrations.—Prothidium was injected into adult rats and rabbits intraperitoneally (7.5 mg./kg.) or intracardially (3.7 mg./kg.). Groups of 2 or 3 animals were killed at intervals and their tissues assayed for Prothidium.

Excretion.—Thirty adult rats, in 10 groups of three, were used in these experiments. Two rats in each group were injected intraperitoneally with Prothidium and the third rat reserved as a control. Immediately after the dose, the bile from the rats in the first group was collected through a cannula in the bile duct over a 24 hr. period, after which the rats were killed. Thereafter, each day for 9 days, a fresh group of rats was treated similarly. The abdomen was opened under ether anaesthesia; the bile duct was identified and freed from the lesser omentum along one inch of its length. The duct was then cannulated using polythene tubing (0.1 cm. diameter) which had been introduced through the side wall of the abdomen. The abdomen was closed and the rat maintained for 24 hr. in a restraining cage whilst the bile was collected. The Prothidium content of the bile (15 to 20 ml.) was estimated after the addition of an equal volume of 2N sulphuric acid and extraction in the usual way.

Perfusion of Rat Liver with Prothidium.—Adult rats were starved overnight and 5 to 8 ml. of blood were removed by cardiac puncture under ether anaesthesia. About 55 ml. of blood was collected in siliconed glass tubes for each perfusion. The plasma was separated from the erythrocytes by centrifugation.

To this plasma was added 30 mg. of powdered Prothidium and the solution continuously stirred using a glass rod. Ten minutes later a clot had formed on the rod; the volume of the clot was decreased by rolling it around the inside of the tube for about 5 min. The plasma was then centrifuged at about 600 *g* for 2 min.; 1 ml. was removed for fluorimetric estimation of Prothidium, and the *p*H of the remainder adjusted to 7.4 with sodium bicarbonate. The treated plasma was then remixed with the erythrocytes, which had been washed once with saline to remove any traces of plasma, and the whole used as the liver perfusate.

The technique of liver perfusion used was that described by Cohen and Gordon (1958), and the surgical technique and the perfusion apparatus were basically as described by Miller, Bly, Watson, and Bale (1952). The liver donor (hooded male rat) was starved overnight. The abdomen was opened along the mid-line, the bile duct and the portal vein identified and cannulated without previous ligation of the gastric and duodenal blood vessels. Perfusion of the liver was commenced as soon as the superior vena cava had been cannulated; the liver was then removed from the animal. Initially, the liver was perfused with Ringer solution to wash out any traces of the donor blood, and the Ringer was then replaced with the treated blood described above. The perfusion was allowed to run for 5 hr., and during this time the bile was collected.

At the end of the experiment the blood was collected for extraction of Prothidium. A 3 mm. cube of liver was frozen in dry ice for histological sectioning at −20°; a 5 mm. cube of liver was fixed in Carnoy and another fixed in formal saline, both for histological sectioning. The remainder of the liver was homogenized in 2N sulphuric acid and the Prothidium was extracted and determined. In some experiments, plasma and bile were extracted by various methods in an attempt to detect any metabolites. Samples were also subjected to paper electrophoresis and chromatography.

A homogenate of the perfused liver was subjected to electrodialysis using the method of Molle (1956). An acid liver brei (8 g. liver in 30 ml. 0.1 N sulphuric acid) was placed in the central cell and the two outer cells were filled with water. Platinum electrodes were used and 100 mA. was passed through the apparatus until the current had fallen to a steady minimum (after about 2 hr.) indicating that all the ions present had travelled to the electrodes. The contents of the cathode cell were removed into 2 ml. of 40% sodium hydroxide, extracted with 10 ml. butanol and back-extracted into N sulphuric acid, and the ultra-violet absorption spectrum determined.

Distribution of Prothidium in a Calf.—Liver biopsies were carried out on a 6-months calf. I am indebted to Mr. Ford of the A.R.C. Unit, Babraham Hall, Cambridge, who kindly demonstrated the technique of liver biopsy. The method used was that described by Loosmore and Allcroft (1951). The tissue samples thus obtained were homogenized in acid and assayed for Prothidium in the usual way. The first liver biopsy, taken 3 days after intraperitoneal injection of 140 mg. of Prothidium in 7 ml. water, yielded 650 mg. of liver. A second liver biopsy (700 mg.) was taken 18 days after injection. Thirteen days later (1 month after injection) the calf was slaughtered and the liver, kidney, and a sample of bile were removed. Samples of the liver, kidney, spleen, muscle, lung, and heart were fixed in formal saline and examined histologically.

Absorption in Cattle.—Three adult barren Ayrshire cows were each given a subcutaneous injection of a 4% solution of Prothidium in water (2 mg./kg.). The site of injection was about a 6-in. square over the eleventh and twelfth ribs about 8 to 10 in. down from the dorsal mid-line.

Prophylactic Activity Against T. vivax *in Rats.*—Adult or young (60 to 80 g.) hooded rats bred in this Institute were given 7.5 mg./kg. of Prothidium by intraperitoneal injection. This dose is close to the toxic range. A group of 3 treated rats, and 2 control rats, were infected immediately with 8 to 12m. parasites. Further groups were inoculated at weekly intervals. Tail blood films were taken daily for six

TABLE I

THE DISTRIBUTION OF PROTHIDIUM AFTER INTRAPERITONEAL AND INTRACARDIAL INJECTION INTO RATS

Route	Intervals after Injection	Percentage of Dose Recovered (Mean Values in Brackets)	
		Liver	Kidneys
Intraperitoneal	2 hr.	11, 26, 24 (20·1)	
	6 ,,	21, 15, 17 (17·5)	
	9 ,,	16, 25, 21 (20·5)	
	12 ,,	18, 23, 25 (21·8)	
	24 ,,	30, 28, 38 (29·0)	5, 2 (3·45)
	48 ,,	16, 9, 11 (11·5)	4, 2 (3·0)
	72 ,,	11, 5, 11 (8·7)	3, 2 (2·5)
	96 ,,	5, 7, 1 (5·3)	0·5, 3 (1·75)
	120 ,,	Trace	3, 3 (3·0)
	192 ,,	0	3, 2 (2·5)
	216 ,,	0	2, 1 (1·5)
Intracardial	2 ,,	23, 15, 12 (16·6)	
	6 ,,	21, 18, 20 (19·5)	
	9 ,,	26, 42, 29 (32·2)	
	12 ,,	37, 48, 27 (36·2)	
	24 ,,	26, 29 (31·0)	
	48 ,,	22, 15 (22·0)	
	72 ,,	3, 10 (3·2)	
	96 ,,	7, 4 (5·4)	
	120 ,,	Trace	
	192 ,,	0	
	216 ,,	0	

days after infection and thereafter weekly for 7 to 11 weeks, and examined for the presence of trypanosomes. The strain of *T. vivax* in this work was the Ilorin (unsupplemented line) strain described by Desowitz and Watson (1953); this strain was made available to me by the courtesy of Mr. Reed of the Wellcome Laboratories of Tropical Medicine, London. The parasite produces a severe and usually lethal infection in laboratory rats and mice.

RESULTS

Absorption and Excretion in Rats and Rabbits

The fluorescence of Prothidium was used to follow visually the fate of the drug after injection. Freshly killed rats were observed under an ultra-violet lamp 2 to 6 hr. after intraperitoneal injection of Prothidium. Under these conditions the liver and kidneys took on an orange fluorescence; the xiphisternum and a small area of the abdominal wall around the site of injection also fluoresced. No orange fluorescence was observed elsewhere in the animals.

The percentages of the dose recovered from rat liver after intraperitoneal and intracardial injections and from rat kidneys after intraperitoneal injections are recorded in Table I; the rates of disappearance of Prothidium from the liver and kidneys of rats and rabbits are shown in Figs. 3 and 4. There were considerable differences between individual animals, but it is clear that the drug appeared in considerable quantities in the liver and kidneys quite soon after injection. Little or no drug was detectable in the gut, spleen, heart, lung or muscle of rats. The rate of disappearance of the drug from the liver and kidneys of both rats and rabbits was rapid for the first 24 hr. after injection, but the drug was detectable in these organs for 7 days in rats and for 10 days in rabbits. A typical ultra-violet absorption curve of these extracts is shown in Fig. 5, demonstrating that unchanged Prothidium was present in the tissues.

Histological sections of treated liver (cut at $-20°$), when viewed with the fluorescence microscope, showed a concentration of fluorescent material in the nucleoli, nuclear membrane and basophilic granules of the hepatic cells. The Kupffer cells were loaded with fluorescent

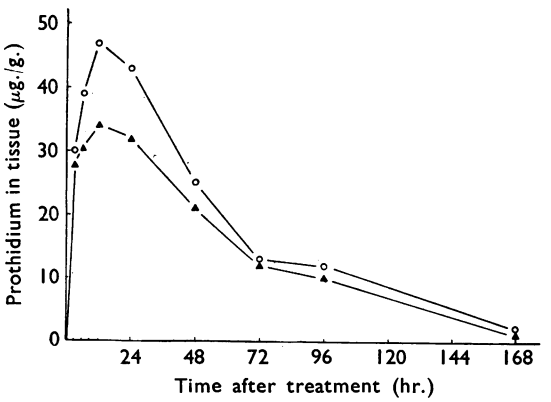


FIG. 3.—The rate of elimination of Prothidium by rat liver and kidneys. O—O = liver (each point on the curve represents the average of 6 rats; 3 rats were injected intraperitoneally and 3 were injected intracardially, but, as the results from each route were similar, the average of the 6 rats was used). ▲—▲ = kidneys (each point on the curve represents the average of 3 rats after intraperitoneal injection).

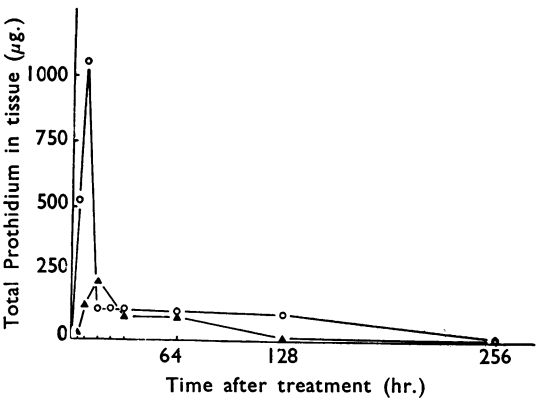


FIG. 4.—The rate of elimination of Prothidium from rabbit liver and kidneys. Each point on the curve represents the average of 2 rabbits, after intraperitoneal injection. O—O = liver. ▲—▲ = kidneys.

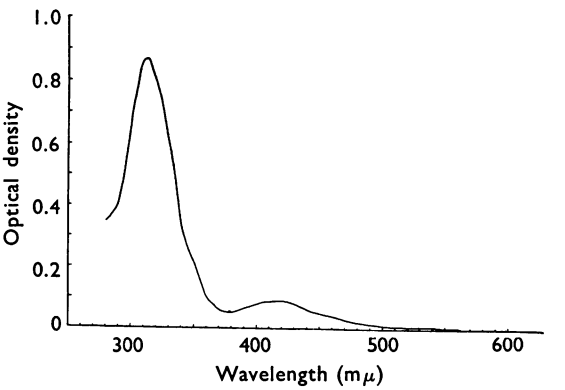


FIG. 5.—The ultra-violet absorption curve of a rat liver extract in N sulphuric acid. The liver of a treated rat was homogenized in 2N sulphuric acid, extracted into butanol and back-extracted into acid.

TABLE II

EXCRETION OF PROTHIDIUM IN THE BILE OF RATS

Each result represents the amount of Prothidium extracted from the bile excreted by one rat in 24 hr. One animal was used for each time point.

Time after Injection (hr.)	Prothidium in Bile (μg.)	Prothidium Given (μg.)	Percentage of Prothidium Recovered/ 24 hr.	Mean Percentage Recovery/ 24 hr.
0–24	135·3 209·0	2,000 1,900	6·75 11	8·9
24–48	285·0 104·0	1,500 2,300	19 4·5	11·7
48–72	46·0 137·0	1,600 2,500	2·85 7·0	4·9
72–96	39·6 43·0	1,800 1,900	2·20 2·25	2·2
96–120	25·6 25·0	1,600 1,550	1·6 1·6	1·6
120–192	24·0 24·2	2,200 2,200	1·1 1·1	1·1
192–216	8·64 10·0	1,600 2,300	0·54 0·44	0·5
	Total Prothidium recovered (mean of each 2 rats)= 558·2 μg.	Average μg. of Prothidium given =1,920 μg.	Percentage of Prothidium recovered in 216 hr.= 30·9%	

material and were frequently swollen with it. Sections of treated liver stained with haematoxylin and eosin appeared little damaged by the drug.

Excretion

Urine from rats and rabbits which had received Prothidium contained little if any of the drug. No Prothidium could be detected by heating the urine samples with acid or alkali to hydrolyse any metabolic derivative, by electrophoresis, by continuous ether extraction or by paper chromatography. If any Prothidium is excreted by this route it can only be a very small amount.

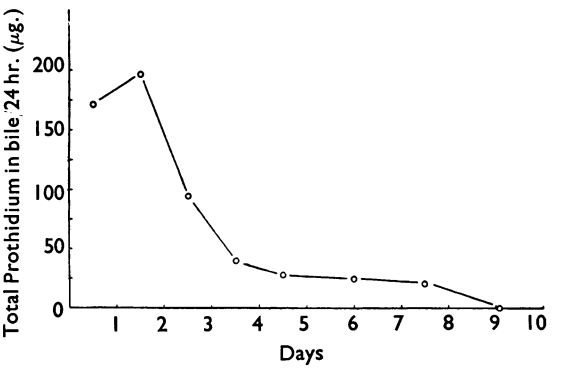


FIG. 6.—The rate of elimination of Prothidium in rat bile. Each point on the curve represents the average of 2 rats.

Since the liver was heavily loaded with Prothidium and since excretion of the drug in the urine appeared to be negligible, the possibility was considered that secretion took place via the bile.

Table II shows the results of experiments in which bile was collected from treated rats; the rate of elimination of Prothidium from the bile is shown in Fig. 6. The drug was rapidly excreted in the bile for the first 3 days and was detectable for 9 days. About 31% of the initial dose of Prothidium was recovered from the excreted bile in 9 days. The ultra-violet absorption curves of the bile extracts corresponded closely with that of the pure drug.

Absorption by Perfused Rat Liver

In view of the concentration of Prothidium in the liver and its rapid elimination from this organ, it was considered possible that this was the site of metabolism of the drug. Plasma, bile and liver tissue taken after 5 hr. of perfusion and examined by various methods of extraction and by paper electrophoresis and chromatography failed to yield evidence of metabolites of Prothidium. A butanol extract of the cathode cell after electrodialysis of a homogenate of perfused liver gave an ultra-violet absorption spectrum which corresponded closely with that of pure Prothidium; no trace of metabolic products could be found.

Free Prothidium was also found in the plasma perfusate and in the bile, but no metabolic product could be detected. Almost all the Prothidium used in the liver perfusion was recovered at the end of the experiment.

TABLE III

THE DISTRIBUTION OF PROTHIDIUM IN CATTLE AFTER INTRAPERITONEAL OR SUBCUTANEOUS INJECTION

i.p.=intraperitoneal; s.c.=subcutaneous.

Animal	Prothidium Given (mg.)	Route	Time after Treatment	Sample	Wt. or Vol. Sample	Conc. of Prothidium in Sample	Total Prothidium Recovered(μg.)	Fluorescence
Calf	140	i.p.	3 days	Liver	0·65 g.	5 μg./0·1 g.	35	
			18 ,,	,,	0·7 ,,	Trace	Trace	
			31 ,,	,,	3·1 kg.	,,	,,	Yellow
				Kidney	660 g.	Nil	Nil	Nil
				Bile	9 ml.	Trace	Trace	
				Muscle	Section			,,
				Lung	,,			,,
				Heart	,,			,,
Cow I (4–5 yr.)	1,026	s.c.	3 weeks	s.c. tissue	28·3 g.	35 μg./g.	994	
				,, fluid	12 ml.	20 μg./ml.	240	
,, II (4–5 yr.)	988	,,	3 ,,	,, ,,	23 ,,	9·9 ,,	227	
,, III (2–3 ,,)	978	,,	3 ,,	,, ,,	31·5 ,,	10·1 ,,	318·2	
,, II			6 ,,	,, tissue	18·2 g.	33 μg./g.	600·6	
,, III			12 ,,	,, ,,	17·3 ,,	15·4 ,,	267	

The Absorption of Prothidium in Cattle

The results of experiments in cattle treated with Prothidium are shown in Table III. The first liver biopsy, taken 3 days after injection of the drug in a calf, contained Prothidium, but no trace of the drug was found in a second specimen taken 18 days after injection. When the calf was slaughtered one month after injection, traces of Prothidium were present in the bile and liver but none was found in the kidneys. The histological sections of all the tissues examined appeared normal; only the (unstained) liver sections showed a yellow fluorescence when viewed with ultra-violet light.

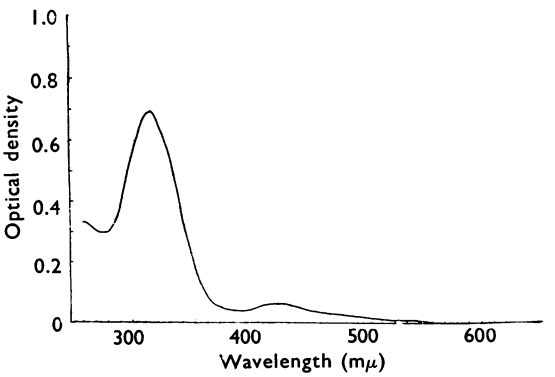


FIG. 7.—The ultra-violet absorption curve of a subcutaneous tissue extract in N sulphuric acid from cow 1. The subcutaneous tissue was homogenized in 2N sulphuric acid, extracted into butanol and back-extracted into acid.

When Prothidium was injected subcutaneously into cows, elongated swellings appeared at the site of injection in all 3 animals; the swellings remained for 6 weeks. They contained a fluid exudate with a high concentration of Prothidium 3 weeks after injection. The subcutaneous tissue at the site of injection also contained a high concentration of drug (Table III; Fig. 7). Six weeks and 12 weeks after injection the subcutaneous tissue at the injection site still contained much unchanged Prothidium, but the swelling had disappeared and there was no fluid.

Prophylactic Action in Rats

A single dose of 7.5 mg./kg. of Prothidium protected rats against *T. vivax* for about 8 weeks and young rats for at least 5 weeks. Young rats developed a transient infection before becoming permanently cured; infected control rats died 2 to 4 days after inoculation. Adult rats were more resistant to infection and untreated infected animals sometimes survived.

DISCUSSION

A single dose of Prothidium will protect cattle against trypanosomiasis for six months (Watkins and Woolfe, 1956). Suramin (Bayer 205) has a

similar prophylactic action in human trypanosomiasis and in this instance the prolonged protection is due to retention of the drug. This retention has been demonstrated for the rat, guinea-pig and monkey (Findlay, 1930). It seemed likely therefore that the protection afforded by Prothidium might also be due to the retention of the drug or its metabolites.

In the present investigation the method used for the estimation of Prothidium was capable of detecting the drug, added to plasma, down to a level of about 2 μg./ml. This is the same order of sensitivity as that of the method used by Boursnell and Wormall (1939) for the estimation of suramin. In contrast to their results with suramin, Prothidium was not detectable in rat blood 1 hr. after intraperitoneal injection of the drug. However, the Prothidium was strongly bound to liver proteins so that the blood concentration was not an adequate measure of the amount of drug retained in the body. When rats were given Prothidium either intraperitoneally or intracardially, the drug could be detected in liver and kidneys for 7 days, and significant levels of Prothidium were detected in the bile secreted between the 7th and 8th days. From a series of experiments in which bile was collected from rats over 24 hr. periods, it was evident that the bile was a major route for the excretion of Prothidium. Examination of the faeces yielded no evidence of the presence of the drug or its metabolites. Prothidium may be broken down by the gut flora or it may be reabsorbed by the small intestine. The concentration of Prothidium in rat's liver and kidney and its excretion *via* the bile show that it is similar in action to another phenanthridinium compound, carbidium ethanesulphonate, which has been shown to have a similar distribution in mice (Goodwin, Goss, and Lock, 1950).

The results of the distribution and excretion experiments in rats suggested that Prothidium would protect these animals against *T. vivax* infection for at least 10 days. Experiments have shown that a single dose of Prothidium protected adult and young rats against *T. vivax* for about 7 weeks in spite of the fact that the young rats developed a transient infection before being fully protected. It may well be that sufficient Prothidium remained bound within the rat tissues to afford protection for this period of time, but there seemed a disinct possibility that Prothidium was converted to an active metabolite which was responsible for the prolonged action of the drug. No metabolite was detected in urine, in blood or in extracts of a liver which had been perfused *in vitro* with the drug, but this evidence is inconclusive because low concentrations of unknown metabolites would almost certainly have escaped detection. In order to obtain significant results on the excretion of metabolites it would be necessary to use radioactive Prothidium and this has not, so far, been available.

The more prolonged prophylactic action of Prothidium in cattle suggested that there might be marked species differences in the rate of excretion of the drug, and accordingly some of these experiments were repeated using rabbits. In these animals Prothidium was detectable in liver and in kidneys for 10 days after a single intraperitoneal injection ; the drug also disappeared at about the same rate from the tissues of a calf after it had received an intraperitoneal injection of the drug. The situation was entirely different when cattle were given Prothidium subcutaneously, the route of injection which is used in the field. The drug formed a depôt at the site of injection and was readily detectable near the site of injection for at least 3 months after treatment. It seems likely therefore that the prolonged prophylactic action of Prothidium is a result of the formation of a local depôt and to the powerful binding of the drug by liver tissue. This would explain the failure to find significant concentrations of Prothidium in the blood of animals which are resistant to trypanosome infection.

Williamson and Desowitz (1956) have shown that a mixture of Prothidium and suramin is less toxic to cattle and shows a greater prophylactic action than either compound alone. When Prothidium and suramin are mixed in the proportions 1:0.77 an insoluble complex is formed and it is probable that the effectiveness of the mixture is due to the formation of a better local depôt at the injection site by such an insoluble salt. Several similar complexes were prepared by these workers by mixing either quinapyramine sulphate, homidium bromide, berenil or RD 2902 with suramin. Extensive field trials were carried out on these substances by Desowitz (1957), who found that the homidium bromide-suramin complex was the most efficient prophylactic in cattle. However, he found that this substance was still highly toxic, whereas no severe local reactions or obvious toxic symptoms were found in the few animals treated with the Prothidium-suramin complex. Since the 3 cows used in the present work also showed little sign of toxic symptoms after treatment with Prothidium, it may be that the Prothidium-suramin complex could be used more safely in the field than the complex of homidium bromide and suramin. No direct comparisons have so far been made.

I would like to thank Dr. F. Hawking for suggesting this problem, and I am deeply grateful to Dr. T. S. Work for his unfailing advice and encouragement in these experiments. I am grateful to Dr. P. Walker, Dr. J. Hitchcock, and Mrs. V. Mijoric for their advice with some of the experiments. I am also much indebted to Miss B. C. Staehelin for her willing and skilful technical assistance.

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THE ANTIMICROBIAL PROPERTIES OF SOME α-AMINO-OXY-ACIDS, α-AMINO-OXY-HYDRAZIDES, ALKOXYAMINES, ALKOXYDIGUANIDES AND THEIR DERIVATIVES

BY

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A series of compounds containing an amino-oxy-group has been examined for antibacterial and antifungal activity. The amino-oxy-acids and the alkoxyamines showed little activity, but the hydrazides, notably the amino-oxydodecyl and the amino-oxytetradecyl compounds, had appreciable activity against *Staphylococcus aureus*. The alkoxydiguanides showed considerable bactericidal activity *in vitro* against Gram-positive and Gram-negative organisms and some activity against *Mycobacterium tuberculosis*. The most active compounds were those with a chain containing 10 to 14 carbon atoms, but the activity was considerably reduced in the presence of serum. They were also active against the few fungi tested. The most active compound, decyloxydiguanide, was moderately toxic when administered intraperitoneally to mice and no therapeutic activity could be demonstrated against an intraperitoneal injection of *Streptococcus pyogenes* administered 15 min. previously.

Canavanine, the *N*-amidino-derivative of an *O*-substituted hydroxylamine, is a powerful inhibitor of certain strains of *Neurospora* (Horowitz and Srb, 1948). Hydroxylamine itself shows slight antibacterial properties (Gray and Lambert, 1948), while certain alkoxyamines (I) and *N*-alkoxyguanidines (II) are active particularly against Gram-positive bacteria (Fuller and King, 1947).

$RONH_2$ (I) $RONH.C(:NH).NH_2$ (II) $RR'C(ONH_2).CO_2H$ (III)

$RONH.C(:NH).NH.C(:NH).NH_2$ (IV)

$RCH(ONH_2).CO.NH.NH_2$ (V)

$RCH(CO_2H).O.NH.C(:NH).NH.C(:NH).NH_2$ (VI)

The preparation of some compounds of types (I) and (III to VI) as potential antibacterials based on hydroxylamine has been described by McHale, Green, and Mamalis (1960) and by Mamalis, Green, and McHale (1960); the antibacterial properties of these compounds are now described. In order to facilitate comparisons with existing data, some of the alkoxyamines (I) of Fuller and King were re-examined. All compounds were tested *in vitro* against representative Gram-positive and Gram-negative organisms; the more active compounds were also screened against a wider range of bacteria including *Mycobacterium tuberculosis* and against some fungi. One compound was examined for its ability to protect mice against *Streptococcus pyogenes* infection.

METHODS

Antibacterial Studies in vitro.—All the compounds were screened against *Staphylococcus aureus* NCTC 4163 and *Escherichia coli* NCTC 8196 using a simple serial dilution test in nutrient broth containing Lab. Lemco 1%, Peptone (Evans) 1%, NaCl 0.5%, at *p*H 7.5. The compounds, in 0.12% aqueous solution adjusted to *p*H 6.8 to 7.0, were added in 5 ml. volumes to 5 ml. of double-strength nutrient broth to give 600 p.p.m. of the substance under test. Twofold dilutions were then made in single-strength broth. The tubes were autoclaved for 10 min. at 10 pounds/square inch pressure and, after cooling, inoculated with 0.02 ml. of a 6 hr. log-phase culture diluted 1 in 10^7. All tubes were incubated at 37° and the minimum inhibitory concentrations (M.I.C.) were observed after 48 hr. Sulphathiazole was included as a reference compound in all tests. Screening against *Streptococcus pyogenes* NCTC 8322, *Corynebacterium diphtheriae* NCTC 3989, *Eberthella typhosa* NCTC 160, *Klebsiella pneumoniae* PCI/602, and *Pseudomonas aeruginosa* NCTC 8203 was similarly carried out both in the absence and presence of 10% normal horse serum.

To determine whether the compounds were bactericidal or merely bacteriostatic, subcultures were

made from the tubes after 18 hr. incubation into large volumes of nutrient broth or on to agar plates. The bactericidal concentration was established as the minimal concentration from which living organisms could not be recovered.

For determination of activity against *Mycobacterium tuberculosis* H 37 Rv, twofold serial dilutions of the compounds in 3 ml. of Dubos and Davis Tween Albumin medium were infected with 0.02 ml. of a 10 day Dubos and Davis culture diluted 1 in 10 and incubated at 37° for 14 days. Isoniazid and *p*-aminosalicylic acid were included as standards for each batch of tests.

Antifungal Studies in vitro.—Antifungal activity was assessed using techniques essentially similar to those of Collier, Potter and Taylor (1955). Dequalinium chloride and chlorhexidine diacetate were used as reference compounds.

Antibacterial Studies in vivo.—Attempts were made to demonstrate systemic activity in mice by protection experiments using *Streptococcus pyogenes* (Richards). A culture of sufficient virulence was obtained after repeated passage through mice, and a 6 hr. log-phase culture used. An infecting dose of 0.2 ml. of a 1 in 10^6 dilution of such a culture (equivalent to 20 lethal doses) was administered intraperitoneally 15 min. before administration of the compound.

Results

Alkoxyamines, α-Amino-oxy-acids, and α-Amino-oxy-hydrazides

In confirmation of the observations of Fuller and King, we have found that the hydrochlorides of alkoxyamines (I) have only feeble antibacterial activity *in vitro* with M.I.C.s of the order 300 to 600 p.p.m. for both *Staph. aureus* and *E. coli* (sulphathiazole under the same conditions has an *in vitro* M.I.C. of 5 to 10 p.p.m.). Slightly greater activity was observed against *M. tuberculosis* (75 to 600 p.p.m.) as compared with the control compounds (0.125 to 0.5 p.p.m.). The following alkoxyamines (I) were examined: R = hydrogen (hydroxylamine), ethyl, n-propyl, isopropyl, n-butyl, n-pentyl, n-hexyl, heptyl, decyl, dodecyl, and benzyl.

The corresponding α-amino-oxy-acids (III) were slightly more active than the alkoxyamines (I) against *Staph. aureus* and *E. coli* (ca. 150 to 600 p.p.m.) while activity against *M. tuberculosis* (ca. 37.5 to 150 p.p.m.) was also somewhat greater. The following α-amino-oxy-acids (III) were examined: R = hydrogen, R′ = hydrogen, methyl, ethyl, n-propyl, isopropyl, n-butyl, n-pentyl, n-hexyl, heptyl, octyl, decyl, dodecyl, 4-cyclohexyl-butyl; R = R′ = methyl.

The possibility existed that the amino-oxy-acids might be amino-acid antagonists and this was investigated with α-amino-oxyisovaleric acid using *Leuconostoc mesenteroides* P. 60, an organism exacting towards valine, the analogous amino-acid. The growth-promoting effect of valine on a valine-deficient synthetic medium was found to be stimulated rather than inhibited by the amino-oxy-analogue, which in fact exerted a slight but significant sparing effect on valine utilization. In the absence of valine, the amino-oxy-compound was itself utilized to a limited extent, about 1,000 μg. being needed to replace 20 μg. valine.

Some α-amino-oxy-hydrazides (V; R = n-hexyl, heptyl, octyl, decyl, and dodecyl) were tested for antitubercular activity *in vitro* and shown to be rather more active (M.I.C.s 9 to 75 p.p.m.) than the analogous acids. All but two of the hydrazides showed little activity against *Staph. aureus* and *E. coli* (about 300 p.p.m.), the exceptions being (V; R = dodecyl and tetradecyl) which inhibited growth of *Staph. aureus* at 30 and 37.5 p.p.m. respectively.

Alkoxy- and Arylmethoxy-diguanides in vitro

While the alkoxyamines (I), the acids (III), and the hydrazides (V) showed little or no increase in activity against *Staph. aureus* and *E. coli* with increasing molecular weight, the same was not true of the alkoxydiguanides (IV). The hydrochlorides of a series of diguanides (IV; R = hydrogen, ethyl, n-butyl, isobutyl, n-pentyl, n-hexyl, heptyl, octyl, nonyl, decyl, undecyl, 2-methyldecyl, dodecyl, tetradecyl, and hexadecyl) were examined. The M.I.C.s for both the above two organisms were found to increase rapidly with increasing alkyl chain length, reaching a maximum (0.6 to 1.25 p.p.m.) at C_{10} to C_{12}. The highest members of the series showed reduced activity, perhaps associated with decreased aqueous solubility. The activity of these compounds against *M. tuberculosis* followed a similar pattern with a maximum effect at C_{10} to C_{12} (5 p.p.m.). Branching of the alkyl chain did not affect activity.

Introduction of a carboxyl group into the diguanides to give (VI; R = n-pentyl, n-hexyl, heptyl, octyl, and decyl) resulted in complete loss of *in vitro* activity.

Hydrochlorides of arylmethoxydiguanides were found, in general, to be less active than the alkoxydiguanides. Of the following which were tested [IV; R = benzyl, *p*-chloro-, *p*-bromo-, and 3,4-dichloro-benzyl, 6-chloro-1,3-benzodioxan-8-yl-methyl, 1- and 2-naphthylmethyl, and 1-bromo-2-naphthylmethyl], 2-naphthyl-methoxydiguanide hydrochloride was the most active against *Staph. aureus* (25 p.p.m.) and *E. coli* (12.5 p.p.m.) [cf. decyloxydiguanide dihydrochloride (1.25 p.p.m.) for both organisms].

TABLE I

MINIMAL EFFECTIVE CONCENTRATIONS (P.P.M.) OF SOME ACTIVE ALKOXYDIGUANIDES IN THE PRESENCE AND ABSENCE OF 10% SERUM

Column a: in broth. Column b: in broth + 10% normal horse serum.

	Alkoxydiguanides RONH.C(:NH).NH.C(:NH).NH_2								Reference Compounds					
	$R=C_8H_{17}$ No. 129		$R=C_{10}H_{21}$ No. 130		$R=C_{12}H_{25}$ No. 133		$R=C_{14}H_{29}$ No. 134		Sulphathiazole		Dequalinium Chloride	Chlorhexidine Diacetate
	a	b	a	b	a	b	a	b	a	b	a	a
Staph. aureus 4163 ..	10	50	0·6	37·5	1·5	75	3	75	5	600		
Strept. pyogenes 8322 ..	2·5	12·5	1·25	12·5	2·5	12·5	10	37·5	>300	>1,200		
C. diphtheriae 3989 ..	2·5	12·5	1·25	12·5	0·6	12·5	0·6	12·5	5	6·25		
E. coli 8196 ..	10	37·5	1·25	37·5	1·5	75	6·25	150	10	>600		
Kleb. pneumoniae 1/602 ..	12·5	40	5	30	5	40	100	150	300	400		
Sal. typhii 160 ..	12·5	30	2·5	40	2·5	50	5	>50	25	40		
Ps. aeruginosa 8203 ..	50	150	12·5	300	>200	>600	>200	>600	>600	>1,200		
Microsporon canis ..	20		2·5		2·5		20				<0·6	0·6
Candida albicans ..	>20		10		20		>20				<0·6	10
Trichophyton mentagrophytes ..	20		5		10		>20				<0·6	5
Epidermophyton floccosum ..	10		2·5		2·5		20				<0·6	5

The table shows the activity of the four most active alkoxydiguanides against a wider range of organisms, the least susceptible being *Ps. aeruginosa*. In the presence of 10% serum, the activities were diminished sharply, the M.I.C.s being increased from 5 to 50 fold. With each of the bacteria listed in the table, it was confirmed that the inhibition was bactericidal and not merely bacteriostatic.

The table also shows that antifungal and antibacterial activities run parallel, the peak effect being reached with the C_{10} and C_{12} compounds.

Toxicities

No adverse effects were noted with any of the four compounds in the table when neutralized suspensions (in 0.5 ml.) were injected subcutaneously into mice (19 to 21 g.) in doses up to 1,000 mg./kg. The mice were killed after 9 days and examined: it was found that the C_{10}, C_{12} and C_{14} compounds remained undissolved in the subcutaneous tissue. The more soluble acetate of decyloxyguanide (C_{10}) when injected subcutaneously at 1,000 mg./kg. produced no systemic effects, but severe necrosis developed at the site of the injection. The intraperitoneal toxicity of the latter compound was determined in mice; the LD50 (Miller and Tainter, 1944) was found to be 20 ± 2.5 mg./kg.

In vivo *Experiments with Diguanides*

The activity *in vivo* of decyloxydiguanide acetate (IV; R=decyl), the most active member of the series, was examined in groups of mice infected with *Strept. pyogenes* (Richards). Fifteen min. after intraperitoneal infection with 0.2 ml. of a 6 hr. log-phase culture, the compound was administered intraperitoneally at 10 mg./kg. The treated and control mice died within three days and no evidence for any prolongation of survival times was observed. Although no attempt has been made to isolate metabolic products, it would appear, therefore, that the compound

is excreted or metabolized too rapidly to show any protective effect; diguanides are known to react rapidly with ketones and keto-esters to form cyclic products.

We are indebted to Dr. J. H. Humphrey for the culture of *Streptococcus pyogenes* (Richards), to Dr. H. O. J. Collier and Dr. F. L. Rose for the samples of dequalinium, chloride and chlorhexidine diacetate respectively, and to Mr. K. J. Stevens for valuable technical assistance.

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ANALGESIC ACTION OF ETHYL 4-PHENYLPIPERIDINE-4-CARBOXYLATES WITH OXYGENATED 1-SUBSTITUENTS

BY

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WITH AN ADDENDUM BY R. E. LISTER AND R. P. STEPHENSON

(RECEIVED DECEMBER 17, 1959)

The analgesic potency of a series of new compounds related to pethidine has been measured in rats. The replacement of the methyl group on the nitrogen atom of pethidine by groups containing ether linkages produces an increase in potency. Two different positions for the oxygen atom are particularly beneficial, but the effects, in these two positions, appear to be exerted in different ways and are not additive. Simple toxicity tests in mice and rats and tests on respiration in rats of five of the more potent compounds revealed no unexpected adverse action.

Until about 1955 it was accepted (see Braenden, Eddy, and Halbach, 1955) that in analgesics of the pethidine type, that is, in 4-phenylpiperidines with an alkoxycarbonyl, acyl, or acyloxy group in the 4-position, the most desirable substituent on the nitrogen atom was a methyl group. More recently, it has been shown that more active compounds may be prepared by introducing other substituents on the nitrogen atom (Millar and Stephenson, 1956; Perrine and Eddy, 1956; Weijlard *et al.*, 1956; Elpern, Gardner, and Grumbach, 1957; Janssen *et al.*, 1959).

The present study is concerned with derivatives of pethidine in which the *N*-methyl group is replaced by substituents carrying at least one ether or thioether function. The substances tested were synthesized by Frearson and Stern (1958) and Frearson, Hardy, and Stern (1960), and are listed in Tables I to IV.

METHODS

Analgesia (Green and Young, 1951).—The pressure on the tail needed to make a rat squeak was measured: drug was injected subcutaneously in the flank in 0.2 ml. saline per 50 g. body weight, and the measurement repeated 6 times at 10 min. intervals. Analgesia was assessed in terms of the index of analgesia (Millar and Stephenson, 1956), which was obtained by dividing the pre-injection pressure by each post-injection pressure. The mean of the 6 indices gave a measure of the effect in that rat. The highest pressure applied to the tail was 3 times the average control value; if this did not produce a squeak the index was taken as zero.

The new compounds were first tested, at several dose levels, in groups of 3 rats. From the results of these tests, 2 doses, expected to be equipotent with 2 and 4 mg./kg. of morpheridine (TA 1), were selected. These 4 doses were each administered to 4 or 5 rats. This test was repeated, so that each dose was given to about 9 animals. Most of the results listed in Tables I to IV were obtained from these simple tests.

To obtain a more accurate measure of the potency of some of the more active compounds and of the relations within several small series of close analogues, four-way cross-over tests were performed. High and low doses of 4 drugs were used in each trial. Each of 24 rats received each drug, 2 at the high and 2 at the low dose level. Each dose was thus administered to 12 rats and the design confounded the variance due to differences in slope and the variance due to differences between rats. Since there were 8 doses, the rats were housed 8 to a cage, and on each day of the test each dose was given to 1 rat from each of the 3 cages. Three randomized versions of the design given by Finney (1952) were used to determine the order of treatment.

In Tables I to III the results from the cross-over tests are indicated by the inclusion of fiducial limits ($P=0.95$).

Respiratory Depression.—Rats were anaesthetized with urethane. Respiration was recorded by a modification of Gaddum's method (1941) using a Krogh volume recorder converted into a sensitive pressure recorder (Paton, 1949). Drugs were injected into the femoral vein.

Peristaltic Reflex.—Trendelenburg preparations of the guinea-pig ileum were set up as described by Schaumann (1955). Fresh tissue and also pieces

stored for 24 hr. at low temperature were used. The substances were added to the bath (40 ml.) 1 min. before the intraluminal pressure was raised. Three doses of each drug were given so that the contractions were reduced by between 20 and 50%.

Toxicity Tests.—In acute experiments, the drugs in saline, 4 ml./kg., were injected intravenously in mice. Each dose was injected into 5 mice, and if possible the LD50 for each drug was determined. Some of the hydrobromides could only be dissolved in warm saline and the LD50s given for these substances may not be very accurate. (Attempts to use ethanol/water mixtures were abandoned when it was found that morpheridine, injected in ethanolic solution, showed increased toxicity.)

In subacute experiments, 2 groups of 10 newly weaned rats were weighed and given subcutaneous injections daily over 12 days. One group, kept as controls, received saline 4 ml./kg. The other group received 5 times the larger dose used in the analgesic cross-over tests. To obtain some indication of the development of tolerance, an analgesic test was performed on the thirteenth day; suitable doses of the drug under test were given to equal numbers of control and treated animals.

The animals were then killed and blood smears taken. Samples of liver, kidney, spleen, heart, lung, brain, and bone marrow were removed and fixed for pathological examination. Some rats died during these tests and were usually eaten by the survivors, so that they were not available for examination.

Results

Analgesia.—Since the compounds in the present series are similar to morpheridine (TA 1) (Millar and Stephenson, 1956) and since, in the test used, pethidine and morpheridine do not give parallel dose-effect curves, we have compared the new substances with morpheridine rather than pethidine.

A number of compounds in which the *N*-substituent was of the type $-[CH_2]_2.OR$ were tested. The results on those in which R was aliphatic, together with other aliphatic substituents of the type $-[CH_2]_n.OC_2H_5$, are listed in Table I. In the first category, the most active compound was the ethoxyethylnorpethidine ($R=C_2H_5$; TA 24), which was as active as morpheridine. This compound is also one of the more active of the series $-[CH_2]_n.OC_2H_5$. The most active compound, up to and including $n=6$, is TA 33 where $n=4$. Independent observations by Morren and Strubbe (1957) agree with our results for TA 24, TA 25 and TA 36.

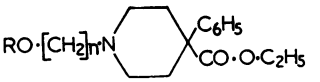
In Table II are listed compounds in which the *N*-substituent is again $-[CH_2]_n.OR$, but where R now contains an aromatic residue. Both the phenoxy- (TA 27) and the benzyloxy-ethylnorpethidine (benzethidine; TA 28) were rather more potent than morpheridine, but the 2-naphthyloxynorpethidine (TA 54) was much less potent. A number of substituted phenoxy-compounds were tested; all were less potent than the parent compound, TA 27. The phenoxybutylnorpethidine (TA 62) was about one-third as potent as TA 27.

Table III shows the results obtained with a miscellaneous group of compounds in which the substituent on the nitrogen atom contains more than one ether grouping and/or a cyclic ether function. The most active member of the present series, furethidine (TA 48), has both of these properties (but see addendum).

In Table IV are collected the results with the sulphur analogues of TA 24, TA 27, and TA 29,

TABLE I

ANALGESIC ACTIVITY OF ALKOXYALKYLNORPETHIDINES

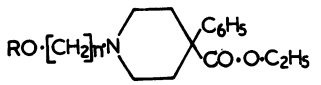


The bases, dissolved in 0.1 N HCl, were examined, except TA 25 and TA 42 which were available as the hydrobromides. Analgesic potency is compared with morpheridine (TA 1) (=100). In Tables I to III, the results based on the 6 cross-over tests may be identified by the inclusion in parentheses of fiducial limits (P=0.95). The superscript numerals identify the number of the cross-over test so that the more immediately comparable figures may be identified. The 3rd and 5th tests did not include morpheridine, since the purpose was to compare small homologous series, and the fiducial limits, though quoted for ease of comparison in terms of the potency of morpheridine, are strictly valid only for comparison within the group of compounds in the particular cross-over test.

TA No.	*n*	R	Relative Analgesic Potency (Limits) in Rats	Intravenous LD50 (Mice) (mg./kg.)
29	2	Methyl	35 (28, 43)[5]	20
24	2	Ethyl	103 (85, 125)[1,3,5]	20
34	2	Propyl	44 (36, 54)[5]	22
35	2	Isopropyl	50	12
36	2	Butyl	42 (35, 50)[5]	15
42	2	Cyclohexyl	25	12
25	3	Ethyl	38 (31, 46)[3]	15
101	3	Methyl	12	—
33	4	Ethyl	265 (191, 368)[3]	15
32	5	,,	73 (56, 94)[3]	17
69	6	,,	47 (38, 61)[6]	—

TABLE II

ANALGESIC ACTIVITY OF ARYLOXYALKYLNORPETHIDINES



The hydrobromides of the bases (dissolved in saline) were examined. An asterisk (*) indicates that the hydrobromide was not sufficiently soluble so the LD50 may be inaccurate.

TA No.	*n*	R	Relative Analgesic Potency (Morpheridine=100) in Rats	Intravenous LD50 (Mice) (mg./kg.)
27	2	Phenyl ..	140 (115, 169)[1]	15
62	4	Phenyl ..	40	17
37	2	*m*-Chlorophenyl	40	30
30	2	*p*-Chlorophenyl ..	15	20
60	2	*o*-Methoxyphenyl ..	10	30
39	2	*m*-Methoxyphenyl ..	40	15
41	2	*p*-Methoxyphenyl	20	25
45	2	*o*-Tolyl ..	11 (7, 18)[4]	20*
44	2	*m*-Tolyl	31 (22, 43)[4]	12*
47	2	*p*-Tolyl ..	17 (13, 24)[4]	15
55	2	*o*-Nitrophenyl	5	—
53	2	*m*-Nitrophenyl	7	50
46	2	*p*-Nitrophenyl	10	25*
51	2	4-Diphenylyl	15	40
40	2	*p*-Acetamidophenyl ..	30	15
43	2	*p*-Ethoxycarbonylphenyl	5	40
54	2	2-Naphthyl	5	—
28	2	Benzyl (benzethidine) ..	145 (117, 177)[1]	7·5

all of which were less active than the corresponding oxygen ethers.

In view of the activity of furethidine and of morpheridine, the "model" substance 2-morpholinoethyl tetrahydrofurfuryl ether was examined: it had no analgesic potency.

In one of the cross-over tests the analysis of variance showed deviation from parallelism significantly greater than chance expectation. This was caused by TA 49, which produced much less increase in effect with dose than the other compounds. In other tests TA 33 and TA 45 also appeared to have lower slopes than the rest of the compounds, but the difference was not significant.

None of the compounds in this series has hitherto been compared directly with morphine and, although many comparisons of morphine and pethidine in rats have been published, we thought it desirable to compare morpheridine and morphine in our conditions. Morpheridine and its analogues usually showed definite peak activity 10 to 20 min. after injection whereas morphine maintained its action longer, so that the result of any comparison depended on its duration. Our test of 1 hr. duration showed that morpheridine was 1.2 times as potent as morphine and furethidine was about 7 times as potent as morphine.

Respiration.—The substances examined all depressed respiratory minute volume when given intravenously in rats anaesthetized with urethane. The rapid onset and short duration of the depression was very similar to that produced by pethidine, but differed markedly from that caused by morphine which was rather slow in onset and prolonged. The potent analgesics morpheridine, benzethidine, furethidine, TA 27, and TA 33 produced respiratory depression in smaller doses than pethidine, but the difference was usually less than the difference in analgesic potency. Indeed, furethidine appeared to have an appreciable advantage over pethidine, though this may reflect the different conditions and route of administration rather than any separation of analgesic action from respiratory depression.

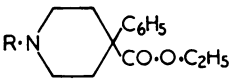
Peristaltic Reflex.—The derivatives depressed both the emptying and the preparatory phases of the peristaltic reflex and the relative activity of some of the compounds was assessed on the preparatory phase. The results, listed in Table V, correspond with those of analgesic tests. Results on fresh tissues differed slightly from those on stored preparations, possibly because of the occurrence of the emptying phase in the fresh tissue.

Toxicity Tests.—The acute intravenous LD50 of most of the substances is listed in Tables I to IV. Death was apparently due to respiratory depression and the convulsions which occur with pethidine were not observed with these derivatives.

In order to obtain some indications of any pathological lesions produced by the more potent substances, doses 5 times as great as the high dose of the analgesic cross-over test were given daily for 12 days. With each of the 6 compounds tested this dose was sufficient to kill at least one of the 12 rats. These deaths were presumed to be

TABLE III

ANALGESIC ACTIVITY OF NORPETHIDINE DERIVATIVES CONTAINING A CYCLIC ETHER GROUPING OR MORE THAN ONE ETHER GROUPING

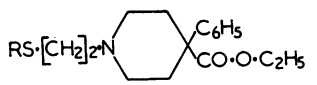


All substances except TA 61 were available as bases and were dissolved in dil. HCl. TA 61 was available as the hydrobromide and was dissolved in saline.

TA No.	R	Relative Analgesic Potency (Morpheridine=100) in Rats	LD50 (Mice) (mg./kg.)
57	Tetrahydrofurfuryl	15	45
20	2-(Tetrahydropyran-4-yl)ethyl ..	90	30–40
48	2-(Tetrahydrofur-furyloxy)ethyl (furethidine) ..	729 (564, 960)[2]	25
70	3-(Tetrahydrofur-furyloxy)propyl ..	35	—
63	4-(Tetrahydrofur-furyloxy)butyl ..	125	7·5
49	2-(Tetrahydropyran-2-ylmethoxy)ethyl	265 (167, 420)[2]	10
65	2-(Tetrahydropyran-2-yloxy)ethyl ..	17 (13, 22)[6]	—
64	4-(Tetrahydropyran-2-yloxy)butyl ..	35 (27, 45)[6]	—
38	2-(2-Ethoxyethoxy)-ethyl	39 (30, 50)[2]	20
61	2-(2-Phenoxyethoxy)-ethyl	30	20

TABLE IV

ANALGESIC ACTIVITY OF NORPETHIDINE DERIVATIVES CONTAINING A THIOETHER FUNCTION



TA No.	R	Relative Analgesic Potency (Morpheridine =100) in Rats	LD50 (Mice) (mg./kg.)
59	Methyl	15	40
58	Ethyl	5	50
56	Phenyl	10	—

due to respiratory depression. The survivors did not show any marked retardation in growth compared with simultaneous controls. The histological examination revealed no evidence that the drugs were harmful. Occasional tissues showed a variety of abnormalities, but these occurred as frequently in the controls as in the treated animals. No signs of kidney, liver, or bone marrow damage were observed.

The analgesic tests on the rats after the 12-day treatment showed signs of some—but usually not very much—tolerance. In Table VI the tolerance may be judged from the difference beween the analgesic effects produced in the treated rats and those in the controls tested simultaneously. The number of animals tested was too small to permit firm conclusions, but TA 24 seemed to produce less tolerance than the other drugs.

DISCUSSION

The substances tested by Millar and Stephenson (1956) were analogues of pethidine in which the *N*-methyl group was replaced by tertiary aminoalkyl groups, most of which were in a ring. The only compounds showing activity were those in which the ring also contained an ether linkage. Our present findings show that neither the additional nitrogen atom (for example, TA 20) nor the ring structure (for example, TA 24, TA 33) are necessary and that enhanced activity may be achieved by inclusion of simple ether substituents at the 1-position.

It is not easy to see why such an ether grouping should have this effect. It is chemically inert and is some distance from what is normally considered to be the active part of the molecule. Two possibilities are that the oxygen atom provides a new point of attachment between the molecule and its site of action, or that it modifies the properties of the molecule as a whole and, for example, facilitates access to the site of action. The latter possibility does not appear likely because: (1) The *p*K values of the bases are all about 7.5, irrespective of potency. (2) One additional carbon atom, while not expected to affect the physical properties greatly, changes the analgesic potency very considerably. (3) Isomers may differ widely in potency (for example, TA 36 and 33, TA 24 and 101, and furethidine and TA 65). Clearly, the efficiency of the oxygen atom in enhancing potency depends rather critically on its position.

In the alkoxyalkyl series (Table I) where only the oxygen atom is involved, two compounds (TA 24 and TA 33) are more active than the rest. In these, the oxygen atom is the third or fifth

TABLE V

RELATIVE DEPRESSANT POTENCY ON PREPARATORY PHASE OF PERISTALTIC REFLEX (MORPHERIDINE=1)

Substance	Fresh Intestine	Stored Intestine
TA 25	0·4	0·5
TA 27	1·0	1·3
Benzethidine (TA 28)	1·0	1·4
TA 33	2·6	3·1
Furethidine (TA 48)	6·5	7·8
Pethidine	0·3	0·7
Morphine	1·9	5·0

atom from the nitrogen atom. TA 33, where the oxygen atom is at much the same distance from the nitrogen atom as in morpheridine and TA 20, is more potent than TA 24.

In the phenoxyalkyl series (Table II), on the other hand, TA 27, in which the oxygen atom is the third atom, is more potent than TA 62, in which it is the fifth atom from the nitrogen atom. The proximity of the phenyl group provided an opportunity to study the effect of changes in the electronic environment of the oxygen atom in TA 27. However, the potency was reduced, whatever the inductive or mesomeric effects of the substituents which were introduced into the phenyl group. This general reduction of potency in the phenoxyethylnorpethidines contrasts with the variation observed by Elpern *et al.* (1957) in the substituted phenylethylnorpethidines. In fact, the presence of the oxygen atom in the phenoxyalkyl series appears to be unnecessary: 3-phenylpropylnorpethidine (Elpern *et al.*, 1957), in which the oxygen atom of TA 27 is replaced by a methylene group, is more potent than TA 27. It thus appears that the phenyl group is more important than the oxygen atom in this series. This may account for the fact that the peak activity in the phenoxyalkylnorpethidine series occurs when the oxygen atom is the fourth atom from the nitrogen atom (Sterling Drug Inc., 1959), in contrast to our finding in the alkyloxyalkyl series.

The most potent of the present compounds is furethidine, which in addition to an open-chain ether linkage has a tetrahydrofuran ring. This cyclic ether grouping is obviously very efficient in enhancing potency; the potency of the corresponding tetrahydropyran (TA 49) is lower and that of the open-chain analogue (TA 38) is lower still. Mr. P. M. Frearson has pointed out to us that this order corresponds to that of the electron

TABLE VI

SUBACUTE TOXICITY AND DEVELOPMENT OF TOLERANCE

* The lower the index the higher the analgesia.

Substance	Daily Dose/ kg.	Deaths	% Increase in Wt. of Survivors	Analgesic Tests after 12 Days' Treatment			
				Low Dose (mg./kg.)	Mean Analgesic Index*	High Dose (mg./kg.)	Mean Analgesic Index*
Saline	4 ml.	0	62·6	1·5	0·72	3	0·43
TA 27	15 mg.	1	54·2	1·5	0·86	3	0·56
Saline	4 ml.	0	65·7	2	0·75	4	0·39
TA 24	20 mg.	3	68·6	2	0·81	4	0·43
Saline	4 ml.	0	94·8	0·25	0·66	0·5	0·34
Furethidine (TA 48) ..	2·5 mg.	1	84·1	0·25	0·85	0·5	0·59
Saline	4 ml.	0	82·6	2	0·80	4	0·44
TA 20	20 mg.	2	66·9	2	0·94	4	0·79
Saline	4 ml.	0	34·4	1·5	0·77	3	0·39
Benzethidine (TA 28) ..	15 mg.	5	45·6	1·5	0·98	3	0·72
Saline	4 ml.	0	37·3	0·5	0·83	1	0·61
TA 33	5 mg.	2	59·5	0·5	0·96	1	0·73

donor properties (Searles and Tamres, 1951), and it may well be that an oxygen atom in this position donates electrons at the site of action, for example, by hydrogen-bond formation.

An oxygen atom nearer to the nitrogen atom, however, does not produce a greater effect if it is part of a ring: TA 57 is much less active than TA 24. Indeed, the relatively high potency of TA 24 seems to be somewhat anomalous; it stands out of the otherwise regular rise and fall of potency as the alkyl group is lengthened in the ethoxyalkylnorpethidines, and this is not just a matter of overall chain length since TA 101 is much less potent than TA 24. Furthermore, when some group conferring increased potency is present at a greater distance, an oxygen atom at the third atom from the nitrogen atom appears to reduce potency. This occurs in the phenoxyalkyl series (see above) and in the case of TA 38 and TA 32. The most active compound we have tested, furethidine, has such an oxygen atom. Dr. Stern and his colleagues have now prepared analogues without this oxygen atom, and these have been tested by Dr. Lister. His results—given in an addendum to this paper—show that the presence of the ether linkage nearer the nitrogen atom slightly reduced the advantage conferred by the more distant tetrahydrofurfuryl group.

The nature and shape of possible receptors for morphine-like analgesics have been suggested by Beckett and Casy (1954) and by Barlow (1955). These must be considered as part of an extended surface. Our findings could well be explained by the presence on this extended surface of an area able to accept electrons, situated at a distance about equal to the length of a 5-carbon chain from the point at which the nitrogen atom is supposed to be accommodated.

Such an additional point of attachment would be expected to provide additional stability or rigidity to the complex formed between drug and receptor. Increased electron density at approximately the right distance from the nitrogen atom appears to be a common feature of all the norpethidine derivatives recently shown to exceed pethidine in potency. These include the phenylpropyl-, 4-pyridylethyl-, cinnamyl-, *p*-aminophenylethyl- (Elpern *et al.*, 1957), phenoxypropyl- (Sterling Drug Inc., 1959), 3-phenyl-3-oxopropylnorpethidine (Janssen *et al.*, 1959), as well as morpheridine, benzethidine, TA 20, TA 33, furethidine, TA 98 and TA 103.

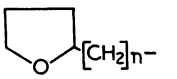
Many of the compounds we have tested are appreciably more potent than pethidine and the question arises whether any one of them might be better than pethidine in clinical use. The capacity to produce undesirable side-effects in analgesics usually runs roughly parallel with the capacity to produce analgesia. High potency in itself is therefore no great virtue (though pethidine is so weak that doses of 100 mg. are often administered and 2 ml. of solution is required to avoid tissue damage). The side-effects can be finally assessed only in man, and even if one compound could be shown to be superior to the others in animal tests, it does not follow that it would also be better in man. We have not attempted to decide which is best in animals and have contented ourselves with showing that five of the compounds did not depress respiration unduly, and did not produce pathological changes in the subacute toxicity tests, in the hope that these five may all be tested in man. Such a test has, in fact, been undertaken (Cahal, D. A., personal communication).

We wish to thank Dr. A. E. Stuart, Department of Pathology, Edinburgh University, for the pathological examinations. We also thank Dr. E. S. Stern and J. F. Macfarlan and Co. Ltd., who supplied the compounds, more particularly since they synthesized a number of compounds which were likely to be of theoretical rather than commercial interest. J. F. Macfarlan and Co. also provided a postgraduate grant which enabled A. M. J. N. B. to do this work.

ADDENDUM

By R. E. LISTER AND R. P. STEPHENSON

Since the completion of the work described in the main body of the paper a further short series of compounds has been prepared and assessed for analgesic activity. The *N* substituent of these compounds is



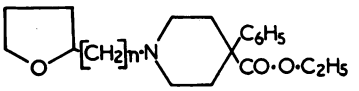
n being 1 to 5 inclusive.

Analgesic activity was determined by a method differing slightly from that described above, namely, by the quantal method of Green and Young (1951), and ED50 for analgesia being determined. Both the method of Millar and Stephenson (1956) and the method for the present series use the same pain stimulus and the same animal, namely, pressure on the tail of young rats, but somewhat different results were obtained in the comparison of morpheridine and furethidine.

All five new compounds possessed marked analgesic activity (Table VII); the activity increased with increasing chain length up to a maximum at $n=3$ and 4, but further increase in n to 5 reduced the activity. The derivatives

TABLE VII

ANALGESIC ACTIVITY AND TOXICITY OF 1-(ω-TETRAHYDROFURFURYLALKYL)NORPETHIDINES



Substance	n	Subcutaneous ED50 in Rats (mg./kg.)	Relative Potency (Morpheridine =100)	Intravenous LD50 in Mice (mg./kg.)
TA 57 ..	1	26	8	45
TA 109 ..	2	3·7	55	23
TA 98 ..	3	0·3	670	11
TA 103 ..	4	0·3	670	11
TA 108 ..	5	1·6	125	12
Pethidine ..	—	7·2	30	58
Morpheridine (TA 1) ..	—	2·0	100	45
Benzethidine (TA 28) ..	—	0·9	220	11
Furethidine (TA 48) ..	—	0·4	500	15

in which $n=3$ or 4 were more potent than furethidine which has an ether linkage in the alkyl side-chain. They may be formally derived from furethidine by omission of the open-chain ether linkage, and by its replacement with a methylene group. Thus in presence of the cyclic ether grouping of furethidine, the open-chain ether oxygen in the 3′-position is of no importance in producing analgesia. This finding supports the view that the most important factor enhancing the analgesic potency is an electron-donating group at a distance of about 5 to 6 carbon atoms from the nitrogen atom.

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Brit. J. Pharmacol. (1960), **15**, 254.

PHARMACOLOGICAL ACTIONS OF TWO NEW PETHIDINE ANALOGUES

BY

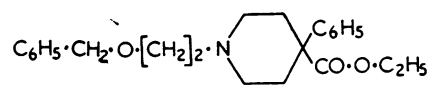
R. E. LISTER

From the Research Department, J. F. Macfarlan & Co., Edinburgh, 8

(RECEIVED DECEMBER 17, 1959)

The behaviour of two new analgesics, benzethidine and furethidine, in a number of different tests has been compared with that of pethidine. Some differences in side-effects at equi-analgesic dosage were observed, particularly a reduction in histamine release.

The synthesis of a number of *N*-substituted derivatives of pethidine has recently been reported by Frearson and Stern (1958) and Frearson, Hardy and Stern (1960). Many of these compounds have been shown by Millar and Stephenson (1956) and by Blair and Stephenson (1960) to have marked analgesic activity. Two potentially useful analgesics of this series have now been compared with pethidine. These compounds are benzethidine (I; ethyl 1-(2-benzyloxyethyl)-4-phenylpiperidine-4-carboxylate) and furethidine (II; ethyl 4-phenyl-1-(2-tetrahydrofurfuryloxyethyl)-piperidine-4-carboxylate).



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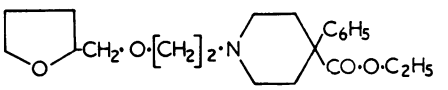

(II)

METHODS

Analgesia.—The elevation of pain thresholds in a group of 10 weanling rats (4 to 6 weeks old) was measured 30 min. after the subcutaneous injection of the analgesic, or 1 hr. after oral administration, by a modification of the method of Green and Young (1951). The pain threshold of each rat was determined before and after administration of the drug; a squeak rather than struggle was taken as the end point. Any animal in which the pain threshold was raised to double or more than that of its initial value was judged to be showing significant analgesia. The ED50 and 95% limits of error were calculated using a standard probit analysis.

Respiration.—The respiratory depressant action on unanaesthetized rats was determined by measuring the frequency of respiration before and 30 min. after the subcutaneous injection of the drugs. The mean respiratory frequency per min. of groups of 10 young rats was determined by counting the respiratory rate of the animals very loosely restrained in a perspex tube.

The effect upon respiratory minute volume and respiratory rate was studied in anaesthetized dogs, cats and rabbits by the method of Paton (1949). The cats and dogs were lightly anaesthetized by intraperitoneal injection of pentobarbitone sodium 35 to 40 mg./kg. Rabbits were anaesthetized with urethane 1.5 g./kg. injected intravenously.

Cardiovascular System.—The direct effect of the drugs on the blood pressure of the anaesthetized rabbit, cat and dog was studied. Heart rate and electrocardiogram were recorded with a direct writing Ediswan electrocardiograph. The effect of the drugs on the normal vascular responses to noradrenaline, adrenaline, histamine, acetylcholine, nicotine and vasopressin was recorded.

Behaviour and Activity.—The effects on normal behaviour and activity of the mouse, rat, cat, dog and monkey were studied. Mice in which hyperkinesia had been induced by $\beta\beta$-iminodipropionitrile after the method of Thuillier and Burger (1954) were treated with benzethidine, furethidine and pethidine and the effect on activity was recorded semi-quantitatively using a jiggle cage.

Antitussive Action.—Cough was induced in cats lightly anaesthetized with pentobarbitone sodium (35 mg./kg.) either by electrical stimulation of the superior laryngeal nerve (Green and Ward, 1955) or by mechanical irritation by means of a polythene tube passed down the trachea as far as the carina. The cough was recorded with a spring-loaded writing lever on a kymograph by means of thread tied to the abdominal wall in the region of the xiphisternum.

Prolongation of Barbiturate-induced Sleep.—Mice were injected subcutaneously with twice the analgesic ED50 20 min. before the intraperitoneal injection of

75 mg./kg. of hexobarbitone sodium. The time from injection of the hexobarbitone to the recovery of the righting reflex was taken as the sleeping time.

Anticonvulsant Action.—Convulsions were induced in mice by the intravenous infusion of leptazol 20 min. after the subcutaneous injection of the ED50 of the analgesic.

Emetic Action.—The drugs were injected in ascending doses into the gluteal muscle of mongrel dogs 0.5 hr. after feeding. The criterion of vomiting was taken as the expulsion of stomach contents.

Mydriatic Effect.—The pupil size of mice before and after subcutaneous and local application of the drug was determined by measuring the pupil diameter with a low power microscope.

Local Anaesthetic Action.—The local anaesthetic action was determined in guinea-pigs, after the intradermal injection of the analgesics, by the method of Bülbring and Wajda (1945), and compared with procaine as a standard. Qualitative assessments in man were also made.

Histamine Release.—The histamine-releasing power was determined in man by injecting the drugs in 0.1 ml. of normal saline intradermally into the skin of the volar surface of the forearm. The area of the weal produced was measured using squared paper (Bain, 1949). Weal area was plotted against log dose.

Gastro-intestinal Effects.—The antagonistic effects of the three drugs against contractions of the guinea-pig ileum induced by acetylcholine, histamine and barium chloride were determined. The preparation described by Trendelenburg (1917) was used to study effects on the peristaltic reflex.

The influence on intestinal motility in mice was studied by comparing the effects of injection of the drugs and a control solution on the weight of faecal pellets passed by 4 groups of 8 mice. A cross-over design was used and each group was given each treatment. The faeces from each mouse were collected over a period of 2 hr. and the means for each group compared statistically.

The effect on gastro-intestinal propulsion of the three drugs under test was also examined using the method described by Green (1959). For this test young rats were used and were kept on a diet of protein hydrolysate for 2 days, being starved for 2 hr. before the commencement of the experiment.

Antidiuretic Action.—This was determined using rats which had been loaded with water by stomach tube at 5% body weight and 1 hr. later with a further 5% body weight of 12% ethanol. The bladder and jugular vein were cannulated and the urine output was measured at 4 min. intervals. All drugs were given by intravenous injection. Antidiuretic potency was calculated by the method of Dicker (1953).

Acute Toxicity.—The LD50 of the three compounds was determined after intravenous injection in mice and oral and subcutaneous injection in rats.

Subacute Toxicity.—This was determined over a period of 12 weeks on 4 groups of 20 male rats. Newly weaned rats were injected daily with furethidine, 1 mg./kg.; benzethidine, 5 mg./kg.; pethidine, 25 mg./kg.; and normal saline, 4 ml./kg. by the intraperitoneal route.

Each animal was weighed daily and any gross toxic symptoms were recorded. Blood samples were taken from randomly selected rats every 2 weeks. The samples were examined and haemoglobin level, red cell count and total white cells determined. Differential white cell counts were performed for each group at the end of the eleventh week of the test.

After 12 weeks, 10 rats from each group were killed and sections of the liver, spleen, lung, kidney, adrenal gland, bone marrow of the femur and skin at the site of injection were examined for pathological changes.

RESULTS

Analgesia.—Both benzethidine and furethidine had a considerably greater analgesic action in rats than pethidine. The activity of the three compounds is compared in Table I. All these compounds showed a similar duration of action by either route. The ratios of analgesic activities by oral and by subcutaneous administration appear to be similar, and values obtained are in satisfactory agreement with those of Blair and Stephenson (1960).

Respiration.—All three compounds produced respiratory depression in unanaesthetized rats, and in the anaesthetized rabbit, cat and dog (Table II).

In unanaesthetized rats a linear relationship between log dose in mg./kg. and the log of the difference in respiratory rates before and after the drug was obtained. The dose-response curves for the three drugs showed no significant deviation from parallelism ($P>1.0$). Similar relationships between dose and respiratory depression were obtained when the effect on the minute volume of anaesthetized cats was studied. The relative potencies of the drugs as respiratory depressants are shown in Table II.

Nalorphine and levallorphan both reversed the respiratory depression which followed the administration of pethidine, furethidine, and benzethidine.

Cardiovascular System.—All three compounds produced a fall in blood pressure when injected intravenously into the anaesthetized rat, cat and dog. A qualitative difference was observed, however, when the drugs were given in the ratio of their analgesic doses. Pethidine produced an initial short-lasting rapid fall of blood pressure which was followed by a secondary more prolonged fall. With benzethidine and furethidine this secondary fall in blood pressure was much less, or frequently absent. Thus the fall in arterial

TABLE I

COMPARISON OF THE ANALGESIC ACTION OF BENZETHIDINE, FURETHIDINE AND PETHIDINE IN RATS

The limits of error (P=0·95) of the ED50's are shown in parentheses.

Compound	Subcutaneous			Oral		
	ED50 mg./kg.	Duration (min.)	Relative Potency	ED50 mg./kg.	Duration (min.)	Relative Potency
Benzethidine	0·92 (0·48–1·76)	60	8	12·0 (10·0–38)	115	3
Furethidine	0·45 (0·27–0·65)	60	16	2·8 (1·8–4·4)	130	13
Pethidine	7·2 (3·0–12·5)	60	1	37·7 (23·4–45·6)	90	1

TABLE II

COMPARISON OF THE EFFECTS OF BENZETHIDINE, FURETHIDINE AND PETHIDINE ON RESPIRATION

The relative potencies were calculated from the reduction in respiratory rate of rats, and from measurements of minute volume in the rabbit, cat and dog.

	Relative Potencies			
	Unanaesthetized Rats (Subcutaneous)	Anaesthetized (Intravenous)		
		Rabbit	Cat	Dog
Pethidine ..	1	1	1	1
Benzethidine ..	2·8 (1·6–5·6)	5	3	5
Furethidine ..	24·3 (12·8–45·3)	16	8	10

TABLE III

INCREASE IN HEXOBARBITONE-INDUCED SLEEPING TIME

Subcutaneous injection in mice.

Drug	Dose (mg./kg.)	Increase in Sleeping Time (%)
Benzethidine	5	63
Furethidine	1	130
Pethidine	20	265

blood pressure caused by pethidine was more rapid and long-lasting than that caused by benzethidine or furethidine.

A slight reduction in heart rate was observed with higher doses of all three drugs, but no changes in the electrocardiogram were observed. The drugs had no effect on the responses of the vascular system to injected adrenaline, noradrenaline, acetylcholine, histamine, nicotine or vasopressin.

Behaviour and Activity.—In doses producing analgesia, no sedative or hypnotic effects were seen in the mouse, rat, cat, dog or monkey. Higher doses of furethidine (4 mg./kg.), benzethidine (20 mg./kg.), pethidine (40 mg./kg.), that is, up to 10 times the analgesic doses, produced sedation in all animals except the cat which showed a pattern of excitation similar to that caused by morphine ; lower doses produced no excitement.

No reduction in activity in mice made hyperkinetic with $\beta\beta$-iminodipropionitrile was observed in analgesic doses ; higher doses reduced the degree of hyperactivity although the animal still responded normally to nociceptive stimuli.

Antitussive Action. — All three compounds prevented artificially induced cough in anaesthetized cats: the minimal doses needed to abolish cough by intravenous injection were benzethidine, 0.5 mg./kg. ; furethidine, 0.1 mg./kg. ; and pethidine, 2.0 mg./kg.

Prolongation of Barbiturate-induced Sleep.—When given in doses producing equal analgesic effects, benzethidine and furethidine produced less prolongation of hexobarbitone-induced sleep than did pethidine (Table III).

TABLE IV

RELATIVE POTENCIES OF BENZETHIDINE AND FURETHIDINE (PETHIDINE=1) IN DIFFERENT TESTS

Compound	Mydriasis	Local Anaesthesia	Charcoal Meal Test	Inhibition of Peristaltic Reflex	Antagonism of Barium Chloride	Histamine Liberation	Analgesia
Benzethidine ..	1	3·5	5	6	0·6	0·15	8
Furethidine ..	20	3·5	10	14	32	0·02	16

Anticonvulsant Effect.—In doses twice the analgesic ED50, none of the three compounds showed any protective effect against leptazol-induced convulsions in mice.

Emetic Action.—Merlevede and Levis (1958) claim to produce vomiting in 50% of dogs with an intramuscular injection of 10 mg./kg. of pethidine hydrochloride. No emesis was observed in dogs with benzethidine 2 mg./kg. injected intramuscularly or 5 mg./kg. orally or with furethidine 0.5 mg./kg. injected intramuscularly or 1 mg./kg. orally, but 10 mg./kg. of pethidine injected intramuscularly produced emesis in one dog out of three.

Pupillary Action. — Benzethidine, furethidine and pethidine produced mydriasis in the mouse, rat and cat, but myosis in the dog. The potency ratios are shown in Table IV.

Local Anaesthetic Action.—Both benzethidine and furethidine possess local anaesthetic properties and are approximately 3 times more potent than pethidine and procaine (Table IV). All 4 compounds had a duration of action of approximately 30 min. in the guinea-pig. When given intradermally in man, furethidine and benzethidine had a local anaesthetic action which lasted about 60 and 45 min. respectively.

Histamine-releasing Action. — Pethidine, like morphine, has been shown by previous workers to be capable of releasing histamine. When benzethidine and furethidine were compared with pethidine in doses producing an equal analgesic effect a striking difference in the histamine-releasing properties was apparent. Furethidine and normal saline produced weals of the same size and the amount of histamine released by benzethidine was very small.

Table IV shows the relative histamine-releasing properties of the three compounds as measured by the weal area produced by approximately equi-analgesic doses. The plot of weal area against log dose gave parallel lines.

Gastro-intestinal Effects.—In common with pethidine, benzethidine and furethidine showed a general, apparently non-specific depressant effect on smooth muscle. All three drugs inhibited the normal spontaneous contractions of isolated rabbit duodenum. They also antagonized the stimulant actions of acetylcholine, histamine and barium chloride on the guinea-pig ileum.

TABLE V

ACUTE TOXICITY OF BENZETHIDINE, FURETHIDINE AND PETHIDINE

The figures represent LD50s (mg./kg.); limits of errors (P=0·95) shown in parentheses.

	Mice (Intravenous)	Rats (Subcutaneous)	Rats (Oral)
Benzethidine ..	10·9 (10·1–11·5)	600 (400–900)	284 (57–460)
Furethidine ..	15·5 (14·1–17·1)	26·2 (14·5–47·1)	135 (125–146)
Pethidine ..	58·5 (54·2–63·1)	360 (334–388)	760 (510–1,140)

TABLE VI

THE EFFECT OF BENZETHIDINE, FURETHIDINE AND PETHIDINE ON THE GROWTH RATE OF RATS

Each drug was injected intraperitoneally in a group of 20 male rats and compared with a similar group injected with saline.

Drug	Dose (mg./kg.)	F Ratio	*P*
Benzethidine ..	5	1·4	>0·10
Furethidine ..	1	1·0	>0·50
Pethidine	25	1·1	>0·25

Like all potent analgesics benzethidine and furethidine inhibited peristalsis in the isolated guinea-pig ileum preparation. All three drugs given subcutaneously reduced the quantity of faeces passed by normal rats. The potency ratios are given in Table IV.

Antidiuretic Action.—In common with all other potent analgesics tested pethidine, benzethidine and furethidine showed antidiuretic properties when injected intravenously in rats.

The antidiuretic responses of different rats of 200 g. body weight showed a wide variation ; the total doses of drug to produce a 40% inhibition of urine output were benzethidine 11 μg., furethidine 30 μg., and pethidine 100 μg.

The antidiuresis appears to be due to the release of antidiuretic hormone as no antidiuretic response was observed during a saline diuresis.

Acute Toxicities.—Table V shows the acute toxicities for benzethidine, furethidine and pethidine. The toxic effects of benzethidine and furethidine appeared to be a result of depression of the central nervous system ; pethidine, on the other hand, produced excitation and clonic convulsions prior to death, symptoms not seen with benzethidine and furethidine.

Subacute Toxicities.—None of the drugs tested had any significant influence on the growth rate of male albino rats, when compared with a control group of rats treated with saline (Table VI). The mortality rate in the treated groups was not significantly different ($P>0.1$) from that of the control group.

No pathological changes attributable to the administration of the drugs were detected in any of the internal organs ; slight necrotic areas were apparent at the injection site in 3 rats treated with pethidine and 2 from the group treated with benzethidine ; no lesions were seen in the furethidine or the control groups.

Haemoglobin concentration, red cell count and differential white cell count were all within normal limits.

Tanabe and Cafruny (1958) have suggested that the development of tolerance to the analgesic action of morphine is accompanied by adrenal hyperplasia ; in the present investigation, however, no significant difference ($P>0.1$) was found between the adrenal weights of the control and the treated groups.

Discussion

For many years it was believed (Braenden, Eddy, and Halbach, 1955) that for high analgesic potency the optimum substituent on the nitrogen atom in the pethidine series was a methyl group ; within the last four years, however, many norpethidine derivatives with markedly higher potency than pethidine have been prepared. Little advantage is to be gained by increasing the potency of an accepted drug if this increase is paralleled by an increase in the incidence of undesirable side-effects. Present research is therefore aimed at achieving a reduction in side-effects caused by a drug administered at an adequate analgesic dose.

Millar and Stephenson (1956) and Blair and Stephenson (1960) have produced evidence that the dose-response curves for analgesia of a number of substituted norpethidine derivatives are not parallel with that of pethidine. Pethidine is an inadequate analgesic for severe pain, because increase of the dose above a certain level does not produce a corresponding increase in analgesia, that is, a plateau in the dose-response curve is reached. As the dose-response curves for furethidine and benzethidine appear to be steeper than that of pethidine a greater degree of analgesia may be possible with these drugs than with pethidine.

The increased analgesic potency of benzethidine and furethidine compared with pethidine appears to be accompanied by an increase in respiratory depression. Respiratory depression, however, only occurs in doses which are higher than those producing an adequate level of analgesia. Since benzethidine and furethidine are considerably more potent than pethidine, it may be possible to control severe pain with these drugs without causing excessive respiratory depression.

In equi-analgesic doses benzethidine and furethidine produce less potentiation of barbiturate-induced sleep in mice than does pethidine ; this indicates that the latter drug may produce more general central nervous depression than benzethidine and furethidine. Reduced sedation has been found particularly useful in obstetric analgesia when it is desirable that the patient should remain unsedated and fully co-operative.

An interesting finding is that benzethidine and furethidine on intradermal injection release much less histamine from the skin than does pethidine in doses of equal analgesic potency. Gershon and Shaw (1958) have suggested that a number of the undesirable side-effects of morphine are due to its histamine-releasing properties and that many of these effects can be controlled by the administration of an antihistamine ; thus it is possible that benzethidine, and furethidine in particular, may

show a decreased liability to produce these troublesome side-effects. In the limited experiments using dogs, no side-effects attributable to histamine release were produced by benzethidine and furethidine in effective analgesic doses.

In anaesthetized cats the fall in blood pressure which follows the intravenous injection of pethidine is greater than that with benzethidine and furethidine ; part of this hypotensive response can be blocked by the administration of an antihistamine drug, and this lends further support to the finding that benzethidine and furethidine are less potent histamine liberators than is pethidine.

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THE EXCRETION AND STABILITY TO METABOLISM OF BRETYLIUM

BY

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Bretylium (*o*-bromobenzylethyldimethylammonium) is a new type of hypotensive drug. It was estimated in extracts of human urine as the methyl orange complex. From 7 to 45% of a single oral dose was found in human urine within 9 hr. The [^{14}C] labelled drug was used to investigate excretion by cats. A minor proportion of a subcutaneous dose was eliminated by cats in the faeces, probably after secretion into the bile. Most of the dose was excreted unchanged in the urine. No products of metabolism were found in either human or cat urine. The drug suffered negligible change when incubated with rat liver tissue *in vitro*.

Bretylium tosylate (Darenthin ; *o*-bromobenzyl-ethyldimethylammonium toluene - *p* - sulphonate) is a quaternary salt described by Boura, Copp, and Green (1959) that selectively impairs the function of adrenergic neurones and lowers the blood pressure of hypertensive patients (Boura, Green, McCoubrey, Laurence, Moulton, and Rosenheim, 1959). It is notable for its freedom from central effects and for lack of actions on the parasympathetic nerves which mar the use of ganglion blocking agents. For a quaternary salt it is fairly well absorbed as judged by its effects in both man and animals. By the same criterion its absorption by any one patient was found to be fairly constant, but there was considerable variation in the effective dose needed by different patients. This paper gives information on the excretion of the drug and its stability to metabolism.

METHODS

Assay of Bretylium in Human Urine.—A satisfactory specific method of chemical assay was not devised ; bretylium is stable to chemical reagents under mild conditions and lacks physical properties suitable for assay purposes. Moreover, the drug is very hydrophilic, its reineckate rather soluble (about 20 mg./l.) and continuous extraction very slow. The following two methods, based on the well-known association of bases with sulphonic acid dyes, were intended to provide approximate values for assessing the excretion of the drug in human urine.

A preliminary estimate was gained by chromatography of 100 μl. portions of urine in the organic phase of n-butanol/acetic acid/water (4:1:5). The size of the spot revealed at Rf 0.75 by spraying with Dragendorff's reagent was compared with standard spots of 2 to 10 μg. of the cation. Rf values in other solvent mixtures were 0.75 in s-butanol/acetic acid/water (12:5:3) and 0.67 in n-butanol/pyridine/water (1:1:1).

Method A.—The sample was diluted with an equal volume of ethanol and filtered. An aliquot of the filtrate, usually 2 ml., was diluted with 5 vol. of 0.05M phosphate buffer, *p*H 7, and the solution passed down a column (10 × 1 cm.) of Amberlite IRC-50 resin buffered at *p*H 7. The column was washed to *p*H 5 by 300 ml. 0.05M phthalate buffer, and the eluate, containing interfering bases, rejected. Bases retained on the column were eluted by 150 ml. warm 0.5N hydrochloric acid. The eluate was neutralized, evaporated under reduced pressure, and extracted with 3 × 20 ml. portions of ethanol. Ethanolic methyl orange (0.05%, 3 ml.) was added, and the solution dried under reduced pressure. The base-dye complexes were extracted with several portions of warm ethylene dichloride until the washings were colourless. The extract was adjusted to volume, usually 25 ml., and the red colour produced by dilution with an equal volume of ethanolic hydrochloric acid (1%) was measured at 525 mμ.

Recovery of 1 mg. amounts of bretylium iodide from normotensive urine averaged 98 ± 11% in four trials. A major source of error arose from colloidal methyl orange carried over in the ethylene dichloride if evaporation of water was not sufficiently thorough. The lengthy procedure could be shortened for urines containing more than 10 mg. of drug/100 ml. (as shown by chromatography). The neutralized acid eluate from the resin was evaporated to a few ml. and 1 ml. 0.5N sulphuric acid and 5 ml. sodium

phosphate (5%) added. A blue complex was formed after shaking with 10 ml. of a cobalt thiocyanate reagent (Ashbrook, 1959); this complex was extracted by thorough shaking with 10 ml. ethylene dichloride. Emulsions were troublesome. The blue colour was read at 620 mμ.

Method B.—In single-dose experiments, blank values were obtainable from urine specimens collected from the patients at the beginning and end of the experiment and it was convenient to obtain a result rapidly by distilling 1 to 2 ml. of the sample with 3 ml. ethanolic methyl orange (0.05%) and 50 ml. ethylene dichloride, until the distilling temperature had reached 85°, adding more solvent if necessary. The solution in the flask was cooled, filtered, adjusted to 25 ml. and read as in Method A. Recovery of 1 mg. amounts of drug from normotensive urine averaged 92±13% in three trials.

Assay of [^{14}C]-labelled Bretylium. — Urines in appropriate amounts were dried on lens paper on planchettes and counted under an end window counter. The results were referred to standards prepared by adding known amounts of labelled drug to similar urines. Faeces were digested with hot 2N hydrochloric acid, cooled and filtered. The volume was adjusted to 100 ml. and aliquots counted as described for urine. Similar methods were used in some experiments with tissues *in vitro*, but where greater accuracy was desired or where the level of radioactivity was low, the sample was dried and combusted in oxygen, with added glucose if necessary, and the carbon dioxide was counted as gas (Glascock, 1954).

Identity of the Excreted Material.—The mobilities of spots revealed by Dragendorff's reagent and autoradiography of paper chromatograms of whole urines were compared in the solvent systems mentioned above with authentic material dissolved in the same samples.

Attempts to isolate pure specimens of bretylium iodide or bromide after precipitation of urinary bases as the reineckates were unsuccessful, and an isotope dilution method was applied. A cat received 22 mg. of [^{14}C]-labelled bretylium iodide subcutaneously. The 1 to 2 and 3 to 5 day urines were collected separately, made up to volume, and aliquots retained for [^{14}C] assay. Carrier bretylium bromide (400 mg.) was added to each sample followed by evaporation to dryness and extraction with ethanol. A portion of each extract was retained for [^{14}C] assay before evaporation and precipitation of the reineckates. These were crystallized once from dilute acetone, and the bases were liberated by silver sulphate. The bases were chromatographed on Whatman 3 MM paper in n-butanol/acetic acid/water. The region indicated on small strips sprayed with Dragendorff's reagent was eluted with water and the eluate evaporated. Bretylium was finally isolated as the reineckate for analysis of [^{14}C] by combustion to carbon dioxide and counting as gas. The reineckates were crystallized from dilute acetone, m.p. 175–178° (decomp.). (Found: C, 32.0; H, 4.0; N, 17.5. $C_{15}H_{23}N_7S_4CrBr$ requires C, 32.1; H, 4.1; N, 17.5%).

Search for Possible Products of Metabolism of Bretylium

Carbon Dioxide.—A rat (100 g. wt.) received 1 mg. of the labelled drug subcutaneously. It was kept in a metabolism chamber in a slow stream of air, and the issuing gas was scrubbed in two towers containing glass beads moistened with 5N potassium hydroxide. Trapped carbon dioxide was liberated into a vacuum line and counted as gas.

Bromide Ion.—A portion of a 24 hr. specimen of urine from a patient who had received 2,250 mg. of bretylium tosylate the day before was examined for bromide ion by the method of Belote (1927).

o-Bromobenzoic Acid.—A 24-hr. specimen of urine from a patient who was receiving 900 mg. of bretylium iodide daily was evaporated to a syrup and hydrolysed by concentrated hydrochloric acid for 5 hr. at 100°. The solution was diluted to 250 ml. and extracted with ether. Organic acids were dissolved out by alkali and transferred back to ether. Removal of the solvent gave 450 mg. of dark material which yielded 350 mg. of white crystals, m.p. 153 to 156°, to hot ligroin. Neither these nor the crude material contained bromine. The crystals were identified by conventional methods as salicylic acid.

Experiments with Liver Tissue in vitro.—Rat liver slices were incubated for 2 to 3 hr. in Krebs-Ringer glucose phosphate saline containing [^{14}C]-labelled bretylium iodide (10^{-3} or 2×10^{-4}M). The rate of oxygen uptake, 46 μ-moles/g. tissue/hr., did not differ from similar experiments in which bretylium was omitted. Evolved carbon dioxide was recovered either from the pooled filter papers containing potassium hydroxide from the centre wells of manometric vessels or from baryta used to wash the issuing gas from aerated mixtures. Trichloroacetic acid extracts of homogenized incubation mixtures were chromatographed in n-butanol/acetic acid/water for autoradiography. The spots on chromatograms, corresponding to controls where the drug was added after completion of incubation, were excised and counted directly on the paper by a scintillation technique (Roucayrol, Oberhauser, and Schussler, 1957) using a non-volatile scintillator (Buck and Swank, 1958).

Liver homogenates were incubated in the fortified medium containing semicarbazide to trap formaldehyde as described by Axelrod (1956). Any formaldehyde produced during metabolism was distilled into phenylsemicarbazide solution for combustion and counting.

RESULTS

Excretion by Cats.—Estimation of the radioactivity in cat urine after subcutaneous doses of [^{14}C]-labelled bretylium iodide (10 mg./kg.) showed

TABLE I

EXCRETION OF [^{14}C]-LABELLED BRETYLIUM IN CATS

Cats were injected subcutaneously with bretylium tosylate 10 mg./kg. Figures are % of dose excreted. Serial estimations in single cats except the first row of urine figures and the bile figures.

	Hours			Days						
	1	12	18	1	2	3	4	5	6	7
Urine	15	30	74	—	—	—	—	—	—	—
,, ...	—	—	—	46·5	10·2	5·7	4·2	1·3	1·7	1·0
,, ...	—	—	44·7	—	14·1	4·7	—	—	—	—
Faeces	—	—	0	0·5	5·5	6·4	0·2	—	—	—
,, ...	—	—	—	—	1·5	—	—	5	—	—
Bile	1·3	0·5	0·1	—	—	0·006	—	—	—	—

that excretion began within 1 hr. and that about half the dose was excreted by this route within 24 hr. (Table I). In one cat, 83% of the dose was accounted for in urine and faeces within 7 days, though detectable radioactivity was still present in urine on the seventh day. The drug was secreted into bile and this may account for the minor amounts found in the faeces after the subcutaneous dose. In another experiment, in which 50 mg./kg. of bretylium iodide was given subcutaneously, 12.7 mg. was found in the bile after 18 hr.

Excretion in Human Urine.—Because of the unsatisfactory nature of the assay used, the results shown in Tables III and IV can only be regarded as approximations. The standard deviations in recovery experiments from normotensive urines were rather high, but the greatest source of error arose from the high blanks given by urines from hypertensive subjects. Normotensive subjects appeared to excrete less basic material, and their urines by Method B gave values that ranged from the equivalent of 1.4 to 8.3 μg./ml. bretylium tosylate (mean of 6 experiments = 4.1 μg./ml.). However, one further specimen of urine from a normotensive subject gave a value equivalent to 167 μg./ml. bretylium tosylate. Urines from 4 hypertensive subjects gave values equivalent to 50, 158, 214, and 368 μg./ml. These high values could not be traced to previous drug regimens. The resin treatment of Method A reduced these values to an equivalent of between 8 and 32 μg./ml. bretylium tosylate.

Table II gives a comparison of the values obtained by three variants of the methyl orange method on the same series of specimens. The third set of figures refers to the application of Method B to the eluate after an initial purification by chromatography.

The figure for the second day by Method A appears to be high, and recovery of small amounts of drug from paper was unsatisfactory, otherwise there was reasonable agreement considering the very high blank values given by this patient. High blank values were also found in a specimen from the same patient before bretylium treatment. The result suggests that about one third of the total dose was excreted in urine by this patient.

TABLE II

COMPARISON OF METHODS FOR ESTIMATING BRETYLIUM IN URINE

24 hr. urine specimens were collected on 5 successive days from a patient. During the first 2 days the patient received 900 mg. bretylium iodide and for the last 3 days a placebo only. The specimens were assayed by 3 different methods. Each figure represents the total 24 hr. excretion in mg. of total bases or bretylium iodide, and is the mean of two determinations. Total bases were calculated in terms of bretylium iodide. The mean of days 4 and 5 was taken as the blank for calculating the amount of bretylium excreted.

Day No.	Method A		Method B		Method B with Chromatography	
	Total Bases	Bretylium Iodide	Total Bases	Bretylium Iodide	Total Bases	Bretylium Iodide
1	980	505	595	496	448	424
2	707	232	216	117	127	103
3	536	61	168	69	20	0
4	456	—	106	—	24	—
5	494	—	92	—	—	—
Total		798		682		527

Table III shows that excretion of single oral doses reached a peak at 2 to 4 hr. after the dose, and that excretion had virtually ceased after 9 hr. The degree of absorption by different patients was variable. In the one instance studied (Table III), total absorption did not appear to be influenced by a heavy meal though the scatter of the figures after a meal contrasts with the smooth rise and fall seen in the same patient when fasting. Table IV shows excretion of total bases while on continued dosage with bretylium; it indicates that excretion became appreciable only when the total daily dose had exceeded 1 g. The very high value for the 15th day (confirmed by chromatography) shows that sudden absorption may occur for no apparent reason.

Metabolism in Vivo.—Chromatograms and autoradiograms of urine and bile from cats that had received the labelled drug showed spots with the same mobilities as bretylium. Trichloroacetic acid extracts of heart, spleen, lung, and superior cervical ganglion also contained unchanged drug. Examination of these chromatograms, and numerous chromatograms of whole human urine, by ultraviolet light and conventional spray reagents, failed to disclose anything that could be interpreted as a metabolic product of the drug.

A rat that had received labelled bretylium produced radioactive carbon dioxide equivalent to 0.03% of the dose during 6 hr. but none in the next 17 hr. No bromide ion was found in the human urine examined.

An isotope dilution method failed to disclose the presence of metabolic products in the urine of a cat. The reineckate was used as a convenient means of purifying the bretylium. The dilution factor calculated from estimation of [^{14}C] in the purified reineckate did not exceed that calculated from estimation of total [^{14}C] in an alcoholic extract of the urine. For a 1 to 2 days sample, the reineckate and alcohol extract factors were 113 and 116 respectively, and for the 3 to 5 days samples, 137 and 163.

Metabolism in Vitro.—Rat liver slices suspended in saline containing labelled bretylium evolved small amounts of labelled carbon dioxide at a rate of 0.15% added drug/g. tissue/hr. The result parallels the slow rate of evolution of carbon dioxide by the rat after dosage with the labelled drug. Attempts to detect formaldehyde such as could arise by *N*-demethylation were negative. Recovery of labelled drug after incubation with liver tissue was assessed by chromatography of protein-free extracts and

TABLE III

URINARY EXCRETION OF BRETYLIUM DURING A 24 HR. PERIOD

Patients were given oral doses of bretylium at 9 a.m. Thereafter urine samples were collected at intervals and assayed for total bases. Values represent mg./hr. of total bases in terms of bretylium. The % excretion was calculated as the total bases excreted from 9 a.m. to 6 a.m., less the mean of the 6 a.m. to 9 a.m. and 6 p.m. to 5 a.m. values. I=bretylium iodide, T*=bretylium tosylate with a heavy meal. T†=bretylium tosylate in the same patient while fasting.

	Method B			Method A	
Dose:	500 mg. I	300 mg. I	500 mg. I	500 mg. T*	500 mg. T†
Time	mg./hr.	mg./hr.	mg./hr.	mg./hr.	mg./hr.
6 a.m.– 9 a.m. ..	1·2	5·5	2·5	8·8	2·4
9 ,, 10 ,,	41·3	7·3	12·0	10·6	3·8
10 ,, 11 ,,				5·5	6·2
11 ,, –12 noon	41·5			26·4	9·8
12 noon–1 p.m.		21·3	7·3	3·5	7·3
1 p.m.– 3 ,, ..	24·3				
3 ,, – 6 ,, ..	6·8	13·3	9·0	8·0	5·3
6 ,, – 5 a.m. ..	1·3	2·4	3·3	1·0	1·9
% of dose excreted	45%	30%	12%	7%	8%

TABLE IV

URINARY EXCRETION OF TOTAL BASES BY TWO PATIENTS ON CONTINUED DOSAGE WITH BRETYLIUM TOSYLATE

The total bases were assayed by Method A and expressed in terms of bretylium tosylate, mg./24 hr.

Day	Dose	Total Bases	Dose	Total Bases
1	—	12·6	—	—
2	—	10·3	—	20·5
3	375	15·4	—	—
6	750	19·5	875	30·6
7	1,000	13·2	1,125	40·0
8	1,375	36·8	1,125	34·9
9	1,750	43·6	1,500	33·2
14	2,250	49·2	—	—
15	2,250	285·0	—	—
16	2,250	61·5	—	—
17	2,250	40·0	—	25·6
18	2,250	61·5	—	8·3
20	2,250	79·2	—	7·1

counting the appropriate areas of paper directly. The chromatograms from incubated mixtures gave $1,690 \pm 265$ counts/min., those from mixtures where labelled bretylium was added after incubation $1,600 \pm 160$ counts/min. (means of 6 results).

Discussion

No evidence was obtained to suggest that bretylium is metabolized in the body to any marked extent, though evolution of labelled carbon dioxide by rats and from liver incubated with the drug indicates that it is not completely immune from metabolic attack. Complete oxidation or extensive debromination by a dehalogenase would have been revealed by presence of bromide ion in urine. The evidence from chromatography and autoradiography can only apply to products with the quaternary group intact, but removal of this group would be expected to give a benzoic acid. The salicylic acid found in one urine almost certainly arose from ingested acetylsalicylic acid. Metabolites that retain the quaternary nitrogen such as a hypothetical mercapturic acid seem to be unlikely since isotope dilution give no hint of other products. Substituted bromobenzenes do not give rise to mercapturic acids (Williams, 1947).

Compared with many other quaternary salts, bretylium is absorbed fairly readily from the alimentary tract. In unpublished work it has been shown to be absorbed from the intestine and not from the stomach, but the mechanism is obscure. Current theories on drug absorption, that assume solution of unionized bases in lipid, are difficult to apply to quaternary salts, unless they form an unionized complex analogous to the complexes with sulphonic acid dyes, with some constituent of the intestine.

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THE SELECTIVE ACCUMULATION OF BRETYLIUM IN SYMPATHETIC GANGLIA AND THEIR POSTGANGLIONIC NERVES

BY

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A study of the distribution of [^{14}C]-labelled bretylium (*N*-*o*-bromobenzyl-*N*-ethyl-*N*,*N*-dimethyl-ammonium) in cat tissues at various times after subcutaneous injection suggests that the specificity of its blocking action on adrenergic neurones may be related to its selective accumulation in these neurones. The rate of rise and fall of concentration in sympathetic ganglia and postganglionic sympathetic nerves showed a close similarity to the time course of the blocking adrenergic neurones as manifested by relaxation of the nictitating membranes. Concentrations found were similar to those in adrenergic nerve trunks when topical application of the drug had caused a local block of conduction. Conduction in other types of nerve could be blocked by topical application, but in general they were less sensitive, heavily myelinated nerves being the most resistant.

The pharmacology of bretylium (*N*-*o*-bromobenzyl-*N*-ethyl-*N*,*N*-dimethyl ammonium) has been described by Boura and Green (1959). The drug impaired the function of adrenergic neurones in doses which did not affect the parasympathetic or central nervous systems. It has been successfully used to control hypertension in man (Boura, Green, McCoubrey, Laurence, Moulton, and Rosenheim, 1959; Smirk and Hodge, 1959; Dollery, Emslie-Smith and McMichael, 1960). This study of the distribution of the [^{14}C]-labelled drug in cats suggests that the specificity of action of bretylium may be related to its selective accumulation and retention in adrenergic neurones.

METHODS

N-^{14}*C-methyl Labelled Bretylium Iodide.*—Methyl iodide, containing a nominal 3 mC of [^{14}C] (0.77 m.mole), was condensed *in vacuo* on to 0.77 m.mole of *N*-o-bromobenzyl-*N*-ethyl-*N*-methylamine in 1.8 ml. methyl acetate. The mixture was sealed off and allowed to stand at room temperature for 24 hr. The product separated as an oil that crystallized on standing. It was filtered off, washed with methyl acetate, and used without further purification (m.p. 119 to 120° on a hot stage; yield, 219 mg.). The material, suitably diluted with inactive carrier, was combusted in oxygen and [^{14}C] was counted as carbon dioxide (Glascock, 1954). It was calculated that the undiluted material had a specific activity of 3.1 mC/m.mole≡79% of the [^{14}C]-methyl iodide used. Paper chromatography in several solvent systems followed by autoradiography revealed one radioactive spot at the position stained brown by Dragendorff's reagent. R_F values were as follows: in *s*-butanol-acetic acid-water (12:5:3), 0.75; in *n*-butanol-pyridine-water (1:1:1), 0.67; and in *n*-butanol-ethanol-10% ammonia (40:20:13), 0.58. This material was diluted with two or three parts of unlabelled bretylium iodide before use.

Tissue Sampling After Subcutaneous Administration of Bretylium Iodide.—For most experiments cats received 10 mg./kg. in saline. After a suitable time interval they were anaesthetized with ether and bled out by cutting the aorta. The blood was collected and heparin added. Tissues were dissected as rapidly as possible, taking the nerves and ganglia first, then samples of larger organs, and finally samples of the central nervous system. The dissection took about 1 hr.

Ganglia, nerves and other small samples were rapidly trimmed on filter paper, weighed on a torsion balance to 0.5 mg., and laid in porcelain boats for drying at 100°. Larger samples were weighed into tared tubes and dried over phosphorus pentoxide *in vacuo* for two days.

Topical Application to Nerve Trunks.—Labelled bretylium was applied topically to several nerve

trunks in cats under chloralose anaesthesia, in order to determine its uptake by the nerves and its effect on conduction. The latter was assessed from the response of a suitable end organ when the nerve was stimulated proximal to the portion exposed to the drug. The following nerves were examined:

(*a*) Inferior cardiac: the heart rate was followed with a Cushny myocardiograph triggering a Thorp impulse counter.

(*b*) Hypogastric: the relaxant effect of nerve stimulation on the uterus was recorded by means of a Cushny myocardiograph.

(*c*) Preganglionic cervical sympathetic: the end organ response was the contraction of the nictitating membrane, recorded isotonically with a frontal writing lever.

(*d*) Greater splanchnic: carotid blood pressure was recorded with a mercury manometer. Conduction in the nerve was considered to be impaired when the pressor effect of nerve stimulation was decreased.

(*e*) Phrenic: isometric contractions of the diaphragm were recorded.

In each of these preparations the nerve was cut and freed from surrounding tissue for a length of 2 to 3 cm., and then drawn through a glass chamber with rubber dam seals at each end. This was filled with Tyrode. The proximal end of the nerve, under warm liquid paraffin, was stimulated through platinum electrodes with supramaximal shocks at 15/sec., for periods of 30 sec. every 3 or 5 min. When the response of the end organ was constant, the fluid in the nerve bath was replaced with Tyrode containing labelled bretylium. At the times stated in Table III when the end organ response, usually diminished, was again fairly constant, the immersed segment of nerve was excised, quickly rinsed in Tyrode, blotted, weighed and dried at 100° ready for assay. In one experiment the superior cervical ganglion and the adjacent portions of its pre- and post-ganglionic nerves, freed from connective tissue but otherwise intact, were immersed in a small plastic

TABLE I

THE CONCENTRATION OF BRETYLIUM IN PERIPHERAL NERVOUS TISSUES

Values for individual cats at intervals following subcutaneous injection of 10, 6 or 50 mg./kg. of [^{14}C]-labelled bretylium iodide. Concentrations are expressed as mμmoles/g. wet tissue. 1 mμmole=0·37 μg. bretylium iodide.

Tissue	10 mg./kg.					6 mg./kg.	50 mg./kg.
	3 hr.	12 hr.	18 hr.	18 hr.	72 hr.	18 hr.	18 hr.
Adrenergic nerves							
Postganglionic cervical sympathetic	42	78	—	700	31	49	696
Hypogastric	—	174	—	—	41	61	421
Gastric	—	99	—	—	—	62	—
Inferior cardiac	22	136	—	—	19	63	196
Cholinergic nerves							
Preganglionic cervical sympathetic	28	29	—	80	8	23	114
Greater splanchnic	14	30	—	—	8	24	37
Vagus	15	19	—	40	2	—	47
Phrenic	11	94	—	40	—	—	—
Sympathetic ganglia							
Superior cervical	197	270	445	890	61	174	965
Middle cervical	—	—	—	610	—	—	—
Stellate	168	315	303	770	16	185	728
Coeliac	158	374	450	950	41	225	634
Superior mesenteric	—	59	258	700	—	—	—
Inferior ,,	290	362	—	430	49	152	440
Other ganglia							
Nodose	38	15	41	30	1	6	37
Ciliary	—	64	—	30	—	—	—
Otic	—	7	—	1	—	—	—
Dorsal root	—	16	—	10	—	—	—
Semilunar	—	<1	—	1	—	—	—

TABLE II

THE CONCENTRATION OF BRETYLIUM IN VARIOUS TISSUES

Values for individual cats at intervals after subcutaneous injection of 10 or 50 mg./kg. of [^{14}C]-labelled bretylium iodide. Concentrations are expressed as mμmoles/g. wet tissue. 1 mμmole=0·37 μg. bretylium iodide. (a) Other tissue concentrations in this cat were: parotid gland 20 mμmole, ovary 38 mμmole, thyroid 14 mμmole, cervical lymph gland 5 mμmole; (b) and (c) are the concentrations in the cortex and medulla respectively; (d) left ventricle; (e) cerebral and cerebellar cortex and spinal cord.

	10 mg./kg.							50 mg./kg.
	1 hr.	3 hr.	3 hr.	12 hr.	18 hr. (a)	18 hr.	72 hr.	18 hr.
Blood	23	15	—	13	28	17	<1	24
Liver	288	78	201	62	23	27	—	—
Kidney	113	—	—	—	9	13	—	—
Adrenal gland ..						110 (b)		
	77	53	—	20	78	164 (c)	<1	85
Heart (d)	400	131	146	44	22	32	<1	38
Spleen	205	34	102	235	142	108	25	45
Lung	—	88	—	—	—	—	—	—
Diaphragm ..	52	—	—	—	45	50	—	—
Area postrema ..	1	25	—	—	—	20	—	—
C.N.S. (e)	<1	<1	—	<1	—	<1	—	—
Hypothalamaus ..	1	3	—	—	—	—	—	—

trough. Stimulation was applied to the preganglionic nerve. After immersion in bretylium, the concentrations in the ganglion and in the immersed portions of the pre- and the postganglionic trunks were assayed separately.

Assay.—The small samples were combusted in oxygen and [^{14}C] counted as carbon dioxide (Glascock, 1954). Most of the nerve samples weighed between 7 and 10 mg. Where necessary, inactive glucose was added to bring the total weight within this range. From the measured volume of carbon dioxide produced and the specific activity, the total activity in each sample was calculated. Large samples were dried *in vacuo* over phosphorus pentoxide for several days, powdered, and plated out on polythene planchettes for counting at infinite thickness under an end window counter.

The results were calculated by reference to calibrations prepared from samples containing known amounts of activity. Most counts were taken to within 2% standard deviation, but samples of low activity had standard deviations up to ±10%.

RESULTS

Previous experiments (Boura and Green, 1959) have shown that the nictitating membranes of cats injected subcutaneously with 10 mg./kg. bretylium bromide began to relax after 3 hr., were fully relaxed at 12 and 18 hr., and regained their normal tone only after two to three days. The time course of this effect, found also in the present series of experiments with the labelled bretylium iodide, was characteristic of the slow and persistent adrenergic neurone blocking action of the drug. It was in relation to this that the distribution of bretylium at various time intervals after injection (Tables I and II) is of particular interest.

The levels of radioactivity found in sympathetic ganglia and their postganglionic trunks showed that these tissues attained far higher concentrations of the drug than did any others examined (Tables I and II). At 18 hr. after subcutaneous injection of 10 or 50 mg./kg., when the nictitating membranes were fully relaxed, these ganglia contained as much as 250 to 1,000 mμmoles/g., and their postganglionic trunks 200 to 700 mμmoles/g. The rate of accumulation of these concentrations was slow, especially by the nerve trunks. The values at 72 hr. indicate that the rate of decline was also slow. The other nerves and ganglia examined accumulated moderate concentrations, in contrast to the high concentrations found in adrenergic nerves and sympathetic ganglia.

The concentration of bretylium in blood and in some other organs is shown in Table II. One hour after injection the blood contained about 20 mμmoles/ml., and similar levels were found at 3, 12 and 18 hr., but much less at 72 hr. Differential centrifugation of the blood in siliconed tubes showed that the concentration was

approximately the same in plasma and red cells. There was no more bretylium in the platelet fraction than could be accounted for by residual plasma. Other organs, for example, liver, kidneys, spleen and heart, accumulated concentrations far exceeding that in the blood within 1 hr. Levels in most organs had greatly declined within 12 to 18 hr., but those in the adrenal gland and spleen were well maintained. No drug was found in the central nervous system except for small amounts in the hypothalamus and area postrema.

After a dose of 6 mg./kg. only partial relaxation of the nictitating membranes occurred; 18 hr. after this dose the concentrations of bretylium in the tissues were considerably less, those in sympathetic ganglia being a half to one-quarter of those in the cats given 10 mg./kg. After 50 mg./kg. of bretylium all tissue levels at 18 hr. were roughly the same as those in cats given 10 mg./kg.

The possibility was considered that bretylium might penetrate into nerve axoplasm during the course of the ionic fluxes associated with impulse propagation and that this mode of entry would account for the rather slow rate of accumulation. A cat in which the right preganglionic cervical sympathetic nerve had been cut a month beforehand was given 10 mg./kg. labelled bretylium subcutaneously. Eighteen hours later the concentration of bretylium in the superior cervical ganglion of the sectioned side (122 mμmoles/g.) was similar to that of the intact side (117 mμmoles/g.) and there was no noteworthy difference in the amounts of bretylium in the postganglionic nerves (79 and 41 mμmoles/g. respectively). In an acute experiment, where the nerve of one side was cut and the other side left intact but stimulated supramaximally for 3 hr. after intravenous injection of the drug, there was again little difference between the bretylium contents of the superior cervical ganglia with their attached postganglionic nerves (cut, 232 mμmoles/g.; stimulated, 388 mμmoles/g.).

Topical Application.—Table III summarizes experiments in which various nerves were immersed in labelled bretylium, the degree of impairment of conduction assessed from the response of end organs, and the uptake of drug measured. The inferior cardiac and the hypogastric nerves took up the drug to concentrations exceeding those in the fluid in which they were immersed, within 20 to 100 min. Stimulation of the inferior cardiac nerve containing 600 mμmoles/g. no longer affected the heart rate, but, while conduction in the hypogastric nerve was apparently impaired when it contained an overall concentration of 90 mμmoles/g., blockade was still incomplete with 1,270 mμmoles/g. The preganglionic cervical

TABLE III

CONCENTRATION OF BRETYLIUM IN NERVES AFTER TOPICAL APPLICATION

The nerve, immersed in Tyrode solution containing [^{14}C]-labelled bretylium iodide, was stimulated at 15 pulses/sec. for 30 sec. every 3 or 5 min. Concentration of bretylium calculated from radioactivity determination.

	Response Tested	Bath Concentration of Bretylium Iodide mμmoles/ml.	Immersion Time in Min.	Inhibition of End Organ Response	Concentration of Drug in Nerve, mμmoles/g.
Adrenergic nerves					
Inferior cardiac	Heart rate	500	20	Complete	600
Hypogastric	Uterus	50	100	Partial	90
,,	,,	500	75	,,	1,270
Cholinergic nerves					
Preganglionic cervical sympathetic	Nictitating membrane	500	30	None	520
,,	,,	500	30	,,	500
,,	,,	1,000	30	,,	340
,,	,,	1,000	90	Partial	310
Greater splanchnic	Rise in B.P.	500	30	,,	90
,, ,,	,, ,,	500	35	,,	50
Phrenic	Diaphragm	500	30	None	90

sympathetic nerve also readily absorbed the drug, but an impairment of conduction was apparent in only 1 of 4 preparations. It may be significant that the nerve which showed blockade, though containing no greater concentration of the drug, had been immersed 90 min. instead of 30 min. The longer period of immersion might perhaps have allowed deeper penetration into the nerve. In an experiment not shown in the Table, the drug was taken up readily not only by the preganglionic cervical sympathetic trunk but also by the superior cervical ganglion and its postganglionic nerve trunk; these structures contained 850, 420, and 540 mμmoles/g. respectively after they had been immersed together for 30 min. in bretylium at a concentration of 500 mμmoles/ml. The response of the nictitating membrane to preganglionic stimulation was abolished.

The greater splanchnic and the phrenic nerve (Table III) took up bretylium to only about a fifth of the concentration of the solution in which they were immersed, within 30 min. Some impairment of the pressor response to stimulation of the splanchnic nerve containing 50 to 90 mμmoles/g. was apparent, but supramaximal stimulation of the phrenic nerve containing 90 mμmoles/g. continued to cause maximal contractions of the diaphragm.

Similar results were obtained with topical application of unlabelled bretylium bromide. The inferior cardiac nerve was blocked by immersion in a solution of 185 mμmoles/ml. for 13 min. (one experiment), and the postganglionic cervical sympathetic nerve was blocked by 450 mμmoles/ml. in 25 min. (one experiment). No impairment of conduction was apparent in preganglionic cervical sympathetic nerves immersed for 30 min. in bretylium at 1,000 to 2,700 mμmoles/ml. (five experiments).

Discussion

The following conclusions are based on the assumption that the radioactivity found in samples was accounted for solely by unaltered drug. Bretylium has been found to suffer negligible metabolic alteration in contact with tissues (Duncombe and McCoubrey, 1960).

The most interesting feature in the distribution of bretylium after subcutaneous injection in the cat is the slow accumulation of very high concentrations of the drug by sympathetic ganglia and their postganglionic nerve trunks. Moreover, the results for various time intervals show that the levels in these tissues are temporally related to the degree of relaxation of the nictitating membrane, a manifestation of the adrenergic neurone blocking action of the drug (Boura and Green, 1959). Other ganglia and nerve tissues never attained such high concentrations of the drug, and the specificity of the blocking action of bretylium on adrenergic nerves (Boura and Green, 1959) may therefore be related to the preferential accumulation of the drug by the adrenergic neurones. The sympathetic ganglia contained higher concentrations than did their postganglionic trunks, and this may be due to their having a relatively smaller proportion of supporting tissue.

Several of the major organs in the body accumulated tissue levels far exceeding those in the blood, but the concentrations attained were considerably less than those in adrenergic neurones and, except in the spleen, reached their peak and declined before the onset of sympathetic block. In experiments in guinea-pigs and rats (McCoubrey, unpublished), the spleen did not retain high concentrations of bretylium. In general, organs known to contain high concentrations of catechol amines accumulated the drug, but there is no clear relationship between their catechol amine concentrations and their degree of retention of the drug. In particular, the concentration in the adrenal medulla was far less than that in adrenergic neurones. At the highest concentration found in the superior cervical ganglion, there were about 10 molecules of bretylium present for every molecule of noradrenaline. It is interesting that, although bretylium did not penetrate into the cerebral cortex and spinal cord, small amounts were detected in the hypothalamus and area postrema, regions known to contain noradrenaline (Vogt, 1954).

Bretylium has a very persistent local anaesthetic action and blocks conduction in the adrenergic nerve trunks supplying the rabbit intestine, uterus and ear vessels when topically applied in a concentration of 37 to 370 mμmoles/ml. (Boura and Green, 1959). The present experiments show that with topical application block occurs in the postganglionic (adrenergic) nerves of the cat when they contain an overall concentration similar to that found when blockade has been produced by a subcutaneous dose. However, the distributions within the nerve cannot be expected to be the same in the two cases. Nerves of other types could also be blocked by topically applied drug but, in general, those examined were less sensitive. The available evidence is not sufficient to show how far the degree of block is related to the thickness of nerve trunks or to the ease of access to individual fibres. The differences observed after topical application of the drug, for example the high sensitivity of postganglionic fibres and the

insensitivity of the phrenic nerve, are compatible with myelin acting as a barrier to the bretylium ion. Fibre size may also be important in relation to sensitivity, as it is with other local anaesthetics. These factors are presumably of importance not only when the drug is applied topically but also when it is given systemically.

We conclude that bretylium may act in a manner analogous to that of the local anaesthetic drugs, as was originally postulated to explain the adrenergic nerve blocking action of choline 2,6-xylyl ether bromide (Hey and Willey, 1954). Its specific action on adrenergic neurones may be related to their ability to selectively accumulate the drug, as well as to the greater sensitivity of finer and less protected fibres. It seems reasonable to assume that the whole of the adrenergic neurone is susceptible to bretylium, but that the ease of access and therefore of block will be influenced by differences in the richness of the local blood supply. The vascularity of the ganglia appears to be greater than that of nerve trunks, and this is in keeping with their more rapid attainment of a blocking concentration. The drug may also have a comparatively ready access to the nerve terminals, and after subcutaneous injection these may well suffer impairment of function first.

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THE RELATIONSHIP BETWEEN THE CHEMICAL STRUCTURE AND NEUROTOXICITY OF ALKYL ORGANOPHOSPHORUS COMPOUNDS

BY

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Thirty-six alkyl organophosphorus compounds have been tested for neurotoxicity in the chicken. The individual compounds were chosen to enable the importance of each portion of the molecule to be assessed in relation to the property of neurotoxicity. Seventeen substances were found to be neurotoxic, fifteen for the first time. All of these contained fluorine. On the basis of the results reported, certain predictions have been made about the chemical structure of compounds which would be expected to be neurotoxic. The importance of fluorine suggests that it plays a direct role in the development of the biochemical lesion, and this may occur as the result of its being carried by the molecule as a whole to specific areas in the nervous system. By the action of cholinesterase, the P–F bond may be ruptured and ionic fluorine liberated where it blocks some metabolic cycle.

Ginger paralysis, Jake paralysis, Jamaica ginger polyneuritis or triorthocresyl phosphate poisoning were some of the names given to a condition which afflicted a very large number of people throughout the Southern States of the United States of America during 1930 (Kidd and Langworthy, 1933). It was caused by the ingestion of an alcoholic extract of Jamaica ginger adulterated with tri-*o*-cresyl phosphate (Smith, Elvove and Frazier, 1930).

Since a similar condition has been observed in man after poisoning by *NN'*-diisopropylphosphorodiamidic fluoride (mipafox) (Bidstrup and Bonnell, 1954) and in chickens following diisopropyl phosphorofluoridate (dyflos), mipafox (Barnes and Denz, 1953) and a number of aryl phosphates other than tri-*o*-cresyl phosphate (Hine, Dunlap, Rice, Coursey, Gross and Anderson, 1956; Henschler, 1958), the names above are no longer satisfactory for this condition. Because the histological lesions produced by these substances are confined to the nervous system (Smith and Lillie, 1931; Barnes and Denz, 1953; Fenton, 1955; Cavanagh, 1954) it is proposed to refer to this condition as organophosphorus neurotoxicity or, for brevity, neurotoxicity.

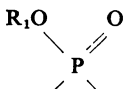
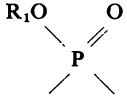
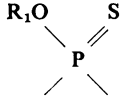
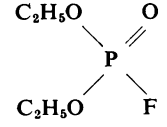
Detailed accounts of both the clinical and histological picture have been given by many writers, and the following due to Kidd and Langworthy (1933) is typical of the clinical picture in man after poisoning by tri-*o*-cresyl phosphate. Following a symptomless period of 5 to 30 days there occurs a tingling numbness or cramping pain in the calves, which lasts for 1 or 2 days. This is followed by a rapid onset of complete or partial paralysis of the extensor muscles of the toes and feet, together with variable degrees of weakness, but always with loss of ankle jerks. A week later the muscles of the hand and forearm develop a sudden weakness and bilateral paralysis involving both sides to an equal extent. The course of the illness is chronic and little improvement can be observed for months after onset. Even when it occurs, progress is slow.

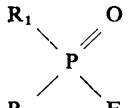
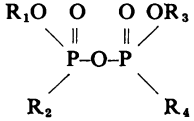
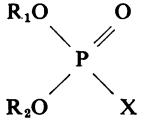
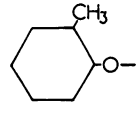
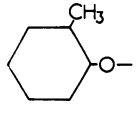
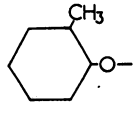
There is considerable variation in both the clinical and histological appearances of the disease in various species (Smith, Elvove and Frazier, 1930) and it is only in the hen and the cat that the appearances closely resemble those seen in man (Smith, Engel and Stohlman, 1932). Most experimental work has been carried out in chickens where the histological lesions are limited to the ascending and descending fibres of the sciatic nerve, the spinal cord and the cerebellum. The changes consist of myelin and axon degeneration (Cavanagh, 1954; Fenton, 1955).

Previous to the work to be described in this paper only a limited number of compounds were

TABLE I

CHEMICAL CONSTITUTION AND PHYSICAL CONSTANTS OF ALL THE COMPOUNDS EXAMINED

Type of Compound	R_1	R_2	R_3	R_4	X	b.p./mm.Hg	Refractive Index n_D^{25} Found	Remarks
 Phosphorofluoridates	CH_3–	CH_3–				46°/12	1·3553	
	C_2H_5–	C_2H_5–				59°/10	1·3708	
	C_3H_7–	C_3H_7–				54°/2·0	1·3875	100% pure by Schoneman reaction
	i-C_3H_7–	*i*-C_3H_7–				40°/1·0	1·3795	
	C_4H_9–	C_4H_9–				68°/0·2	1·3993	C. & H. analysis. Found C. 45·44%. H. 8·39%. Calculated C. 45·28%. H. 8·55%
	i-C_4H_9–	*i*-C_4H_9–				50°/0·5	1·3955	
	s-C_4H_9–	s-C_4H_9–				59°/1·0	1·3935	
	C_5H_{11}–	C_5H_{11}–				68°/0·1	1·4080	C. & H. analysis. Found C. 50·11%. H. 9·30%. Calculated C. 49·99%. H. 9·23%
	$C_3H_7.CH(CH_3)$–	$C_3H_7.CH(CH_3)$–				63°/0·2	1·4119	
	Cyclohexyl–	Cyclohexyl–				82°/0·01	1·4552	C. & H. analysis. Found C. 54·44%. H. 8·56%. Calculated C. 54·53%. H. 8·39%
	C_2H_5–	C_3H_7–				71°/10·0	1·3787	
 Alkylphosphonofluoridates	*i*-C_3H_7–	CH_3–						
	i-C_3H_7–	C_2H_5–						
	CH_3–	*i*-C_3H_7–				55°/13	1·3869	
	C_2H_5–	CH_3–				64°/23	1·3810	
	i-C_4H_9–	CH_3–				46°/1·5	1·3922	
 Phosphorofluoridothionate	C_2H_5–	C_2H_5–				68°/24	1·4208	Infra-red spectrum shows the presence of approx. 10% impurity probably: 

 Dialkylphosphinic fluorides	C_2H_5– C_3H_7– *i*-C_3H_7– C_4H_9–	C_2H_5– C_3H_7– *i*-C_3H_7– C_4H_9–				48°/0·5 76°/1·5 52°/0·5 74°/0·2	1·4123 1·4185 1·4220 1·4275	
 Dialkylpyrophosphonates	C_2H_5– C_2H_5– C_4H_9– C_2H_5– *i*-C_3H_7– C_2H_5–	C_2H_5O– CH_3– CH_3– CH_3– CH_3– CH_3–	C_2H_5– C_3H_7– C_4H_9– C_2H_5– *i*-C_3H_7– C_2H_5–	C_2H_5O– CH_3– CH_3– C_2H_5– CH_3– CH_3–			1·4166 1·4348 1·4360 1·4350 1·4298 1·4348	Ethyl pyrophosphate
 " Miscellaneous " compounds	C_2H_5– C_2H_5–	C_2H_5– C_2H_5–			Cl– CN–	47°/1·5 43°/0·1	1·4155 1·4009	Nitrogen determination. Found 8·51%. Calculated 8·59%
	C_2H_5– C_3H_7–	C_2H_5– C_3H_7–			C_2H_5O– 	102°/0·035	1·4770	C. & H. determination. Found C. 56·33%. H. 7·78%. Calculated C. 57·34%. H. 7·77%
	i-C_3H_7– C_4H_9– *i*-C_4H_9– C_2H_5–	*i*-C_3H_7– C_4H_9– *i*-C_4H_9– C_2H_5O–			$C_6H_5.NH$– Cl– Cl– $(C_2H_5)_2N.[CH_2]_2.S$–	120° (m.p.) 78°/0·2 76°/0·7	1·4294 1·4254	Refractive index n_D^{15} Oxalate used—solid
					CH_3–	130°/0·05	1·5335	

known to be neurotoxic. These were dyflos, mipafox and certain triaryl phosphates. Consequently it was not possible to recognize any correlation between chemical structure and neurotoxicity.

This paper presents the results of a systematic study of the relationship between the structure of certain organophosphorus compounds and neurotoxicity. A number of these compounds have been shown for the first time to be neurotoxic. All of these contain fluorine ; in addition the significance of the other groups in the molecule has been worked out.

METHODS

Chemical Methods

Phosphorofluoridates.—The majority of these were prepared by the method of Ford-Moore, Lermit and Stratford (1953). This was modified for the preparation ethyl propyl phosphorofluoridate (British Intelligence Objectives Subcommittee, 1945) and for di(1-methylbutyl) phosphorofluoridate (Goldwhite and Saunders, 1955).

Diethyl Phosphorofluoridothionate. — This was prepared from the phosphorochloridothionate, replacing the chlorine atom by a fluorine atom, by the action of antimony trifluoride in the presence of antimony pentachloride.

Alkylphosphonofluoridates.—These were obtained by the action of phosgene on the appropriate alkyl alkylphosphonate to give the alkylphosphonochloridate, and this on treatment with sodium fluoride gave the alkylphosphonofluoridate (Coe, Perry and Brown, 1957).

Dialkylphosphinic Fluorides.—Treatment of the corresponding chlorides with a mixture of sodium fluoride and sodium bifluoride gave the dialkylphosphinic fluorides.

Dialkylpyrophosphonates.—These were obtained pure by reacting the silver salt of an alkyl hydrogen alkylphosphonate with an alkyl alkylphosphonochloridate (Kosolapoff, 1950).

Tests of Purity and Identification.—The purity and identity of all the compounds were established by examination of the infra-red spectrum, determination of the refractive index and, in some cases, determination of the carbon and hydrogen. With one exception, which is discussed in the text, all the compounds were at least 95% pure at the time of testing. The chemical structure and physical constants of the 36 compounds examined are shown in Table I.

Biological Methods

Chickens of mixed breed and both sexes were used throughout, and since age is significant in the development of neurotoxic effects all the birds used were at least 18 months old (Barnes and Denz, 1953).

The agents were given intramuscularly as a 1% dilution in 10% ethanol saline (Austin and Davies, 1954), within 0.5 hr. of preparation of the solution. Where an agent was known to be unstable or insoluble in water the solution was made up in isopropanol. Slight deviations from this procedure were made when the dose to be administered was greater than 10 mg./kg. Under these circumstances the concentration of the agent was adjusted to keep the volume of the injected solution between 0.2 and 1.0 ml.

Many of the agents used were so lethal that each bird had to be protected by the intramuscular injection of 2-hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate (P2S) 100 mg./kg. and of atropine sulphate 1 mg./kg. 10 min. before the injection of the agent. Preliminary experiments with dyflos showed that, whilst such treatment saved the lives of birds given many times a lethal dose, it had no effect upon either the incidence, the severity or the onset of neurotoxic effects. Despite the protective effect of P2S and atropine some compounds were so toxic that the dose levels necessary for adequate testing could only be given in 20% daily aliquots of the total.

Occasionally, for very specific reasons, P2S was not administered. In these instances the dose of atropine was increased and this is indicated either in the tables or the text.

In the chicken the interval between poisoning and the onset of paralysis is always between 9 and 14 days ; all birds were therefore observed for a period of 21 days from the completion of the injections. The absence of bilateral leg weakness and ataxia at the end of this period was taken as a negative result and the compound was considered inactive. With a number of the compounds, particularly those which did not produce leg weakness or ataxia, the period of observation was longer. The onset of such signs in a shorter interval than 7 days was regarded as atypical and the test was repeated.

All the birds were killed either at the end of the observation period or when they developed unequivocal clinical signs. The sciatic nerves, spinal cord and cerebellum of many of these clinically positive birds were examined histologically.

RESULTS

The Acute Effects of Organophosphorus Compounds in the Presence of P2S and Atropine

With the exception of four compounds, all the agents were tested in the presence of P2S and atropine, with the result that very much larger doses of agent could be administered than was otherwise possible. For example, the LD50 of sarin injected intramuscularly to hens is approximately 50 μg./kg. whereas 20 times this dose was given in the presence of P2S and atropine without lethal effects.

Despite protection against the lethal action of these substances acute signs of anticholinesterase poisoning invariably occurred, and at the highest

dose levels some birds died. The effects varied in severity with the class of compound and with the individual members of any class. Immediate collapse followed by violent convulsions was a characteristic feature of poisoning by doses of 0.5 mg./kg. or more of both phosphonic and phosphinic fluorides. The protective effect of P2S and atropine was more marked in the case of the phosphorofluoridates. Although the rate of recovery was more rapid after this class of compound it was mainly dependent upon the actual substance.

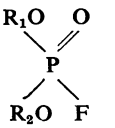
Large doses of dialkylpyrophosphonates could be given after P2S and atropine, without causing much distress. Although collapse and convulsions occurred, these were much less frequent and much less severe. For example, after the administration of ethyl pyrophosphate 50 mg./kg., only slight signs were observed and recovery was complete within 1 hr.

The Neurotoxicity of Organophosphorus Compounds

The Phosphorofluoridates.—Eleven compounds of this type were examined (Table II). All of them, with the exception of dimethyl phosphorofluoridate, were neurotoxic at dose levels between 0.3 mg. to 2.5 mg./kg. The dimethyl compound was neurotoxic at 30 mg./kg., but this may reflect the hydrolytic instability of this compound. In general, however, all the phosphorofluoridates were neurotoxic at about the same level, and neither the character of the alkyl groups nor the fact that they may be straight or branched chains seemed to influence this neurotoxic property. The onset of ataxia was markedly uniform, first signs

TABLE II

THE NEUROTOXICITY OF PHOSPHOROFLUORIDATES IN CHICKENS



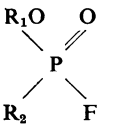
Each chicken was injected intramuscularly with P2S 100 mg./kg. and atropine sulphate 1 mg./kg., 10 min. before intramuscular injection of the agent.

R_1	R_2	Range of Doses Tested (mg./kg.)	Maximum Dose at which No Hens were Ataxic (mg./kg.)	Minimum Dose at which Ataxia was Observed (mg./kg.)	Proportion of Birds Positive at Minimum Dose or Greater	Mean Time of Onset of Ataxia (Days)	Remarks
CH_3–	CH_3–	1·5–40	20	30	1/1	11	
C_2H_5–	C_2H_5–	0·25–5·0	0·5	0·75	26/27	10	1 bird died from acute effects
C_3H_7–	C_3H_7–	0·1–1·0	0·1	0·25	12/13	12	,, ,,
i-C_3H_7–	*i*-C_3H_7–	0·2–1·0	0·2	0·3	10/12	10	2 birds showed no signs at 0·3 mg./kg.
C_4H_9–	C_4H_9–	0·5–5·0	—	0·5	12/14	9	1 bird showed no signs. 1 bird died from acute effects
i-C_4H_9–	*i*-C_4H_9–	1·5–5·0	—	1·5	6/6	14	
s-C_4H_9–	s-C_4H_9–	1·5–5·0	—	1·5	5/6	12	1 bird died from acute effects
C_5H_{11}–	C_5H_{11}–	1·0–40·0	1·0	2·5	7/11	10	4 birds showed no signs
$C_3H_7.CH(CH_3)$–	$C_3H_7.CH(CH_3)$–	2·5–10·0	—	2·5	4/6	9	2 birds died from acute effects
cyclohexyl-	cyclohexyl-	1·0–5·0	1·0	2·5	3/4	10	1 bird died from injury
C_2H_5–	C_3H_7–	1·0–5·0	—	1·0	5/6	12	1 bird died from acute effects

TABLE III

THE NEUROTOXICITY OF ALKYLPHOSPHONOFLUORIDATES IN CHICKENS

Dose schedule as described in Table II.



R_1	R_2	Range of Doses Tested (mg./kg.)	Maximum Dose at which No Hens were Ataxic (mg./kg.)	Minimum Dose at which Ataxia was Observed (mg./kg.)	Proportion of Birds Positive at Minimum Dose or Greater	Mean Time of Onset of Ataxia (Days)	Remarks
i-C_3H_7-	CH_3-	0·5–2·5	0·5	1·0	9/28	12	In general the total dose was built up by giving 20% daily aliquots. 19 birds died during period of dosing
i-C_3H_7-	C_2H_5-	1·0–5·0	—	1·0	5/6	13	1 bird died of acute effects
CH_3-	i-C_3H_7-	2·5–15·0	2·5	5·0	3/8	10	2 birds died of acute effects at 15 mg./kg. 3 birds showed no signs
C_2H_5-	CH_3-	1·0–2·9	2·25	2·9	5/7	11	2 birds showed no signs
i-C_4H_9-	C_2H_5-	2·9	—	2·9	2/6	6	4 birds died from acute effects. Dosing was spread over 7 days. The delay period was therefore difficult to assess

always being noticeable 9 to 15 days after poisoning (Table II).

The Alkylphosphonofluoridates.—The toxicity of the alkylphosphonofluoridates was so great that demonstration of their neurotoxicity was very difficult. Treatment with P2S and atropine was consistently effective in protecting birds against the lethal effects of 0.5 mg./kg., but not against higher doses. These were therefore given in divided doses, usually 20% daily aliquots of the total dose to be tested. Where this was done is indicated in Table III. Five compounds were examined at doses ranging from 0.5 to 15.0 mg./kg. All five were active between 1.0 and 5.0 mg./kg. (Table III).

The Phosphorofluoridothionates.—Diethyl phosphorofluoridothionate was examined in order to determine the significance of the oxygen atom directly attached to the phosphorus atom. It was neurotoxic at 5.0 mg./kg., but the sample contained 10% of an impurity, which was probably the oxygen analogue.

Diethyl phosphorofluoridate was therefore re-tested under comparable conditions. It was non-neurotoxic at 0.5 mg./kg. and neurotoxic at 0.75 mg./kg. Thus even if all the impurity present in the sample of diethyl phosphorofluoridothionate was the oxygen analogue it would not be sufficient to account for the neurotoxicity of the sulphur compound.

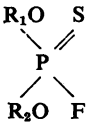
The Dialkylphosphinic Fluorides.—Four compounds of this type were tested. Their formulae are shown in Table I. In a preliminary experiment the di-*n*-propyl and the di-*n*-butyl analogues were examined, in the presence of P2S and atropine at maximum doses of 5.0 and 2.5 mg./kg. respectively. Neither was neurotoxic. Since it was thought that the oxime might catalyse the breakdown of the agent, all four compounds were tested in the absence of the P2S, but the dose of atropine was increased to 10 mg./kg. Again the compounds produced no effect, although one bird given 5.0 mg./kg. di-*n*-propylphosphinic fluoride exhibited atypical symptoms 27 days after poisoning.

The Dialkylpyrophosphonates.—Five dialkyl dialkylpyrophosphonates and ethyl pyrophosphate were examined. With the exception of ethyl pyrophosphate they were tested at doses varying from 1 to 10 mg./kg. Ethyl pyrophosphate was tested more vigorously, cumulative doses of up to 100 mg./kg. being administered over a period of a week. The period of observation was also prolonged and in some cases lasted for 56 days. None of these compounds produced neurotoxicity. It is possible that the maximum doses of 10 mg./

TABLE IV

THE NEUROTOXICITY OF PHOSPHOROFLUORIDOTHIONATES

Dose schedule as described in Table II.



R_1	R_2	Range of Doses Tested (mg./kg.)	Maximum Dose at which No Hens were Ataxic (mg./kg.)	Minimum Dose at which Ataxia was Observed (mg./kg.)	Proportion of Birds Ataxic at Minimum Dose or Greater	Mean Time of Onset of Ataxia (Days)	Remarks
C_2H_5–	C_2H_5–	2·5–7·5	2·5	5·0	4/4	7	Sample contained 10% impurity—(infra-red)

kg. were too low to define adequately a negative response. The intrinsic toxicity of these compounds, however, is so high that even with these comparatively small quantities the birds received many times the LD50 dose.

Miscellaneous Compounds.—The formulae of nine miscellaneous compounds investigated are also shown in Table I. These are essentially "phosphorofluoridates" in which the fluorine has been replaced by other radicals. These were also non-neurotoxic, even though some were tested at 50 to 100 mg./kg., for example, diethyl phosphorocyanidate, diisopropyl phosphoramidate, di-*o*-cresyl methyl phosphate and diethyl phosphorochloridate.

DISCUSSION

Thirty-six organophosphorus compounds have been examined for neurotoxicity. The various classes of compounds and the individual members of each were chosen with the object of assessing the importance which the individual groups, attached to the phosphorus atom, have in producing these effects. The results are summarized in Table V.

All the phosphorofluoridates tested were neurotoxic, from the dimethyl to the di(1-methylbutyl) homologue, suggesting that the actual nature of the ester alkyl group is not critical; this is supported by the observation that dicyclohexyl phosphorofluoridate and the mixed alkyl ester, ethyl propyl phosphorofluoridate, also produce neurotoxicity.

At least one alkoxyl group seems to be necessary, for the alkylphosphonofluoridates are neurotoxic, whereas the dialkylphosphinic fluorides are not. The ester oxygen of the phosphorofluoridates may be replaced by an amino-group as in mipafox, but

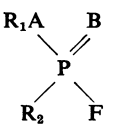
TABLE V

NEUROTOXICITY OF VARIOUS CLASSES OF ORGANOPHOSPHORUS COMPOUNDS

Class of Compound	No. of Compounds Tested	No. Positive
Phosphorofluoridates ..	11	11
Alkylphosphonofluoridates	5	5
Phosphorofluoridothionate	1	1
Dialkylphosphinic fluorides	4	0
Dialkylpyrophosphonates and ethyl pyrophosphate ..	6	0
Miscellaneous compounds ..	9	0

it is not known whether an alkyl *N*-alkylphosphoramidofluoridate would be neurotoxic. The phosphoryl oxygen may be replaceable by sulphur, but, in view of the possible presence of the oxygen analogue of diethyl phosphorofluoridothionate as an impurity in the only compound of this type examined, this is by no means established.

The most significant structure is the fluorine atom, for replacement of this atom with a wide variety of groups always results in loss of neurotoxicity. Thus a compound with the general formula, I, will possess neurotoxic properties, provided that R_1 is an alkyl group, R_2 is an alkyl, alkoxyl or alkylamino-group, A is an oxygen or secondary amino-group, and B is an oxygen or possibly a sulphur atom.



(I)

The significance of the fluorine atom suggests that it plays a direct role in the development of the biochemical lesion. This may result from its being carried by the compound as a whole to specific areas in the nervous system. At these sites ionic fluorine may be liberated by rupture of the P-F bond. This may occur by the action of cholinesterase, but this need not be the only mechanism involved. The need to deposit the fluorine at these specific sites determines the character of the rest of the molecule.

Substitution of the fluorine atom by a chlorine or bromine atom results in a very unstable molecule which almost certainly does not penetrate the central nervous system intact. This is borne out by the lack of toxicity of such compounds. Compounds like the dialkyl dialkylpyrophosphonates are more stable, and there is little doubt as judged by their acute toxic effects that they enter the central nervous system. An explanation for their lack of neurotoxicity must therefore be sought elsewhere. The hypothesis offered above is capable, however, of explaining the absence of neurotoxicity of these substances, for the rupture of the P-X bond in different compounds will result in the production *in vivo* of a variety of substances with markedly different properties. Thus the pyrophosphates would yield derivatives of orthophosphoric acid, and these do not possess properties similar to hydrofluoric acid.

Whilst this working hypothesis offers at least a partial explanation of the neurotoxicity of organophosphorus compounds of type I, it must not be forgotten that triaryl phosphates are similarly neurotoxic. This hypothesis, however, can be modified to cover such compounds, but its adaptation to these cases will be considered in a later communication.

The neurotoxic hazard which these substances constitute to man is a difficult problem to assess because of (*a*) known species differences in the response to organophosphorus compounds, and (*b*) the absence of any direct evidence that organophosphorus compounds other than tri-*o*-cresyl phosphate and mipafox are neurotoxic in humans. Both of these have been extensively examined in the chicken. The delay period, the clinical character of the condition and the distribution and nature of the histological lesions are all the same as those found with dyflos and the compounds which have, in this study, been reported active for the first time. It must therefore be assumed that all the organophosphorus compounds shown to be neurotoxic in chickens will, under appropriate conditions, produce neurotoxicity in man.

The authors wish to acknowledge the help given by Mr. W. Pearce with the animal experiments.

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A NOTE ON THE DEMYELINATION PRODUCED IN HENS BY DIALKYLFLUORIDATES

BY

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From the Microbiological Research Establishment, Porton, Salisbury, Wilts

(RECEIVED DECEMBER 22, 1959)

Hens were treated with organophosphorus compounds until signs of neurotoxicity developed. The sciatic nerves, spinal cord, and medulla were then examined histologically by the Marchi method, to determine the extent and distribution of demyelination. The lesions were proportional to the dose of compound injected, and the spinal cord was more sensitive than the sciatic nerves. It is suggested that the spinal cord, and not the sciatic nerves, is the main site of damage in clinical paralysis following dyflos poisoning.

In order to confirm the clinical findings described in the previous paper (Davies, Holland, and Rumens, 1960), a number of active compounds were examined for their ability to produce demyelination in the hen. The distribution of lesions in the peripheral and central nervous system following organophosphorus administration has been described by Barnes and Denz (1953), Fenton (1955), and Cavanagh (1954), who found demyelination in the sciatic nerve, spinal cord, medulla, and cerebellum.

METHODS

The sciatic nerves, spinal cord, and medulla or cerebellum from seriously ataxic or paralysed birds were taken for histological examination. The birds were killed by the injection of pentobarbitone, perfused, and tissues taken as described by Barnes and Denz (1953). They were stained by the modified Marchi staining methods of Swank and Davenport (1935), Poirier, Ayotte and Gauthier (1954), and Adams (1958). Using these methods the degenerating myelin stains black, and, for recording, all the positive sections were scored from + to +++ depending upon the intensity of the staining. This method is used in Table I in which are summarized the effects of nine compounds to indicate the severity of the lesions. All sections were made at comparable levels in each tissue.

Normal controls and diisopropyl phosphorofluoridate(dyflos)-poisoned birds were similarly treated for comparison with other compounds.

RESULTS

Although all these compounds produced demyelination in the spinal cord, some failed to do so in the sciatic nerve, but there was no essential difference in the distribution of lesions

TABLE I

DEMYELINATION OF THE SCIATIC NERVE, SPINAL CORD AND MEDULLA IN HENS AFTER POISONING WITH CERTAIN ORGANOPHOSPHORUS COMPOUNDS

All comparisons of Osmium staining were made at similar levels in corresponding tissues.

	Dose (mg./kg.)	Clinical Effects	Demyelination		
			Sciatic Nerve	Spinal Cord	Medulla
Phosphoro-fluoridates					
1. Diisopropyl	0·2	None	−	−	−
	0·4	Slight	−	+	−
	0·6	Definite	−	++	−
	0·9	Severe	+	++	+
2. Diethyl ..	2·5	,,	+	++	+
3. Dibutyl ..	2·5	,,	++	++	+
4. Dipentyl	20·0	,,	++	++	+
Phosphoro-fluorido-thionate					
5. Diethyl ..	7·5		++	++	−
Alkylphosphono-fluoridates					
6. Isopropyl ethyl-	1·0	Slight	−	+	−
	5·0	Severe	+	+++	++
7. Ethyl methyl-	2·9	,,	+	+++	+
8. Isobutyl methyl-	2·9	,,	+	++	−
9. Methyl isopropyl-	5·0	,,	+	+++	+

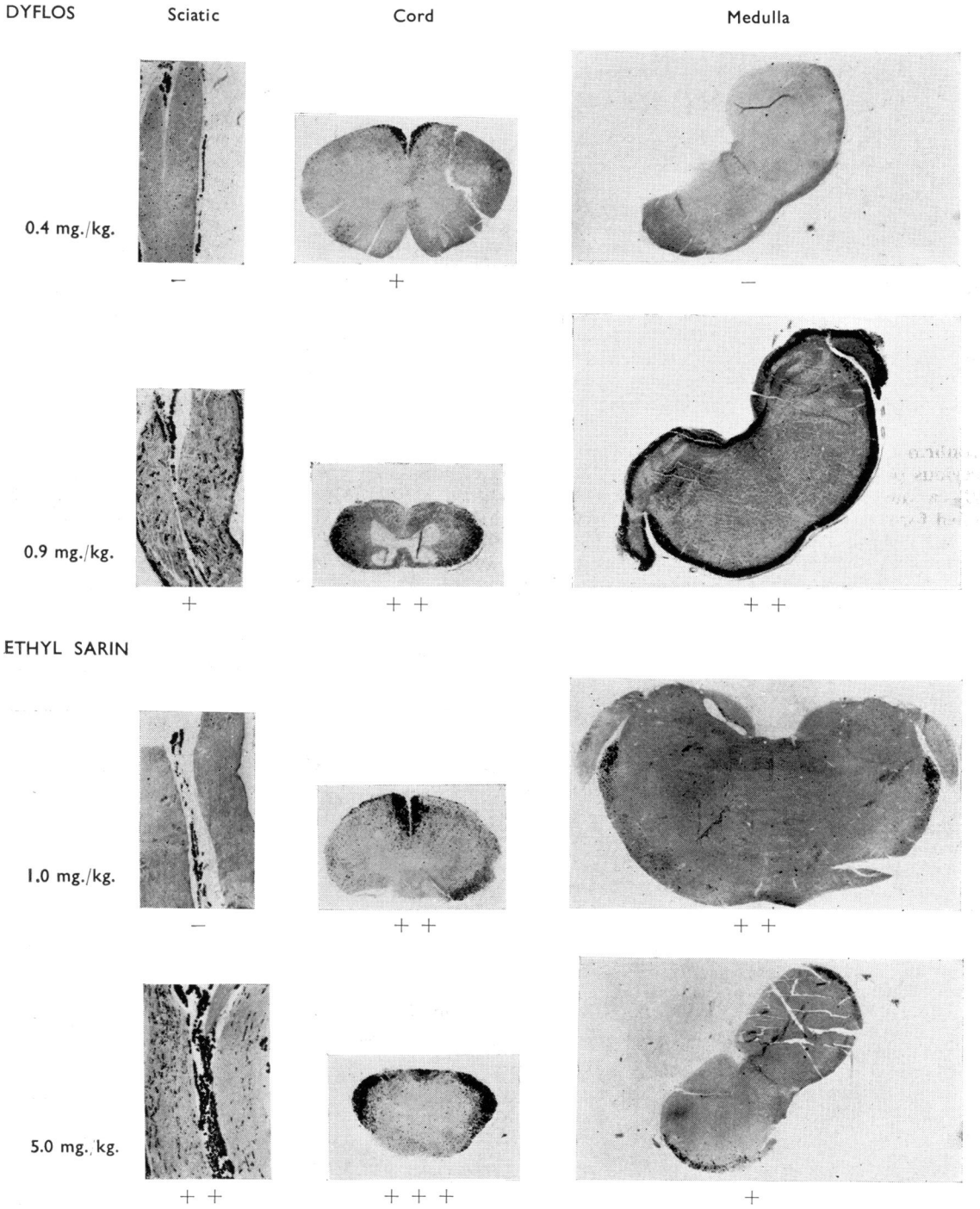

FIG. 1.—Sections at different levels of sciatic nerve, spinal cord and medulla illustrating demyelination in hens poisoned with dyflos and ethyl sarin (magnification × 8).

from that produced by dyflos. The distribution and severity of lesions produced by dyflos are summarized in Table I. The effects are proportional to the dose over the range 0.2 mg./kg. to 0.9 mg./kg. Their extent and distribution are similar to that described by Fenton (1955). It should be noted that the low dose levels produced lesions in the spinal cord but not in the sciatic nerve, and this clearly demonstrates the greater sensitivity of the spinal cord to damage by the fluoridates.

For comparison the distributions of Marchi staining after dyflos 0.4 mg./kg. and 0.9 mg./kg. and isopropylethylphosphonofluoridate (ethyl sarin) 1.0 mg./kg. and 5.0 mg./kg. are shown in Fig. 1, and illustrate the comparable distribution of demyelination obtained with both these compounds.

The importance of the sciatic nerve lesions in the clinical paralysis with dyflos seems to have been over-emphasized, for the present results indicate that it is the spinal cord which is the main site of damage.

The distribution of the histological lesions produced by the other fluoridates is identical to that seen after dyflos poisoning, and since the clinical picture is also closely similar to that caused by these other compounds, it is highly probable that all the fluoridates produce an identical clinical and pathological neurotoxic syndrome.

The author wishes to thank Miss G. Meaden for preparing the Marchi sections, Mr. G. B. Carter for the Adams method, and Mr. E. Cuff for the photomicrographs.

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THE INFLUENCE OF THIOPENTONE ANAESTHESIA ON THE BLOOD LIPID AND BLOOD SUGAR LEVEL

BY

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(RECEIVED JANUARY 1, 1960)

Thiopentone anaesthesia in dogs and rats was accompanied by a sharp fall in blood non-esterified fatty acids and a small increase in blood sugar. No pronounced changes in the blood concentrations of cholesterol, phospholipids or fatty acid esters were observed. Ether anaesthesia had no effect on the blood non-esterified fatty acids in rats. The fall in non-esterified fatty acids during thiopentone anaesthesia is therefore not related to the state of anaesthesia itself.

Thiopentone has a special position among anaesthetics of the barbiturate series. It acts almost immediately and its action is of very short duration. This latter property of thiopentone is due to its ready solubility in body fat (Brodie, Bernstein, and Mark, 1952), and the amount of body fat is the main factor influencing the duration of thiopentone anaesthesia. A decrease in body fat prolongs the duration of anaesthesia (Hermann and Wood, 1952). On the other hand, alimentary lipaemia in rats reduces the duration of anaesthesia due to a partial binding of thiopentone by blood lipids (Anderson and Magee, 1953).

In view of the great affinity of thiopentone for body fat, the possibility that other metabolic changes, particularly in lipid metabolism, might occur during thiopentone anaesthesia has been investigated. Valuable information in this respect can be obtained from investigations of the level of non-esterified fatty acids, an important lipid fraction in the mobilization, transport and utilization of fats.

METHODS

The experiments were carried out on dogs of both sexes, fasted for 12 hr. prior to experiments, and on 7-month-old albino rats kept on a standard laboratory

TABLE I

CONCENTRATIONS (M.EQUIV./L.) OF NON-ESTERIFIED FATTY ACIDS IN THE BLOOD OF CONTROL DOGS (1–5) AND OF DOGS (6–10) ANAESTHETIZED WITH THIOPENTONE

The initial sample (0) was taken just before the injection. Subsequent samples were taken at the times indicated up to 2 hr.

Treatment	Dog No.	Time in min.								
		0	10	30	40	60	70	90	100	120
Intravenous saline	1	0·37	—	0·37	—	0·42	—	0·42	—	0·48
	2	0·58	0·53	—	0·74	—	0·79	—	0·69	0·93
	3	0·89	0·85	—	—	0·90	—	0·89	—	0·93
	4	1·01	0·98	—	1·03	—	0·98	—	1·03	0·98
	5	0·74	0·90	0·85	—	0·85	—	0·90	—	0·95
Intravenous thiopentone	6	0·37	0·37	0·21	—	0·19	—	—	—	0·16
	7	0·77	0·74	0·58	—	0·48	—	0·42	—	0·37
	8	0·66	0·45	—	0·40	—	0·40	—	0·34	—
	9	0·62	0·53	—	0·37	—	0·37	—	0·32	—
	10	0·69	—	0·48	0·42	—	—	—	—	—

TABLE II

MEAN CONCENTRATIONS (±S.E.) OF LIPID FRACTIONS AND GLUCOSE IN THE SERUM OF 5 DOGS DURING THIOPENTONE ANAESTHESIA

Time (min.)	Non-esterified Fatty Acids m.equiv./l.	Total Cholesterol mg./100 ml.	Phospholipids mg./100 ml.	Fatty Acid Esters mg./100 ml.	Blood Sugar mg./100 ml.
0	1·36 ± 0·34	133 ± 44	363 ± 87	335 ± 68	70 ± 15
30	0·85 ± 0·18	125 ± 40	339 ± 85	306 ± 63	90 ± 8
60	0·81 ± 0·19	125 ± 44	349 ± 93	307 ± 74	102 ± 17
90	0·82 ± 0·27	125 ± 44	347 ± 81	310 ± 69	102 ± 8
120	0·69 ± 0·40	125 ± 46	348 ± 88	305 ± 77	104 ± 9

diet. Anaesthesia was induced by injection of Thiopental-Spofa, a Czechoslovak preparation of thiopentone.

Series 1.—Thiopentone was administered intravenously to 5 dogs. Each dog received first an initial dose of about 30 mg./kg. thiopentone as a 2.5% solution in saline; later, supplementary injections were given to maintain anaesthesia. Each dog received a total of about 60 mg./kg. An equivalent amount of saline was administered to 5 control dogs. Venous blood was drawn from a limb and 1 ml. portions were immediately pipetted into an extraction mixture and the non-esterified fatty acids were estimated by Dole's method (Dole, 1956), using an alcoholic solution of Nile blue as indicator. At first, whole blood was employed to avoid spontaneous lipolysis which might occur during the separation of serum.

In a further group of 5 dogs blood was allowed to coagulate, since spontaneous lipolysis was found to be negligible under these conditions. In the corresponding serum samples, total cholesterol was estimated according to Abell, Levy, Brodie, and Kendall (1952), phospholipids by King's method (King, 1951) and fatty acids by the method of Stern and Shapiro (1953). Blood sugar was estimated by a ferricyanide-titrimetric method.

Series 2.—Six female rats were injected intraperitoneally with thiopentone 40 mg./kg. as a 2.5% solution in saline. The control animals received saline only. The animals were killed by decapitation 60 min. after thiopentone and the blood was collected into test tubes. After coagulation and centrifuging, sugar and non-esterified fatty acid were estimated in the serum.

Series 3.—Fourteen experimental rats were kept for 1 hr. under ether anaesthesia. The control group consisted of 16 untreated rats. Subsequent procedures were similar to those of series 2.

RESULTS

Series 1

The results of this series of experiments are summarized in Table I. It is evident that thiopentone anaesthesia caused a marked fall of non-esterified fatty acid concentrations in all the dogs. In the 5 control dogs the concentration showed a slight tendency to increase.

In another 5 dogs (Table II) the serum lipid and sugar concentrations during thiopentone anaesthesia were investigated. A marked decline of non-esterified fatty acid was observed, whereas the blood sugar values increased. These changes were greatest during the first hour of anaesthesia. Total cholesterol and phospholipid concentrations

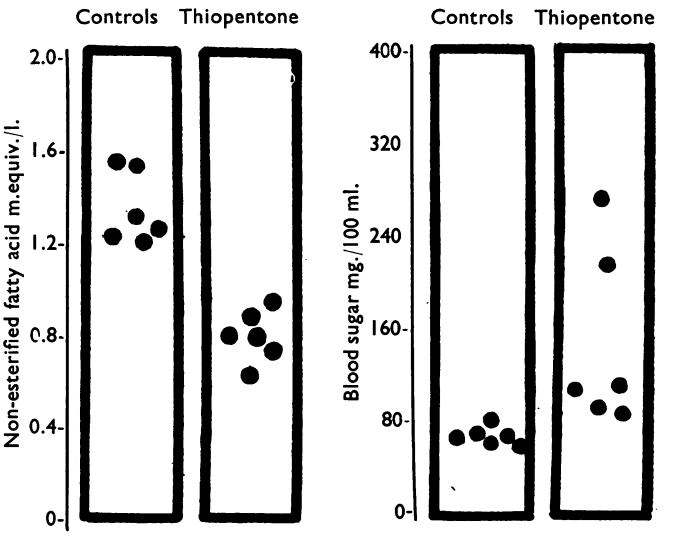

FIG. 1.—The effect of thiopentone on the concentrations of non-esterified fatty acids and sugar in rat blood. Six rats were injected intraperitoneally with thiopentone 40 mg./kg. and six control rats with saline. Blood samples were collected 1 hr. after the injections.

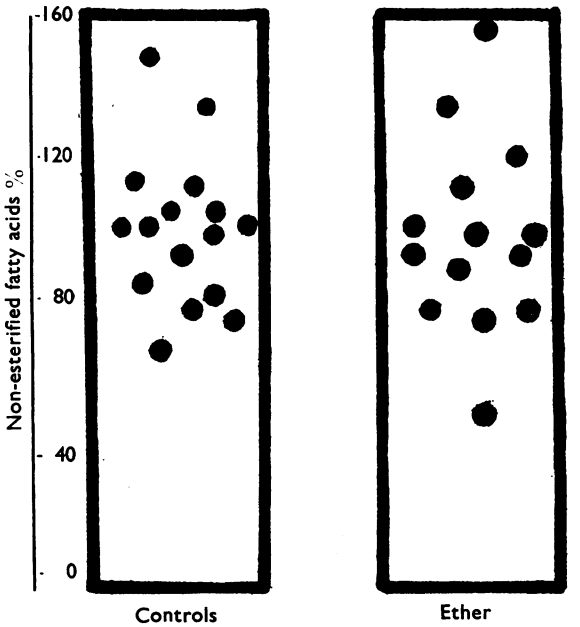

FIG. 2.—The effect of ether on the non-esterified fatty acid concentration in rat serum. 14 rats were anaesthetized with ether and 16 rats were untreated. Blood samples were collected at the end of 1 hr.

remained practically unchanged throughout the experiment. The concentrations of fatty acid esters had a tendency to fall, but as the standard errors were high the change cannot be considered significant.

Series 2

As can be seen from Fig. 1 a similar action of thiopentone on the concentration of non-esterified fatty acids and sugar was found in rats. The effects on fatty acid concentrations were highly significant ($P<0.001$, $t=6.359$); those on sugar significant ($P<0.05$, $t=2.534$; $P<0.01$ by the approximate rank test H).

Series 3

Fig. 2 presents the combined results of two experiments, the concentrations of non-esterified fatty acids being expressed as percentages of the mean value for the control group. It was found that 60 min. ether narcosis was without effect.

DISCUSSION

Our results show that during thiopentone anaesthesia the blood concentrations of non-esterified fatty acid in dogs and rats decline, whereas the blood sugar rises. Non-esterified fatty acids are important as intermediates in the utilization of fat, and as a mobile fraction necessary for fat resynthesis and deposition. In view of their significance study of their concentrations under different conditions has attracted much interest in recent years. When such experiments are carried out under thiopentone anaesthesia a primary response may be combined with side-effects of the thiopentone anaesthesia.

Results so far do not allow an unequivocal explanation of the mechanism leading to the drop of non-esterified fatty acids during thiopentone anaesthesia. One possible explanation might be a direct action of thiopentone on lipolysis in adipose tissue. On the other hand it is possible that during thiopentone anaesthesia the catabolism of fat is enhanced, and that the non-esterified fatty acids are in consequence removed more rapidly from the blood stream. However, a similar effect could also be accounted for by increased lipogenesis.

Blood sugar changes due to thiopentone anaesthesia have already been reported many times. Most authors report a slight increase of blood sugar (Blackberg and Hrubetz, 1937; Rašková and Vlček, 1947) while a decrease has been reported only after the cessation of anaesthesia (Benešová, Koutenská and Wenke, 1959).

An unequivocal interpretation of our results is not possible since among other factors our control animals, which were not under anaesthesia, were allowed to move, in contrast to the animals under anaesthesia. The question as to what extent the fall of non-esterified fatty acids is caused specifically by thiopentone, or whether it is a change primarily due to anaesthesia, is therefore left open at present. Our results with ether suggest that the former alternative is more reasonable.

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APOMORPHINE-INDUCED PECKING IN PIGEONS

BY

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From the Department of Pharmacology, K.G. Medical College, Lucknow, India

(RECEIVED JANUARY 12, 1960)

Apomorphine produced persistent pecking in pigeons, the latent period, intensity and duration of which were related to the dose. The ED50 was estimated as 78.1 ±11.1 μg./kg. On chronic administration of apomorphine there was a significant decrease in latent period and weight which quickly returned to normal on stopping the drug. No conditioning and no tolerance were observed. The uncertain emetic effect of apomorphine in pigeons has been confirmed. Ten other centrally acting agents tested (caffeine, cocaine, 5-hydroxytryptamine, lysergic acid diethylamide, methamphetamine, morphine, nalorphine, pentylenetetrazol, strychnine, and yohimbine) failed to produce similar effects in pigeons.

While engaged in a comparative evaluation of anti-emetic drugs in pigeons (Gujral, Saxena and Dhawan, 1956), an attempt was made to produce emesis by apomorphine. It was seen that instead of emesis the drug constantly produced persistent pecking. The birds pecked rapidly at the tin floor and wires in the side and ceiling of the cage as if pulling off particles from them and swallowing them. They ignored any grain that was put in the cage. The effect came on very quickly, persisted for 0.5 to 1 hr. and was so compulsive that in many cases the beak got injured and started bleeding. The pigeons were depressed for some time after the pecking had stopped. This type of fruitless pecking was not seen in normal pigeons. No reference could be found in the literature about this effect of apomorphine in pigeons. Our interest in the problem was revived by a report of similar effects of apomorphine in pigeons by Koster (1957). It was decided to undertake a detailed study of apomorphine-induced pecking in pigeons and to see if a similar behaviour could be induced by some other drugs as well.

A preliminary report of this work was presented before the 1959 session of the Association of Physiologists and Pharmacologists of India at Poona (Dhawan and Saxena, 1960).

METHODS

Three hundred and fifty pigeons of either sex weighing between 200 and 400 g. were used in this study. The birds were housed in groups of 8 to 10 (regardless of sex) in large cages, and had free access to water and mixed grains. They were not used more than twice a week except when studying the effects of chronic administration. At the time of use they were placed separately in small wire mesh cages, and food and water were withdrawn. Experiments were carried out in a quiet room diffusely illuminated with daylight.

The drugs were dissolved in distilled water or suspended in a 4% homogenized gum acacia solution. Whenever possible solutions or suspensions were prepared immediately before use. Each dose was tested in at least one batch of 10 birds. Intramuscular injections were made in the pectoral muscles, intravenous injections through alar veins, and oral administration by letting the suspension drop in the mouth from a tuberculin syringe.

The birds were observed for at least 1 hr. after administration of drugs, and pecking was considered positive only if the bird pecked more than 10 times during this period. Active expulsion of crop contents was employed as the criterion for vomiting. The latent period was the period elapsing between the drug administration and the first act of pecking or vomiting respectively. The duration of pecking was the period during which at least 50% pigeons were pecking. Only an approximate estimate of the duration could, therefore, be made. The pecking was so rapid that it was difficult to count the exact number in each bird and an accurate gradation of the intensity could not be done.

RESULTS

Effects of Graded Doses of Apomorphine.— In a few preliminary experiments it was observed that there was little difference in the onset, duration or intensity of pecking following intravenous or intramuscular administration of apomorphine. Hence it was decided to give the drug intramuscularly. Graded doses of apo-

morphine hydrochloride (British Drug Houses) from 12.5 to 12,000 μg./kg. were administered and the effect was assessed in terms of (a) the percentage of birds affected, (b) the latent period, (c) the duration of pecking, (d) the intensity of pecking and (e) the presence or absence of vomiting.

The final regression line obtained by the method of Bliss (1935a and b) is shown in Fig. 1. It was tested for goodness of fit by the χ^2 test (Finney, 1952) and found to be homogeneous. The ED50 was found to be 78.1 ± 11.1 μg./kg. When higher dose levels of apomorphine were tested, an occasional bird was met with which did not respond at certain dose levels (Table I, 600, 800 and 2,400 μg./kg. respectively). With still larger doses, however, all the birds responded. The latent period progressively decreased as the dose of apomorphine was increased. The duration of pecking also was more in batches receiving higher doses of apomorphine. It was not possible quantitatively to evaluate the severity of pecking in various groups, but it was observed that pecking was more vigorous with increasing doses. None of the pigeons vomited in response to doses of apomorphine below 2,400 μg./kg. Even in higher doses the response was erratic (see effects of 4,800 μg./kg., Table I).

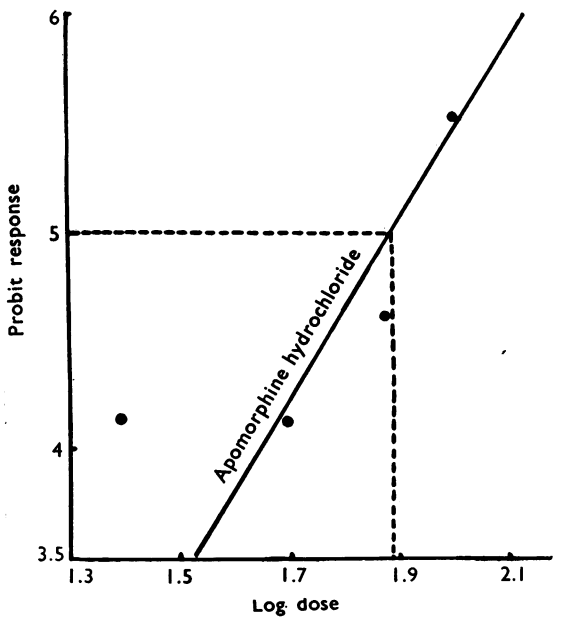

FIG. 1.—Showing the final regression line for apomorphine-induced pecking in pigeons. The ordinate shows the transformed probit values for percentage of birds responding. The ED50 value has been marked. The points refer to the experimental data obtained.

TABLE I

THE EFFECTS OF APOMORPHINE ON PIGEONS

Groups of pigeons were treated with apomorphine in doses ranging from 12·5 μg. to 12 mg./kg. The incidence of pecking and vomiting was recorded. A pecking frequency greater than 10/min. was considered a positive response. The duration was the period during which 50% or more of the birds were pecking. The latent period was the time between drug administration and the onset of pecking or vomiting respectively. The mean latent period for pecking was calculated for each group and the standard error is given in parentheses.

Apomorphine μg./kg.	No. of Pigeons Tested	% Pecking	Duration (min.)	Mean Latent Period in min.	Vomiting	
					%	Latent Period (min.)
12·5	10	0				
25	10	20	7	6·0 (1·0)		
50	10	20	7	6·0 (1·0)		
75	20	35	8	8·0 (1·1)		
100	10	70	25	6·0 (0·8)		
150	10	100	35	6·1 (0·9)		
200	10	100	40	3·3 (0·4)		
250	20	100	40	4·0 (0·4)		
400	10	100	40	5·5 (0·8)		
600	7	86	40	6·5 (0·9)		
800	8	88	60	4·0 (0·6)		
2,400	10	90	105	4·3 (1·2)	10	4
3,600	10	100	120	2·4 (0·3)		
4,800	10	100	120	2·0 (0·5)	70	18
4,800	10	100	120	2·1 (0·8)		
6,000	10	100	120	2·0 (0·3)	40	18
12,000	10	100	120	1·4 (0·4)	20	10

Effects of Chronic Administration.—In one batch of 10 pigeons effects of daily administration of 250 μg./kg. (approximately $2 \times$ ED99) apomorphine for 15 days were studied. The results obtained are shown in Fig. 2. The percentage of birds affected and the duration of pecking were unaltered by daily administration. There was, however, a significant decrease ($P = 0.05$) in the latent period from the third day of the start of the experiment (Fig. 2). After stopping apomorphine the latent period returned towards the control level and a week later no significant difference from the initial value could be seen (Fig. 2). The weights of the birds also started falling, and by the tenth day a significant ($P = 0.05$) loss in weight was observed. After stopping the apomorphine,

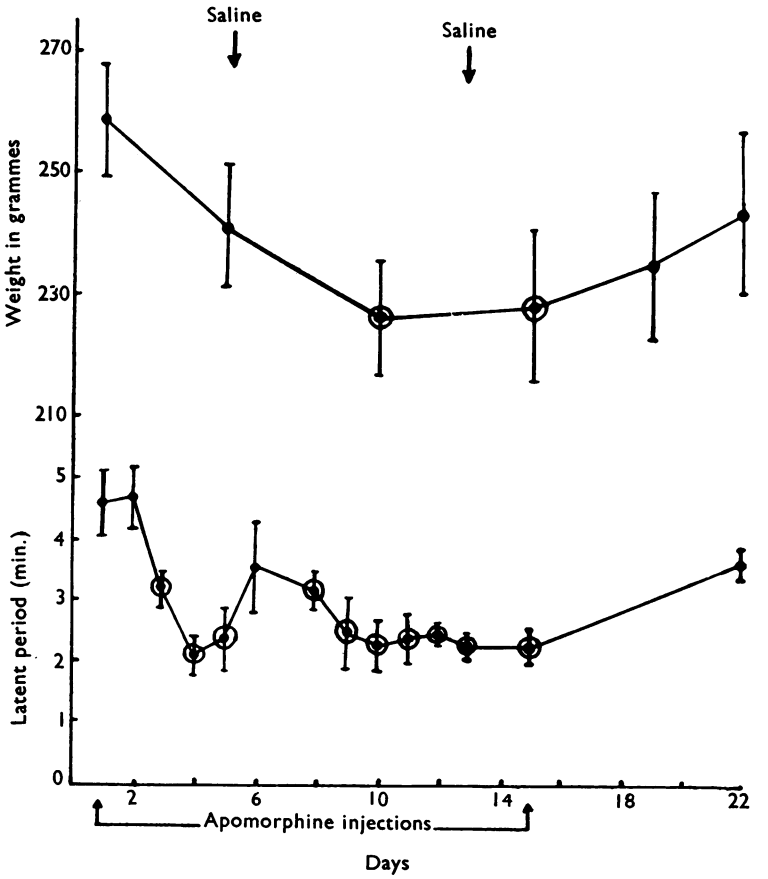

FIG. 2.—Showing the effect of chronic intramuscular injection of apomorphine (250 μg./kg. daily) on the mean weight and latent period for pecking of a group of 10 pigeons. The points surrounded by a circle (O) differ significantly (P=0.05) from the starting values. The vertical lines represent standard errors of the mean. At Saline a sham procedure was performed with saline injection. Note the quick recovery on stopping apomorphine.

the weight returned towards the initial level. The difference in weight on the 19th and 22nd day is not significant statistically from control values. A sham procedure with normal saline injections was performed on 2 occasions (fifth and thirteenth day) during the study (Fig. 2). There was, however, no conditioned pecking after saline injection.

Effects of Various Factors on Pecking.—Hypoglycaemia produced by fasting, administration of insulin (0.2 unit/kg. injected subcutaneously) or tolbutamide (100 mg./kg. orally) failed to alter the incidence, duration or latency of subsequent pecking induced by 250 μg./kg. of apomorphine. The response to apomorphine was also unaffected by previous administration of a carbonic anhydrase inhibitor, acetazoleamide (150 mg./kg. orally). The results have been summarized in Table II.

Intensity of pecking was considerably reduced if the room was made dark and in some cases the pecking stopped altogether in darkness. The pecking could be interrupted by vigorous thumping on the table, loud noise, etc., but was always resumed with the same intensity after a few minutes. Occurrence of vomiting also subdued

TABLE II

THE EFFECTS OF VARIOUS PROCEDURES ON APOMORPHINE (250 μG./KG.) INDUCED PECKING IN PIGEONS. THE CONTROL GROUP RECEIVED APOMORPHINE ALONE

P values refer to difference between treated and control groups. L.P.=latent period.

Procedure or Drug	Number of Birds	Apomorphine Challenge After	% Positive Responses to Apomorphine	Mean L.P. in min. ±S.E.	P	Approximate Duration in min.
Control	10		100	4·9±0·5		40
Fasting	10	24 hr.	100	4·6±0·4	0·5	40
Insulin	10	0·5 ,,	100	5·0±1·0	0·1	40
Tolbutamide	10	3 ,,	100	4·9±1·0	0·9	40
Acetazoleamide	10	3 ,,	90	3·5±0·44	0·5	40

pecking for some time. Pecking could be induced even in pigeons where both eyes had been destroyed.

Testing of Other Drugs for Pecking Effect.— Ten centrally acting agents, caffeine citrate (100 mg./kg.), cocaine hydrochloride (24 mg./kg.), 5-hydroxytryptamine creatinine phosphate (250 and 1,000 μg./kg.), lysergic acid diethylamide (25 and 100 μg./kg.), methamphetamine hydrochloride (5 mg./kg.), morphine tartrate (1 and 10 mg./kg.), nalorphine hydrochloride (1 and 20 mg./kg.), pentylenetetrazol (25 mg./kg.), strychnine hydrochloride (500 μg./kg.) and yohimbine hydrochloride (75, 150 and 600 μg./kg.), were tested for their ability to produce pecking in pigeons. All drugs were given intramuscularly. Occasional pecking (frequency never more than 10/hr.) was observed in some of the birds receiving yohimbine (150 μg./kg.) and cocaine. Lysergic-acid-diethylamide-treated birds appeared unusually sedated as contrasted with its excitatory effect in other species. Many of these agents produced emesis in some of the pigeons.

DISCUSSION

The results obtained clearly demonstrate that pecking is a characteristic and well defined behavioural disturbance induced by apomorphine in pigeons. The phenomenon can be produced by such minute amounts that the procedure might be useful for detecting small amounts of apomorphine. Results of chronic administration (Fig. 2) suggest that tolerance does not develop to this effect of apomorphine. There is a definite facilitation after a few days which may be due to accumulation of the drug in the body or due to a sensitization of the receptors concerned. That facilitation is not due to conditioning is shown by the fact that sham procedures with saline injections are ineffective in eliciting a pecking response (Fig. 2). The loss in weight seen during the chronic administration of apomorphine could be due either to the excessive muscular activity involved in the pecking or to some toxic effect of the drug itself.

It is difficult to explain the mechanism of this effect of apomorphine on the basis of the existing data. The reaction does not seem to be identical with pre-emetic nausea of other emetic agents. Our data confirm the findings of several other workers that apomorphine is an uncertain emetic in pigeons (Madjarek and Stern, 1956; Zeehuisen, 1895). This inability of apomorphine to produce consistent emesis in pigeons strongly suggests that the chemoreceptor trigger zone for emesis (Wang and Borison, 1950) is either rudimentary in pigeons or is qualitatively different from that in other species. The results of Hanzlik and Wood (1929) with digitalis emesis in pigeons also support this contention. Again tolerance has been reported to emetic action of apomorphine in other species (Co Tui, 1931), while no tolerance is observed to its pecking effect. Finally, large doses of apomorphine are capable of preventing apomorphine-induced vomiting in dogs (Dordoni, 1951; Hatcher, 1924; Koppanyi, 1930; Leake, 1922). The pecking effect is, however, more intensive with increasing doses of apomorphine.

Koster (1957) has suggested that the disturbance might be a feeding hallucination. However, some known hallucinogenic agents (lysergic acid diethylamide, cocaine, yohimbine, nalorphine) tested fail to produce pecking. The response is unaffected by hypoglycaemia induced by fasting, insulin or tolbutamide. Hypoglycaemia is known to increase excitability of nervous structures (Kety, Polis, Nadler, and Schmidt, 1948).

Similarly decreased brain excitability produced by acetazoleamide (Millichap, Woodbury, and Goodman, 1955) has not altered the pecking response. The response is also unaffected by prior administration of azacyclonol, an antihallucinogenic agent (unpublished observations).

It is possible that the phenomenon may be identical with chewing movements produced by apomorphine in rat (Du Toit and Christensen, 1948) and rabbit (Brucke, Petsche, Sailer, and Stumpf, 1957). Brucke *et al.* (1957) have shown that there is a pronounced and long-lasting desynchronizing effect on precentral cortical electroencephalograph accompanying it in rabbits. A similar electroencephalographic pattern is produced by methamphetamine. Methamphetamine is, however, unable to produce a pecking response in pigeons. Some valuable data about the mechanism of apomorphine pecking may be obtained by studying the effect of various groups of pharmacological agents on this response. Work on these lines is already in progress and will be published in a separate communication.

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THE BRONCHOCONSTRICTOR ACTION OF BRADYKININ IN THE GUINEA-PIG

BY

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Bradykinin was found to be a potent bronchoconstrictor agent in the guinea-pig anaesthetized with urethane. This action was not affected by vagotomy, or by treatment of the animal with mepyramine, atropine, lysergic acid diethylamide, or cortisone. Adrenaline and isoprenaline suppressed the bronchoconstrictor responses to bradykinin and histamine. Small doses of acetylsalicylic acid, however, suppressed only that to bradykinin. Bradykinin also produced bronchoconstriction in the isolated perfused lungs of the guinea-pig. The closely related peptide, wasp kinin, was also a potent bronchoconstrictor.

Bradykinin is known to affect smooth muscles of intestine, uterus and blood vessels. When the action of bradykinin on bronchiolar muscle of the guinea-pig was investigated, it was found to be a potent bronchoconstrictor agent. A preliminary report of this observation has already been made (Collier, Holgate, Schachter and Shorley, 1959), and the present account describes the findings in detail.

METHODS

Materials.—Bradykinin was prepared by the action of crystalline trypsin on heated bovine serum globulin and was purified chromatographically (Lockhart and Jones, personal communication). The potencies of the various samples used, assayed on guinea-pig ileum against a standard preparation (Rocha e Silva, Beraldo and Rosenfeld, 1949), ranged from 7 to 900 units/mg. After the work described below was completed, Elliott, Lewis and Horton (1960) kindly provided us with a sample of pure bradykinin; and comparison with our reference preparation indicated that about 12,000 to 12,500 units were equivalent to 1 mg. pure bradykinin, or that 1 unit was equivalent to about 80 ng. Wasp kinin was prepared by washing dried venom glands with alcohol, extracting the kinin from the residue with glacial acetic acid and precipitating it with ether (Holdstock, Mathias and Schachter, 1957). Chymotrypsin and trypsin (Armour) were crystalline preparations. The following salts were used: acetylcholine chloride, adrenaline hydrochloride, atropine sulphate, DL-cysteine hydrochloride, 5-hydroxytryptamine creatinine sulphate, isoprenaline hydrochloride, lysergic acid diethylamide tartrate, mepyramine maleate, sodium phenylbutazone. All weights are expressed in terms of the active acid or base. Acetylsalicylic acid was suspended in 5% gum acacia for intraperitoneal administration. For intravenous injection, 10 parts by weight of acetylsalicylic acid were mixed with 3 parts of calcium carbonate and 1 part of citric acid, and the mixture dissolved in water immediately before use. Cortisone, as the alcohol, was suspended in saline for intramuscular or subcutaneous injection. Amidopyrine was administered as the base.

Whole Animal Preparations.—The method was based on that of Konzett and Rössler (1940). Guinea-pigs (300 to 700 g.) were anaesthetized with urethane (1.0 to 1.25 g./kg. i.p.). Further urethane was given intraperitoneally when necessary to maintain suppression of spontaneous respiratory movements. The trachea was cannulated and the lungs inflated with air by a miniature Starling pump operating on a partially-closed circuit (5 to 10 ml. stroke volume, generally at 72 strokes/min.). A sidearm from the cannula permitted some air to escape through a water valve offering a resistance of 10 cm. water, and the escaping air operated a piston recorder. When resistance of the lungs to inflation increased, more air passed through the sidearm, causing a greater excursion of the recording lever. Solutions of drugs in saline were injected at 5 to 10 min. intervals through a cannula in the external jugular vein and 0.05 to 0.4 ml. washed in with 0.4 ml. heparinized saline (10 units/ml.). After a marked response of the lungs, increased resistance to inflation might persist for some time. This was overcome by momentarily

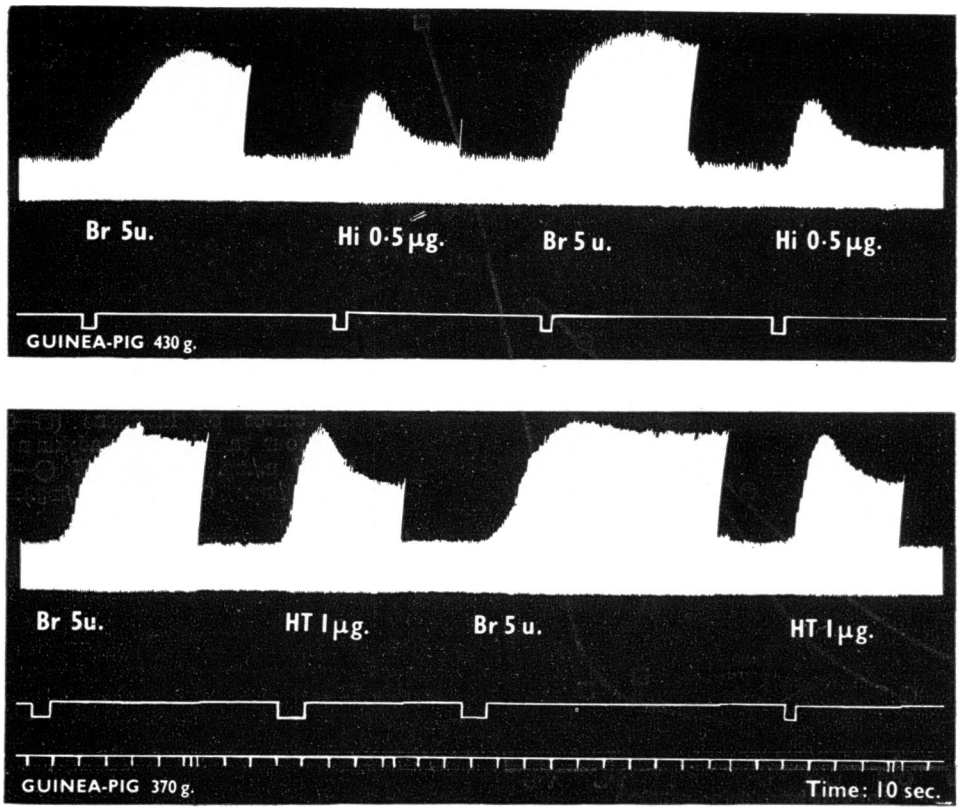

FIG. 1.—Resistance to inflation of guinea-pig lungs *in vivo*. Increased resistance caused by bradykinin (Br), histamine (Hi), and 5-hydroxytryptamine (HT). Doses were administered intravenously at 10 min. intervals.

closing the sidearm of the cannula, thus more forcibly inflating the lung.

Isolated Perfused Lungs.—Isolated lungs were perfused with Tyrode solution through a cannula in the pulmonary artery. Drugs were injected into the perfusion fluid, and inflation and recording were as in whole animal preparations.

RESULTS

Action of Bradykinin

In Whole Animals.—In the course of experiments on 87 guinea-pigs, prepared for recording resistance of the lungs to inflation, bradykinin was administered intravenously in doses ranging from 1.25 to 80 units. In all animals, bradykinin increased resistance to inflation, doses of 2.5 to 10 units normally being effective (Fig. 1). This figure shows that the response to bradykinin differs slightly from those to histamine and 5-hydroxytryptamine in its slower onset and longer persistence at or near peak level. In four experiments, cutting the vagi in the neck produced no significant alteration of the pulmonary response to bradykinin. In a number of guinea-pigs, the chest wall was opened to permit visual inspection of the lungs. This procedure also failed to influence significantly the recorded response to bradykinin. We observed that effective doses of bradykinin or histamine invariably reduced lung excursion. After larger doses of either drug, there appeared in the lungs cyanotic patches, some of which persisted. These patches could be removed by closing the sidearm of the tracheal cannula and so forcing air into the lungs.

When bradykinin was given repeatedly, refractoriness usually developed. This was more noticeable than it was with histamine, to which refractoriness developed less readily or not at all.

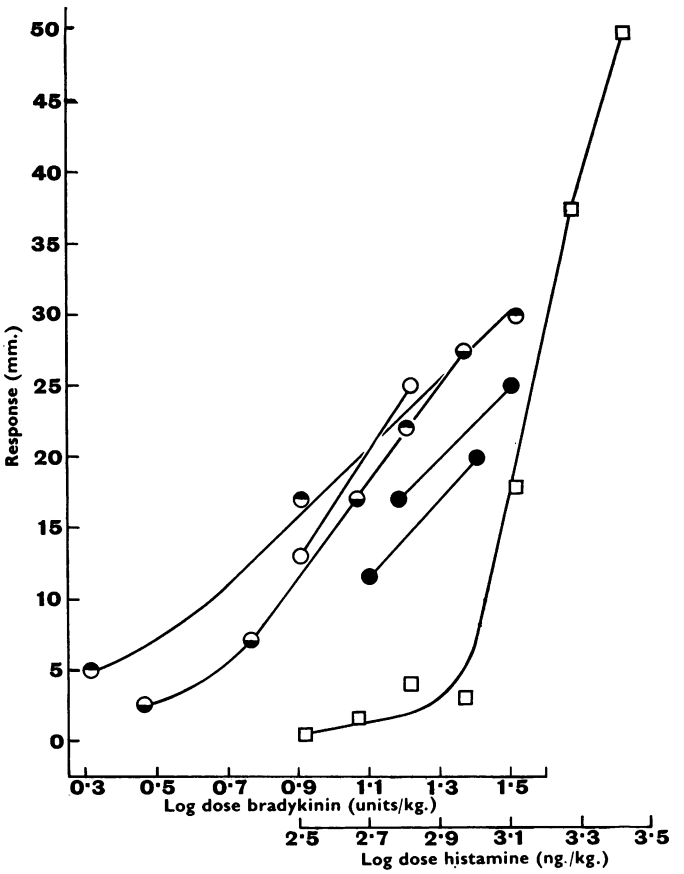

FIG. 2.—Resistance to inflation of guinea-pig lungs *in vivo*. Dose-response curves of histamine (□—□) and four samples of bradykinin (●—●, 7 u./mg.; ◒—◒ and ◒—◒, 200 u./mg.; O—O, 900 u./mg.).

Before refractoriness became serious, however, increasing the dose of bradykinin produced increased resistance to inflation, enabling dose-response curves to be obtained. In Fig. 2 are illustrated a number of such curves, each from a different animal, obtained with samples of bradykinin assayed at 7, 200 and 900 units/mg. on the guinea-pig ileum. All curves were less steep than that of histamine in a similar experiment. It is evident from Fig. 2 that the samples of bradykinin containing 200 and 900 units/mg. showed the same relative potencies on lung as on ileum. In order to ascertain whether the potencies of the 7 and 200 unit samples also ran parallel on lung and ileum, we administered doses of 5 and 10 units of each to alternate guinea-pigs in a series of 12 animals. Statistical analysis of the results showed that the relative potency of these preparations on lung did not differ significantly from their activity ratio on the guinea-pig ileum.

The activity of bradykinin on the lungs was abolished by incubating it with chymotrypsin (25 μg. in 0.1 ml. saline + 10 units bradykinin in 0.1 ml. saline) for 10 min. at room temperature. Incubation of bradykinin with trypsin in the same way, however, did not abolish its activity. Incubation with guinea-pig serum (0.05 ml. + 5 units bradykinin in 0.05 ml. saline) for 30 min at 35° also inactivated bradykinin. This effect was reduced if 1 mg. cysteine was added to the mixture incubated (5 units bradykinin in 0.05 ml. saline + 0.1 ml. serum). Records of these experiments are shown in Fig. 3.

In Isolated Perfused Lungs.—Bradykinin and histamine increased the resistance to inflation of isolated perfused lungs, as in whole animals (Fig. 4). To evoke responses from isolated lungs, larger amounts of both agents were required, but their relative potencies were unchanged.

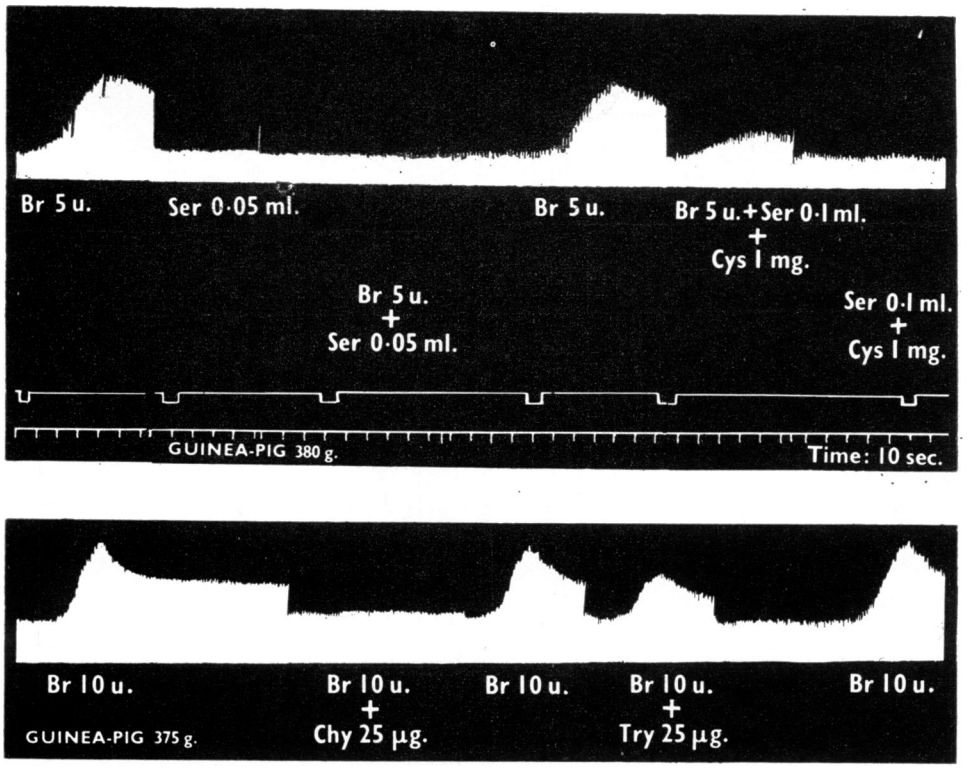

FIG. 3.—Resistance to inflation of guinea-pig lungs *in vivo*. Inactivation of bradykinin on incubation with guinea-pig serum (Ser) or with chymotrypsin (Chy), but not on incubation with trypsin (Try). Protection of bradykinin by cysteine (Cys).

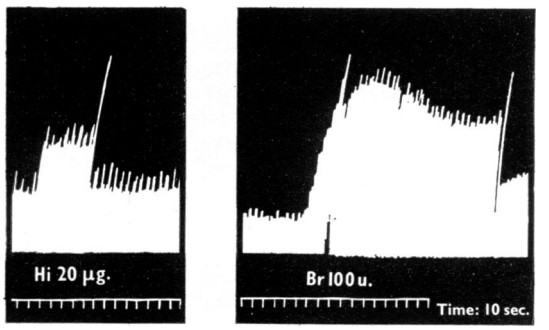

FIG. 4.—Resistance to inflation of guinea-pig lungs *in vitro*. Responses of isolated perfused lungs to histamine and bradykinin. In the histamine experiment, lungs were inflated at 60 strokes/min.; in the bradykinin experiment, in another preparation, at 72 strokes/min.

Action of Wasp Kinin

Intravenous injection of wasp kinin in 10 guinea-pigs increased resistance to inflation. In the experiment illustrated in Fig. 5, the partially purified preparation of wasp kinin exhibited about one-third of the potency of histamine on a weight basis. Like the response to bradykinin, that to wasp kinin was slower in onset and more sustained than the histamine response. As with bradykinin, opening the chest wall did not significantly alter the effectiveness of wasp kinin, which also produced in the lungs cyanotic patches that could be removed by more forcible inflation.

Effects of Drugs on the Response to Bradykinin

The action of bradykinin was unaffected by intravenous doses of mepyramine or lysergic acid diethylamide that abolished the responses to equivalent doses of histamine or 5-hydroxytrypt-

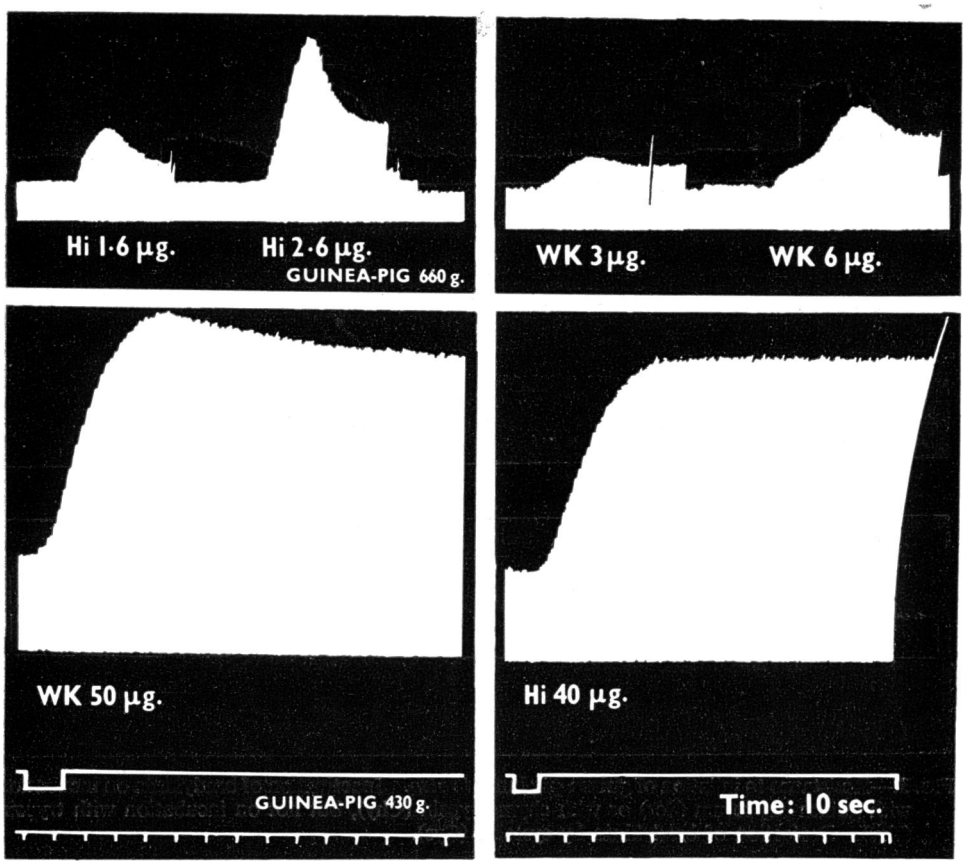

FIG. 5.—Resistance to inflation of guinea-pig lungs *in vivo*. Increased resistance caused by wasp kinin (WK) and histamine.

amine. In the experiment illustrated in Fig. 6, small doses of antagonists were used ; but in other experiments as much as 0.5 mg./kg. mepyramine failed to affect the response to bradykinin. Doses of atropine that suppressed the response to acetylcholine likewise did not affect that to bradykinin.

Intravenous injection of adrenaline (2 μg./kg.) or isoprenaline (0.6 μg./kg.) given together with histamine or bradykinin completely suppressed their actions on the lungs. The response to adrenaline was of brief duration, and, a few minutes after its injection, responses to histamine and bradykinin could again be elicited.

Acetylsalicylic acid, injected intraperitoneally or intravenously, completely suppressed the response to bradykinin without materially affecting those to histamine or 5-hydroxytryptamine (Fig. 7). This effect could be obtained with doses as low as 2 mg./kg. when given intravenously. Amidopyrine and sodium phenylbutazone, given intravenously, had comparable effects, but possessed only about one-half to one-eighth the potency of acetylsalicylic acid. Acetylsalicylic acid also suppressed the response to wasp kinin.

Cortisone was administered subcutaneously to a guinea-pig in three successive daily doses of 2 mg./kg. After the last dose, the animal was prepared for recording pulmonary resistance to inflation, and injected with bradykinin and histamine. The responses to these two agents were unaffected by pre-treatment with cortisone. In another experiment pre-treatment of a guinea-pig, with successive intramuscular doses of 2, 10 and 25 mg./kg. cortisone, administered within 2.5 hr., failed to influence the bronchoconstrictor action of bradykinin or histamine.

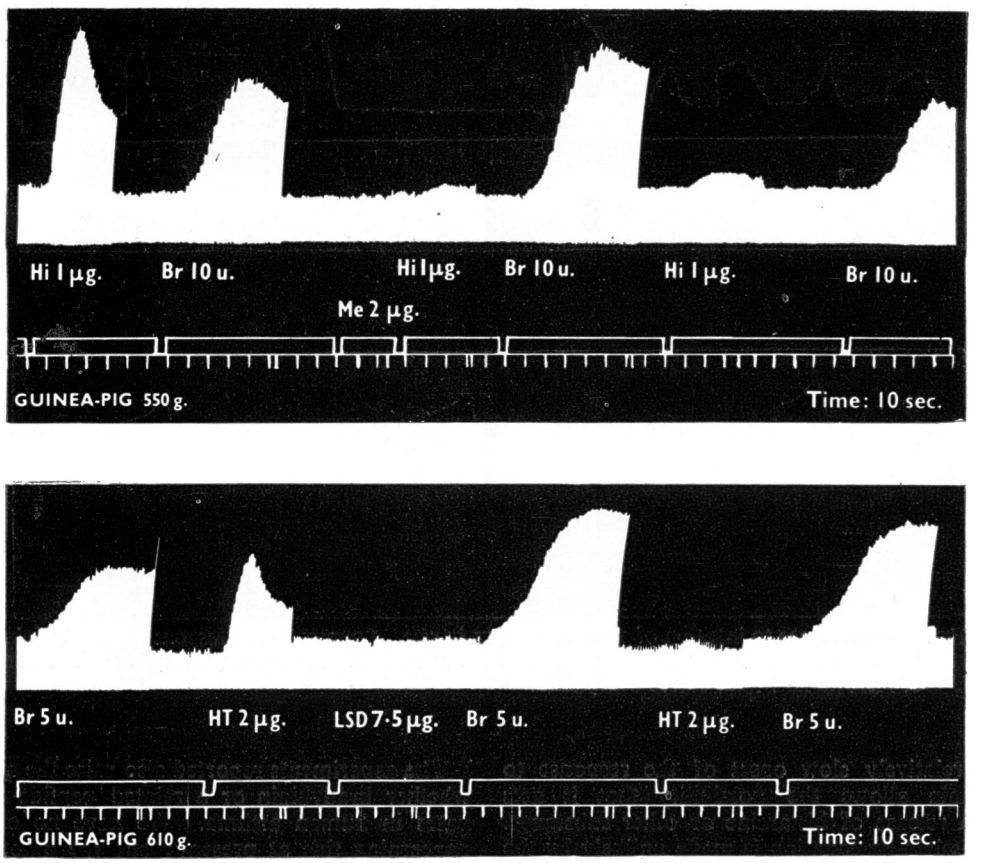

FIG. 6.—Resistance to inflation of guinea-pig lungs *in vivo*. Failure of mepyramine maleate (Me) and lysergic acid diethylamide (LSD) to reduce response to bradykinin.

DISCUSSION

Increased resistance of the lungs to inflation after bradykinin might be due to (1) increased resistance of the chest wall; (2) an increase of fluid in the lungs; or (3) bronchoconstriction. The first possibility can be eliminated because the response occurs after opening the chest wall and in the isolated lungs. The second possibility is very unlikely because (*a*) the response was obtained in the isolated lung without obvious change in the rate of flow of perfusion fluid, and (*b*) at the height of response the lung could sometimes be seen to be deflated, with no sign of fluid engorgement. We are left with the conclusion that the effect of bradykinin is due to bronchoconstriction. This is supported by the close resemblance of the response, except in small details of time-course, to that to histamine, which is an established bronchoconstrictor. The view that the effect of bradykinin is due to bronchoconstriction is also supported by its suppression by adrenaline and isoprenaline, which are well known to dilate bronchioles. The same evidence was obtained for a bronchoconstrictor action of wasp kinin, except that it was not tested on isolated lungs.

Since 1 unit is equivalent to about 0.08 μg. pure bradykinin, it is evident from Fig. 1 that, weight for weight, bradykinin is at least as potent as histamine. Wasp kinin, of which an impure preparation was used, must also be of this order of activity. In terms of molecules, therefore, the two peptides must rank among the most potent known stimulants of bronchiolar smooth muscle. It may well be that other natural peptides also possess bronchoconstrictor properties.

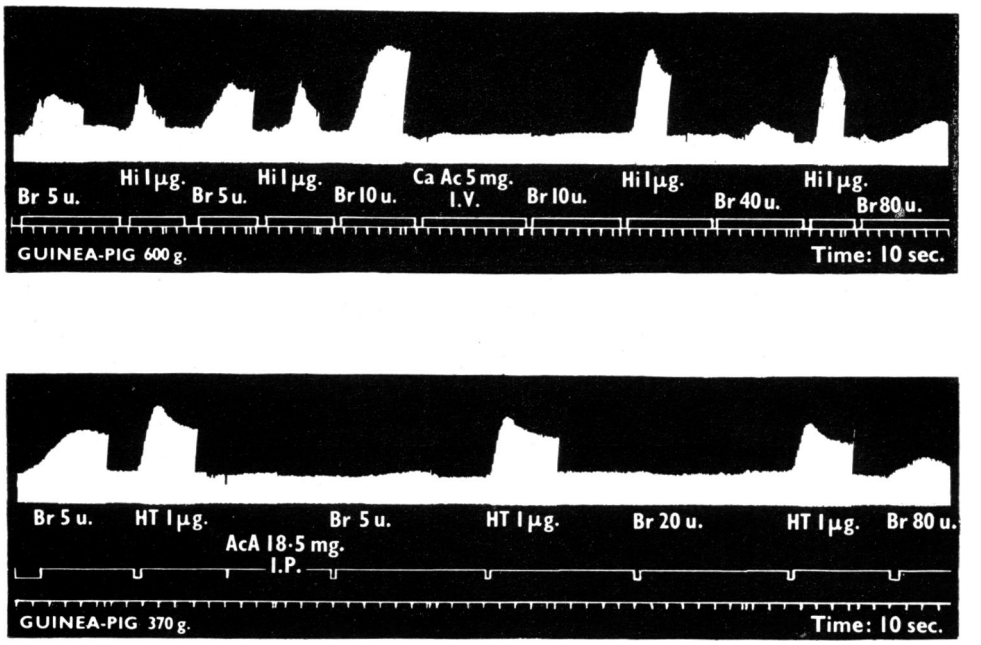

FIG. 7.—Resistance to inflation of guinea-pig lungs *in vivo*. Suppression of response to bradykinin by aspirin. Ca Ac=calcium acetylsalicylate, dose as acid. AcA=acetylsalicylic acid. Dose of AcA given 4 min. before bradykinin.

The relatively slow onset of the response to bradykinin allows the possibility that it acts through release of histamine or other substance. This possible explanation is made unlikely by the fact that mepyramine, atropine and lysergic acid diethylamide, in doses which antagonized known endogenous bronchoconstrictor substances, did not suppress the response to bradykinin.

Until a pure preparation is available, it will be impossible to state with certainty that the principle which constricts bronchiolar muscle is the same as that which contracts guinea-pig ileum. None the less, two facts strongly suggest that the two principles are identical. First, throughout purification, the potencies of bradykinin preparations on guinea-pig ileum and on lung ran parallel. Secondly, like the ileum-stimulating principle (Werle, 1955; Schachter, 1960), the bronchoconstrictor principle is inactivated by guinea-pig serum and by chymotrypsin, though not by trypsin, and is stabilized by cysteine.*

The experiments reported also raise the question whether bradykinin or a related peptide participates in human asthma. We have no evidence concerning this at present; but the amounts of kinin that might be present in plasma in certain circumstances are sufficient to allow the question to remain open.

The action of acetylsalicylic acid in suppressing the response to bradykinin is interesting for several reasons. First, this is a potent *in vivo* effect of acetylsalicylic acid which occurs at concentrations in the blood that are much lower than those reached in man during salicylate therapy. Also, amidopyrine and phenylbutazone, two other antirheumatic substances which were tested, suppressed the bradykinin response. This raises the question whether suppression of a response to bradykinin or to a related peptide contributes to the antirheumatic action of non-steroid agents.

We wish to thank Dr. I. M. Lockhart and Mr. W. A. Jones for supplies of bradykinin, Dr. B. T. Warner for statistical advice and Miss L. Deeming, Miss S. Horwood-Barrett and Miss M. Nicholas for technical assistance. We also wish to thank Dr. D. F. Elliott, Dr. G. P. Lewis, and Dr. E. W. Horton for a sample of pure bradykinin.

* *Note added in proof.* Subsequent experiments with pure bradykinin by Elliott, Lewis, and Horton, and by ourselves, confirm that the pure material exerts the expected bronchoconstrictior action and that this is specifically suppressed by aspirin.

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POTENTIATION OF SULPHADIMIDINE BY 2,4-DIAMINO-6,7-DI-ISOPROPYLPTERIDINE AND OTHER 6,7-DISUBSTITUTED 2,4-DIAMINOPTERIDINES AGAINST *EIMERIA* INFECTIONS OF CHICKS

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Six 2,4-diaminopteridines substituted in the 6 and 7 positions were tested as potentiators of sulphadimidine against *Eimeria tenella*. 2,4-Diamino-6,7-di-isopropylpteridine and 2,4-diamino-1′-propylindolo(2′,3′:6,7)pteridine were selected for further study. Both potentiated sulphadimidine against *E. tenella* and the di-isopropylpteridine potentiated it against *E. necatrix*. A 10% mixture of the di-isopropylpteridine with sulphadimidine (or other sulphonamides) appears to offer advantages in the treatment of *E. tenella* and still more of *E. necatrix*.

Coccidia are protozoal parasites of the alimentary tract of fowls. *Eimeria tenella* is the cause of caecal coccidiosis, a common and often lethal disease of young chickens. There are also several forms of intestinal coccidiosis which are becoming increasingly important in Britain as diseases of older fowls. The different forms of intestinal coccidiosis are caused by different species. A form of intestinal coccidiosis caused by *E. necatrix* is often responsible for a high death rate in flocks. Owing to their economic importance, the species *E. tenella* and *E. necatrix* were selected as the infective agents in the present work.

Certain sulphonamides exhibit pronounced anticoccidial properties and have been used in the control of field outbreaks of caecal coccidiosis for a number of years. Horton-Smith and Taylor (1945) showed that sulphadimidine could be used successfully to treat established infections of *E. tenella* in chickens. Later Horton-Smith and Boyland (1946) and Waletzky and Hughes (1946) found that its inhibitory effect could be reversed by *p*-aminobenzoic acid. These observations suggested that "more potent inhibitors of this parasite (*E. tenella*) might be found among other more active structures known to interfere with the *p*-aminobenzoic acid-folic acid sequence" (Lux, 1954). Lux obtained encouraging results when 2,4-diaminopyrimidines or 2,4-diamino-dihydro-*s*-triazines were mixed with sulphonamides, pyrimethamine being a particularly effective synergist. Joyner and Kendall (1955, 1956) confirmed this observation and showed that full therapeutic control of caecal coccidiosis was obtained with 0.005% pyrimethamine mixed with 0.05% sulphadimidine. Kendall and Joyner (1958) extended this work and showed that 2,4-diamino-6,7-di-isopropylpteridine and other folic acid antagonists potentiated sulphadimidine against *E. tenella*.

Collier, Campbell and Fitzgerald (1950) first described 2,4-diamino-6,7-di-isopropylpteridine as a vibrostatic agent, which potentiated sulphaguanidine in antibacterial activity, but not in toxicity to mice. Collier and Waterhouse (1950) described the antagonism between this pteridine and folic acid, and Collier and Phillips (1954) the antagonism between folinic acid and the pteridines used in the present investigation. Pteridines of this type were also shown to be active inhibitors of *Streptococcus faecalis* and *Staphylococcus aureus* (Collier and Waterhouse, 1952) and of *Plasmodium gallinaceum* (Bishop, 1954) and *P. berghei* (Thurston, 1954). The pteridines selected for the present trial were those shown most active in the above experiments.

We describe below extended observations showing potentiation of sulphadimidine by admixture with pteridines against *E. tenella*. We also describe the successful treatment with a

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mixture of 2,4-diamino-6,7-di-isopropylpteridine and sulphadimidine of chicks infected with a strain of *E. necatrix* not particularly susceptible to sulphonamides alone (Horton-Smith and Long, 1959).

METHODS

Chemicals.—Sulphadimidine and the six 2,4-diaminopteridines were administered as bases and *p*-aminobenzoic acid as the acid. All the pteridines were substituted in the 6 and 7 positions, and are listed

TABLE I

EFFECTS OF SIX DIFFERENT PTERIDINES ON THE SULPHADIMIDINE TREATMENT OF CHICKS INFECTED WITH *E. TENELLA*

Groups 1 to 7 were given diets containing 0·05% sulphadimidine, and groups 1 to 6 diets containing 0·005% of the appropriate pteridine.

Group	Treatment	Experiment 1	
		No. of Birds	No. of Deaths
1	2,4-Diamino-6,7-di-isopropylpteridine+sulphadimidine	12	4
2	2,4-Diamino-6,7-di(cyclohexylmethyl)pteridine+sulphadimidine ..	12	8
3	2,4-Diamino-1′-methylindolo(2′,3′: 6,7)pteridine+sulphadimidine ..	12	8
4	2,4-Diamino-1′-propylindolo(2′,3′: 6,7)pteridine+sulphadimidine ..	12	2
5	2,4-Diamino-6,7-dibutylpteridine+sulphadimidine	12	9
6	2,4-Diamino-6,7-dipentylpteridine+sulphadimidine	12	9
7	Sulphadimidine alone	12	7
8	No treatment	12	12

TABLE II

EFFECTS OF 2,4-DIAMINO-6,7-DI-ISOPROPYL- AND 2,4-DIAMINO-1′-PROPYLINDOLO(2′,3′: 6,7)-PTERIDINE ON THE SULPHADIMIDINE TREATMENT OF CHICKS INFECTED WITH *E. TENELLA*

Pteridine	Pteridine % in Diet	Sulphadimidine % in Diet	Experiment 2		Experiment 3		Total		
			No. of Birds	No. of Deaths	No. of Birds	No. of Deaths	No. of Birds	No. of Deaths	% Mortality
Di-isopropyl	0·00625	0·0C0625	9	7			9	7	78
	0·00125	0·0125	9	6			9	6	67
	0·0025	0·025	9	7	9	2	18	9	50
	0·005	0·05	9	1	9	2	18	3	17
	0·01	0·1			9	0	9	0	0
	0·005	—	9	7			9	7	78
	0·01	—			9	7	9	7	78
Propylindolo	0·000625	0·00625	8	6			8	6	75
	0·00125	0·0125	8	5			8	5	63
	0·0025	0·025	8	3	9	1	17	4	24
	0·005	0·05	8	1	9	1	17	2	12
	0·01	0·1			9	0	9	0	0
	0·005	—	9	7			9	7	78
	0·01	—			9	8	9	8	89
—	—	0·05	9	6	9	5	18	11	61
	—	0·1			9	2	9	2	22
	—	—	9	6	9	7	18	13	72

in Table I. The methods of synthesis of these pteridines were described by Campbell, Dunsmuir, and Fitzgerald (1950).

Tests in Chicks.—Rhode Island Red × White Leghorn chicks aged 8 to 16 days at time of infection were used in the experiments. Groups of chicks were maintained in electrically heated metal brooders with wire floors and were kept for 13 to 18 days after infection with oocysts. *E. tenella* and *E. necatrix* cultures were derived from single oocyst infections. Cultures of oocysts used for infecting chicks in these experiments were freshly collected where possible and never stored for longer than 45 days. The infective dose was 50,000–100,000 oocysts of either species. Medication commenced 48 hr. after infection with oocysts and continued for 3 days, that is, until the 5th day of the infection.

Full details of methods used in chemotherapy experiments and the composition of the diet have already been described (Horton-Smith and Long, 1956, 1959). In toxicity tests drugs were administered in the diet to uninfected birds of the same age and type, food intake being measured.

Results

Therapeutic Tests against E. tenella

Comparison of Pteridines as Potentiators of Sulphadimidine.—In a preliminary experiment involving 96 chickens, the six pteridines listed in Table I were administered at a concentration of 0.005% in the diet, together with sulphadimidine at 0.05%. Only those groups of birds receiving the di-isopropyl- and propylindolo-pteridine in addition to sulphadimidine had lower mortalities than the control group receiving sulphadimidine alone (Table I).

The effects of these two active pteridines mixed with sulphadimidine were compared in two experiments involving 203 chicks (Table II). In these experiments the pteridines alone were ineffective, but mixtures of either pteridine and sulphadimidine were effective if given in sufficient concentration in the diet. The propylindolopteridine appeared slightly more active than the di-isopropylpteridine.

TABLE III

SUMMARY OF FOUR EXPERIMENTS ON 2,4-DIAMINO-6,7-DI-ISOPROPYLPTERIDINE AND SULPHADIMIDINE AND THEIR MIXTURES IN THE TREATMENT OF CHICKS INFECTED WITH *E. TENELLA*

Pteridine % in Diet	Sulphadimidine % in Diet	Experiment 4		Experiment 5		Experiment 6		Experiment 7		Total		
		No. of Birds	No. of Deaths	No. of Birds	No. of Deaths	No. of Birds	No. of Deaths	No. of Birds	No. of Deaths	No. of Birds	No. of Deaths	% Mortality
0·01	0·05			12	1	11	1			23	2	9
0·02	0·025							12	5	12	5	42
0·02	0·05			12	0	11	0	12	0	35	0	0
0·03	0·025							12	6	12	6	50
0·03	0·05			12	0	11	1	12	1	35	2	6
0·01	—	12	4			11	9			23	13	57
0·02	—	12	8			11	11	12	10	35	29	83
0·03	—					11	10	12	10	23	20	87
0·04	—	12	9							12	9	75
0·05	—			12	11					12	11	92
0·06	—	12	11							12	11	92
0·08	—	12	4							12	4	33
0·1	—	12	7	12	11					24	18	75
0·12	—	12	4							12	4	33
0·15	—			12	5	11	5	12	6	35	16	46
0·2	—			12	8					12	8	67
—	0·025							12	10	12	10	83
—	0·05					11	7	12	7	23	14	61
—	0·08			12	5	11	7			23	12	52
—	0·1	12	0	12	1	11	3	12	1	47	5	11
—	0·12	12	0	12	1					24	1	4
—	0·14	12	1							12	1	8
—	—	12	8	12	10	11	10	12	11	47	39	83

TABLE IV

EFFECTS OF 2,4-DIAMINO-6,7-DI-ISOPROPYL- AND 2,4-DIAMINO-1′-PROPYLINDOLO(2′,3′:6,7)-PTERIDINE ON THE SULPHADIMIDINE TREATMENT OF CHICKS INFECTED WITH *E. NECATRIX*

Pteridine		Sulpha-dimidine	Experiment 8		Experiment 9		Total		
Name	% in Diet	% in Diet	No. of Birds	No. of Deaths	No. of Birds	No. of Deaths	No. of Birds	No. of Deaths	% Mortality
Di-isopropyl	0·0025	0·025	10	6			10	6	60
	0·005	0·05	10	9			10	9	90
	0·01	0·1	10	2	10	1	20	3	15
	0·015	0·15			10	0	10	0	0
	0·02	0·2	10	0	10	0	20	0	0
	0·02	—	9	4			9	4	44
Propylindolo	0·0025	0·025	9	6			9	6	67
	0·005	0·05	9	6			9	6	67
	0·01	0·1			11	9	11	9	82
	0·015	0·15			11	3	11	3	27
	0·02	0·2			11	7	11	7	64
	0·005	—	9	7			9	7	78
	—	0·2	9	7	11	8	20	15	75
	—	—	10	6	11	8	21	14	67

TABLE V

EFFECTS OF 2,4-DIAMINO-6,7-DI-ISOPROPYLPTERIDINE, SULPHADIMIDINE AND THEIR MIXTURES ON *E. NECATRIX* INFECTIONS OF CHICKS AND THE EFFECT OF *p*-AMINOBENZOIC ACID ON THEIR ACTION

% Drug in Diet			Experiment 10		Experiment 11		Experiment 12		Total		
Pteridine	Sulpha-dimidine	*p*-Amino-benzoic Acid	No. of Birds	No. of Deaths	No. of Birds	No. of Deaths	No. of Birds	No. of Deaths	No. of Birds	No. of Deaths	% Mortality
0·01	0·1	—			9	1	8	1	17	2	12
0·01	0·15	—					8	0	8	0	0
0·01	0·2	—			9	1	8	0	17	1	6
0·015	0·1	—					8	0	8	0	0
0·015	0·15	—					8	0	8	0	0
0·015	0·2	—					8	0	8	0	0
0·02	0·1	—			9	1	8	1	17	2	12
0·02	0·15	—					8	0	8	0	0
0·02	0·2	—	8	0	9	0	8	0	25	0	0
—	1·0	0·01	8	7					8	7	88
0·02	0·2	0·01	8	4					8	4	50
0·01	—	—			9	4			9	4	44
0·02	—	—	8	8	9	8			17	16	94
0·04	—	—			9	5			9	5	56
—	1·0	—	8	2					8	2	25
—	0·2	—	8	8	9	5	8	4	25	17	68
—	—	—	7	7	8	6	9	5	24	18	75

2,4 - Diamino - 6,7 - di - isopropylpteridine with Sulphadimidine.—It will be shown below that the di-isopropylpteridine is more active than the propylindolopteridine as a sulphadimidine potentiator against *E. necatrix*. For this reason and because supplies were more readily avilable, the di-isopropylpteridine was selected for further study against *E. tenella*. The results of four experiments, involving 517 chickens, with this pteridine, sulphadimidine and their mixtures are summarized in Table III, from which it will be seen that each drug alone exerted some therapeutic action, though that of the pteridine was only just detectable at high dose levels. Mixtures of the two drugs were very much more effective, roughly equiactive treatments being: the pteridine alone, 0.15%, sulphadimidine alone, 0.08%, and a mixture of the pteridine 0.02% and sulphadimidine 0.025%.

Therapeutic Tests against E. necatrix

Comparison of the Di-isopropyl- and Propyl-indolo-pteridine as Potentiators of Sulphadimidine.—In two experiments, involving 180 chickens, 10% mixtures of each of the pteridines with sulphadimidine were compared. The results expressed in Table IV indicate clearly that the di-isopropylpteridine strongly potentiated sulphadimidine and that the propylindolopteridine potentiated it only slightly if at all. Sulphadimidine alone at 0.2% in the diet was inactive.

In a further three experiments, involving 224 chicks (Table V) the activities against *E. necatrix* infections of the di-isopropylpteridine, sulphadimidine and their mixtures were again compared and the results confirmed those shown in Table IV. Also as *p*-aminobenzoic acid is known to antagonize sulphonamides, we investigated in one of these experiments its effect on the therapeutic action of sulphadimidine and of a mixture of the di-isopropylpteridine and sulphadimidine. There were 7 deaths in 8 birds treated with sulphadimidine and *p*-aminobenzoic acid, and 2 in the corresponding group treated with sulphadimidine alone. There were 4 deaths in a group of 8 infected birds treated with *p*-aminobenzoic acid as well as the pteridine-sulphadimidine mixture, as compared with no deaths in the group treated with the pteridine-sulphadimidine mixture alone (Table V, Experiment 10).

TABLE VI

DIETARY INTAKE OF 2,4-DIAMINO-6,7-DI-ISOPROPYLPTERIDINE AND SULPHADIMIDINE BY TWO-WEEK-OLD CHICKS

Groups of 10 chicks were tested at each dose level.

% in Diet		Amount of Drug Consumed in mg.	
Pteridine	Sulphadimidine	Pteridine	Sulphadimidine
0·005	0·05	3·47	34·7
0·01	0·1	7·35	73·5
0·02	0·2	11·4	114
0·04	0·4	21·4	214
0·08	0·8	56·0	560

Toxicity Studies in Chicks

Diets containing various concentrations of a mixture of 1 part of the di-isopropylpteridine with 9 parts of sulphadimidine were fed to groups

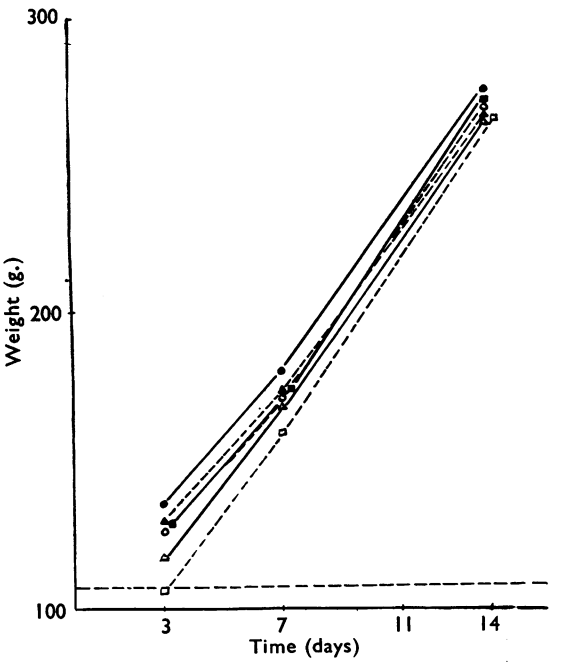

FIG. 1.—The effect of 2,4-diamino-6,7-di-isopropylpteridine-sulphadimidine mixtures in the diet on the weight gain of chicks during and after treatment. Treatment from Day 0 to Day 3. The horizontal line indicates the initial mean weight of all birds at Day 0 (107·8 g.). ●———●= Untreated control. ▲ — — — ▲ = Pteridine 0.005%, sulphadimidine 0.05%. ■ ——— ■ = Pteridine 0.01%, sulphadimidine 0.1%. O— — —O = Pteridine 0.02%, sulphadimidine 0.2%. △ ——— △ = Pteridine 0.04%, sulphadimidine, 0.4%, □— — —□ = Pteridine 0.08%, sulphadimidine 0.8%.

of 10 two-week-old chicks for 3 days. The amount of drug consumed was calculated and the body weight of the chicks was observed. The amount of drug consumed is given in Table VI and the weight gain in Fig. 1, which shows that drug treatment at high concentrations depressed weight gain and this gradually recovered after the withdrawal of treatment.

Discussion

In discussing the combined action of sulphadimidine and pteridines, the terminology described by Gaddum (1940) will be used. In this, the term "synergism" refers to any kind of joint action that is not antagonism, and "potentiation" refers to synergism that is greater than additive. From the experiments reported above it would be difficult to provide formal proof of potentiation by constructing a diagram of the type illustrated by Gaddum. However, a number of experiments strongly suggest that the pteridines potentiate sulphadimidine against both species of *Eimeria*. For *E. tenella* these experiments are Nos. 3, 5, 6, and for *E. necatrix* No. 11. In the other experiments, the mixtures generally show therapeutic action, but the absence of results at appropriate dose levels made it difficult to conclude that potentiation is present.

As strains of *E. necatrix* which do not respond to sulphonamide have been described (Horton-Smith and Long, 1959), the therapeutic action of the mixture of 2,4-diamino-6,7-di-isopropylpteridine and sulphonamide against this species might be of considerable importance. Mixtures of these drugs seem worthy of toxicological investigation with a view to field trial.

The brief experiment on toxicity of the di-isopropylpteridine-sulphonamide mixture in the diet of chicks reported above indicates some depression of growth, from which birds afterwards recovered, by a mixture fully active in the therapeutic tests. It is of interest that Collier, Hall and Waterhouse (1950) observed no potentiation by di-isopropylpteridine of sulphaguanidine toxicity in mice.

We are grateful to Allen and Hanburys Ltd. for the supply of pteridines used in this investigation.

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THE PHARMACOLOGY OF CHYMOTRYPSIN ADMINISTERED BY INHALATION

BY

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The ability of chymotrypsin to reach the limits of the bronchial tree has been studied in cats receiving the enzyme by inhalation as a very fine powder. For this purpose derivatives of chymotrypsin were used which had been labelled with a fluorescent molecule or with [^{131}I]. Quantitative measurements of the absorption and distribution of inhaled chymotrypsin- [^{131}I] revealed a rapid removal of radioactivity from the lungs over the first 24 hr. and corresponding excretion of labelled inorganic iodide in the urine. High levels of activity were not attained in the blood or thyroid. Subcutaneous administration of labelled enzyme led to more rapid accumulation of radioactivity in the blood and thyroid. Consideration of these and other results leads to the conclusion that, while some enzyme ascends the respiratory tract by ciliary movement of mucus, a substantial part is absorbed into the lungs and the [^{131}I] subsequently detached from it.

The changes in tidal air accompanying inhalation of labelled trypsin and chymotrypsin were followed in anaesthetized cats. Trypsin brings about a decrease in tidal air in distinctly lower doses than does chymotrypsin. Prior administration of mepyramine had an antagonistic effect, and it is suggested that the change in tidal air is essentially the result of bronchial spasm.

Where a drug is required to act locally within the lung it is advantageous to administer it by inhalation as a particulate cloud. However, one must ensure that an adequate proportion of the particles—by weight rather than by number—are of the requisite size for the site within the lungs which the powder is intended to reach and in which it has to be retained. Thus, one of the factors controlling the effectiveness of the drug is the homogeneity of the powder and particularly the low proportion of particles outside the desired range of sizes.

These considerations apply to the use of chymotrypsin as a mucolytic agent in the treatment of chronic bronchitis. Here the advantage is one of direct action on mucoprotein fibrils within the bronchial lumen. At present nothing is known of the subsequent disposal of the enzyme. We have reported here our attempts to ascertain whether the enzyme penetrates to all parts of the bronchial tree, what its subsequent distribution might be, and the degree of intolerance to the inhaled enzyme powder, in comparison with that shown to crystalline trypsin.

METHODS

Enzymes.—In the earlier stages crystalline chymotrypsin (Worthington) was employed, but the majority of the experiments were carried out with crystalline chymotrypsin as used in the therapeutic preparation "Lomudase." By the Anson procedure (Anson, 1938; Northrop, Kunitz, and Herriott, 1948) this was found to assay at 0.002 Anson units per mg. enzyme. The characteristics of this material are such that when it is dispersed in air with the clinical applicator ("Lomulizer") the resulting powder inhalation consists of particles of which about 80% by weight have diameters between 2 and 6 μ. Of the remainder about one-third by weight are in the range 1 to 2 μ and two-thirds 6 to 9 μ (Fowler, personal communication).

A check was also made on photomicrographs of particles collected on a microscope slide from a low density smoke produced by the "Lomulizer" in a chronic toxicity apparatus (to be described in a later publication). Under these conditions the majority of the particles were seen to be 3 to 7 μ in size. In each high power field there were one or two aggregates, each consisting of a chain of three or four particles.

The trypsin used was a commercial preparation (Armour) of the pure crystalline enzyme (0.011 Anson units per mg. enzyme). The particle size distribution of the inhaled trypsin powder was in the same range as that described for chymotrypsin.

Fluorescent labelling of chymotrypsinogen was carried out by a modification of the method of Hartley and Massey (1956). The final material, 1 - dimethylaminonaphthalene - 5 - sulphonylchymotrypsin, as used in the insufflation experiments was

sparingly soluble in water and contained, in different batches, 80 to 90% protein, 2.5 to 3.5 moles of label per mole of protein, and 76 to 79% of the proteolytic activity of the unlabelled enzyme. Its particle size distribution lay within the limits set for chymotrypsin.

Labelling of trypsin and chymotrypsin with [^{131}I] by the method of Veall, Pearson, and Handley (1955) involved the use of 2 mc. [^{131}I] per 200 mg. enzyme protein. The temperature was maintained below 5° throughout all the operations. To remove free iodine, the iodinated protein was precipitated with 4 volumes of acetone, the precipitate dissolved in water and dialysed against tap water. Samples were taken from the dialysis tubing and chromatographed, using butanol:pyridine:water (3:2:1.5) as a solvent. The chromatograms were scanned to determine the concentration of ionic iodine; when the ionic iodine spot contained less than 1% of the total radioactivity, dialysis was discontinued. It is important not to dialyse over too long a period, since the enzymes diffuse through the Visking membrane. The preparation was then freeze-dried, the active product diluted with 4 times its weight of the original enzyme, and converted to a very fine powder having the same characteristics as those described for chymotrypsin. The resulting chymotrypsin and trypsin powders possessed specific activities of 76,000 and 43,500 counts/min./mg. and 94% and 92.5% respectively of the proteolytic activities of the crystalline enzymes. The radioactive enzymes contained less than 1% ionic iodine.

Determination of Metabolic Fractions.—For protein-bound [^{131}I] in plasma, urine, and alimentary tract washings, the protein was precipitated by the addition of an excess of 20% trichloracetic acid to a known volume of urine or plasma. The precipitate was removed by centrifugation, washed with 20% trichloracetic acid, and dissolved in 10 ml. N sodium hydroxide to determine the activity.

To determine the inorganic iodine, it was precipitated from the trichloracetic acid supernatant by the addition of an excess of a solution containing 20% silver nitrate and 10% nitric acid. The precipitate was suspended in water and its activity determined. Butanol-soluble iodine (thyroxine, diiodotyrosine, and inorganic iodine) was estimated by extracting 5 ml. of plasma or urine with 5 ml. *n*-butanol, and measuring the activity of the butanol layer. Since this was low, it was decided not to fractionate the extract into its component forms of iodine.

Insufflation Procedures.—All the enzymes were converted to very fine powders approximating as closely as possible to the chymotrypsin of "Lomudase." They were packed into cartridges and dispersed precisely as was done with chymotrypsin.

Indirect method A.—Cats under chloralose and urethane anaesthesia were connected by intratracheal cannula to the insufflation apparatus shown in Fig. 1. Here a measured volume of air (0.2 to 20 ml., according to the dose of powder required) was blown from a syringe through the dispersion unit B in which was placed a cartridge containing the powdered enzyme. The dense cloud of powder impinged on the baffle plate C, the purpose of which was to break up aggregates of powder, and passed into a large jar fitted with a magnetic stirrer D. The airborne suspension of powder was mixed with air entering the jar from a respiration pump at F and passed to the cat through tube G or to an excess volume recorder at E. The tracheal cannula had an outlet to I through the electro-magnetic clamp H which was closed on inspiration, thus making it possible to lead out of the laboratory all expiratory air containing radioactive powder.

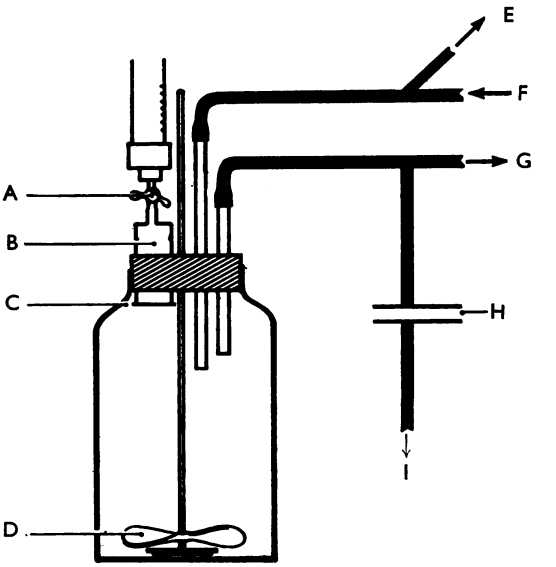

FIG. 1.—Diagram of apparatus used in the administration of powders by inhalation to anaesthetized cats by the indirect method A. A, Syringe with two-way tap; B, dispersion unit; C, baffle plate; D, stirrer (magnetic); E, tube to excess volume recorder; F, air inlet from respiration pump; G, tube to tracheal cannula; H, electro-magnetic clamp closed on inspiration; I, expiratory air outlet.

Changes in the distensibility of the cat lungs at a constant pressure of 9.5 cm. of water were measured by the overflow method of Konzett and Rossler (1940); blood pressure was simultaneously recorded from the carotid artery. The tidal air change was calibrated for each experiment in terms of the pressure, in cm. of water required to produce an equivalent excursion of the recorder.

Direct method B was used to attain higher lung concentrations for autoradiography and other radioisotope studies. The apparatus shown in Fig. 1 was modified by connecting the outlet of the dispersion unit B directly to the tube leading to G. A 50 ml.

syringe replaced the excess volume recorder at E. To insufflate powder, the respiration pump was stopped and, with the lungs of the cat momentarily deflated, clamps were applied to the tube leading from F and to the side-tube leading to H and I. Immediately air was expelled through the dispersion unit B and from the syringe at E into the lungs. By means of this syringe the lungs were deflated and inflated several times before the clamps were removed, and artificial respiration resumed with the pump at F.

Post-mortem Observations.—The cats were killed at varying times after insufflation of enzyme. The lungs and tracheae of animals which had received fluorescent labelled chymotrypsin were examined macroscopically under ultraviolet light. In some experiments frozen sections of the lung were studied by ultraviolet microscopy.

The lungs and tracheae of animals which had inhaled radioactive enzymes were used either for quantitative determination of the amount of enzyme present or for autoradiography. In carrying out radioassay of the labelled enzymes, the trachea was cut off at the carina and the trachea, main bronchi, and lungs separately homogenized in a known volume of 5% sodium hydroxide. The activity of the homogenate was determined in an EKCO N530 Scintillation Counter fitted with an annular type crystal. In some animals, determinations were also made of the radioactivity present in the liver, thyroid, stomach, small intestine, blood, and urine.

Autoradiographs were prepared by embedding the trachea and lungs in paraffin wax, cutting the blocks so as to obtain a flat, transverse section through the lung and trachea and placing each block in contact with an Ilford B *x*-ray film for 7 days.

RESULTS

Experiments with Fluorescent Enzyme.—Untreated cat lungs exhibit a blue fluorescence in ultraviolet light. In cats killed immediately after insufflation of 3.2 mg. fluorescent chymotrypsin the terminal portion of the trachea, bronchi, and lung tissue showed a faint yellow fluorescence in ultraviolet light. In lung sections examined by ultraviolet microscopy (Lempert, 1944) the fluorescence was seen to emanate from discrete particles present throughout the lung right to the periphery, but was too weak to photograph in our apparatus. Moreover, attempts to use fluorescent counterstains resulted in quenching of the enzyme fluorescence. In Fig. 2 is shown a photomicrograph of one such lung section, for which we are indebted to Dr. A. Jarrett and Mr. T. Bligh of University College Hospital Medical School, London. It demonstrates that the inhaled powder has penetrated deeply into the lung, as far as the bronchioles; one bronchiole is shown whose lumen is lined by fluorescent enzyme.

When these experiments were repeated, but the cats killed 1 hr. after enzyme inhalation, no fluorescence was detected macroscopically in the smaller bronchi. Accordingly the procedure was modified by ligating the hilum of one lung after the insufflation of enzyme. One hour later the cat was killed and now, while no fluorescence was detected in the unligated lung, there was a uniform distribution of fluorescence in the bronchi and bronchioles of the ligated lung. In further experiments a polythene annulus was introduced into one main bronchus in order to prevent ciliary transfer of mucus past the ring while maintaining normal blood flow and aeration to the lung distal to it. The enzyme was then inhaled by the cat, which was killed 1 hr. later. No fluorescence was detectable in either lung, but an accumulation of fluorescent material was found to have taken place in the bronchial lumen immediately beyond the ring.

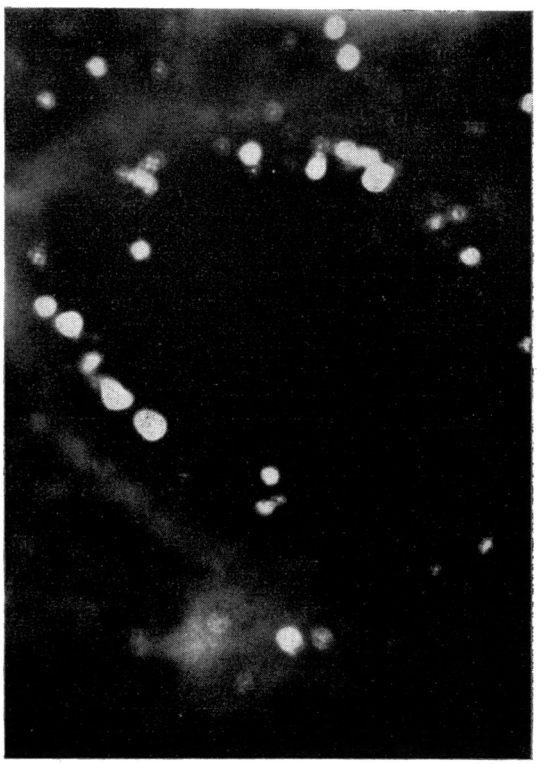

FIG. 2.—Ultraviolet photomicrograph of a section through cat lung following inhalation of fluorescent-labelled chymotrypsin, showing a bronchiole whose lumen is lined by fluorescent particles of enzyme. (Original magnification ×840.)

It was concluded from these experiments that the particulate enzyme, when administered by inhalation, was able to reach to the periphery of the lung but that it was rapidly cleared from the lung. In part at least, such clearance was taking place by the upflow of mucus in the bronchial tree. Attempts were then made to place these observations on a quantitative basis by fluorimetry of the labelled enzyme. However, the intrinsic fluorescence of the lung tissue precluded such measurements.

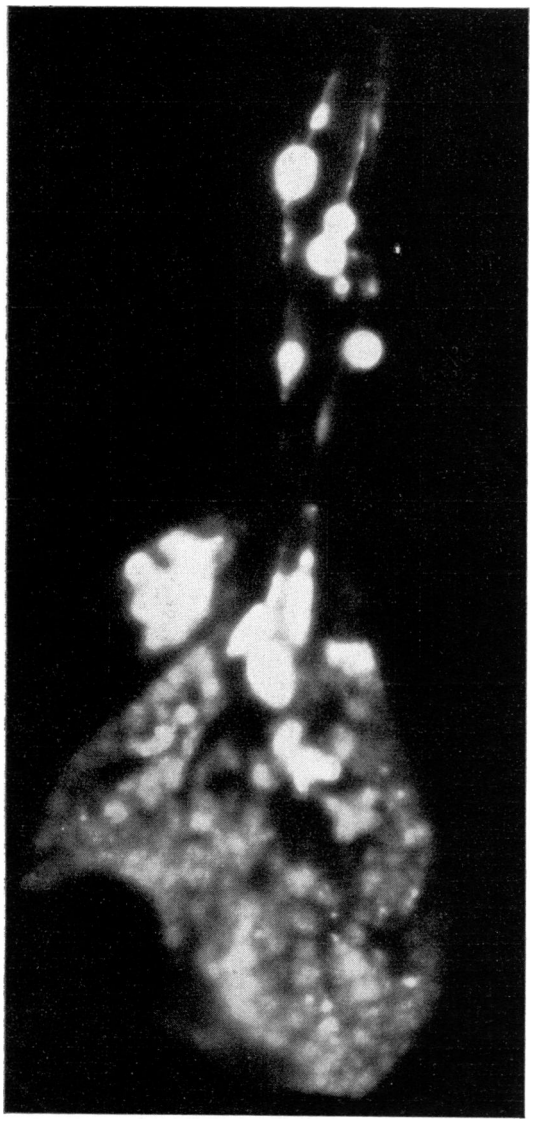

FIG. 3.—Autoradiograph of a section of the trachea, main bronchus and lung of a cat showing the penetration of inhaled chymotrypsin-[^{131}I]. The remarkable uniformity with which the radioactive material has been distributed throughout the lung is clearly demonstrated. Above is seen the heavy deposit, presumably of larger-sized particles, in the more proximal parts of the trachea.

Chymotrypsin Labelled with [^{131}I].—Preliminary experiments showed iodine-labelled chymotrypsin to be eminently suitable for distribution studies. Autoradiography was also possible since normal cat lung produced no artefact under the conditions used. An autoradiograph of a section through the trachea, main bronchus, and lung of a cat is shown in Fig. 3. The animal had been killed immediately after it had inhaled chymotrypsin-[^{131}I], of which 1.0 mg. was retained in the lungs. There is a uniform distribution of radioactive material throughout the lung, whereas in the more proximal part of the trachea a heavy deposit is present. It is possible that such tracheal sedimentation involves the larger particles.

Although there was considerable variation from one experiment to the next in the quantity of enzyme retained in the lungs, approximately 10% of this amount was always found in the trachea. In order to follow the changes in distribution of radioactivity with time, a series of experiments

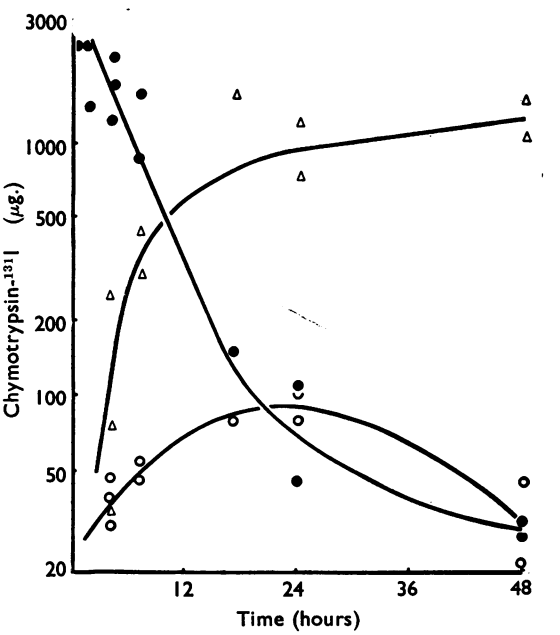

FIG. 4.—Distribution of radioactivity in lungs ●——●, liver O——O, and urine Δ——Δ of cats at various times following the inhalation of chymotrypsin-[^{131}I].

TABLE I

DISTRIBUTION OF RADIOACTIVITY IN TEN CATS FOLLOWING ADMINISTRATION OF INORGANIC [^{131}I] OR CHYMOTRYPSIN-[^{131}I]

The results are expressed in μg. chymotrypsin corresponding to the observed radioactivity, or as a percentage of the radioactive dose administered. In inhalation experiments the dose of chymotrypsin refers to the weight of powder expelled from the dispersion unit. The quantities in the total blood of the cat have been calculated on a basis of blood volume (ml.)=52 × body weight (kg.).

Method of Administration				Time after Administration (hr.)	Results of Analysis (μg.)				
Material	Dose		Route		Thyroid	Lungs	Trachea	Whole Blood	Urinary Excretion (from Start)
	mg.	μc.							
Iodide ..		1·0	Subcutaneous	5·5	8·7%			4·4%	25·5%
,, ..		1·0	,,	5·5	6·3%			4·7%	
Chymotrypsin ..	3·0	1·0	,,	5·5	70 (2·3%)			214 (7·1%)	99 (3·3%)
,, ..	3·0	1·0	,,	5·5	359 (12·0%)			132 (4·4%)	267 (8·9%)
,, ..	28·8		Inhalation (Method A)	5·5	21	1,000	28	108	619
,, ..	30·8		,,	5·5	24	1,232	28	93	525
,, ..	17·6		,,	24	200	31	1	38	408
,, ..	42·0		,,	24	295	145	19	92	1,020
,, ..	14·3		Inhalation (Method B)	6·0		2,320	263	350	249
,, ..	13·2		,,	24		377	40	130	780

was carried out, with the results shown in Fig. 4. It is clear that activity is present in the liver and urine within 4 hr. after insufflation of the enzyme. A second dose of radioactive enzyme was absorbed very much like the first. A comparison was made of the behaviour of ionic [^{131}I] and chymotrypsin-[^{131}I] given subcutaneously and by insufflation. This study revealed a rapid uptake of [^{131}I] by the thyroid from both materials given subcutaneously. Equally, there was rapid excretion in the urine (Table I). When the labelled chymotrypsin is given by inhalation, thyroid uptake is slow, in parallel with the slow clearance from the lung (see also Fig. 4). Only at 24 hr. does appreciable accumulation take place in the thyroid; by this time over 90% of the enzyme has been cleared from the lungs. It is worth noting that over the period 6 to 24 hr. there is no increase in tracheal radioactivity, as might be expected if the labelled material had been swept up from the lung by ciliary action.

In three preliminary experiments using direct method B for insufflation, 9.8, 8.5, and 10.1 mg. of chymotrypsin-[^{131}I] were expelled from the dispersion unit. The weights of enzyme present in the lungs were 2.30, 2.67, and 2.44 mg. respectively. The mean pulmonary retention was 26.4%, and the results emphasize the heavy, variable, and unpredictable loss of material on its way to the lungs and by exhalation. It is thus impossible to administer accurately a predetermined dose in any experiment; hence it is essential to measure the weight of enzyme retained in the lungs and trachea in each experiment. In Table II are given two typical sets of data for the distribution of radioactive fractions in some tissues of the cat at 6 and 24 hr. after it had inhaled chymotrypsin-[^{131}I] (method B). In view of the appearance of radioactivity in the stomach washings, the experiments were repeated, with division of the oesophagus between ligatures prior to insufflation of the enzyme. Radioactivity still appeared in the stomach washings within 4 hr., and consisted largely of iodide ion, thus readily accounting for its secretion into the gastric juice.

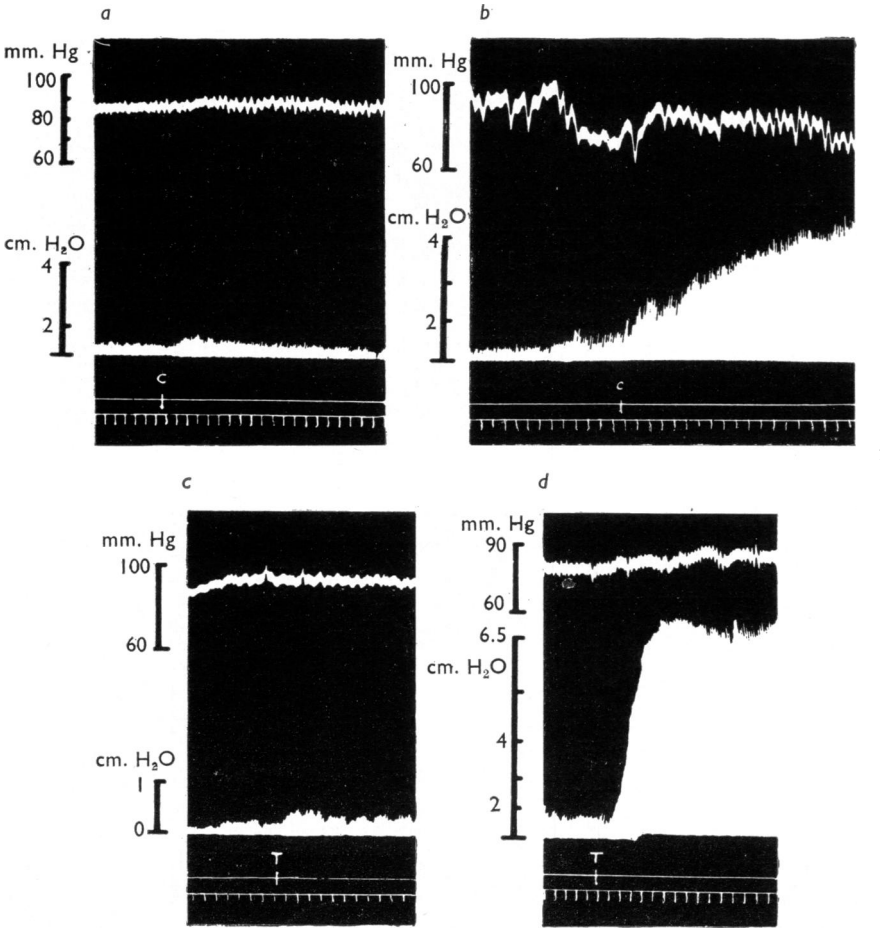

FIG. 5.—Records of tidal air and blood pressure following administration of trypsin and chymotrypsin to anaesthetized cats. The time scale is in min. The tidal air change was calibrated for each experiment in terms of the pressure, in cm. of water, required to produce an equivalent excursion of the recorder. (*a*) No significant alteration of tidal air after the insufflation of chymotrypsin-[^{131}I] at C. Radioassay of the trachea and lungs showed retention of 0.37 mg. enzyme. (*b*) Reduction of tidal air after the insufflation of chymotrypsin-[^{131}I] at C. Radioassay of the trachea and lungs showed retention of 1.09 mg. enzyme. (*c*) No alteration of tidal air after the insufflation of trypsin-[^{131}I] at T. Radioassay of the trachea and lungs showed retention of 0.023 mg. enzyme. (*d*) Reduction of tidal air after the insufflation of trypsin-[^{131}I] at T. Radioassay of the trachea and lungs showed retention of 0.08 mg. enzyme.

Tidal Air Changes.—Using anaesthetized cats as before, changes in tidal air were measured following the insufflation of [^{131}I]-labelled trypsin or chymotrypsin. The maximum dose was sought which could be administered without bringing about a reduction in tidal air. Whereas small amounts of trypsin decreased tidal air (Table III), preliminary tests showed that much greater doses of chymotrypsin could be given without eliciting such changes. Accordingly efforts were made to deposit several hundred micrograms of chymotrypsin within the lungs. From the results in Table III it is clear that smaller amounts of trypsin than of chymotrypsin are capable of inducing an appreciable reduction in tidal air. In Fig. 5 are shown tracings from 2 experiments in which the amounts of trypsin retained in the lungs were 23 and 80 μg. respectively. In the second experiment

there was a rapid decrease in tidal air (represented by an increased excursion of the lever measuring ventilation overflow), reaching its maximum in 5 min. Corresponding tracings with 370 and 1,090 μg. chymotrypsin are also shown in Fig. 5.

TABLE II

DISTRIBUTION OF RADIOACTIVE FRACTIONS IN SOME TISSUES OF THE CAT FOLLOWING INHALATION OF CHYMOTRYPSIN-[^{131}I]

The results are expressed in μg. chymotrypsin equivalent to the radioactivity observed. In the 6 and 24 hr. experiments 14.3 and 13.2 mg. respectively of chymotrypsin-[^{131}I] were expelled from the dispersion unit (method B). The total plasma volume (ml.) of the cat has been taken as 30 × body weight (kg.).

Specimen Analysed	Time after Administration (hr.)	Radioactive Fraction (μg.)			
		Inorganic Iodide	Protein-bound Iodine	Butanol-soluble Iodine	Total
Plasma ..	6	195·0	57·0	23·6	275·6
,, ..	24	—	—	—	99·5
Urine ..	6	236·0	0	12·7	248·7
,, ..	24	737·8	23·1	19·2	780·1
Stomach (washings)	6	202·2	—	—	—
Duodenum (washings)	6	42·5	—	—	—

TABLE III

CHANGES IN TIDAL AIR IN CATS FOLLOWING INHALATION OF TRYPSIN-[^{131}I] AND CHYMOTRYPSIN-[^{131}I]

Each result represents an experiment on one cat. Tidal air changes were measured by the method of Konzett and Rossler at a constant pressure of 9.5 cm. of water. The change in each experiment was calibrated in terms of the pressure, in cm. of water, required to produce an equivalent excursion of the recorder.

Decrease in Tidal Air (cm. Water)	Weight of Enzyme Present in Lungs and Trachea (μg.)	
	Trypsin	Chymotrypsin
0	5, 10, 23, 55	160, 370, 400, 480
1·0	5, 25	260, 530, 630
2·0	—	630
2·5	25	—
4·0	80, 320	1,090
6·5	80	—

The higher dose had a slow effect; about 13 min. elapsed before the maximum change was recorded. Repeated doses of either enzyme brought about no further change.

The carotid pressure was not affected by trypsin or chymotrypsin inhalation. It may be noted here that Werle, Kehl, and Koebke (1950) attributed the slight fall of blood pressure in dogs given crystalline trypsin to the presence of small amounts of adsorbed kallikrein.

The reduction in tidal air is most probably the result of bronchoconstriction, but its exact nature and mechanism have yet to be elucidated. It could be overcome in part by intravenous aminophylline (10 mg./kg.) or isoprenaline (0.5 to 2.0 μg./kg.) given by the same route. Prior administration of mepyramine (2 mg./kg. intravenously) in some cats completely abolished, and in others very substantially suppressed, the response to heavy doses of trypsin (195 to 300 μg. present in the lungs) or chymotrypsin (870 to 1,134 μg. present in the lungs). With mepyramine given only when the plateau of response to the enzyme had been reached, the same consistent effect was not seen.

DISCUSSION

Evidence of Lung Penetration.—The problem of ensuring pulmonary penetration of particulate drugs is primarily one of adequate dispersion of existing aggregates and prevention of formation of new ones. Since the objective was bronchiolar rather than alveolar retention of the powder, the low proportion by weight of particles below 2 μ in diameter was a decided advantage. On the other hand, the range selected as most suitable for human clinical use, and used in these experiments, is not necessarily the ideal one when the intention is to deposit the particles in the bronchioles of cats or other species of laboratory animals.

The evidence presented makes it clear that under the experimental conditions employed chymotrypsin does penetrate throughout the lungs. For technical reasons the precise localization and relative distribution of the enzyme particles in alveoli, alveolar ducts, various orders of bronchioles and bronchi could not be accurately determined. Ultraviolet fluorescence microscopy revealed a very generalized and singularly uniform distribution of the enzyme, but all attempts to counterstain the tissue quenched the weak fluorescence of the enzyme. Similarly [^{131}I], while it is a convenient label for quantitative studies, does not lend itself to high resolution in autoradiography.

Disposal of Inhaled Enzyme.—The speed with which the enzyme dissolves will to a great extent determine the relative parts played by ciliary clearance of enzyme-impregnated mucus and by direct absorption of chymotrypsin into the lung. The experiments with the relatively insoluble fluorescent enzyme suggested that some of the material was being cleared by centripetal movement up the bronchi—and very rapidly too, for within 1 hr. no fluorescence was seen in the lung macroscopically unless its activities had been suspended by a ligature at the hilum. The use of an intrabronchial polythene ring led to an accumulation of fluorescent material in the bronchus on the side distal to the ring, again pointing to ciliary clearance. (Small amounts of exogenous fluorescent material were no doubt still present in the lung but were impossible to detect owing to the intrinsic fluorescence.)

On the other hand, the clearance and distribution data with the readily soluble chymotrypsin-[^{131}I] are more precise. These results were relatively unaltered when the oesophagus was divided between ligatures prior to insufflation of the enzyme, so they cannot be attributed to ciliary clearance, swallowing and gastro-intestinal absorption. For at least 12 hr. after insufflation there is an exponential rate of disappearance of radioactivity from the lung, continuing at a low level over the next 36 hr. The possibility that liberation of [^{131}I] takes place within the bronchial lumen cannot be entirely excluded, but *in vitro* experiments with cat mucus make it clear that such a mechanism can contribute little to the overall picture. If the conclusions of Barnes and Trueta (1941) regarding subcutaneous absorption are applicable to the lung, chymotrypsin (composed mainly of α-chymotrypsin dimer with molecular weight of about 42,900) should be absorbed by the lymphatic route ; but it is conceivable that its proteolytic activity would bring about an increase in permeability to facilitate its own absorption, as has been suggested for trypsin (Martin, Bogner, and Edelman, 1957).

The rapidity of uptake from the lung and excretion into the urine suggests an artefact brought about by splitting off the radioactive iodine, which would then be readily removed. On the other hand, similar results have been obtained by us with water-soluble carboxylic acids labelled with [^{14}C] and administered in the same way (unpublished observations). The extent of liberation of inorganic iodine from the labelled enzyme was measured by incubating cat lung homogenate with chymotrypsin [^{131}I] for periods of up to 24 hr. at 37°. The supernatant after precipitation of the proteins contained only up to 18% of the total radioactivity present ; of this, one-half was inorganic iodide demonstrable by chromatography (unpublished observations). It is safe to conclude that no rapid splitting of the label from the enzyme takes place. This conclusion is borne out by the low activity appearing in the thyroid after the first 5.5 hr.

The subsequent disposal of the radioactive material, whatever it may be, appears to follow similar lines to those after subcutaneous injection —whether of chymotrypsin-[^{131}I] in cats or trypsin-[^{131}I] in rats (Bogner *et al.*, 1959). Unfortunately no data for urine are provided by these authors. On the other hand, the subcutaneous route does lead to a more rapid accumulation of activity in the blood and in the thyroid gland.

The question also arises of the secretion of free radioiodine into the lungs and especially the mucous glands of the trachea and bronchi. Eichler and Schütterle (1958) claimed that preferential concentration of ^{131}I occurs in the trachea of the guinea-pig and rat within 2 hr. after intraperitoneal administration. Subsequently (Eichler and Sebening, 1959) the results in rats were not confirmed, but in the cat trachea an enrichment of radioiodine of 1.25 over the plasma value was observed. Following inhalation of chymotrypsin-[^{131}I] under the conditions described here there was no evidence to suggest preferential uptake of [^{131}I] by the trachea whereas secretion into the stomach was readily demonstrable at 4 hr. Pulmonary secretion of the label is unlikely to be playing a significant part in its disposal during the first 12 to 24 hr.

Tidal Air Changes.—Barer and Nusser (1953) and Konzett (1956) have emphasized the difficulty of attributing a decrease in tidal air to bronchoconstriction rather than pulmonary congestion. With histamine or 5-hydroxytryptamine the response is immediate and certainly points to a direct action on the bronchial musculature (Konzett, 1956). The tidal air change elicited by trypsin is of the same type, whereas chymotrypsin, in high doses, produces its effect gradually, reaching a plateau only after several minutes. Despite the different character of these changes in the anaesthetized cat, both were largely or completely abolished by pretreatment with mepyramine.

Our thanks are due to Drs. R. E. C. Altounyan and W. G. Smith for helpful suggestions, and to the Directors of Benger Laboratories Ltd. for permission to publish the results of our investigations.

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PHARMACOLOGICAL STUDY OF A NEW ANTIBIOTIC OF BACILLARY ORIGIN

BY

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The purified extract of a new bacterial mutant, which exhibits antibiotic activity against paramecia and amoebae, was tested on various smooth muscle preparations. Spontaneous activity as well as the response to a variety of smooth muscle stimulants was diminished or completely suppressed. Intravenous injection of the extract lowered the blood pressure of the anaesthetized cat or dog. These properties may explain the beneficial effect of the extract on the intestinal symptoms of human amoebiasis, before eradication of the parasites.

In 1946, Reitler and Boxer isolated a new bacterial strain from the mesenteric lymph glands of a patient who succumbed to ileus with multiple intussusceptions of the jejunum. This strain grew in the form of short, Gram-negative rods, which produced spores abundantly. It also showed interesting antibiotic properties and exhibited a marked tendency to mutate under a variety of experimental conditions. Systematic selection led to mutants, which possessed not only antibacterial activity but produced also antiprotozoal substances in increasing quantities. One of these mutants, labelled RB-103, distinguished itself by its filamentous growth, its weak tendency to sporulation and its regular production of material, active against paramecia and even to a higher degree against amoebae (Reitler and Berner, 1960).

Purified extracts from the sub-strain RB-103 have been tried on several hundred patients suffering from acute or chronic infection with *Entamoeba histolytica*. Marked improvement of the intestinal symptoms was usually noted after a few days of treatment, but examination of the stools at this stage still revealed the presence of numerous amoebae and cysts. Analysis of the faeces became negative only after 2 to 3 weeks of continuous administration of the antibiotic. Such observations led to the idea that, in addition to the antibiotic substances, the bacterial extract may contain principles that act on the smooth muscle of the intestine and thus could be responsible for the pronounced change of the clinical picture long before elimination of the parasites (Reitler, 1950). The experiments described in this paper were designed to test this hypothesis and to investigate in general the pharmacodynamic properties of the antibiotic of the new strain.

METHODS

Antibiotic Material.—A sterile extract of the mutant RB-103, representing the last stage in the purification process, immediately before adsorption on to a solid carrier, was put at our disposal by Messrs. Hillel Remedies Inc., Haifa. The antibiotic activity of the preparation was tested against *Paramecium caudatum*, which is first immobilized and, during 24 hr., undergoes lysis. The extract used caused complete lysis at a dilution of 1 in 200. For the sake of brevity, we shall use in this paper the commercial name "Colisan" for the antibiotic preparation.

Isolated Organs.—Smooth muscle organs were suspended in an organ bath at 38°, containing aerated Locke solution. When the response to graded doses of a stimulant was irregular (for example, with the ileum of the guinea-pig), it was advantageous to store the organ in the refrigerator overnight. In the experiments described, figures about reagents refer to the final concentration of the substance in Locke solution.

Whole Animals.—Cats and dogs were anaesthetized with intravenous pentobarbitone, after induction with ether. Blood pressure was recorded from the left carotid artery, using an Hg manometer, and respiration by a membrane manometer, connected to the side arm of the tracheal cannula. Contractions of the left gastrocnemius were produced by stimulation of the sciatic nerve, placed on shielded silver electrodes, and registered by attaching the muscle to an isometric lever.

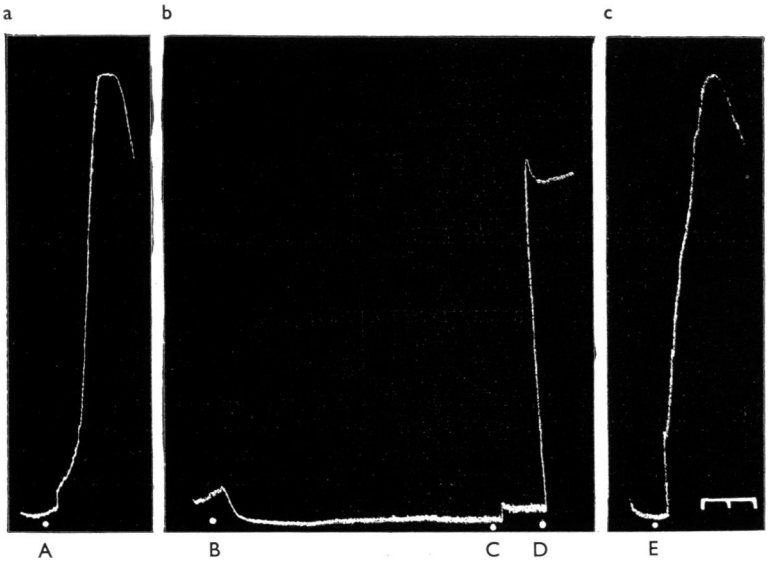

FIG. 1.—Antagonism of acetylcholine and colisan on the isolated guinea-pig ileum. A, Acetylcholine, 0.004 μg./ml. B, Colisan, 1 in 100 final dilution. C, Without washing, addition of acetylcholine, 0.004 μg./ml. D, Without washing, addition of acetylcholine, 0.16 μg./ml. Between D and E, repeated washings. E, Acetylcholine 0.004 μg./ml. Time marker, 1 min.

RESULTS

Effect of Colisan on the Guinea-pig Ileum.—An intestinal loop, incubated with colisan, showed a progressively diminishing response to acetylcholine. After 5 to 7 min. complete inhibition was obtained (Fig. 1). This effect was reversible; after repeated washing, the loop returned to its original sensitivity. The effect of colisan is competitive with the stimulant drug, because concentrations of acetylcholine, 10 to 50 times higher than the one used before inhibition, provoked a response even after 10 min. incubation with the bacterial extract.

The inhibitory effect can also be demonstrated in the reverse manner. Addition of colisan at the height of the contraction, due to stimulation by acetylcholine, led to immediate relaxation of the ileum (Fig. 2). This reverse reaction is distinguished from the effect of previous incubation not only by the time factor but also by the lower dose of colisan needed for complete relaxation (about 1/3 to 1/5 of the dose required in the incubation method).

The reverse method, because of its immediate effect, lends itself to a quantitative estimation of the activity of colisan. Increasing doses of the drug produce an increasing spasmolytic action (Fig. 3). By plotting log colisan concentration against the percentage decrease of the amplitude of the contraction induced by acetylcholine, a straight line is obtained (Fig. 4).

Effect of Colisan on the Guinea-pig Colon.—Analogous observations were made on the isolated colon. The spontaneous peristaltic movements of the organ, as well as the contractions produced by various stimulants, were abolished by colisan, which clearly exerts a threefold action (Fig. 5): (*a*) It lowers the tonus of the smooth muscle; (*b*) it diminishes the amplitude of the contractions; and (*c*) it reduces the frequency of the peristaltic movements.

Non-specificity of the Antagonism Between Colisan and Stimulants of Intestinal Smooth Muscle.—Colisan exerts its effect against any one of the following stimulants: acetylcholine, histamine, 5-hydroxytryptamine, nicotine, γ-amino-butyric acid and barium chloride. When equiactive doses of stimulants were compared, approximately the same concentration of colisan was required to counteract their effect. The broad antispasmodic spectrum demonstrates the non-specificity of colisan, compared with the action of other known antagonists of smooth muscle stimulants. Thus, a dose of atropine sufficient to abolish the response to acetylcholine will

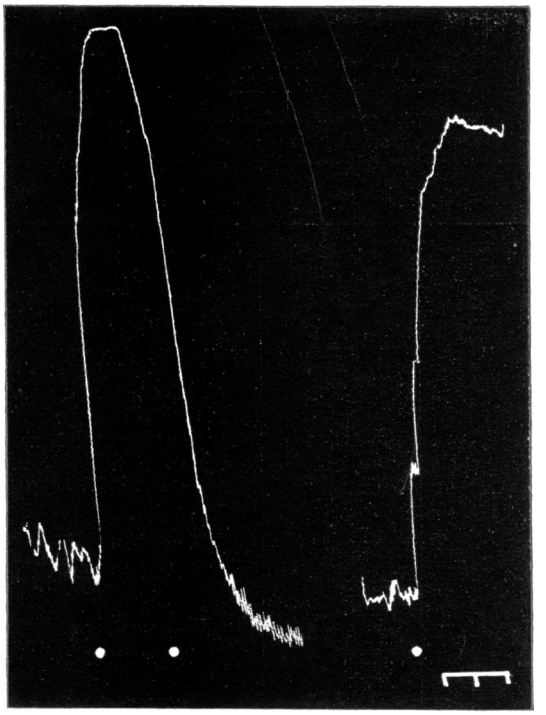

FIG. 2.—Effect of colisan, added at the height of a contraction of the guinea-pig ileum caused by acetylcholine. A, Acetylcholine, 0.02 μg./ml. B, Without washing, addition of colisan, 1 in 50 (final dilution). Between B and C, repeated washings. C, Acetylcholine, 0.02 μg./ml.

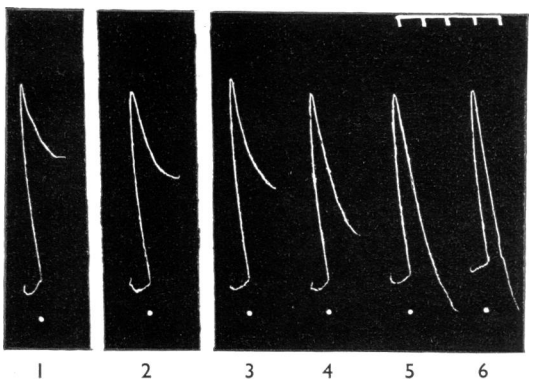

FIG. 3.—Graded effect of colisan on the contractions of the guinea-pig ileum, induced by 0.02 μg./ml. of acetylcholine. 1, Control. 2, Colisan 1 in 200. 3, 1 in 165. 4, 1 in 145. 5, 1 in 125. 6, 1 in 110. Note that 5 already produced maximal relaxation, while 6 produced this effect within a shorter time.

suppress only partially the response to histamine, 5-hydroxytryptamine, nicotine, or barium chloride. A given dose of the antihistamine diphenhydramine, which eliminates completely the contractions of the ileum induced by histamine or nicotine, is only partially effective against acetylcholine or 5-hydroxytryptamine. Florey's factor I shows specific antagonistic activity against acetylcholine and nicotine, but does not influence the response to 5-hydroxytryptamine (Florey and McLennan, 1959). Finally, botulinum toxin abolishes the contraction caused by nicotine, but does not interfere with the effect of acetylcholine or histamine (Ambache and Lessin, 1955).

Effect of Colisan on Other Smooth Muscle Organs.—The above experiments show that the relaxing effect of colisan is independent of the

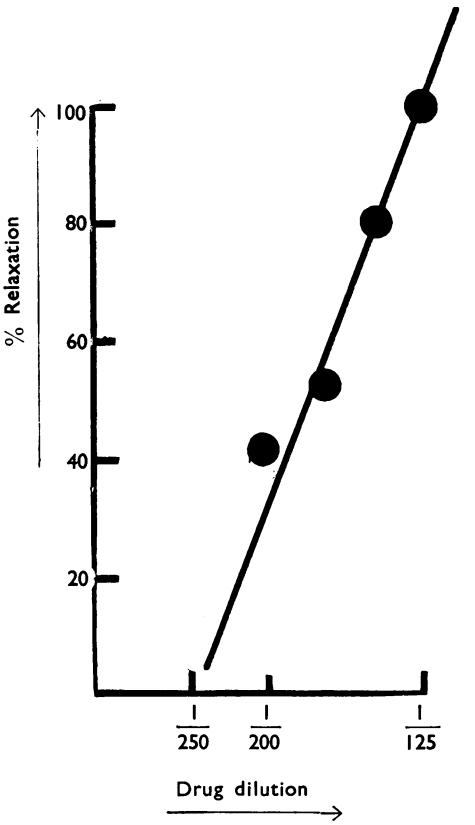

FIG. 4.—Percentage relaxation of guinea-pig ileum as function of log drug concentration. Taken from the data of Fig. 3. Ordinate,

$$\frac{\text{amplitude of descending limb of trace} \times 100}{\text{maximal amplitude of ascending limb}}$$

specific transmitter mechanism which operates in a given organ. This view is further supported by the observation that all smooth muscle organs tested showed essentially the same response to colisan as does the intestine. A survey of the organs examined and the agents used as most effective stimulants in each case is given in Table I, which also shows the species independence of the drug effect. For example, the non-pregnant uterus of the guinea-pig, which responds best to acetylcholine and histamine, and the uterus of the rat in the third week of pregnancy, which contracts after acetylcholine, 5-hydroxytryptamine (Fig. 6), and oxytocin, were both completely inhibited by colisan.

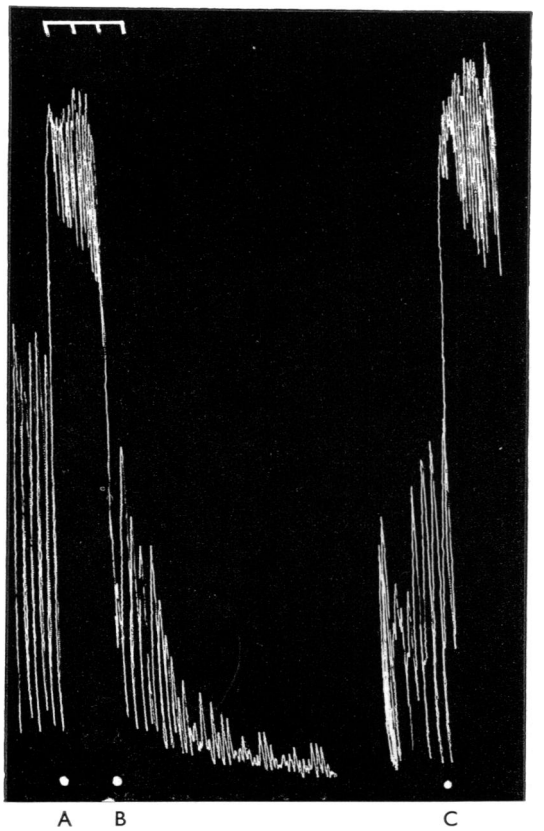

FIG. 5.—Antagonism of acetylcholine and colisan on the guinea-pig colon. The movements, left of A, represent the spontaneous peristalsis of the colon. A, Acetylcholine, 0.01 μg./ml. B, Without washing, addition of colisan 1 in 25 final dilution. Between B and C, repeated washings. Left of C, recovery of spontaneous peristalsis. C, Acetylcholine, 0.01 μg./ml.

TABLE I

SMOOTH MUSCLE ORGANS TESTED FOR THE ANTAGONISTIC EFFECT OF COLISAN

The tests with γ-aminobutyric acid were carried out according to Hobbiger (1958).

Species	Organ	Stimulants Used
Guinea-pig	Ileum	Acetylcholine, histamine, 5 - hydroxytryptamine, nicotine, γ-aminobutyric acid, barium chloride
,,	Colon	Acetylcholine, histamine, 5 - hydroxytryptamine, nicotine, barium chloride
,,	Uterus (non-pregnant)	Acetylcholine, histamine, barium chloride
,,	Ureter	Histamine, nicotine, barium chloride
,,	Bladder	Histamine, barium chloride
Rat ..	Uterus (third week of pregnancy)	Acetylcholine, 5-hydroxytryptamine, oxytocin, barium chloride
,, ..	Stomach fundus	5-Hydroxytryptamine

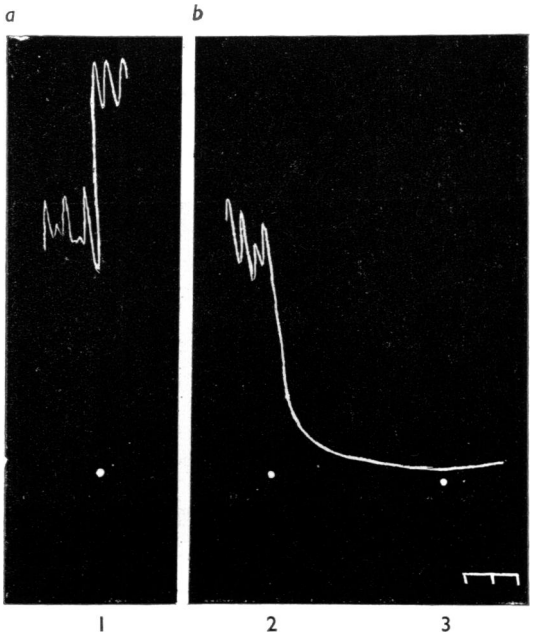

FIG. 6.—Horn of uterus of rat in third week of pregnancy. 1, 0.05 μg./ml. 5-hydroxytryptamine. 2, Colisan 1 in 100. After 7 min. incubation, addition of 0.05 μg./ml. 5-hydroxytryptamine at 3.

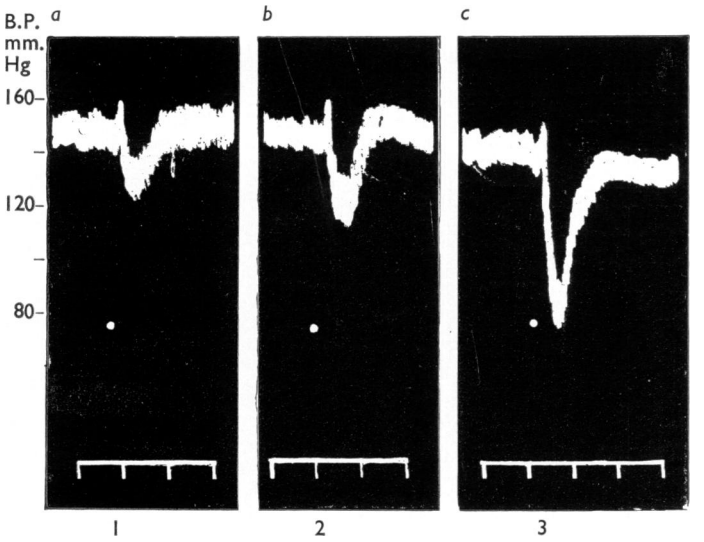

FIG. 7.—Effect of intravenous injections of colisan on the blood pressure of the dog under nembutal anaesthesia. 1, 0.5 ml. colisan. 2, 1.0 ml. colisan. 3, 2.0 ml. colisan.

Effect of Colisan on Striated Muscle.—Intravenous or intra-arterial injection of colisan into the cat had no effect on the amplitude of the contractions of the gastrocnemius, stimulated indirectly. Similar results were obtained with the phrenic-diaphragm preparation of Bülbring (1946).

Effect of Colisan on the Blood Pressure.—The response of the smooth muscle of the arterial wall was tested by measuring the blood pressure in the intact dog or cat. As shown in Fig. 7, after intravenous injection of colisan, the blood pressure fell immediately and returned within 1 to 2 min. to its original level. However, thereafter the blood pressure fell progressively to very low levels. Infusion of adrenaline raised the pressure again and restored the hypotensive response of the animal to colisan.

DISCUSSION

The extract from the bacterial mutant RB-103 contains a smooth muscle relaxant with a spectrum of activity much broader than that of other known antagonists to chemical stimulation, whether of animal, plant, or bacterial origin. The inhibitory efficiency of colisan against a whole array of smooth muscle stimulants, independent of their specific point of attack along the neuronal system or at the effector cell itself, supports the view that the drug acts directly on the muscle cell. The important fact should be stressed that, after incubation with colisan, any of the stimulating drugs tested can provoke contraction, if doses 10 to 50 times higher than the original ones are applied. Apparently, the antagonistic effect takes place at the contractile substance itself, a view supported by the observation that equal concentrations of colisan were required to neutralize the effect of equiactive doses of different stimulants.

The peculiar character of colisan is also revealed by comparison with other antagonists against smooth muscle stimulants. Florey (1954) isolated from brain extracts Factor I, which inhibits movements of the gut caused by acetylcholine or nicotine, but has no effect on the contraction provoked by 5-hydroxytryptamine (Florey and McLennan, 1959). In addition, these authors noted that about 25% of their biological preparations were insensitive to the brain extract. These properties distinguish Factor I from colisan, in view of the broad antagonistic spectrum of the latter (see Table I) as does the fact that its solutions, if active against one specific organ, are effective against all organs of the same or different species as far as has been tested.

The point of attack of colisan is also clearly demarcated from that of the botulinum toxins (Ambache, 1951). The latter abolishes contractions caused by nicotine, but does not impair the effect of acetylcholine, thus suggesting the neuronal parts of the preparation, that is, the synapse of the outer ganglion cells, the postganglionic neurons in the wall of the gut, or the nerve terminals at the muscle surface as locus of action. Furthermore, after about 10 min. incubation, the toxin cannot be washed out any more, whereas the effect of colisan is completely reversible by washing.

The situation is different with respect to staphylococcal β-toxin, which suppresses completely the spontaneous peristalsis of the isolated rabbit intestine as well as its response to acetylcholine (Anderson, James, and Marks, 1954; Kelsey and Hobbs, 1954). However, a comprehensive study of the localization of this antagonistic action has not been carried out. Therefore, it cannot be decided at present whether the toxin belongs to the same group as the active principle present in the extract of the mutant RB-103.

The present experiments support the hypothesis that the beneficial effect of colisan on human amoebiasis is due, at least in part, to the presence of a smooth muscle relaxant, which reduces the motility of the gut and thus alleviates the intestinal symptoms some time before the antiprotozoal effect becomes apparent. The pharmacodynamic observations with colisan naturally raise the question whether the antispasmodic principle of the bacterial extract is a separate entity or is identical with the antibiotic present in colisan. Experiments pertinent to this question will be reported in due course.

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THE PHARMACOLOGICAL ACTIONS OF 3,4-DIHYDROXYPHENYL-α-METHYLALANINE (α-METHYLDOPA), AN INHIBITOR OF 5-HYDROXYTRYPTOPHAN DECARBOXYLASE

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α-Methyldopa (3,4-dihydroxyphenyl-α-methylalanine) is an inhibitor of 5-hydroxytryptophan decarboxylase which is effective *in vitro* and *in vivo*. The inhibition is complex and shows coenzyme reversal. Evidence is presented that it acts by coenzyme inactivation. Its administration to mice reduced brain 5-hydroxytryptamine but had no effect on noradrenaline. After repeated doses, intestinal 5-hydroxytryptamine concentrations were also reduced. Co-ordinated activity was diminished coincidently with the enzyme inhibition and the reduction in brain 5-hydroxytryptamine. Rectal temperatures were reduced and the possibility that this resulted from inactivity is discussed. Treated animals showed miosis and narrowing of the palpebral fissures. A comparison is drawn between the actions of α-methyldopa and reserpine. The present results support the idea that the sedative effects of both drugs are due to the fall in brain 5-hydroxytryptamine concentration that they produce.

The endogenous formation of 5-hydroxytryptamine in body tissues depends on the activity of the enzyme 5-hydroxytryptophan decarboxylase (Clark, Weissbach and Udenfriend, 1954). This enzyme is widely distributed in the body and is found with a few exceptions wherever 5-hydroxytryptamine itself is present (Gaddum and Giarman, 1956). 3,4 - Dihydroxyphenyl - α - methylalanine (α-methyldopa), an inhibitor of dopa decarboxylase described by Sourkes (1954), is effective also against 5-hydroxytryptophan decarboxylase *in vitro* and *in vivo* (Westermann, Balzer and Knell, 1958).

This paper concerns the action of α-methyldopa on 5-hydroxytryptophan decarboxylase *in vitro* and *in vivo*, and the pharmacological effects of its administration to mice and other animals. Results of preliminary experiments have been reported to the Physiological Society (Smith, 1959).

METHODS

Estimation of Decarboxylase.—Tissues were homogenized cold in physiological saline and were stored, when necessary, for up to 2 hr. in the refrigerator. Aliquots containing 250 mg. of tissue were incubated in 25 ml. conical flasks at 37° and shaken. The reaction mixture in the flasks was made up as follows:

1. Tissue homogenate	1 ml.
2. 0.5M phosphate buffer (*p*H 8.0) ...	2 ml.
3. Additions	1 ml.
4. DL 5-hydroxytryptophan 1.1 mg. (final concentration =1 μmole/ml.)	1 ml.

The mixture always contained iproniazid 0.89 mg. (final concentration 1 μmole/ml.) to inhibit amine oxidase. The substrate, 5-hydroxytryptophan, was added last after preincubation of the mixture for 15 min. 5-Hydroxytryptamine was assayed in the reaction mixture immediately after the addition of the substrate and again 30 min. later. Preliminary experiments showed that the rate of 5-hydroxytryptamine production was linear up to 1 hr.

Manometric Estimation of Decarboxylase.—In 1 experiment, 5-hydroxytryptophan decarboxylase was prepared from guinea-pig kidney by the method of Clark *et al.* (1954) as far as stage 3. CO_2 evolution was measured manometrically in Warburg flasks. The reaction mixture was made up as follows:

1. Enzyme preparation	2.0 ml.
2. 0.2M phosphate buffer (*p*H 8.0)	0.5 ml.
3. Pyridoxal 5-phosphate 30 μg. ...	0.1 ml.
4. Substrate (in side-arm 1) ...	0.2 ml.
5. 4N sulphuric acid (in side-arm 2)	0.2 ml.

The incubation periods were 20 or 40 min. The procedure was otherwise as described by Clark *et al.* (1954).

Assay of 5-Hydroxytryptamine. — Aliquots of incubation mixtures and of tissue homogenates were assayed fluorimetrically by the method of Bogdanski, Pletscher, Brodie, and Udenfriend (1956). Of the substances used in the experiments, α-methyldopa and pyridoxal 5-phosphate were found not to interfere with the fluorescence of 5-hydroxytryptamine. Iproniazid in the concentration used in the incubation mixtures quenched fluorescence, and a correction of +15% was found to compensate for the difference. This correction was applied when appropriate.

Assay of Noradrenaline.—The pressor action on the pithed rat (Shipley and Tilden, 1947) was employed. The extraction procedure used for tissues was as follows: brains were homogenized in 3 volumes (w/v) 0.02N hydrochloric acid, placed in deep freeze overnight and centrifuged the following day. The clear supernatant (*p*H 5.0) was injected directly into the test rats and assayed against noradrenaline. Although such a preparation probably contains other pressor and interfering substances, it seems unlikely that they interfere with the assay. Dopamine, which is present in the brain in about equal concentration, has only 1/100 of the pressor activity, and adrenaline, which is present in much lower concentration, has less activity than noradrenaline. Similar quantities of 5-hydroxytryptamine have a slight pressor activity on the preparation.

Mouse Activity.—The method of Dews (1953) was used, employing groups of 5 mice. This method appears to depend to some extent on the interest that the test animals display in their surroundings, and the activity counts diminish if animals are tested too frequently. In the experiments described no group was tested more than once in 48 hr., an arbitrary time chosen for lack of interference, and control groups were always tested at the same time as treated groups. Where the influence of α-methyldopa on activity has been followed against time, the results have been obtained with different groups over a period of many weeks.

Mouse Rectal Temperature.—This was measured with a small thermistor, inserted 2 cm. into the rectum, and supplied with current from a hearing-aid battery. The resistance of the thermistor was measured on a galvanometer, the full range of which was calibrated from 30 to 40°. Ambient temperatures varied from 22.6 to 25.3°.

Intestinal Passage of a Meal.—This was done by a modification of the method of Brittain and Collier (1958). The test meal given intragastrically consisted of 0.4 ml. of aqueous carmine solution 1%, instead of charcoal. This modification was applied so that an estimate could be made of the extent of gastric emptying during the test period. The animals were killed after 20 min., the stomachs removed, extracted with 0.1 N sodium hydroxide, filtered, and the carmine estimated colorimetrically against a standard curve using a green filter (Ilford 404). The percentage of the small intestine traversed by the meal was measured as described by the above authors.

RESULTS

In Vitro *Experiments*

Action of α-Methyldopa on Mouse Brain 5-Hydroxytryptophan Decarboxylase. — The experiments confirmed that α-methyldopa is an effective inhibitor, 0.1 μmole/ml. producing 100% inhibition. The results are shown in Fig. 1. In the presence of added pyridoxal 5-phosphate, the natural coenzyme, 0.008 μmole/ml., it was much less effective and 10 μmole/ml. was now needed for complete inhibition. By interpolation, the doses needed to produce 50% inhibition were

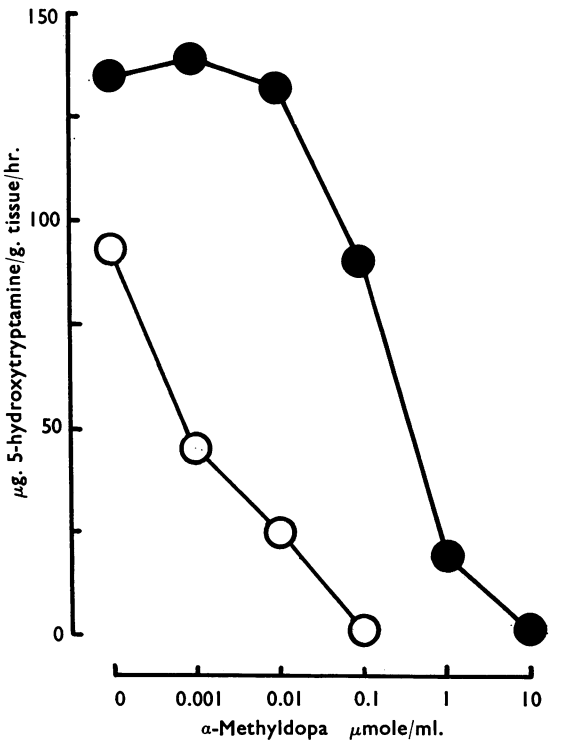

FIG. 1.—Mouse whole brain homogenate 5-hydroxytryptophan decarboxylase *in vitro*. Action of α-methyldopa. 5-Hydroxytryptophan and iproniazid concentrations each 1 μmole/ml. Ordinates: μg. 5-hydroxytryptamine formed/g. tissue/hr. Abscissae: α-methyldopa concentration in μmole/ml. incubation mixture. ●——● with added pyridoxal 5-phosphate 0.008 μmole/ml. O——O without addition.

0.0012 and 0.20 μmole/ml. respectively. In the absence of inhibitor, the addition of coenzyme increased the rate of decarboxylation from 87 to 135 μg. 5-hydroxytryptamine formed/g. tissue/hr. Further addition of coenzyme up to a concentration of 1 μmole/ml. had no effect.

Interaction of Substrate Concentration, Coenzyme Concentration and α-Methyldopa on Mouse Brain Decarboxylase.—Homogenates were incubated with varying concentrations of 5-hydroxytryptophan and pyridoxal 5-phosphate in the presence of a fixed concentration of α-methyldopa. The results are shown in Fig. 2. Increasing the concentration of coenzyme reduced the inhibition at any substrate level. Increasing the concentration of substrate also reduced the inhibition at any coenzyme level. The reciprocals of substrate concentrations (S) and of enzyme activities (V) were plotted graphically according to the method of Lineweaver and Burk (1934). Fig. 3 shows the results obtained at a coenzyme concentration of 1 μmole/ml. Regression lines for 1/V against 1/S at all coenzyme concentrations were calculated and the theoretical $1/V_{max}$ found in each case. The figures obtained are given in Table I. At low coenzyme concentrations

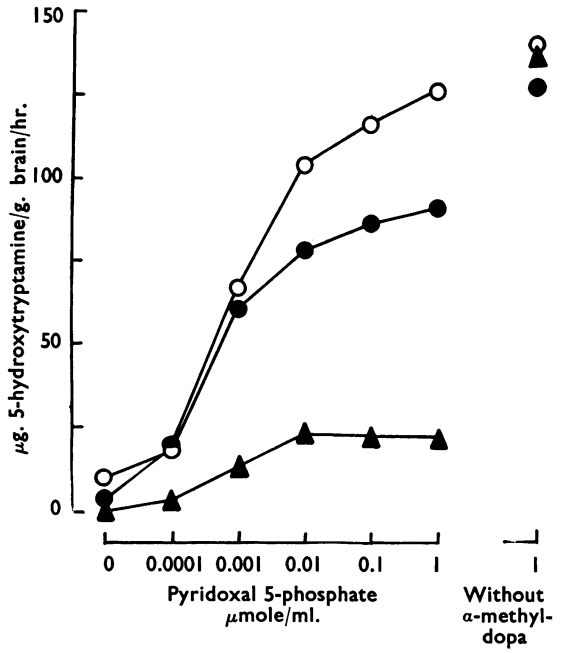

FIG. 2.—Mouse whole brain homogenate 5-hydroxytryptophan decarboxylase *in vitro*. Effect of varied 5-hydroxytryptophan and pyridoxal 5-phosphate concentrations in the presence of a fixed concentration of α-methyldopa 0.2 μmole/ml. incubation mixture. Iproniazid added 1 μmole/ml. Ordinates: μg. 5-hydroxytryptamine formed/g. brain/hr. Abscissae: pyridoxal 5-phosphate concentration in μmole/ml. 5-Hydroxytryptophan concentrations: O——O 10 μmole/ml. ●——● 1 μmole/ml. ▲——▲ 0.1 μmole/ml.

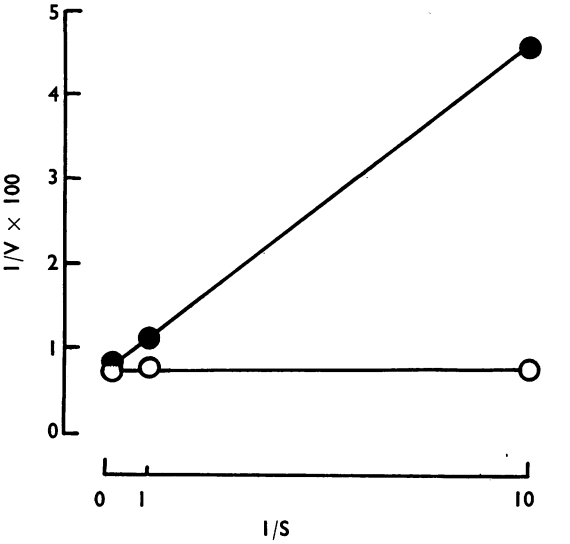

FIG. 3.—Mouse whole brain homogenate 5-hydroxytryptophan decarboxylase *in vitro*. Reciprocal analysis of activities in presence of pyridoxal 5-phosphate 1 μmole/ml. by method of Lineweaver and Burk (1934). See text for details. Ordinates: $1/V \times 100$, where V=reaction velocity in μg. 5-hydroxytryptamine formed/g. tissue/hr. Abscissae: 1/S, where S=substrate concentration in μmole/ml. ●——● with α-methyldopa 0.2 μmole/ml.; O——O without addition.

TABLE I

RECIPROCAL ANALYSIS OF RESULTS FROM FIG. 2

Intercepts and slopes for reciprocals of enzyme activity ($1/V \times 100$) against substrate concentration (1/S).

Inhibitor	Coenzyme Concentration in μmoles/ml.	Intercept ($1/V_{max}$)	Slope
−	0·0001 to 1	0·75	0
+	1	0·74	0·38
+	0·1	0·81	0·37
+	0·01	0·93	0·35
+	0·001	1·22	0·64
+	0·0001	3·87	2·93

the intercept is greater in the presence than in the absence of the inhibitor; this indicates non-competitive inhibition. At high coenzyme concentrations the intercept is identical whether inhibitor is present or not; this indicates competitive inhibition.

Action of Guinea-pig Kidney Decarboxylase on α-Methyldopa.—The preparation was incubated with various mixtures of 5-hydroxytryptophan and α-methyldopa at *p*H 8.0. Carbon dioxide evolution, measured by Warburg manometry, indicated that this enzyme preparation shows a much greater affinity for α-methyldopa than for 5-hydroxytryptophan at this *p*H, and that it decarboxylates α-methyldopa to a small extent. Fig. 4 shows the results obtained.

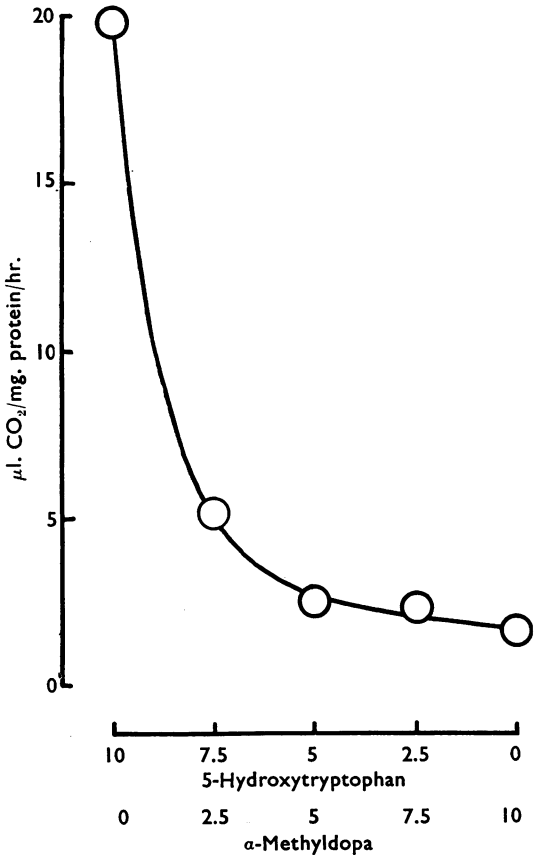

FIG. 4.—Guinea-pig kidney 5-hydroxytryptophan decarboxylase *in vitro*. Warburg manometry. Action on mixtures of 5-hydroxytryptophan and α-methyldopa. See text for details of preparation. Ordinates: μl. carbon dioxide evolved/mg. protein/hr. Abscissae: quantities of substrates added in μmole.

In Vivo *Experiments*

Mouse Brain and Intestinal Decarboxylase.—Groups of male white mice weighing 20 to 28 g. were given α-methyldopa 100 mg./kg. subcutaneously, and killed at intervals thereafter. Brain and intestinal homogenates were assayed for decarboxylase activity, without addition of pyridoxal 5-phosphate. The results, presented graphically in Fig. 5, show that activity was inhibited in both preparations, intestine being affected more than brain. Inhibition was maximal 0.5 to 1 hr. after treatment, and disappeared within 8 hr.

Mouse Brain and Intestinal 5-Hydroxytryptamine Concentration.—The results, given in Table II, show that α-methyldopa 100 mg./kg. sub-

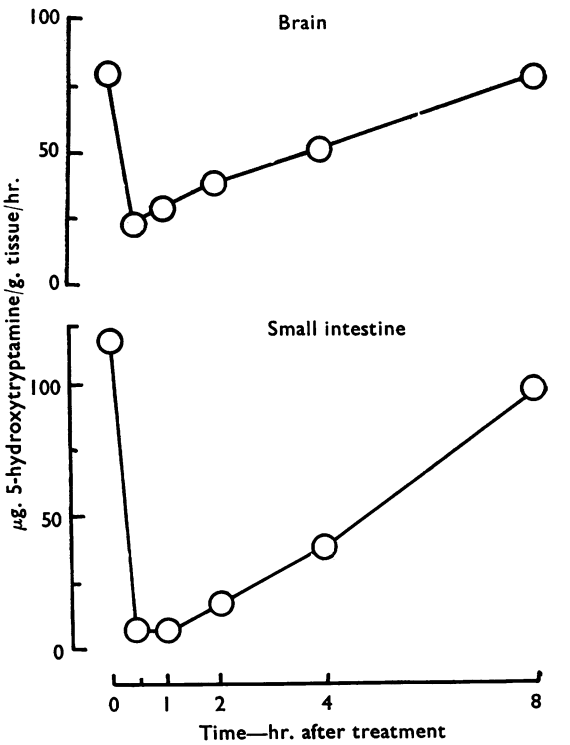

FIG. 5.—Mouse whole brain and small intestine homogenate 5-hydroxytryptophan decarboxylase activities after pretreatment with α-methyldopa 100 mg./kg. subcutaneously. No addition of pyridoxal 5-phosphate to incubation mixture. 5-Hydroxytryptophan and iproniazid concentrations each 1 μmole/ml. Each point obtained from pooled tissues of 2 mice. Ordinates: μg. 5-hydroxytryptamine formed/g. tissue/hr. Abscissae: time in hr. after treatment.

TABLE II

THE CONTENT OF 5-HYDROXYTRYPTAMINE IN MOUSE WHOLE BRAIN AND SMALL INTESTINE AFTER TREATMENT WITH α-METHYLDOPA

The figures represent μg. 5-hydroxytryptamine/g. tissue. Brain results were obtained on pairs of organs. Intestines were assayed singly.

Tissue	Saline 1 Dose at 1 hr.	α-Methyldopa 100 mg./kg. at 1 hr.	Saline 4-hourly for 24 hr.	α-Methyldopa 400 mg./kg. 4-hourly for 24 hr.
Brain	0·96 0·79 0·89	0·86 0·49 0·57	0·96 1·02	0·38 0·48
Intestine	2·51 1·77 2·64 3·17 3·29 3·61	3·79 3·55 2·29 3·01	3·05 5·07 3·33 3·22	2·82 2·61 1·81 2·00
	Mean =2·83 (±0·27)	Mean =3·16 (±0·33)	Mean =3·67 (±0·47)	Mean =2·31 (±0·24)
	p>0·4		p<0·05	

TABLE III

NORADRENALINE CONTENT OF MOUSE WHOLE BRAIN AFTER TREATMENT WITH α-METHYLDOPA

The figures represent μg. noradrenaline/g. brain.

Saline 1 Dose at 1 hr.	α-Methyldopa 100 mg./kg. at 1 hr.	Saline 4-hourly for 24 hr.	α-Methyldopa 400 mg./kg. 4-hourly for 24 hr.
0·90 0·80	0·95 0·80	0·82 1·04	0·92 1·00

TABLE IV

5-HYDROXYTRYPTAMINE CONTENT OF GUINEA-PIG WHOLE BRAIN AFTER TREATMENT WITH α-METHYLDOPA

The figures represent μg. 5-hydroxytryptamine/g. brain.

Saline 4-hourly for 24 hr.	α-Methyldopa 400 mg./kg. 4-hourly for 24 hr.
0·30 0·28	0·03 0·02

cutaneously reduced the brain 5-hydroxytryptamine content, the reduction (about 30%) being maximal 1 hr. after treatment. A much bigger reduction (60%) was found with a larger dose (400 mg./kg.) given at 4-hourly intervals for 24 hr. Intestinal 5-hydroxytryptamine concentrations were not affected by the single doses, but were reduced by about 40% by the repeated doses of α-methyldopa.

Mouse Brain Noradrenaline Concentration.—α-Methyldopa in a single dose of 100 mg./kg. or in multiple dosage of 400 mg./kg. 4-hourly for 24 hr. had no effect on noradrenaline concentrations in the whole brain. Table III shows the results found.

Guinea-pig Brain 5-Hydroxytryptamine Concentration.—α-Methyldopa 400 mg./kg. 4-hourly for 24 hr. produced more than 90% reduction in whole brain 5-hydroxytryptamine content. This reduction was much greater than was found in mice. The results are given in Table IV.

Pharmacological Effects

Mouse Activity.—Groups of mice treated with α-methyldopa 100 mg./kg. subcutaneously were found to be much less active than saline-treated controls. This is shown in Fig. 6. The activity was minimal 1 hr. after treatment, at which time the animals were considerably sedated. Eight hr. after treatment the animals appeared normal and the activity counts had returned to the control level.

In further experiments, the results of which are shown in Fig. 7, groups of mice were treated with α-methyldopa (100 mg./kg.) and/or 5-hydroxytryptophan (100 mg./kg.) or dopa (90 mg./kg.), both of which are substrates for decarboxylase and might be expected to reduce the inhibition produced by α-methyldopa. Other groups received α-methyldopa (100 mg./kg.) and/or iproniazid (50 mg./kg.) or harmaline (20 mg./kg.), both of which are amine oxidase inhibitors and might be expected to prevent destruction of 5-hydroxytryptamine in the brain. All treatment was given subcutaneously, iproniazid 4 hr. before, and all other substances 1 hr. before testing. 5-Hydroxytryptophan reduced activity slightly, though not significantly, but diminished the effect of α-methyldopa ($p<0.05$). Dopa produced a big reduction ($p<0.01$) and exacerbated the effect of α-methyldopa ($p<0.01$). Iproniazid had no effect alone but reduced the effect of α-methyldopa ($p<0.05$). Harmaline produced a big reduction in activity ($p<0.001$), but did not influence the effect of α-methyldopa.

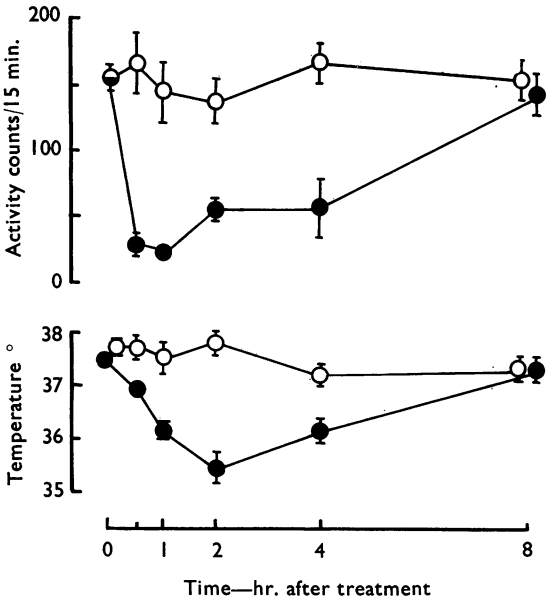

FIG. 6.—Activity counts and rectal temperatures of mice after treatment with α-methyldopa or saline in equivalent volume. Vertical lines show standard errors of the mean. Ordinates—upper graph: mean activity counts of 3 groups of mice/15 min.; lower graph: mean rectal temperature of 10 mice in °. Abscissae: time in hr. after treatment. O——O saline treatment. ●——● α-methyldopa 100 mg./kg.

Mouse Rectal Temperature.—The temperature of mice treated with α-methyldopa 100 mg./kg. subcutaneously was reduced by comparison with saline-treated controls. This is shown in Fig. 6. The temperature fall continued after the maximal reduction in activity, and was greatest 2 hr. after treatment. In further experiments, groups of mice were treated with combinations of α-methyldopa and/or 5-hydroxytryptophan, dopa, iproniazid, or harmaline. Doses and timing were exactly as in the experiments described for activity, and the difference in temperatures from saline-treated animals are shown in Fig. 7. 5-Hydroxytryptophan reduced temperature ($p<0.001$) but did not alter the effect of α-methyldopa significantly. Dopa had no significant action alone, but it exacerbated the effect of α-methyldopa ($p<0.01$). Iproniazid had no action at all. Harmaline reduced temperature ($p<0.001$) and exacerbated the effect of α-methyldopa ($p<0.01$).

Guinea-pig Rectal Temperature.—α-Methyldopa 400 mg./kg. 4-hourly for 24 hr. had a slight effect only on rectal temperature of guinea-pigs. Figures obtained were:

Before treatment	..	37.6°, 37.4°
After treatment	..	36.4°, 36.9°

Other Drugs on Mouse Rectal Temperature.—Hexobarbitone sodium 80 mg./kg. intraperitoneally produced a fall in temperature which was maximal 0.5 to 1 hr. after treatment. The mean decrease at 1 hr. was 2.6° ($p<0.05$). Reserpine 5 mg./kg. subcutaneously produced at 24 hr. a fall in temperature which was usually more than 7.5°, that is too low to be measurable with the thermistor circuit as calibrated.

Intestinal Passage of a Meal.—Test meals of carmine were given to a group of mice 1 hr. after

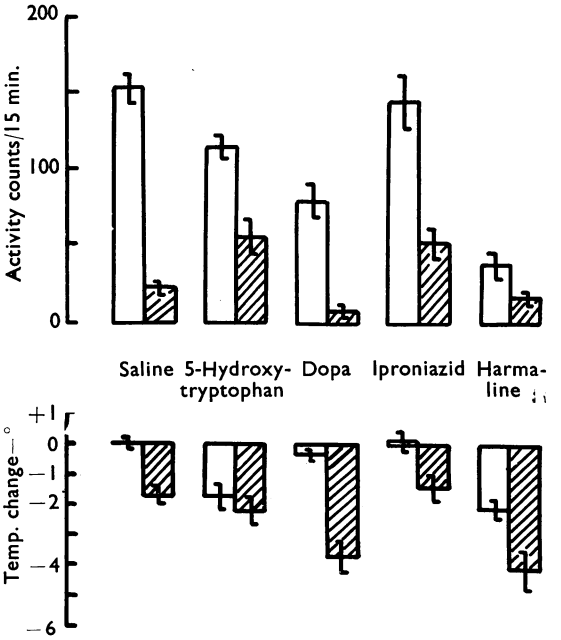

FIG. 7.—Activity counts and rectal temperatures of mice 1 hr. after treatment with combinations of α-methyldopa and/or 5-hydroxytryptophan, dopa, iproniazid, or harmaline. See text for details of times, doses, and routes of treatment. Upper histograms: mean activity counts on 3 groups of mice/15 min. Lower histograms: mean temperature change of 10 mice from saline-treated mice (37.2±0.1°) in °. Vertical lines represent standard errors of the mean. Plain columns: saline-treated; hatched columns: α-methyldopa-treated mice. Other treatments in the figure.

TABLE V
PASSAGE OF CARMINE MEAL IN MICE AFTER VARIOUS TREATMENTS

Details of dosage and routes of treatment in text. Figures are means (±standard error). Figures in italics —$p<0.02$.

Treatment	No. of Animals	% of Carmine Remaining in the Stomach	% of Small Intestine Traversed by the Meal
Saline ..	12	21 (±6)	73 (±3)
α-Methyldopa	12	*55* (±7)	*55* (±3)
Atropine ..	6	50 (±10)	*55* (±8)
Controls for reserpine ..	12	32 (±5)	69 (±5)
Reserpine ..	12	*19* (±2)	*46* (±4)

treatment with α-methyldopa 100 mg./kg. subcutaneously. The results, given in Table V, show that α-methyldopa delayed gastric emptying, for the amount of carmine remaining in the stomach was greater than in the control animals. The percentage of the small intestine traversed by the meal was less after α-methyldopa. Atropine 20 mg./kg. subcutaneously 1 hr. previously had exactly the same effect.

Two groups of mice were given reserpine (in ascorbate solution) 5 mg./kg. or ascorbate solution subcutaneously and starved for 24 hr. Gastric emptying was more complete in the reserpine-treated animals, but in spite of this the percentage of the small intestine traversed by the meal was less.

Hexobarbitone Sleeping Time.—Groups of 10 mice were given α-methyldopa 100 mg./kg. subcutaneously or saline, followed 30 min. later by hexobarbitone sodium 80 mg./kg. intraperitoneally. The mean sleeping times, calculated as the interval between injection and the time of restoration of the righting reflex (± standard error), were:

Hexobarbitone .. .. 20 (±2) min.
α-Methyldopa + hexobarbitone 43 (±6) min.

These results were significantly different ($p<0.01$).

Eye Signs.—Narrowing of the palpebral fissure occurred in all mice treated with α-methyldopa, whether in a single dose or multiple doses, as previously described. Miosis was present in those animals which were treated repeatedly. Both these signs were present to a very marked degree in 2 guinea-pigs given 4 hourly doses of α-methyldopa 400 mg./kg.

Similarities Between Appearances of Animals Treated with Reserpine and α-Methyldopa.—In many respects the appearance of α-methyldopa-treated and reserpine-treated animals was very similar. Both were sedated, inactive, and hypothermic, and the eye signs were the same. One difference, however, was noted. Twenty-four hours after reserpine, mice and guinea-pigs, though sedated, react violently to any handling. This was not so after repeated doses of α-methyldopa. The animals remained very quiet and limp, and were apparently quite oblivious of their surroundings.

DISCUSSION

The Action of α-Methyldopa on 5-Hydroxytryptophan Decarboxylase. — The experiments *in vitro* showed that the effect of α-methyldopa depends on a relationship between the concentrations of inhibitor, substrate, and coenzyme. The most significant finding is that increased concentrations of either substrate or coenzyme or both reverse the inhibition. At high coenzyme concentrations the relationship between substrate and inhibitor is one which indicates a competitive type of inhibition, a concept which is supported by the evidence that α-methyldopa is itself decarboxylated, albeit at a slow rate. At low coenzyme concentrations, however, a different mechanism probably exists. Reciprocal analysis of the results showed that inhibition was partly non-competitive, and the fact that raising the coenzyme concentration was capable of reversing the inhibition suggests that α-methyldopa can inactivate coenzyme. Whether this inactivation is caused by a chemical reaction between the two is not clear. Schott and Clark (1952) and Holtz and Westermann (1957) showed that a number of decarboxylase inhibitors, derivatives of phenylalanine and phenylethylamine, react with pyridoxal, and that the reaction is dependent on the presence of a hydroxyl group in the *meta*-position. They showed that the reaction takes place in two stages, to form first a Schiff base and then a tetrahydroisoquinoline derivative by ring closure. However, Sourkes (1954) demonstrated only a very slow combination between pyridoxal and α-methyldopa, though this substance does contain a *meta*-hydroxyl group. The mechanism of the coenzyme reversal of α-methyldopa inhibition is, therefore, incompletely explained.

One of the implications of the *in vitro* findings is that the measurement of inhibition *in vivo* can never represent a true picture of what occurs in

the tissues themselves. Knowledge of local concentrations of all the variables is necessary before any assumptions can be made. In any case, all known methods of enzyme activity measurement involve the addition to the incubation mixture of relatively large amounts of substrate, so that the substrate/inhibitor ratio is already disturbed. From this viewpoint, the present measurements of decarboxylase inhibition *in vivo* provide only one piece of information: that α-methyldopa reaches the brain. The only absolute indication of inhibition *in vivo* is that brain and intestinal 5-hydroxytryptamine concentrations are reduced.

Tissue Amine Concentrations.—The finding that α-methyldopa treatment led to a reduction in brain 5-hydroxytryptamine but not in brain noradrenaline is of considerable interest. This might be due to a preferential action on 5-hydroxytryptophan as opposed to dopa decarboxylase. Westermann *et al.* (1958) demonstrated such a preference in their experiments, and the possibility that local concentrations of the two substrates in the brain may differ might contribute to this difference. On the other hand, it must be remembered that 5-hydroxytryptamine and noradrenaline do not lie in equivalent biochemical relation to decarboxylase. Whereas 5-hydroxytryptamine is itself the product formed from 5-hydroxytryptophan, dopamine is the product formed from dopa, and noradrenaline is only formed by β-hydroxylation of dopamine. The fact that there are considerable quantities of dopamine present in the brains of different species (Carlsson, Lindqvist, Magnusson, and Waldeck, 1958; Bertler and Rosengren, 1959) suggests that this β-hydroxylation is a limiting reaction by comparison with the decarboxylation which precedes it. It is perhaps not surprising, therefore, that noradrenaline concentrations were not affected in the present experiments. So far, no estimations of dopamine have been performed here, but these are planned for the near future. The effect of α-methyldopa on them should be of great interest.

Pharmacological Effects of α-Methyldopa.—It is evident that α-methyldopa produced many of the effects of reserpine, though presumably in a completely different manner. The implications of this finding are considerable. Reserpine is known to deplete the brain of 5-hydroxytryptamine (Hess, Shore, and Brodie, 1956), noradrenaline (Vogt, 1957; Shore, Mead, Kuntzman, Spector, and Brodie, 1957), and dopamine (Carlsson *et al.*, 1958), and it has been suggested at various times that these depletions are responsible for the sedative and ocular effects that reserpine produces. Hess *et al.* (1956) suggested that these effects are due to the presence of free 5-hydroxytryptamine acting on the brain. The present experiments make this very unlikely, as it is inconceivable that inhibition of decarboxylase should cause the presence of free 5-hydroxytryptamine. The experiments also suggest that the depletion of noradrenaline is of less importance than that of 5-hydroxytryptamine, for α-methyldopa had no effect on noradrenaline concentrations. The possibility remains that depletion of dopamine has occurred also after α-methyldopa. Whether this has contributed to its pharmacological effects remains to be seen.

The experiments in which 5-hydroxytryptophan, dopa, iproniazid, or harmaline were given in addition to α-methyldopa illustrate some points of interest. 5-Hydroxytryptophan alone reduced activity to a slight extent and it is possible that a high concentration of this substrate in the brain inhibits decarboxylase by coenzyme removal. This has been found *in vitro* with dopa (Schott and Clark, 1952). On the other hand, 5-hydroxytryptophan diminished the reduction in activity produced by α-methyldopa, presumably reversing its inhibitory action on the enzyme. By contrast, dopa had the same effect as, and potentiated the action of, α-methyldopa. This suggests that dopa inhibits 5-hydroxytryptophan decarboxylase *in vivo* as it does *in vitro* (Yuwiler, Geller, and Eiduson, 1959), and that the effects of dopa and α-methyldopa are, in this respect, additive. Iproniazid diminished the effect of α-methyldopa, as would be expected if activity is related to the concentration of 5-hydroxytryptamine in the brain. The effects of harmaline were unexpected and suggest that this substance has some other action beside its amine oxidase inhibitory one. What this action is remains obscure.

The temperature changes in all the treated mice followed very approximately the observed changes in activity. This suggests that the two are related. That falls in temperature were consequent upon inactivity, and not vice versa, is suggested by two findings: first, that temperatures of α-methyldopa treated mice continued to fall from 1 to 2 hr. after treatment, at a time when activity counts were already returning to normal; and secondly, that guinea-pigs treated with α-methyldopa were sedated and showed marked eye signs, though their temperatures fell only very slightly. This difference may be a reflection of the different surface area/heat capacity ratios in the two

species, a factor which would make mice particularly prone to changes in temperature. Lessin and Parkes (1957) showed that keeping mice at an ambient temperature of 32° prevents the sedative action of reserpine, and concluded that the sedation was a result of the hypothermia. The present findings are not in keeping with such an idea.

The experiments on the passage of a test meal in mice showed that α-methyldopa treatment produced an apparent slowing, thus imitating the effect of reserpine. There was, however, one striking difference between the effects of the two substances. Whereas reserpine produced a slight acceleration of gastric emptying, α-methyldopa delayed it. The effects of α-methyldopa were exactly mimicked by those of atropine, which suggests that they might have been mediated via the vagus and possibly resulted from a decrease in parasympathetic tone.

The Use of Decarboxylase Inhibitors.—The use of α-methyldopa, and probably other substances with similar actions, provides a method of depleting tissue of 5-hydroxytryptamine without the use of releasing agents such as the Rauwolfia alkaloids. Also, its greater effect on 5-hydroxytryptamine as opposed to noradrenaline formation provides a way of distinguishing some of the functions of these two amines in the body. Lastly, it provides a possible mode of treatment for human subjects suffering from the effects of excessive 5-hydroxytryptamine production by carcinoid tumours.

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EFFECT OF DENERVATION AND OF COCAINE ON THE ACTION OF SYMPATHOMIMETIC AMINES

BY

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The secretory effect of sympathomimetic amines on the submaxillary gland of cats was increased after section of the chorda tympani (preganglionic, parasympathetic supply). After sympathetic denervation of the gland the secretory response to tyramine and phenylethylamine was absent, the response to dopamine and ephedrine decreased and the response to adrenaline and noradrenaline increased. Large doses of cocaine, given locally into the gland, produced changes similar to those observed after sympathetic denervation. The sensitization towards adrenaline and noradrenaline was obtained with smaller doses. Tyramine did not cause a release of catechols from the suprarenal glands of the cat.

It is known that sympathetic denervation has different effects on the response of the nictitating membrane to different sympathomimetic amines (Fleckenstein and Burn, 1953). Some amines are no longer effective shortly after denervation and these are considered not to have a direct action upon the effector organ. The response to other amines is increased, and these are supposed to exert a direct action on the effector organ. Thirdly, there is an intermediate group of substances which exert some effect even after denervation; these are believed to have both a direct and indirect mode of action.

In a number of tissues cocaine affects the response to sympathomimetic amines in the same way as sympathetic denervation: the effect of an indirectly acting substance, like tyramine, is decreased, while the response to substances which act directly, like adrenaline, is increased (Fröhlich and Loewi, 1910; Tainter and Chang, 1927; Burn and Tainter, 1931; Burn, 1932; Emmelin and Muren, 1951; Fleckenstein and Bass, 1953; Fleckenstein and Stöckle, 1955).

The salivary glands offer a special advantage for a comparison of the effects of cocaine and denervation; a high dose of cocaine can be given locally, via the secretory duct, to affect only one of the two submaxillary glands.

METHODS

Cat submaxillary glands were denervated sympathetically by excision of the superior cervical ganglion under ether anaesthesia. Acute experiments were carried out under chloralose anaesthesia.

The drugs were given intravenously into the femoral vein or via a cannula in the lingual artery (in 0.1 ml. saline during 10 sec.) after ligating all arterial branches except that to the submaxillary gland. When substances were given by the submaxillary duct, the injections were done as described by Emmelin, Muren, and Strömblad (1954).

The drops of saliva falling from the cannula inserted in the submaxillary duct were recorded by a signal on a smoked drum. The cannulae used delivered 1 ml. of distilled water in 40 drops.

The suprarenal glands were perfused according to Emmelin and MacIntosh (to be published).

RESULTS

Secretory Responses Due to Drug Action.—The effects of DL-phenylalanine, DL-tyrosine, dopa, β-phenylethylamine, tyramine, dopamine (3-hydroxytyramine) and l-ephedrine were studied. The three amino acids, tested by intravenous injection, phenylalanine (20 mg./kg.), tyrosine (20 mg./kg.), and dopa (2 mg./kg.), did not have any secretory activity.

With phenylethylamine, tyramine, dopamine, and ephedrine a secretion was obtained both by intravenous and close-arterial injection. The threshold doses, in normally innervated submaxillary glands, for intravenous injections were: for phenylethylamine about 5 mg./kg., for tyramine and dopamine 50 to 200 μg./kg. and for ephedrine about 2 mg./kg.

The responses refer to the first dose given; it was found that all these compounds exhibited

tachyphylaxis and cross-tachyphylaxis. In Fig. 1 an experiment is shown in which cross-tachyphylaxis between phenylethylamine and tyramine occurred. This figure is taken from an experiment on an animal in which both chordae tympani had been cut three weeks earlier; this led to the sensitization of the glands to these substances which will be discussed below. Phenylethylamine (1 mg.) was given close-arterially to the left gland and 14 drops were secreted; the same dose given to the right gland led to a secretion of 9 drops. After a second dose of phenylethylamine to the left gland, the response was 12 drops, that is, a slightly reduced amount. An intravenous infusion of tyramine was then started, with 100 mg. given during 1 hr. When a second dose of phenylethylamine was then applied to the right gland no secretion occurred. Acetylcholine, tested before and after the infusion, gave identical responses from the right gland. Thus the tyramine had inhibited the response to phenylethylamine on the right side; without the infusion the effect would only have been diminished by as little as on the left side.

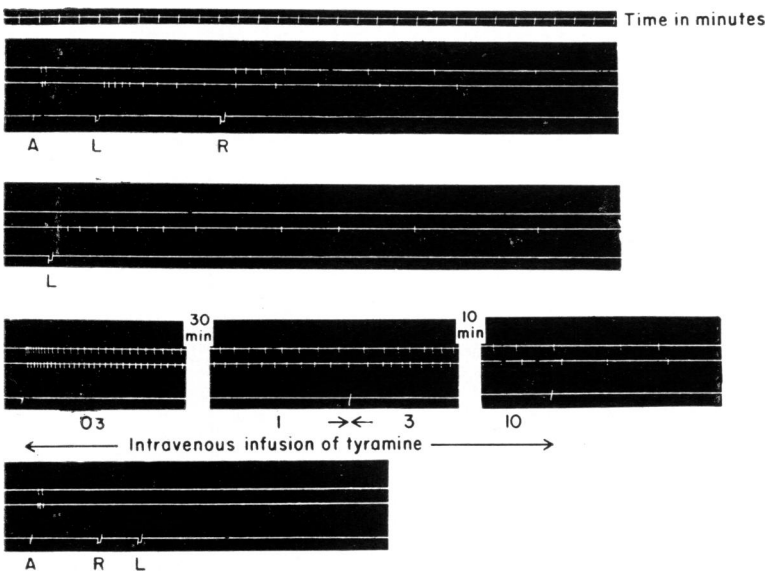

FIG. 1.—Cross-tachyphylaxis for phenylethylamine and tyramine in cat submaxillary gland. Cat 2.9 kg. Right and left chorda tympani cut 3 weeks earlier. Top, time tracing in min. In each of the other tracings the three records are from above: secretion, in drops, from right gland; secretion, in drops, from left gland; signal. A, Injection of 10 μg./kg. of acetylcholine intravenously. L and R: injection into the left (L) and right (R) lingual artery of 1 mg. of phenylethylamine during 10 sec. in a volume of 0.1 c.c. The infusion rate of tyramine in mg./min. is given under the record. A total of 100 mg. tyramine was infused during one hour.

Similar results were obtained when dopamine or l-ephedrine were tested in single doses with tyramine infusions, and also with tyramine as the test substance and an infusion of 100 mg. ephedrine in 1 hr. A single dose of ephedrine (3 mg./kg.) did not abolish the response to tyramine.

Section of Chorda Tympani.—Three to five weeks after section of the parasympathetic (preganglionic) supply to the submaxillary gland, supersensitivity was always present towards phenylethylamine, dopamine, tyramine, and ephedrine. In a cat, operated on one side three weeks before the experiment, the secretory response to an intravenous injection of 5 mg./kg. of phenylethylamine was 27 drops on the operated side and a just perceptible secretion, amounting to less than a drop, on the normally innervated side. Dopamine (100 μg./kg.), tested in another animal, gave 25 drops on the operated, and a trace of secretion on the normally innervated side. Similar observations have already been reported for ephedrine (Emmelin and Muren, 1951) and for tyramine (Strömblad, 1956).

Sympathetic Denervation.—Three weeks after ganglionectomy the secretory responses to both tyramine and phenylethylamine were abolished even when these substances were given in large doses (up to 5 mg./kg. of tyramine and 20 mg./kg. of phenylethylamine). An acute excision of the superior cervical ganglion did not affect the secretory response of the gland.

A secretion from the gland in response to dopamine and ephedrine was still obtained three weeks after sympathetic denervation. With these substances, the threshold on the denervated side was lower than on the innervated side, but the secretory response to larger doses was greater for the gland with its sympathetic nerve supply intact.

Effects of Cocaine.—Advantage was taken of the increased responsiveness of the submaxillary gland after section of the chorda tympani. In these experiments, cocaine was

given either intravenously or by the glandular duct. It is known that 2 mg./kg. of cocaine, given intravenously, causes a supersensitivity to adrenaline and noradrenaline (Emmelin and Muren, 1951). This was confirmed in the present experiments. On the other hand, it was found that the response to tyramine decreased. In two cats previously given cocaine (2 mg./kg.) intravenously, 100 μg./kg. of tyramine did not cause any secretion; this is a dose to which all other parasympathetically denervated glands had responded. After this dose of cocaine there was, however, no complete loss of responsiveness to tyramine, since 500 μg./kg. of tyramine still caused a flow of saliva. No detailed quantitative analysis of the effect of cocaine given intravenously was attempted in view of the tachyphylaxis already referred to.

Cocaine, 0.1 mg. administered by the submaxillary duct, regularly caused a supersensitivity to adrenaline and noradrenaline. A further increase in sensitivity was usually noted when 1 mg. of cocaine was given by the same route; when this dose was followed, in 2 cats, by an intravenous injection of 2 mg./kg. of cocaine, no further increase in sensitivity was seen. In 3 cats, 3 successive injections of cocaine, 0.1 mg., 1 mg., and 10 mg., were made in intervals of 1 hr. In one of these experiments, the secretory responses were measured after 5 μg./kg. of noradrenaline intravenously, and after sympathetic stimulation for 1 min. they were as follows:

	After noradrenaline	After sympathetic stimulation
Before cocaine	1 drop	16 drops
After 0.1 mg. cocaine ...	15 drops	14 „
After further 1 mg. cocaine	23 „	15 „
After further 10 mg. cocaine	19 „	0 „

Thus the sensitization of the response to noradrenaline was fully developed with doses of cocaine much lower than those necessary to block completely the effect of sympathetic stimulation.

Tyramine was similarly tested. Secretion was seen as long as stimulation of the sympathetic nerve was effective. When no secretion occurred on stimulation of the sympathetic, even large doses of tyramine were ineffective; it was confirmed that small doses of acetylcholine were still effective.

In some of the experiments, the large doses of cocaine necessary to block the effect of sympathetic stimulation also abolished the response to acetylcholine; such experiments were discarded. To avoid such an unspecific effect of cocaine, a procedure was eventually adopted in which the big dose of cocaine was replaced by two smaller doses given with a time interval. The following observations were made in one of these experiments: before any cocaine was given the secretion after 20 μg./kg. of noradrenaline intravenously was 1 drop on each side. After 5 mg. of cocaine given by the right submaxillary duct, the response of the right gland to the same dose of noradrenaline was 20 drops, the response from the left gland was 2 drops. One hour later 5 mg. of cocaine was given, via the duct, on the right side. The response to the intravenous test dose of noradrenaline was now 17 drops from the right gland and 19 drops from the left gland. Obviously, by now cocaine had reached the left gland and had rendered it supersensitive to noradrenaline. Sympathetic stimulation on the right side was now tested and found to be ineffective, but on sympathetic stimulation of the left side, secretion of saliva occurred. Tyramine (1 mg./kg. intravenously) was then tested: 6 drops of saliva were counted on the left side; no secretion occurred on the right side. This experiment reinforces the conclusion drawn from the experiments already described: with cocaine it is possible to achieve maximal sensitization towards adrenaline and/or noradrenaline without blocking the response to sympathetic stimulation. The experiment also shows that a block of the sympathetic nerve by cocaine abolishes the effect of tyramine.

The responses to both dopamine and ephedrine were diminished, but not abolished, when the response to sympathetic stimulation was blocked by cocaine. The effects of section of the chorda tympani, of sympathetic denervation, and of small and big doses of cocaine on the secretory response to various amines are summarized in Table I.

Perfusion of the Suprarenal Glands.—It has been suggested by Burn and Rand (1958a) that the action of tyramine is mediated by a release of catecholamines. It was therefore of interest to study the effect of tyramine on the perfused suprarenal gland, in order to find out if in this location evidence could be obtained for a releasing action of tyramine.

Three experiments were performed in which the suprarenal glands of cats were perfused with oxygenated Tyrode solution. Each perfusion lasted 5 min.; the volume perfused was between 4 and 6 ml. For two periods in each animal, either 1 μg./ml. or 10 μg./ml. or 100 μg./ml. of

TABLE I

THE EFFECT OF VARIOUS PROCEDURES ON THE SECRETORY RESPONSE FROM THE SUBMAXILLARY GLAND OF CATS

+ = Increased secretory response, − = decreased secretory response, +− = increased response to small doses and decreased response to bigger doses, 0 = no secretory effect even with very big doses.

Drug	Threshold Dose on Normally Innervated Glands	Secretory Response After			
		Para-sympathetic Denervation	Sympathetic Denervation	Cocaine 2 mg. Intravenously	Cocaine 10 mg. into the Sub-maxillary Duct
β-Phenylethylamine ..	2–5 mg./kg.	+	0	−	0
Tyramine	50–200 μg./kg.	+	0	−	0
Dopamine	50–200 ,,	+	+−	+−	+−
l-Ephedrine	2–5 mg./kg.	+	+−	+−	+−
Noradrenaline	5–50 μg./kg.	+	+	+	+
Adrenaline	5–50 ,,	+	+	+	+

tyramine were added. The perfusates were tested against adrenaline on the cat submaxillary gland with the chorda tympani cut and the superior cervical ganglion excised 3 weeks before; in this way a contributory effect on the gland of the tyramine present in the perfusate could be avoided.

In one of these experiments the activity found in the perfusates, expressed in μg./min. of adrenaline, was as follows:

Perfusion Fluid
Tyrode 1.2 (1st period) 0.9 (5th period)
Tyrode containing 1 μg./ml. of tyramine
0.9 (2nd period) 0.8 (6th period)
Tyrode containing 10 μg./ml. of tyramine
1 (3rd period) 0.9 (7th period)
Tyrode containing 100 μg./ml. of tyramine
1 (4th period) 0.6 (8th period)

The results obtained with the two other animals were very similar to those just given. The activity of the perfusates was not affected by atropine (1 mg.), but it was blocked by dihydroergotamine (1 mg.).

DISCUSSION

Excision of the superior cervical ganglion affects the secretory response of the submaxillary gland as well as the response of the nictitating membrane to sympathomimetic amines. Tyramine and phenylethylamine completely lost the power of causing secretion, while, as is well known, the secretory responses to adrenaline and noradrenaline were augmented. Dopamine and ephedrine represented an intermediate group: doses near the threshold were more effective after sympathetic denervation, while bigger doses were less effective than on normally innervated glands; this could be explained by assuming that the denervated gland had become sensitized to the direct effect of these two compounds, but that the indirect action had been abolished.

The observations on cocaine are of particular interest. In a high dose cocaine not only blocked the effects of sympathetic stimulation, but it also had other effects identical with those seen after excision of the superior cervical ganglion, that is, a sensitization of the gland to the direct effects of the amines and an abolition of their indirect actions. Sensitization was also fully developed after small doses of cocaine, but these did not extinguish the effects of substances believed to act indirectly. These observations are in accordance with those of Fleckenstein and Stöckle (1955) on the nictitating membrane; these authors found it possible to sensitize the membrane to substances like adrenaline or noradrenaline with smaller doses of cocaine than are necessary to diminish the response to indirectly acting substances, for example, tyramine. These two effects are probably brought about by different mechanisms because different doses of cocaine are required to cause a super-sensitivity to noradrenaline on the one hand, and to abolish the response to tyramine on the other.

These mechanisms are unknown, but the experiments reported demonstrate that a functionally intact sympathetic supply is necessary for the indirect effect of tyramine. Experiments with reserpine (Burn and Rand, 1958a) suggest that a local store is required for the indirect action. Consequently, it was suggested that tyramine and similar amines act by release of catechol amines.

It is conceivable that cocaine acts on these stores, which presumably are located in or near the nerve terminals. The experiments reported suggest that the sensitization of the effector organ to adrenaline and similar compounds is not brought about by the same mechanism.

In the experiments on the perfused suprarenal glands, tyramine did not cause a release of catechol amines. Thus, either the indirect action of tyramine and similar amines is not to release catechol amines, as discussed above, or the mechanism for release in the medulla of the suprarenal gland differs from that in the submaxillary gland and the nictitating membrane.

It seems possible that the chromaffin tissue present in the adrenal medulla, or in the skin of the rabbit ear (Burn and Rand, 1958b), or in the human skin (Adams-Ray and Nordenstam, 1956) represents one type of store that can be released by nicotine; this has already been discussed elsewhere (Strömblad, 1959). The adrenergic neurones, on which tyramine and similar substances might act as releasers, may represent a second type of store of catechol amines. Some support for this hypothesis might be found in the experiments by Ginzel and Kottegoda (1953), who observed that the constrictor action of nicotine in the rabbit ear was still present three weeks after sympathectomy. On the other hand, the present experiments have shown that the effect of tyramine is abolished by factors which interfere with the sympathetic nervous supply.

The finding of a cross-tachyphylaxis for all the sympathomimetic amines which act indirectly suggests a similar mechanism of action for all these compounds.

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EFFECTS OF ERGOMETRINE (ERGONOVINE) ON THE ISOLATED ATHEROSCLEROTIC HEART OF THE CHOLESTEROL-FED RABBIT

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Ergometrine usually depresses the S-T segment as in coronary insufficiency, when injected intravenously in rabbits with experimental coronary atherosclerosis and in patients with effort angina, but not in normal animals and man. To explain this difference, we carried out Langendorff perfusion studies in 32 normal and 29 atherosclerotic isolated rabbit hearts. Preliminary tests with ergometrine were done to ensure that advanced coronary atherosclerosis had developed in the rabbits fed a cholesterol diet; pathological examination of the heart after perfusion confirmed the result of the final test with ergometrine. Before drugs were perfused, the basal rate of coronary flow was greater, the heart rate was slower and the contractile amplitude was smaller in the atherosclerotic than in the normal hearts; nitroglycerin markedly increased flow in both normal and atherosclerotic groups. Ergometrine consistently caused a reduction in contractile amplitude with negligible changes in heart rate in both normal and atherosclerotic hearts. On coronary flow, however, the effects of ergometrine differed significantly in these groups; in doses of between 0.2 and 0.4 mg., the average decrease in flow was 8% in normal and 22% in atherosclerotic hearts. The effect was more variable in normal hearts and an increase in flow sometimes occurred. The difference in the response of normal and atherosclerotic hearts was particularly striking when ergometrine was given during recovery from a reduction of coronary flow which had been induced by vasopressin. Ergometrine then uniformly increased flow in the normal, but usually had the opposite effect in the atherosclerotic heart. In normal and atherosclerotic hearts, cardiac effects of vasopressin were similar. Tachyphylaxis to vasopressin, but not to ergometrine, was observed.

Ergometrine injected intravenously produces transient S-T segment depression characteristic of coronary insufficiency in patients with coronary artery disease (Stein, 1949; Stein and Weinstein, 1950, 1953; Rinzler, Stein, Bakst, Weinstein, Gittler, and Travell, 1954), and in rabbits with experimental coronary atherosclerosis (Rinzler, Travell and Karp, 1955; Rinzler, Travell, Karp, and Charleson, 1956). Such electrocardiographic changes after ergometrine are not seen in man or animal with a normal coronary circulation.

Although effects of ergometrine on heart rate and amplitude had been investigated in the normal frog (Davis, Adair, Chen, and Swanson, 1935), rabbit (Brown and Dale, 1935) and cat (Rothlin, 1935), we found no reports concerning the actions of this drug directly on the coronary circulation. We therefore investigated the mechanism of the electrocardiographic effects of ergometrine by perfusion of the isolated heart in normal rabbits and in those rendered atherosclerotic by cholesterol feeding.

It was found that the atherosclerotic isolated heart can be satisfactorily perfused and serves like the normal heart for studying drug effects on coronary flow, heart rate, and amplitude of contraction. Furthermore, satisfactory pathological detail of the coronary arteries is obtained on section of the heart after perfusion. Preliminary reports (Karp, Penna, Rinzler, and Travell, 1956; Karp, Travell, and Rinzler, 1956; Travell, Karp, and Rinzler, 1957) have been made of what we believe are the first perfusion studies of the isolated atherosclerotic heart. The present

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report deals with the methods, the state of the isolated atherosclerotic heart, and the effects of ergometrine on this preparation.

METHODS

Animal Care.—Male Dutch rabbits (Loryctolageus Cuniculus Domesticus) were housed in single cages in an air-conditioned room. They were placed first on a stock diet for 10 days, and if conjunctivitis, coryza, or diarrhoea developed, they were given sulphamethylthiazole (Sulmet-Lederle) intraperitoneally every other day for 3 doses of 200 mg. each. Two groups of rabbits were then started at the same time either on the stock diet or on a similar diet with added cholesterol. The diets were fed ad libitum.

Stock and cholesterol diets were commercially prepared (Vi-D-Co). The stock diet comprised: rolled oats 7.5 g.; ground corn 27.6 g.; dried skim milk 7.5 g.; grey shorts 7.5 g.; 17% dehydrated alfalfa 37.2 g.; bone meal 2.0 g.; calcium carbonate 1.5 g.; mineral salt 1.0 g. Either 2% cholesterol with 6% corn oil, or 2% cholesterol alone, was added to the stock diet. When the rabbits nibbled pellets prepared with corn oil, these crumbled into a powder which could not be ingested. A firmer pellet was made by coating the stock pellets with crystalline cholesterol dissolved in ether.

Rabbits may contract coccidiosis if the drinking water becomes contaminated with faecal matter. Water was supplied from hanging bottles with metal drinking spouts. Bottles, feed containers, and cages were sterilized by steam once a week. No insecticide sprays were used. Removable pans beneath the wire mesh floor were washed with a disinfectant solution of substituted dimethylbenzyl ammonium chloride (Hytron).

Ergometrine Test.—Serial ergometrine tests on cholesterol-fed and control animals were done after 6 to 8 weeks on the diets and thereafter usually at weekly intervals until the test became positive. The general procedure has been described (Rinzler *et al.*, 1955, 1956). Rabbits were anaesthetized with pentobarbitone sodium (Nembutal Sodium Veterinary Abbott) 15 mg./kg. intravenously, or 10 mg./kg. in some atherosclerotic rabbits. The corrosive action of this alkaline solution caused sclerosis of ear veins and created technical difficulties on repeated testing until we reduced the *p*H slightly by adding dilute hydrochloric acid just before injection and also diluted the dose with an equal part of 0.85% sodium chloride.

The general condition of the cholesterol-fed rabbits was usually good, although they were sluggish in behaviour and tended to lose weight gradually after the initial period of a few weeks on the diet (Rinzler *et al.*, 1956). Thus, when placed on the diet, the average weight of the 29 cholesterol-fed animals was 1.87 kg. (1.47 to 2.29 kg.) and 1.67 kg. (1.07 to 2.73 kg.) when sacrificed for coronary perfusion. In contrast, the average weight of the 32 control rabbits initially was 1.76 kg. (1.27 to 2.47 kg.), and 1.93 kg. (1.19 to 2.63 kg.) when the experiment was terminated.

Control electrocardiograms were taken before, and 5 and 10 min. after, pentobarbitone injection. Lead II and a precordial lead approximating to V_4 were taken. Ergometrine maleate 0.05 mg./kg. was injected intravenously, and leads II and V_4 were repeated after 1, 3, 5, 7 and 10 min. or longer, depending on the duration of the effect. Depression of the S-T segment, if it occurred, was usually most marked 3 to 5 min. after injection, and as a rule lasted 15 to 30 min. but in some instances 1 to 2 hr. The ergometrine test is positive if the S-T segment is depressed transiently by 0.5 mm. or more below the isoelectric level.

One or two days after the ergometrine test became positive, the atherosclerotic heart was isolated and the coronary circulation perfused. In control rabbits which were fed on the stock diet for approximately equal periods of time, the heart was similarly isolated and perfused.

Perfusion. — A modification of the basic Langendorff perfusion apparatus (Anderson type) was used; the modified apparatus has been described by Garb, Penna, and Scriabine (1955), and is shown in Fig. 1. For the operation, rabbits were anaesthetized with pentobarbitone sodium 30 mg./kg. intraperitoneally. We followed Garb's surgical technique for isolating the heart, with minor modifications. In the atherosclerotic rabbit, care was needed not to perforate the often rigid and fragile aorta when the cannula was inserted. The heart was suspended in air by the cannula and amplitude of contraction was recorded from the apex of the heart. Perfusion with oxygenated Ringer-Locke solution (NaCl 7.0 g./l.; $NaHCO_3$ 2.1 g./l.; KCl 0.42 g./l.; $CaCl_2$ 0.21 g./l.; $MgCl_2$ 0.2 g./l.; and dextrose 1.8 g./l.; *p*H 7.2 to 7.4 at 37°) at a pressure of 52 cm. of water was started as soon as the cannula was connected with the perfusion system. The aortic valve remained closed at this perfusion pressure, and the solution flowed into the coronary arteries. The perfusate dripped from the pulmonary artery and was not recirculated. Water at 37° was circulated between the double walls of the glass chamber. The heart rate and amplitude of contraction were read from an ink writing kymograph. The rate of coronary flow was measured by a flowmeter with a glass ball (Emil Greiner Co.); readings on an arbitrary scale were converted to ml./min. by reference to the calibration curve of the flowmeter for water. In analysing the data, diastolic values were used for coronary flow.

It usually required about 15 min. for stabilization of baselines after starting the perfusion with Ringer-Locke solution. While this was continuously perfused, drugs were injected into the system close to the heart by means of a syringe attached to the upper end of a fine polyethylene catheter, the lower end of which opened within the bulb of the aortic cannula. The total time of injection was approximately 15 sec.

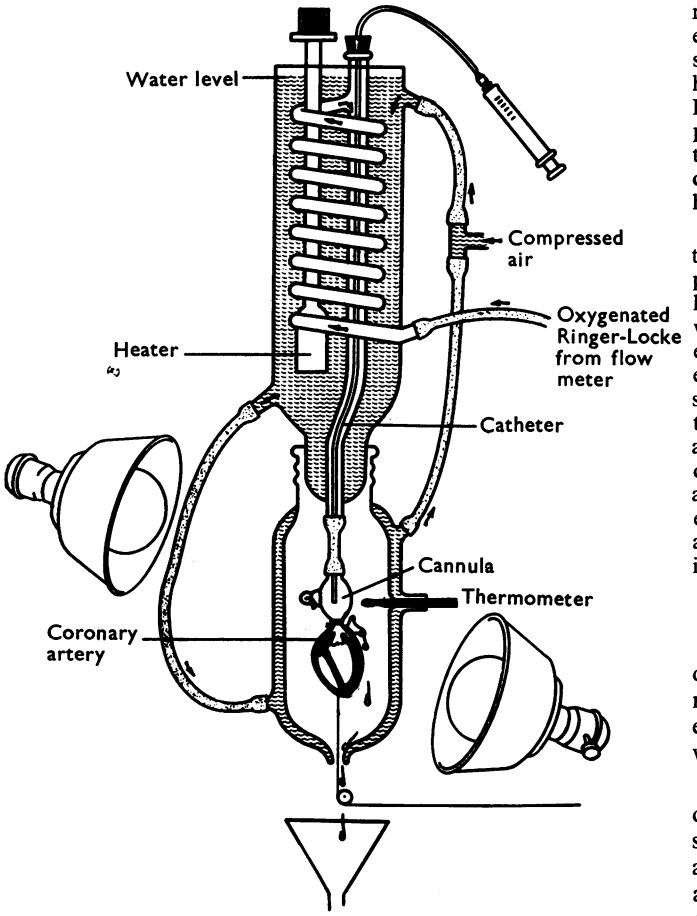

FIG. 1.—Diagram of the modified Langendorff perfusion apparatus.

for each dose. This method simulates the peak concentration of an intravenous injection in the intact animal. Each drug was injected in a volume of about 1 ml., and was followed at once by Ringer-Locke solution in a second syringe to make the volume 2 ml. and to wash the drug through the catheter. The capacity of the catheter was 0.3 ml. Control injections of 2 ml. of Ringer-Locke solution were made during every experiment. The effects of drugs were observed for at least 4 min., and longer if the effects lasted longer. Ringer-Locke solution alone was then perfused for 4 to 5 min. before the next dose or drug was given, except when ergometrine was injected while vasopressin acted. Continuous records were taken throughout the experiment, which usually lasted from 2 to 3 hr.

Drugs.—The drugs perfused in this study were: ergometrine (U.S.P. ergonovine maleate); purified arginine-vasopressin free of oxytocin, supplied by Dr. Vincent du Vigneaud as a solution (0.8 u./ml.) recently assayed by the blood pressure effect in the rat and without any preservative; and nitroglycerin, as 0.65 mg. hypodermic tablets dissolved in Ringer-Locke solution just before injection. In preliminary experiments, we determined the approximate threshold and maximal doses of these drugs for the isolated rabbit heart.

Pathology.—After perfusion was ended, the heart was fixed in formalin. For pathological study, cross-sections at 3 levels (base, centre, and apex) were stained with haematoxylin-eosin, Weigert (for elastin) and oil-red-O (for fat). In the ergometrine-positive rabbits with atherosclerosis of the small coronary arteries, the arterial changes were roughly graded as follows: 1+ subintimal foam cells without encroachment on the lumen of any artery sectioned; 2+ subintimal plaques extending partially into the arterial lumen; and 3+ obliteration of the lumen by subintimal atheroma in one or more arteries.

RESULTS

A total of 29 atherosclerotic hearts of cholesterol-fed, ergometrine-positive rabbits and 32 normal hearts of ergometrine-negative control rabbits were perfused with the various drugs.

Pathological Changes.—Most (96%) of the ergometrine-positive rabbit hearts showed advanced (2+ to 3+) intimal atherosclerosis of the small intramural arteries (Figs. 2 and 3); one heart showed 1+ atherosclerosis. The larger arteries seldom showed such changes. In the ergometrine-negative control hearts, atherosclerotic changes in the coronary arteries were not seen.

After perfusion, in both groups the myocardium appeared oedematous with separation of muscle bundles. However, in the non-perfused hearts of similar ergometrine-positive cholesterol-fed rabbits (Rinzler *et al.*, 1955, 1956), the myocardium showed diffuse fatty infiltration, spotty hyaline degeneration, and occasionally the fibrosis of old infarction (Fig. 3). Fragmentation of the elastic membrane beneath the subintimal plaques was also evident at many sites (Fig. 2).

Basal State of Isolated Heart.—Initially and before any drug, the coronary flow, heart rate, and amplitude of contraction were different in the normal and atherosclerotic hearts (Table I). In the atherosclerotic hearts, the average rate of coronary flow was about 4 ml./min. (24%)

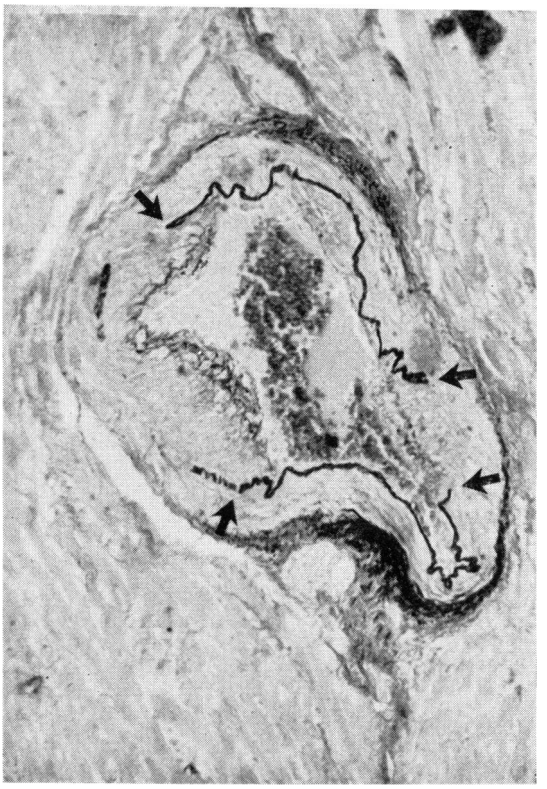

FIG. 2.—Photomicrograph of a small coronary artery showing lumen partially occluded by subintimal plaques (2+ atherosclerosis) and fragmentation of underlying elastic membrane at margins of plaques, as indicated by arrows (Weigert stain). In this ergometrine-positive rabbit (11 weeks on cholesterol diet), the heart was sectioned immediately after death and was not perfused.

greater, the average heart rate was 25 beats/min. (17%) slower, and the average amplitude of contraction was 6 mm. (19%) less than in the normal hearts. No correlation was evident between basal coronary flow and heart weight, which ranged from 6.7 to 12.2 g. for 9 atherosclerotic, and from 6.6 to 11.6 g. for 23 normal hearts.

To determine whether the higher level of basal coronary flow in the atherosclerotic heart showed a correlation with the maximal capacity of the coronary bed to dilate, nitroglycerin in graded doses was injected in 8 experiments on atherosclerotic hearts, and, for comparison, in 7 experiments on normal hearts. Fig. 4 illustrates the typical effects of a maximal dose of nitroglycerin

TABLE I

INITIAL STATE OF ISOLATED PERFUSED HEART OF RABBIT

Figures in parenthesis indicate range.

Group	Total Hearts (No. of Expts.)	Coronary Flow (ml./min.)	Heart Rate (Beats/min.)	Amplitude of Contraction (mm.)
Normal	32	15·7 (9–28)	151 (98–200)	33·0 (7–79)
Atherosclerotic	29	19·5 (8–32)	126 (36–196)	26·3 (6–52)
	t test	$p<0·01$	$p<0·01$	$p<0·001$

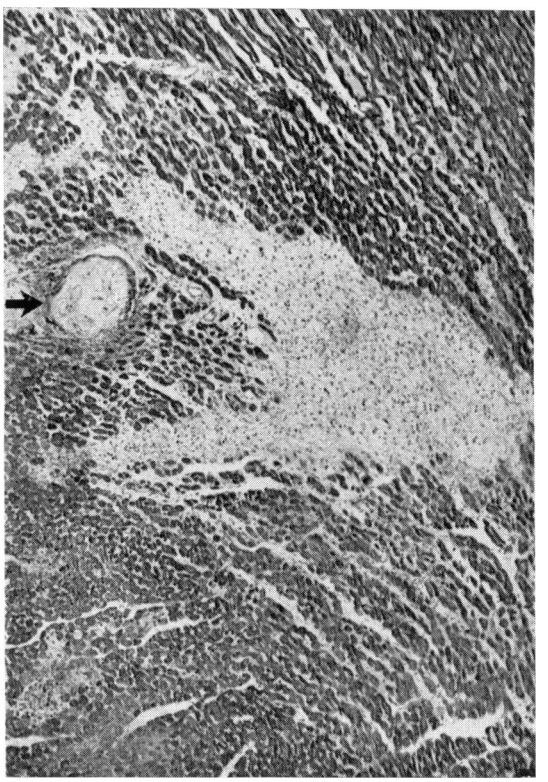

FIG. 3.—Photomicrograph of an old myocardial infarct with adjacent occluded small coronary artery (3+ atherosclerosis) at arrow (Masson stain). The heart was perfused before it was sectioned (rabbit 18 weeks on cholesterol diet).

in a normal and an atherosclerotic heart. In both, a marked increase in coronary flow occurred; in the normal, flow rose to 33 ml./min., and, in the atherosclerotic heart, to 29 ml./min., with little change in heart rate and slight reduction in amplitude of contraction. The highest level of coronary flow achieved with the maximal dose of nitroglycerin in the 7 normal hearts averaged 28.8 ml./min., and for the 8 atherosclerotic hearts 25 ml./min., a difference of 3.8 ml./min., or 13% higher in the normal group. In these small groups of animals, this difference was not statistically significant.

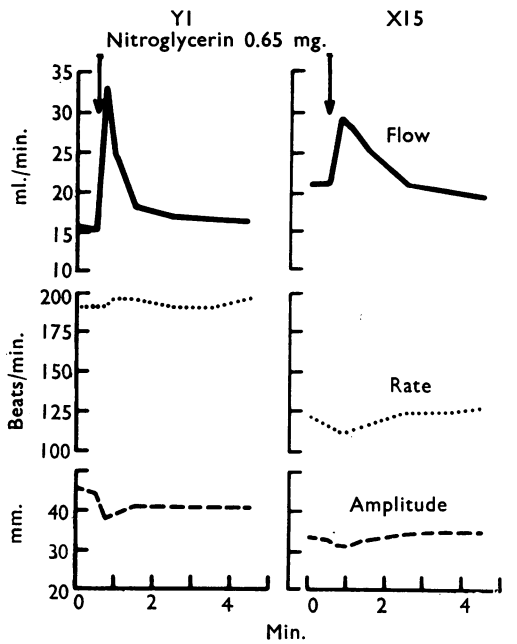

FIG. 4.—Response of perfused *normal* heart (Rabbit Y_1 at left) and *atherosclerotic* heart (Rabbit X_{15} at right) to a maximal dose of nitroglycerin. Note higher basal coronary flow in the atherosclerotic heart, but similar drug effects in both: increased flow and reduced amplitude of contraction with minor changes in heart rate.

Ergometrine.—Effects of graded doses of ergometrine (0.1 to 2.0 mg.) were studied in 15 atherosclerotic and 14 normal hearts; the average changes in coronary flow, heart rate, and contractile amplitude are shown in Fig. 5, and illustrative individual experiments on normal and atherosclerotic hearts in Fig. 6. Maximal changes usually occurred within 1 min. after injection of the dose, with a return to the control level within 2 min. In any given heart duplicate tests with the same doses gave results which agreed well (Fig. 7A). For example, in 25 comparisons with 61 doses of ergometrine on 13 hearts the mean reduction in coronary flow was 20.1% and the average difference from the mean 2.8% (S.E. ± 0.35).

Coronary flow was the only parameter that showed a significant difference in the response of the normal and atherosclerotic heart to ergometrine. Thus (Table II), after doses of 0.2 to 0.4 mg. in 47 tests on normal hearts, the average decrease in coronary flow was 8% as compared with 22% in 50 tests on atherosclerotic hearts. The greatest decrease in flow observed in any normal heart was 52%, whereas in the atherosclerotic group 3 hearts showed a decrease of 97%, 98%, and 100%, respectively. Furthermore, in the dosage range of 0.2 to 0.4 mg. ergometrine sometimes caused a significant increase (15% to 55%) in coronary flow in the 14 normal hearts, whereas in none of the 15 atherosclerotic hearts was there an increase in flow greater than 8%, which is comparable to the occasional effect of a control injection of Ringer-Locke solution.

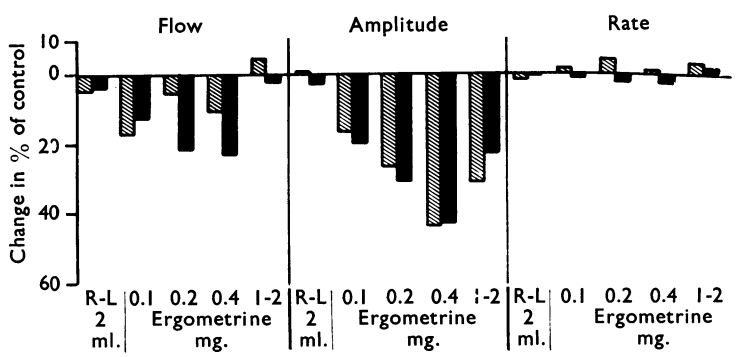

FIG. 5.—Average effects of graded doses of ergometrine on coronary flow, amplitude of contraction and heart rate in 14 *normal* (cross-hatched columns) and 15 *atherosclerotic* (black columns) perfused rabbit hearts. A control dose of Ringer-Locke 2 ml. was injected initially.

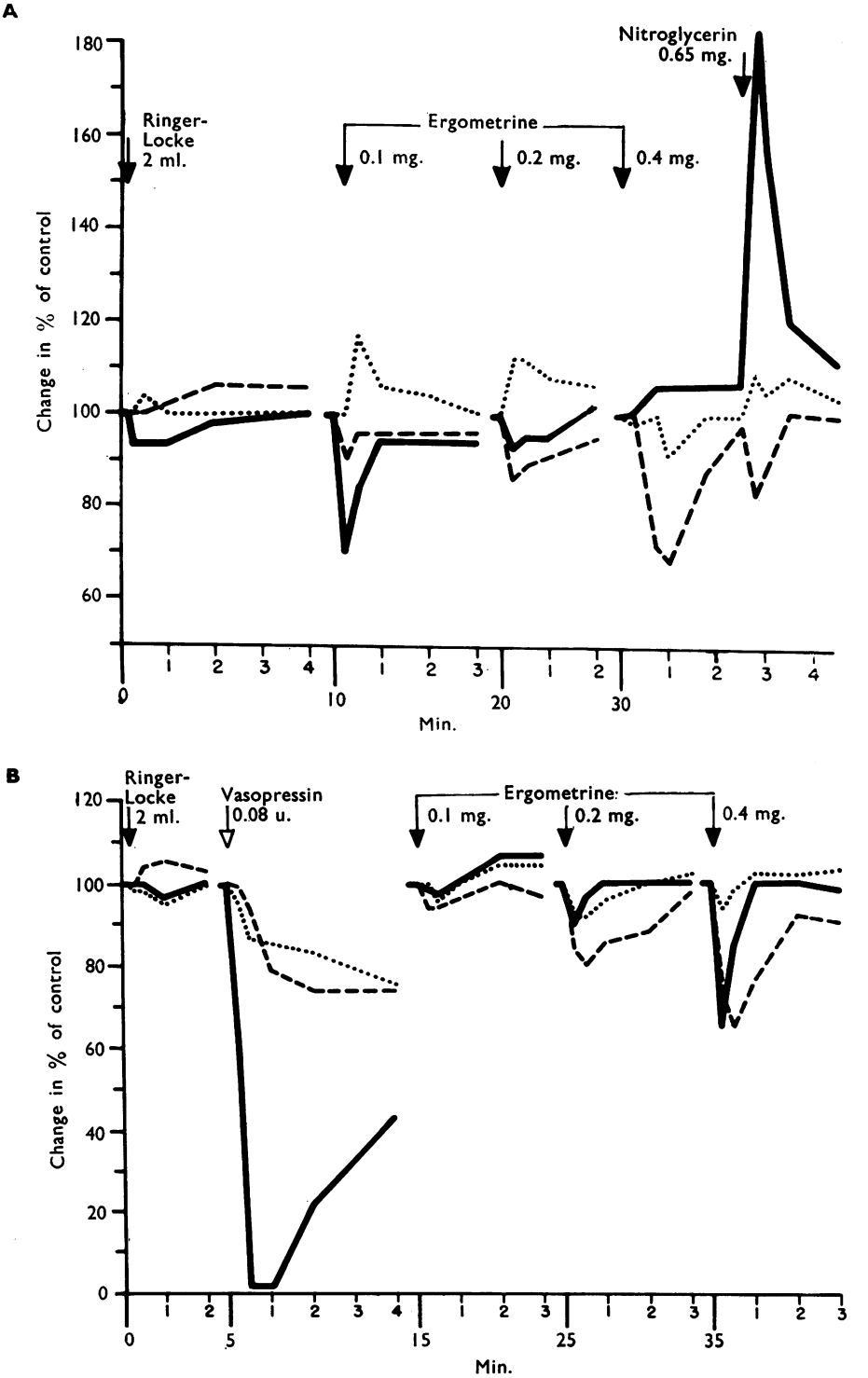

FIG. 6.—Illustrative experiments showing graded responses of flow ——, rate . . . . , and amplitude – – –, to 0.1 mg., 0.2 mg., and 0.4 mg. of ergometrine in (A) *normal* and (B) *atherosclerotic* hearts. Note also marked dilator effect of nitroglycerin after ergometrine in (A), and profound constrictor effect of vasopressin in (B).

Fewer tests were conducted with the smaller (0.1 mg.) and larger (1 mg. and 2 mg.) doses of ergometrine, and with these doses differences in the response of coronary flow in the normal and atherosclerotic groups were not statistically significant. However, for the small dose (0.1 mg.) in 19 tests on normal hearts, there was an average decrease of 17% in coronary flow, and in 20 tests on atherosclerotic hearts an average decrease of only 12%. With the largest doses (1 mg. and 2 mg.) the trend was toward a reversal of the vasoconstrictor effect; thus, in 11 tests on normal hearts coronary flow increased 6%, whereas in the atherosclerotic hearts it decreased only 2% (Fig. 5).

Amplitude of contraction decreased after all doses of ergometrine, and to about the same extent in normal and atherosclerotic hearts (Fig. 5, Table III). The % change was similar in both groups even though the average control amplitude for the atherosclerotic hearts was approximately one-half that for normal hearts before each dose of ergometrine. For example, after the 0.4 mg. dose the average decrease in amplitude in normal hearts was 44% from a level of 17.6 mm., and 43% from a level of 9.3 mm. in atherosclerotic hearts.

Graded responses in amplitude of contraction were obtained for doses of 0.1 mg., 0.2 mg., and 0.4 mg. of ergometrine. The average decrease for

TABLE II

COMPARISON OF EFFECTS OF ERGOMETRINE AND VASOPRESSIN ON CORONARY FLOW

Ergometrine effects were studied in 14 normal and 15 atherosclerotic hearts. Vasopressin effects were studied in 9 normal and 9 atherosclerotic hearts. Maximal change within 1 min. after injection is expressed in % of control (pre-injection) value. When duplicate tests were done, the average for each heart was used.

Coronary Arteries	Ringer-Locke 2 ml.		Ergometrine 0·2 to 0·4 mg.		Vasopressin 0·08 u.	
	Control Flow	Maximal Change	Control Flow	Maximal Change	Control Flow	Maximal Change
	ml./min.	%	ml./min.	%	ml./min.	%
Normal	15·2	−4·5	13·0	−8·1	12·6	−90·4
Atherosclerotic	21·0	−3·9	17·6	−22·0	17·5	−82·3
t test	$p>0·8$		$p<0·05$		$p>0·8$	

TABLE III

EFFECT OF GRADED DOSES OF ERGOMETRINE ON CARDIAC CONTRACTION AND RATE IN 14 NORMAL AND 15 ATHEROSCLEROTIC RABBIT HEARTS

Maximal change within 1 min. after injection is expressed in % of control (pre-injection) value. When duplicate tests were done, the average for each heart was used.

Drug and Dose	Amplitude of Contraction				Heart Rate			
	Control Level		Maximal Change		Control Level		Maximal Change	
	Normal	Athero.	Normal	Athero.	Normal	Athero.	Normal	Athero.
	mm.	mm.	%	%	Beats/min.	Beats/min.	%	%
Ringer-Locke 2 ml.	21·3	15·3	−0·1	−2·5	138	113	−1·5	−0·4
Ergometrine 0·1 mg. ..	24·6	13·9	−16·4	−19·9	141	115	+1·6	−1·8
0·2 ,, ..	21·4	11·6	−26·3	−30·3	132	113	+4·5	−2·5
0·4 ,, ..	17·6	9·3	−43·6	−42·9	150	115	+0·9	−2·8
1–2 ,, ..	16·7	8·7	−13·1	−22·6	138	114	+3·5	+2·1

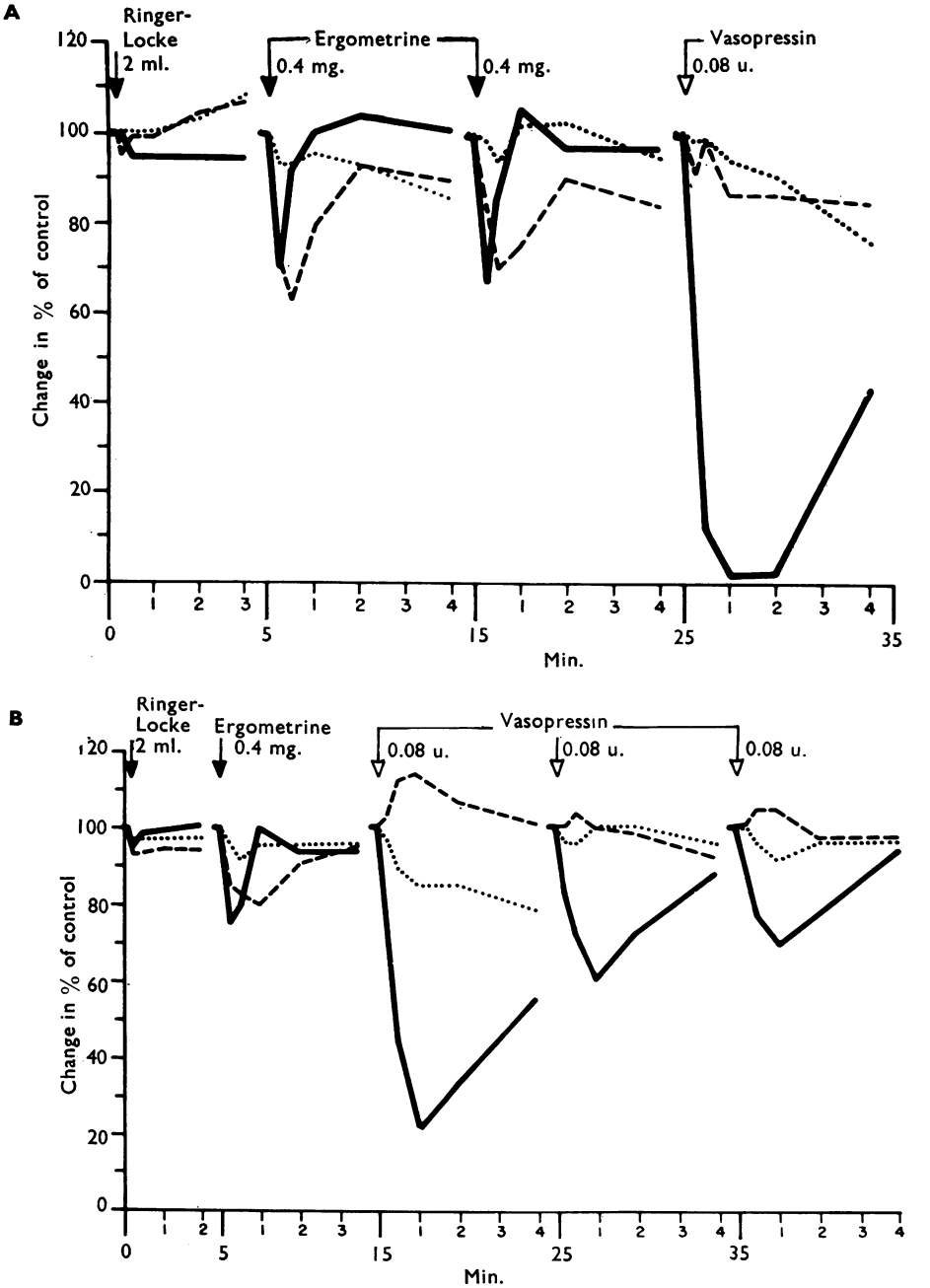

FIG. 7.—*Atherosclerotic* hearts. Flow ——, rate ...., and amplitude – – –. (A) Note reproducible effects of duplicate injections of ergometrine on all three parameters; also typical profound effect on flow of an initial dose of vasopressin, given after two doses of ergometrine. (B) Note limited reduction in flow after ergometrine as compared with initial dose of vasopressin, and tachyphylaxis to vasopressin when same dose was repeated three times.

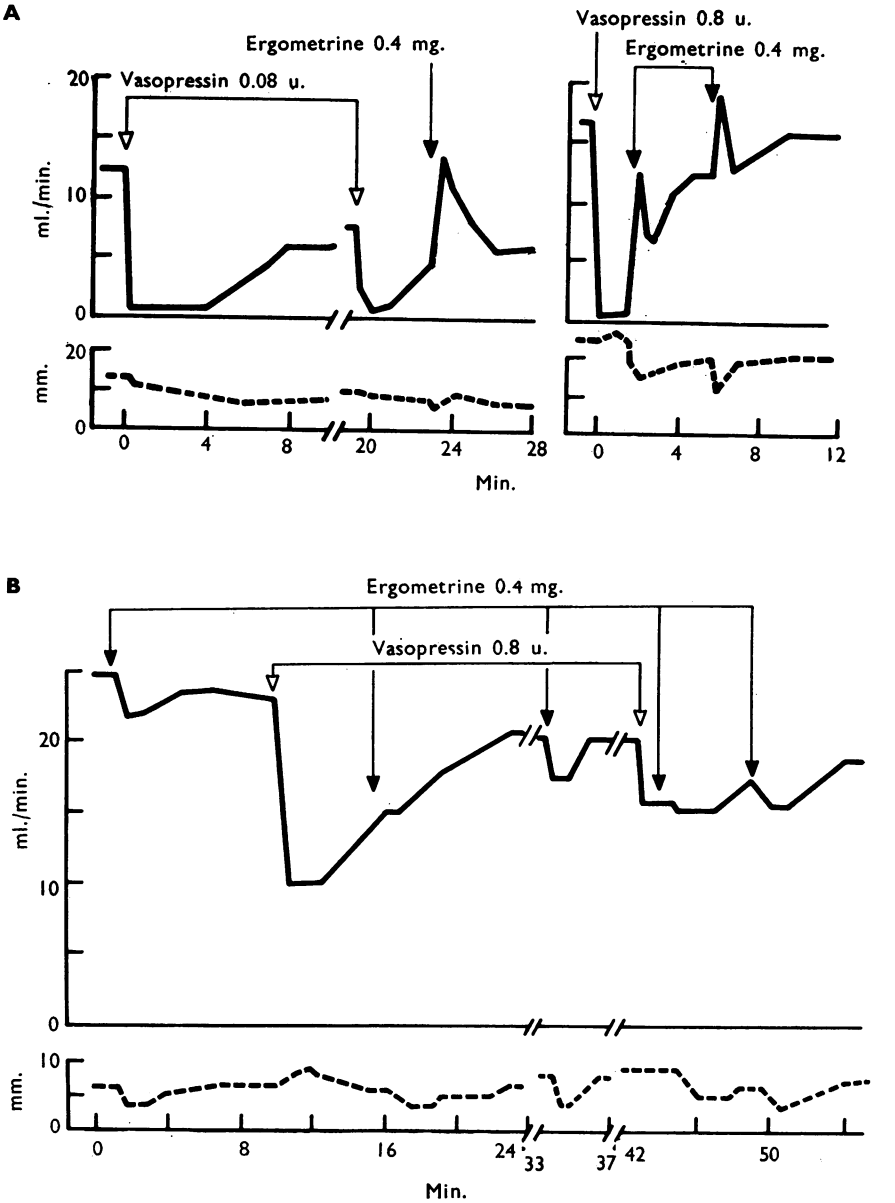

FIG. 8.—Responses of coronary flow —— and of amplitude - - -. (A) *Normal* hearts. Note coronary constrictor and negative inotropic effects of three doses of ergometrine (0.4 mg.) given during different phases of vasopressin action. (B) *Atherosclerotic* heart. Note coronary constrictor and negative inotropic effect of three doses of ergometrine (0.4 mg.) given during similar phases of vasopressin action. For comparison, control doses of ergometrine were given before and after the first dose of vasopressin. Heart rate was essentially unchanged in both *normal* and *atherosclerotic* hearts, and is not charted.

these doses in normals was −16.4%, −26.3%, and −43.6%, and in the atherosclerotic hearts, −19.9%, −30.3%, and −42.9%, respectively. However, with the larger doses (1 mg. and 2 mg.) the negative inotropic effect was less intense, namely, −13.1% and −22.6% in the respective groups (Table III).

The heart rate showed no consistent change after ergometrine (Fig. 5).

Vasopressin.—All hearts, normal and atherosclerotic, that were injected with ergometrine were tested with vasopressin; the dose of vasopressin ranged from 0.008 to 0.8 units. The effects of vasopressin showed striking differences from those of ergometrine.

Coronary flow was more profoundly reduced by the initial dose of vasopressin than by ergometrine both in normal and atherosclerotic hearts (Table II, Figs. 6B, 7 and 8). Thus, 0.08 units of vasopressin, maximal dose, decreased the average coronary flow by 90% in 9 normal hearts, and by 82% in 9 atherosclerotic hearts; this difference was not statistically significant (Table II).

Unlike ergometrine, successive identical doses of vasopressin yielded diminishing effects (Fig. 7B). This tachyphylaxis to vasopressin was observed for both atherosclerotic and normal hearts. The typical profound reduction in flow after the first dose of vasopressin was not blocked if ergometrine was given first (Fig. 7), nor did a prior dose of vasopressin modify the effect of ergometrine given after the vasopressin effects had finished (Figs. 6B and 8B). The reduction in coronary flow by vasopressin could be momentarily reversed by nitroglycerin.

Amplitude of contraction after vasopressin sometimes increased initially, but usually decreased if the reduction in coronary flow was profound and prolonged (Figs. 6B, 7 and 8A). In this respect, no essential difference was observed between atherosclerotic and normal hearts.

Heart rate after vasopressin usually decreased in both atherosclerotic and normal hearts (Figs. 6B, 7 and 8). Both contractile amplitude and heart rate returned to control levels more slowly than did coronary flow.

Ergometrine During the Action of Vasopressin.—When ergometrine (0.4 mg.) was given about 1 min. after vasopressin, when the coronary flow was most reduced, ergometrine caused no change in flow in either normal or atherosclerotic hearts (Table IV). However, when ergometrine was given while the vasoconstrictor action of vasopressin was wearing off, usually in 1.5 to 2 min. after vasopressin injection, the effect of ergometrine on coronary flow differed significantly for normal and atherosclerotic hearts (Table IV). In the normals, a transient rise in flow after ergometrine (8 doses) was uniformly superimposed on the rising slope of recovery from the vasopressin reduction (Fig. 8A). On the other hand, in the atherosclerotic hearts, only 1 of 8 similar doses of ergometrine caused any increase in flow; 6 of the doses caused a transient reversal of the vasopressin recovery curve, and in the remaining 1 the rising slope was temporarily flattened (Fig. 8B).

TABLE IV

EFFECT OF ERGOMETRINE ON CORONARY FLOW WHEN INJECTED DURING VASOPRESSIN CONSTRICTION IN 7 NORMAL AND 5 ATHEROSCLEROTIC HEARTS

Ergometrine given early, 0·25 to 1 min. after vasopressin, in column at right; other doses given later, 1·5 to 23 min. after vasopressin, during rising flow.

Heart	Total Doses Ergometrine (0·4 mg.)	Ergometrine-induced Flow Change during Vasopressin Action		
		Increased	Decreased	Unchanged
	No.	No.	No.	No.
Normal	10	8	0	2
Atherosclerotic	12	1	7	4

In these experiments, the dose of vasopressin ranged from 0.008 to 0.8 units, and showed no correlation with the direction of the ergometrine effect on flow during vasopressin action.

When ergometrine was given while vasopressin acted, the usual reduction in amplitude was seen regardless of whether coronary flow increased or decreased (Fig. 8). The heart rate showed negligible changes.

DISCUSSION

Basal State of Isolated Heart.—The finding that the atherosclerotic heart in its initial state, before any drugs were perfused, had a higher coronary flow than normal was unexpected. Since these observations were made, however, it has been reported (Gorlin, Brachfeld, Macleod, and Bopp, 1959) that in man the resting coronary flow determined by cardiac catheterization likewise is significantly higher in patients with coronary insufficiency (effort angina) than in normal subjects.

Wegria (1951) showed in the normal dog that blood flow is determined by the diastolic pressure at the coronary ostia and by the resistance of the coronary bed. Since the aortic (perfusion) pressure is constant in our isolated rabbit heart preparation, the observed differences in basal coronary flow must depend on differences in coronary resistance. Theoretical factors which might alter coronary resistance of the atherosclerotic heart in the direction of an increased basal coronary flow include: (1) an increase in collateral circulation during the course of atherogenesis with enlargement of the total coronary bed; (2) increased energy expenditure of the heart owing to impaired myocardial efficiency; (3) a hyperaemic response to localized areas of anoxic myocardium, mediated by the intrinsic neurohumoral mechanisms of the heart; (4) yielding of the atherosclerotic vascular wall owing to fragmentation of the subintimal elastic membrane in the small coronary arteries; and (5) weakening of the extravascular support of the coronary bed owing to extensive damage to the myocardium.

In considering mechanical factors in the rate of coronary flow, Duguid and Robertson (1957) point out that it is the large coronary arteries which receive most of the impact of ventricular systole, and thus their functional state rather than the resistance of the terminal arterial bed largely determines the maximal capacity of the coronary bed to dilate. In the atherosclerotic rabbit heart, the large coronary arteries are relatively free of atherosclerotic plaques even when the intramural vessels are extensively occluded. It is not surprising, therefore, that the coronary dilator effect of nitroglycerin in the atherosclerotic rabbit heart is only moderately or little impaired. At the same time, lowered resistance in the smaller arteries would be compatible with a higher basal coronary flow in the atherosclerotic group.

The known depletion of myocardial catecholamines in coronary artery disease (Raab and Gigee, 1955) would explain the diminished amplitude and slower rate of contraction observed initially in the atherosclerotic, as compared with the normal isolated rabbit heart. Lee and Shideman (1959) have demonstrated in cats that a reduction in myocardial catecholamines by sympathectomy or reserpine does indeed cause diminished contractility of isolated cardiac (papillary) muscle.

Differences in Ergometrine Effects on Normal and Atherosclerotic Hearts. — Mechanisms by which ergometrine causes electrocardiographic changes typical of coronary insufficiency in man and the intact rabbit with latent coronary artery disease, but not in normal persons or animals, are suggested by the results of this investigation.

The consistent and considerable negative inotropic effect of ergometrine, although equal in normal and atherosclerotic isolated rabbit hearts, may nevertheless play a role in the production of electrocardiographic changes in the intact atherosclerotic animal. In the isolated heart, ergometrine causes a similar percentage decrease in contractile amplitude for both groups, but the absolute level to which the amplitude falls is much lower for atherosclerotic hearts since the amplitude is initially less, only half that of the normals. If this reduction represents a deleterious effect on heart muscle with impaired oxygen utilization, it may well contribute to the S-T segment depression induced by ergometrine in the intact animal, especially when a reduction in coronary flow is superimposed.

On the average, the quantitative effect of ergometrine on coronary flow differs significantly in the normal and atherosclerotic groups at the dose range of 0.2 to 0.4 mg.; in the normals the reduction in flow was 8% as compared with 22% in the atherosclerotic. For the smaller and larger doses of ergometrine that we employed, the numbers of experiments were too small for differences to be statistically significant.

In the atherosclerotic heart, ergometrine quite uniformly causes a transient decrease in coronary flow. In the normal heart, the response is more variable; increases as well as decreases in flow occurred. The coronary dilator action of ergometrine in normal hearts is most consistently seen when the coronary bed is partially constricted by vasopressin. In contrast, under these conditions ergometrine has a predominantly constrictor effect in the atherosclerotic heart.

A reduction in coronary flow of about 25% has been shown not to cause electrocardiographic changes in the normal intact animal (Wegria, Segers, Keating, and Ward, 1949). On the other hand, in the presence of occlusive disease of the small coronary arteries and myocardial damage, a flow reduction of this magnitude may be critical and so contribute to anoxic changes in the electrocardiogram. The delay of several min. in the appearance of S-T segment depression after intravenous ergometrine in the intact atherosclerotic rabbit and in the patient with effort angina suggests that this effect depends on myocardial ischaemia secondary to constriction of the diseased coronary arteries, rather than solely on a direct action of the drug on the myocardium.

In contrast to the reproducible effects of a given dose of ergometrine, vasopressin induces tachyphylaxis in the isolated heart. Furthermore, the effects of vasopressin are essentially similar for normal and atherosclerotic isolated rabbit hearts. These observations suggest fundamental differences in the mechanism of action of these two drugs on the heart. One may speculate that the effects of ergometrine on coronary flow are mediated at least in part through mobilization of catecholamines, whereas vasopressin acts more directly on receptors. When catecholamines are depleted during atherogenesis, a direct action of ergometrine on receptors may be unmasked.

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THE EFFECT OF TEMPERATURE ON THE DIRECT MUSCLE TWITCH RESPONSE AND THE ACTION OF DRUGS ON THE ISOLATED DENERVATED RAT DIAPHRAGM

BY

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The effect of temperature on the direct twitch response of the normal and denervated rat diaphragm, and the sensitivity of the contractural response of the denervated muscle to acetylcholine, suxamethonium, decamethonium, and potassium salts, was studied. Cooling the normal diaphragm from 39 to 20° increased the size of the direct twitch, but below this temperature the twitch response of the muscle was progressively depressed. The denervated muscle twitch was, by contrast, maximal at 39° and decreased as the temperature was lowered. This property of the denervated muscle first appeared about the third day after denervation. The contractural response of the denervated diaphragm to applied acetylcholine was enhanced by cooling, while the response to potassium salts was depressed. The depression of the contracture induced by potassium followed closely the depression of the direct muscle twitch of the denervated muscle over the same temperature range.

Previous work on the relation between temperature and the size of the direct muscle twitch response has indicated that generally the tension developed in a twitch is greater at low temperature. This has been found for the frog sartorius over temperature ranges from 0 to 20° (Hill, 1951), whereas studies on the rat triceps (Walker, 1949) have indicated that an 80 to 90% increase in the size of the indirect muscle twitch occurs on cooling from 37 to 24°. These findings have been confirmed by Doudoumopoulos and Chatfield (1959), who found that the twitch tension of the rat gastrocnemius was greatest between 26 and 18°. Work on the cat tibialis anterior and rat diaphragm muscles (Maclagan and Zaimis, 1957; Bigland, Goetzee, Maclagan, and Zaimis, 1958), however, indicates that in these muscles lowering of temperature decreases the size of the twitch response. The present studies were made to investigate whether both the normal and denervated rat diaphragm showed similar or dissimilar changes in the size of the direct twitch response when cooled.

Recently it has been shown that cooling markedly increases both the magnitude and duration of action of depolarizing blocking agents in cat muscle (Zaimis, 1958; Bigland *et al.*, 1958), whereas the blocking action of competitive agents is reduced by cooling (Holmes, Jenden, and Taylor, 1951). In view of these findings it seemed important to establish whether the contractural response produced by acetylcholine (Frank, Nothmann, and Hirsch-Kaufmann, 1922; Gasser and Dale, 1926; Dale and Gaddum, 1930; Knowlton and Hines, 1937; Brown, 1937) and potassium salts (Frank, Nothmann, and Guttmann, 1923; Gasser and Dale, 1926; Brown, 1937) in denervated mammalian muscle was materially affected by cooling.

METHODS

The experiments were made on the isolated rat diaphragm preparation (Bülbring, 1946), denervated some days previously by removal of the right phrenic nerve under ether anaesthesia. The muscle was suspended in a bath of 50 ml. capacity and bathed with Krebs solution aerated with 95% oxygen and 5% carbon dioxide. Stimulation of 3 milliseconds duration at 120 volts was delivered at 5 per min. Twitch responses to electrical stimulation, together with contractural responses to various drugs, were recorded by means of a semi-isometric lever on a smoked drum. The doses of drugs are expressed as their final bath concentrations.

In the experiments on the effect of temperature on the direct twitch response of the denervated muscle, the normal diaphragm of the opposite side was used as a control.

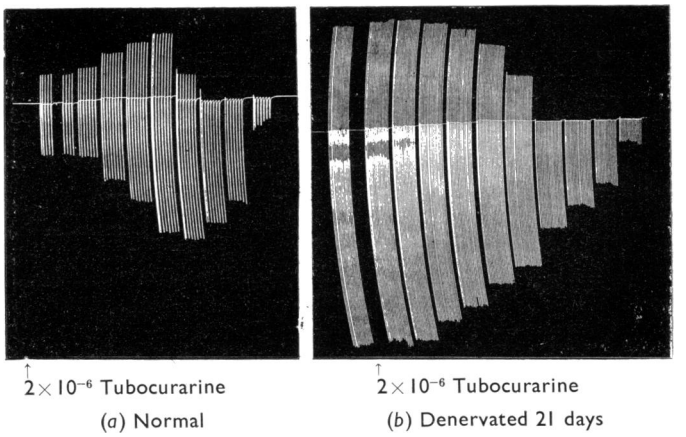

FIG. 1.—The effect of cooling on the direct twitch response of (*a*) a normal rat diaphragm, and (*b*) a 21-day denervated diaphragm. Both muscles were suspended in Krebs solution containing 2×10^{-6} tubocurarine. The normal muscle was cooled in 4° steps (left to right) from 39° to 3°. The denervated muscle was cooled in 3° steps from 39° to 12°.

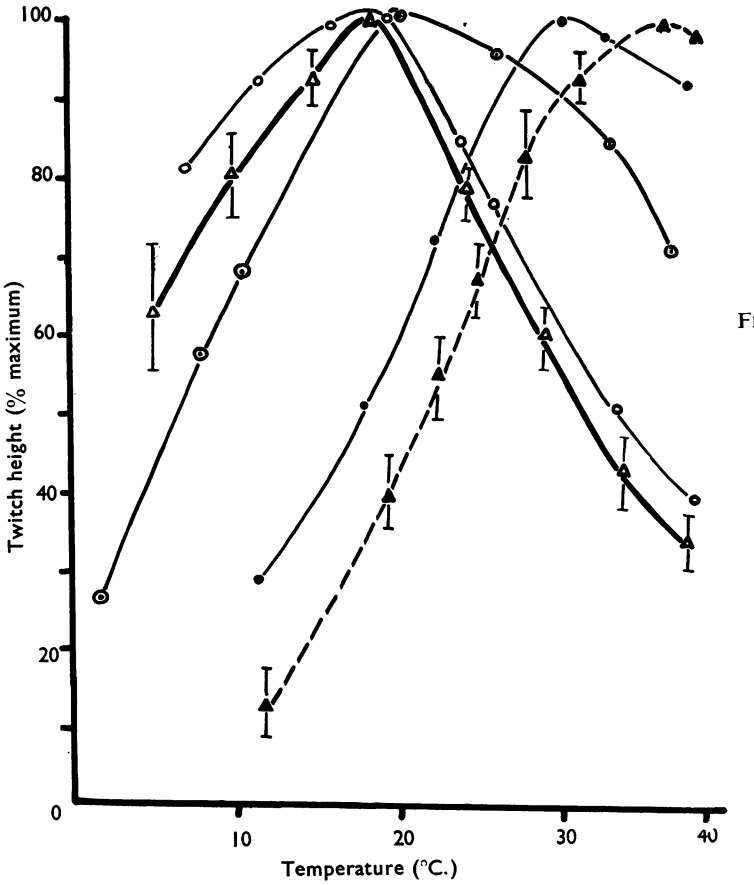

FIG. 2.—The relation between the direct twitch response and temperature for normal and denervated diaphragms in the presence of tubocurarine. The curves represent: (△——△) the mean for normal muscles, (▲- - -▲) the mean for muscles denervated from 3 to 28 days, (O——O) 1-day denervated muscle, (⊙——⊙) 2-day denervated muscle, and (●——●) 3-day denervated muscle.

RESULTS

The Effect of Temperature on the Size of the Direct Twitch Response of the Normal and Denervated Diaphragm

In all experiments on both normal and denervated muscles a concentration of 2×10^{-6} tubocurarine was maintained to abolish any neurally mediated effects, thereby ensuring that the twitch responses recorded were direct muscle responses.

Fig. 1 shows records from (*a*) a normal muscle, and (*b*) a muscle denervated for 21 days. The size of the direct twitch response of the curarized denervated muscle at 39° was much greater than that of the curarized normal muscle at the same temperature. As the bath temperature was lowered the size of the direct twitch response of the normal muscle increased down to a temperature of approximately 20°, while below this temperature the twitch response became sluggish (Li, 1958). In marked contrast to the normal diaphragm, the direct twitch response of the denervated muscle underwent a progressive decline as the temperature was lowered; also the twitch itself became sluggish in form at a higher temperature in the denervated muscle than in the normal muscle.

The results of a large number of experiments on normal and denervated diaphragms are graphically presented in Fig. 2. The mean curves which relate the size of the direct muscle twitch to temperature for the normal and denervated muscles (from 3 to 28 days) are significantly different. The normal diaphragms showed their greatest twitch responses at nearly 20°, whereas the denervated muscles gave their greatest twitch responses around 39°. The curve for the muscle denervated for 1 day shows but little departure from the normal, unlike that for the muscle denervated 3 days which corresponds closely to the mean curve for muscle denervated from 3 to 28 days. These findings therefore show that denervation brings about a characteristic change in the relation between the size of the direct muscle twitch response and temperature; this change first appears about the second day after denervation and is complete soon after the third day.

The Effect of Temperature on the Contractural Response of the Denervated Diaphragm to Acetylcholine, Suxamethonium, and Decamethonium

Cooling the denervated muscle to 30, 20, and 10°, respectively, progressively increased the magnitude and duration of the contractural response which the standard (2×10^{-6}) dose of acetylcholine elicited (Fig. 3). Further, the threshold dose of acetylcholine which excited the denervated muscle at 39° was reduced by at least one log-dose on cooling to 20°. When the muscle was rewarmed the original sensitivity to acetylcholine was regained, indicating that the sensitization of the muscle to acetylcholine by cooling was a reversible process. Similar experiments carried out in the presence of 5×10^{-7} neostigmine have shown that the presence of an anti-cholinesterase in no way modifies the sensitization of the muscle to acetylcholine at low temperature, an observation which suggests that the hypersensitivity was not caused by the inactivation of the muscle cholinesterase by cooling. The contractural responses of the denervated diaphragm to acetylcholine were incompletely blocked by tubocurarine, 2×10^{-6}.

Suxamethonium and decamethonium also showed increased activity on the denervated diaphragm at low temperature. Generally, it was found that the degree of sensitization seen for

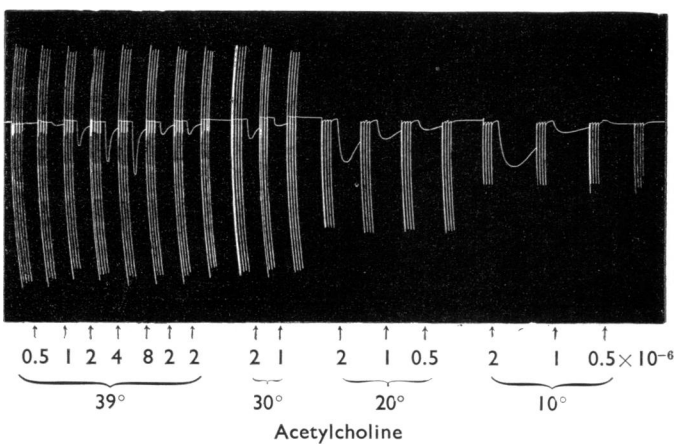

FIG. 3.—Contractural responses of the denervated diaphragm to acetylcholine are interposed between groups of direct twitch responses. On the left are contractures to doses of 1, 2, 4, and 8×10^{-6} acetylcholine. The test dose of 2×10^{-6} acetylcholine was then given to the muscle at 39°. Cooling the preparation to 30, 20, and 10° progressively enhanced the contractural response of the denervated muscle to the test dose of acetylcholine.

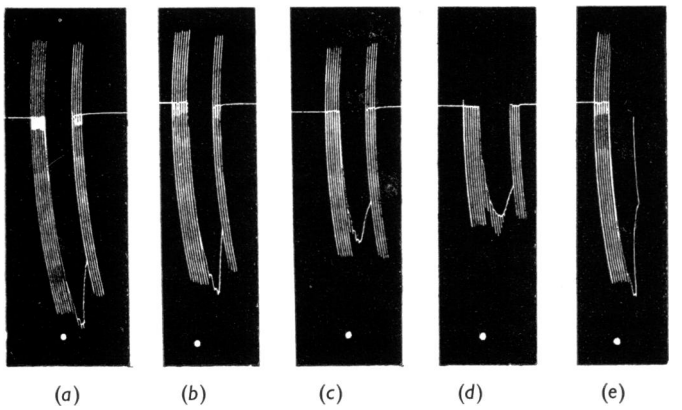

FIG. 4.—At (O) potassium chloride was added to the bath to give a concentration of 5×10^{-3}. Contractural responses of the same denervated diaphragm to 5×10^{-3} potassium chloride at (*a*) 38°, (*b*) 34°, (*c*) 30°, and (*d*) 22°. Rewarming the muscle to 39° (*e*) restored the original sensitivity of the preparation to the test dose of potassium chloride.

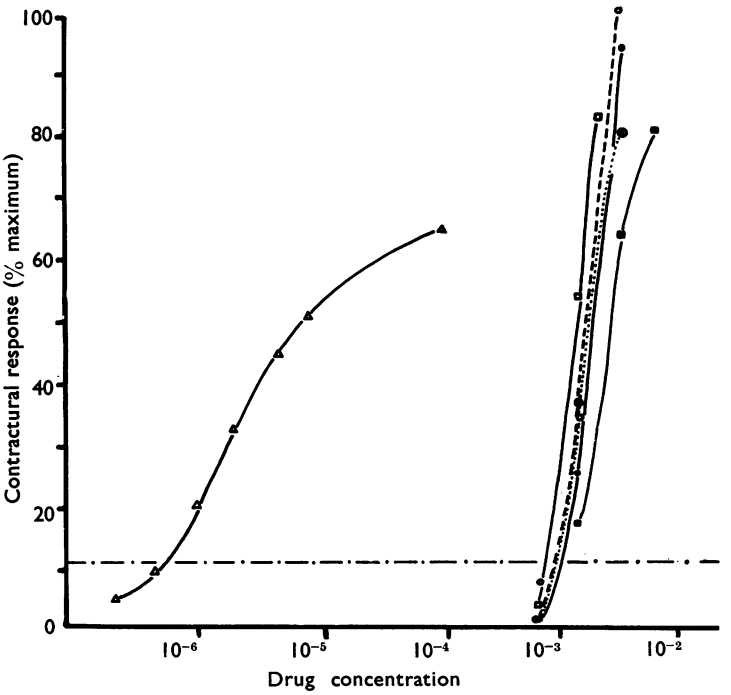

FIG. 5.—The dose-response relations for the contractures produced in the denervated rat diaphragm at 37° by acetylcholine (△——△), potassium chloride (●——●), potassium bromide (⊙ - - ⊙), potassium iodide (O - - O), potassium nitrate (□——□) and potassium sulphate (■——■). The contractural response elicited by a sucrose solution isosmotic with a solution of 5×10^{-3} potassium chloride is represented (—·—·).

suxamethonium was about the same as that found for acetylcholine. The sensitization seen with decamethonium was usually much smaller than that with acetylcholine.

The Effect of Temperature on the Contractural Response of the Denervated Diaphragm to Potassium Salts

Large doses of potassium salts (1×10^{-3}) were found to depress the electrical excitability of the denervated muscle, confirming the findings of Hajdu, Knox, and McDowall (1950). However, still larger doses, 5×10^{-3}, in addition to depressing the electrical excitability of the muscle also produced a typical contracture (Frank, Nothmann, and Guttmann, 1923; Gasser, 1930). These doses of potassium salts produced contractures which were attributed specifically to the presence of the potassium ion since the nature of the potassium salt used only slightly influenced the responses obtained. Control experiments showed that the effect was not caused by any effect of the potassium salts on the *p*H of the Krebs solution, or an osmotic action, since an osmotic solution of sucrose produced only a very small contracture (Fig. 5).

Cooling, instead of producing enhancement of the contractural response as was found to be the case with acetylcholine, suxamethonium, and, to a lesser extent, with decamethonium, depressed the contractural response to potassium chloride (Fig. 4). The depression of the contractural response to potassium salts on cooling paralleled the simultaneous depression of the twitch response.

The contractural responses of the denervated diaphragm to doses of 5×10^{-3} potassium chloride were unaffected by tubocurarine, 2×10^{-6}.

Discussion

Frog skeletal muscle is known to give a greater twitch at low temperature attributed to the temperature coefficient of the velocity of shortening being less than the temperature coefficient of relaxation (Hill, 1951). It is possible to account for some of the present findings in the normal rat diaphragm in this way. This might well be the reason for the increase in size of the twitch response of the normal diaphragm on cooling to 20°, but additional factors probably come into play when the muscle is cooled below 20° to cause the twitch response to gradually decline. Thus below 20° the oxygen consumption of the rat is decreased and at the same time there is a rise in the blood sugar and in the level of the plasma inorganic phosphate (Andjus and Smith, 1955). In addition to these findings, Li (1958) reported that the efficiency of the rat neuromuscular junction was greatest at temperatures above 15° while below this temperature changes in the size and duration of the muscle action potentials could be observed.

It would seem from Hill's (1951) work that, in the rat, denervation may, in effect, cause the temperature coefficient of the velocity of shortening to become greater than the temperature coefficient of relaxation, possibly by bringing about fundamental changes in the biochemical processes of the muscle related to contraction. Whatever the true explanation of these phenomena may be, it is of considerable interest that the appearance of these new properties of the denervated muscle, with respect to the change in its twitch response on cooling, should be on or about the third day after denervation, as it is at this time also that the denervated rat diaphragm is known to develop contractural properties to depolarizing drugs (Paterson, 1957). This would seem strong evidence for regarding the progressive depression of the twitch response of denervated muscle on cooling as a property *per se* of denervated muscle. Axelsson and Thesleff (1959) have recently drawn attention to the fact that denervated mammalian muscle will display increased sensitivity to humoral agents and other drugs only when there is a complete lack of connexion between the muscle and living nerve tissue. In support of this view, Jefferson, Phillips, and Necheles (1950), Jefferson, Ogawa, and Necheles (1958), and Jefferson, Ogawa, Toman, Scruggs, and Necheles (1959) have shown that the degenerative changes which normally appear in the diaphragm of the dog after phrenic nerve section can be prevented by either anastomosing the peripheral cut end of the phrenic to the other cut end of the same phrenic, or to the vagus nerve. These findings suggest that the degenerative changes appear in denervated mammalian muscle only when the trophic influence of a functional, or potentially functional, nerve supply has been lost. If this is so, the appearance of the characteristic twitch responses of the denervated rat diaphragm might well be associated with the loss of the trophic influence of the phrenic nerve innervation.

The probable explanation why cooling enhances the contractural responses elicited with acetylcholine and suxamethonium and yet depresses those produced by potassium salts is that these two groups of compounds produce contracture in denervated muscle by different mechanisms. Some evidence in favour of the dissimilar modes of action of acetylcholine and potassium ions has already been put forward in the literature. Brown and Feldberg (1936) found that 7×10^{-4} tubocurarine blocked the response of the perfused superior cervical ganglion to pre-ganglionic impulses and to applied acetylcholine but not to potassium chloride. Buchthal and Lindhard (1939) were also impressed by the fact that lizard muscle could still be stimulated with potassium chloride when it was refractory to nerve stimulation and applied acetylcholine. They suggested, on this basis, that acetylcholine and potassium chloride acted upon different regions of the muscle endplate. In agreement with these workers it was found in the present study that the contractural responses of the denervated diaphragm produced by acetylcholine were blocked by a dose of 2×10^{-6} tubocurarine whereas those produced by doses of 5×10^{-3} potassium chloride were completely unaffected by the tubocurarine. Examination of the log-dose curves for acetylcholine, potassium chloride, potassium bromide, potassium nitrate, and potassium sulphate for this preparation (Fig. 5) shows that the curve for acetylcholine is not parallel to those for the potassium salts which are grouped closely together, and are themselves parallel. Further, the graph indicates that acetylcholine can evoke a contracture from the denervated muscle in doses approximately 1,000 times smaller than those required for potassium salts (Brown, 1937).

The evidence presented above indicates that acetylcholine and related substances trigger a contracture in denervated mammalian muscle by combining with receptors which, according to Axelsson and Thesleff (1959), are situated diffusely over the whole of the outer surface of the muscle membrane. Potassium salts appear to produce contracture in the denervated muscle either by

acting directly on some other region of the former endplates or muscle membrane generally, or by an intracellular mechanism. An intracellular mechanism cannot be ruled out since massive doses of potassium salts have to be given before any contracture is seen, and potassium sulphate, the least permeable of the potassium salts used, was the weakest of the potassium salts in producing contracture in the denervated rat diaphragm.

The results suggest that the biochemical processes concerned with the production of a mechanical twitch after an electrical stimulus, together with those which produce a contracture in the denervated muscle on the application of potassium salts, are similarly depressed by cooling. That enhancement of the contractures produced by acetylcholine is seen on cooling may therefore be explained by specific sensitization of the muscle receptors by cooling.

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THE EFFECT OF ENZYME INHIBITORS ON HISTAMINE CATABOLISM IN MAN

BY

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The catabolism of histamine was studied in three female psychiatric patients by analysis of the [^{14}C]-labelled metabolic products occurring in the urine after a subcutaneous injection of [^{14}C]histamine. Each patient was studied before and during treatment with aminoguanidine or iproniazid.

Without treatment the patients had a normal histamine catabolism. Aminoguanidine and iproniazid inhibited the oxidation of histamine to imidazoleacetic acid; iproniazid produced a 50% inhibition of the oxidation of methylhistamine to methylimidazoleacetic acid. After iproniazid a large proportion of the injected [C^{14}]histamine was excreted as methyl [^{14}C]histamine.

The catabolism of histamine in various species, including man, has been elucidated by Schayer and his co-workers by means of [^{14}C]-labelled histamine (for references see Schayer, 1959). The pathways for the breakdown of histamine have been studied in normal men (Schayer and Cooper, 1956) and in non-pregnant and pregnant women (Nilsson, Lindell, Schayer, and Westling, 1959). So far, there has been no study, in man, of the effect of substances which inhibit the enzymes involved in the catabolism of histamine. From animal experiments (Schayer, 1959) it is known that histaminase (diamine oxidase) can be inhibited very effectively by aminoguanidine in doses which appear to be non-toxic. The enzyme that methylates histamine cannot yet be effectively inhibited *in vivo*, but the oxidation of the methylated histamine is blocked in mice by iproniazid (Rothschild and Schayer, 1958).

Mitchell (1956) measured the urinary excretion of non-labelled histamine in humans before and after subcutaneous injection of histamine. Treatment by mouth with aminoguanidine in large doses, or with iproniazid, caused only small changes in the urinary histamine and in the percentage of injected histamine recovered from the urine. Mitchell therefore suggested that the enzymes concerned in histamine catabolism in man were either different from those in animals, or less sensitive to the inhibitory actions of aminoguanidine and iproniazid than could be expected from the animal experiments of Schayer.

In the present study the catabolism of [^{14}C]-labelled histamine was studied in three psychiatric patients before and during the administration of aminoguanidine or iproniazid. It was felt that such a study might give more information about the effectiveness of the inhibitors. Furthermore, iproniazid is now much used in psychiatry, and information about its various effects in humans is therefore of interest.

METHODS

Three female patients at Lillhagen's Mental Hospital were studied. The patients were:

A. Born in 1904. Schizophrenia-like condition since 1941. At present no symptoms of "active" disease, but dementia with periods of auditory hallucinations. Duodenal ulcer 1928 and 1931; rheumatoid arthritis 1938; erythema nodosum 1941. Since then somatically healthy. Menopause 1956. After 0.5 mg. histamine dihydrochloride subcutaneously, normal reactions (facial flush and tachycardia). Treatment with iproniazid gave no mental changes.

B. Born in 1908. Schizophrenia-like condition since 1936. Mental condition similar to that of patient A, with no signs of "active" disease. Auditory hallucinations. Somatically healthy. Low muscular "tone." Menopause 1954. Normal reaction to 0.5 mg. histamine. Treatment with iproniazid gave no mental changes.

C. Born in 1902. 1934 treated for acute encephalitis (diagnosis not quite certain). Between

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1934 and 1957 periods of depression. Chronic depression since August, 1957, that is, 1.5 years before given iproniazid. Paraesthesia in lower legs and feet, but neurologically normal. Otherwise somatically healthy. Menopause in 1934 in connexion with the encephalitis. Normal reaction to 0.5 mg. histamine. Significant mental improvement during treatment with iproniazid 50 mg. 3 times daily. Improvement maintained on half of this dosage. Five months after stopping the iproniazid treatment, the patient was given aminoguanidine by mouth in a dose of 50 mg. 4 times daily for 9 days, and then at twice this dosage for another 12 days. This treatment caused no mental improvement.

Iproniazid (" Marsilid," Roche) was given in doses of 50 mg. 3 times daily. Aminoguanidine sulphate (Eastman Organic Chemicals), dissolved in distilled water and sterilized by filtration, was injected subcutaneously in a dose of 0.1 mg./kg. (patient B), or 1.0 mg./kg. (patients A and C) at 3-hourly intervals.

[^{14}C]Histamine (The Radiochemical Centre, Amersham, England), dissolved in 0.9% saline containing 0.2% glucose and sterilized by filtration, was injected subcutaneously at about 8 a.m. The dose was 10.2 to 11.5 μg. histamine base, specific activity 42.7 μC/mg. base. Each patient received three injections of [^{14}C]histamine, in all about 1.5 μC, the first one before any treatment, the second one after 3 weeks of treatment with iproniazid, and the third one 3 to 6 months after stopping the iproniazid administration. Injections of aminoguanidine were begun 30 min. before the third injection of [^{14}C]-histamine and continued for 12 hr. in all 4 injections of aminoguanidine.

Patient C was given a fourth injection of [^{14}C]histamine, 2 months after the third one. The patient was not given any treatment during these 2 months. The fourth injection was given when the patient had taken aminoguanidine sulphate, 100 mg. 4 times daily by mouth for 7 days.

Urine was collected in 2 portions, 0 to 12 hr. and 12 to 24 hr. after the injection of [^{14}C]histamine. Great care was taken to obtain all urine from the patient during the 24 hr. after injection.

The analysis of the [^{14}C]-containing compounds in the urine was carried out as described earlier (Lindell and Schayer, 1958a; Nilsson *et al.*, 1959). The following substances were determined: histamine, methylhistamine [4-(2-aminoethyl)-1-methylimidazole], total imidazoleacetic acid (imidazol-4(5)-ylacetic acid), and methylimidazoleacetic acid (1-methyl-imidazol-4-ylacetic acid). Briefly the analytical procedure is as follows: a carrier amount (non-radioactive) of the substance to be determined is added to an aliquot of the urine specimen; a preliminary extraction is made (butanol extraction for histamine, chloroform extraction for methylhistamine, and absorption on an anion-exchange resin for the acids); the compounds are crystallized as picrates, and the picrates of histamine and imidazoleacetic acid are converted to derivatives of *p*-iodophenylsulphonyl chloride (pipsyl chloride). The picrates or pipsyl compounds are counted on standard plates at infinite thickness in a gas flow counter. Repeated recrystallizations and treatments with activated charcoal are then performed. When the radioactivity is stable through three recrystallizations purity is considered to have been achieved. The samples to be assayed for total imidazoleacetic acid are first hydrolysed in a sealed glass tube for 5 hr. at 160° in N hydrochloric acid with the carrier included. This hydrolysis removes the ribose that is conjugated to part of the imidazoleacetic acid.

The excretion of total [^{14}C] was measured as described by Schayer and Cooper (1956).

TABLE I

EXCRETION OF [^{14}C] IN URINE AFTER INJECTION OF [^{14}C]HISTAMINE

The [^{14}C] activity is expressed in terms of μg. [^{14}C]histamine.

Patient	A			B			C		
Injected [^{14}C] histamine (μg.)	11·5	10·2	11·5	11·5	11·5	11·5	11·5	11·5	11·5
Treatment	None	Ipro-niazid	Amino-guanidine	None	Ipro-niazid	Amino-guanidine	None	Ipro-niazid	Amino-guanidine
[^{14}C] activity in urine 0–12 hr. after injection	7·3	5·7	7·9	7·3	7·6	7·2	8·7	4·9	9·2
[^{14}C] activity in urine 12–24 hr. after injection	1·6	2·7	1·3	1·6	1·1	1·0	1·5	4·7	1·2
Total: [^{14}C] activity in urine 0–24 hr. after injection	8·9	8·4	9·2	8·9	8·7	8·2	10·2	9·6	10·4

Results

Table I shows that by far the largest amount (71 to 90%) of the injected [^{14}C] was excreted during the first 24 hr. after the injection. The greater part of this [^{14}C] came out during the first 12 hr. However, in patients A and C, during treatment with iproniazid, the excretion of [^{14}C] seemed to be somewhat delayed. This may have been due to the fact that in these patients the urinary volumes were comparatively small during the first 12 hr. after the injection of [^{14}C]histamine during iproniazid treatment.

The analyses for radioactive metabolites of histamine in the two urine specimens are shown in Table II. Table III summarizes the main pathways for the catabolism of injected [^{14}C]-histamine, and the effect of aminoguanidine and iproniazid on them.

The Metabolism of [^{14}C]Histamine Without Treatment.—The pattern of urinary metabolites of [^{14}C]histamine in these three psychiatric patients was not significantly different from that in non-pregnant women (Nilsson *et al.*, 1959) or from that in normal men (Schayer and Cooper, 1956). The methylated compounds occurred in slightly higher amounts in the present study, but the difference is rather small. It is seen from the tables that imidazoleacetic acid and methyl-imidazoleacetic acid are the most important metabolites.

The Effect of Aminoguanidine.—It may be seen that aminoguanidine treatment blocked the transformation of histamine to imidazoleacetic acid. The inhibition was the same with 0.1 mg./kg. as with 1.0 mg./kg. Patient C, who was given a fourth injection of [^{14}C]histamine while receiv-

Table II

RADIOACTIVE METABOLITES OF [^{14}C]HISTAMINE IN URINE AS % OF EXCRETED [^{14}C]

The upper figures are those from the 0 to 12 hr. urine samples, the lower figures from the 12 to 24 hr. samples.

Patient	A			B			C		
Treatment	None	Iproniazid	Aminoguanidine	None	Iproniazid	Aminoguanidine	None	Iproniazid	Aminoguanidine
Histamine	1	5	3	2	4	0	2	4	0
	0	0	0	0	2	0	1	2	0
Imidazoleacetic acid (total)	26	3	2	26	5	2	23	4	2
	15	4	6	11	6	9	19	8	12
Methylhistamine	6	70	22	8	57	10	9	70	18
	0	41	5	1	34	4	1	46	3
Methylimidazoleacetic acid	60	13	71	68	30	61	60	22	77
	51	47	94	53	61	96	56	41	80
% of radioactivity recovered	93	91	98	104	96	73	94	100	97
	66	92	105	65	103	109	77	97	95

Table III

PATHWAYS IMPORTANT FOR [^{14}C]HISTAMINE CATABOLISM AND THE EFFECTS OF AMINOGUANIDINE AND IPRONIAZID

The figures show the range of values for the three patients studied, and refer to the whole 24-hr. period after injection of [^{14}C]histamine.

Enzyme	Metabolic Product Found in Urine	As % of Injected [^{14}C]		
		No Treatment	Iproniazid	Aminoguanidine
Histaminase (diamine oxidase)	Imidazoleacetic acid	17–20	2–5	2–3
Methylating enzyme	Methylhistamine	4–7	41–52	6–16
Methylating enzyme+oxidase	Methylimidazoleacetic acid	45–53	20–26	46–70

ing the same amounts of aminoguanidine by mouth as had been previously given subcutaneously, excreted [^{14}C]imidazoleacetic acid representing 2% and 0% respectively of the amount of radioactivity excreted during the two periods of urine collection. Thus, aminoguanidine sulphate seems to be effective when given by mouth.

The inhibitory effect of aminoguanidine indicates strongly that histaminase (diamine oxidase) is involved in the transformation of histamine to imidazoleacetic acid. It has been postulated from *in vitro* experiments that monoamine oxidase is important in this reaction (Zeller, Stern, and Blanksma, 1956), but monoamine oxidase is not inhibited by aminoguanidine.

The inhibition of histaminase by aminoguanidine does not lead to an increase in the urinary excretion of unchanged histamine. Instead more histamine is methylated as indicated by increased conversion to methylhistamine and methylimidazoleacetic acid.

The Effect of Iproniazid.—Treatment with iproniazid for 3 weeks causes conspicuous changes in histamine catabolism. The excretion of imidazoleacetic acid is reduced considerably, nearly as much as after treatment with aminoguanidine. The excretion of methylimidazoleacetic acid, not inhibited by aminoguanidine, is reduced by about 50% by iproniazid. The methylation of histamine increases just as much as it does after aminoguanidine, but since the oxidation to methylimidazoleacetic acid is partially blocked, methylhistamine is found as the most important urinary metabolite. These results indicate that iproniazid treatment inhibits both histaminase and the enzyme that oxidizes methylhistamine, while the methylating enzyme is not affected. Iproniazid has been found to be an efficient inhibitor of diamine oxidase *in vitro* (Lindahl, Lindell, Westling, and White, 1957; Blaschko, Friedman, Hawes, and Nilsson, 1959).

Discussion

The results are in full agreement with previous findings with the two enzyme inhibitors in animals (Schayer, 1959). We did not attempt to obtain a more complete inhibition by iproniazid with an increased dosage, since this drug has toxic effects. It appears that the inhibitor specificities of the enzymes concerned in histamine catabolism in man are not different from those in animals, which refutes the suggestion by Mitchell (1956).

As mentioned, the catabolism of [^{14}C]histamine in these psychiatric patients appeared to be normal. Some cases of chronic mental disease, notably schizophrenia, can tolerate large doses of histamine (Lucy, 1954), but in the patients studied here the response to histamine was entirely normal.

Iproniazid has profound effects on the catabolism of many amines in man. Thus Sjoerdsma, Gillespie, and Udenfriend (1959) found the conversion of 5-hydroxytryptamine, administered orally, to 5-hydroxyindoleacetic acid to be diminished by 33 to 55% after iproniazid, 50 mg. three times daily; this indicates that such a dose of iproniazid produces an inhibition of monoamine oxidase. Resnick (1959) concluded, from studies with radioactive adrenaline, that this dose level of iproniazid produces a 50% decrease of monoamine oxidase activity in man.

It appears therefore that iproniazid, 50 mg. three times daily, will reduce monoamine oxidase activity by about 50%. In the present study a 50% reduction of the oxidation of methylhistamine to methylimidazoleacetic acid was seen after iproniazid. This may be taken as indirect evidence that methylhistamine is oxidized by monoamine oxidase (Lindell and Westling, 1957). The lack of effect of aminoguanidine speaks against the possibility that histaminase (diamine oxidase) oxidizes methylhistamine in the body. *In vitro* methylhistamine is a good substrate of histaminase (Lindell and Westling, 1957).

Various investigators have tried to explain the central nervous effects of iproniazid as a result of an interference with the metabolism of 5-hydroxytryptamine and/or the catechol amines. It is therefore important to remember that iproniazid interferes with the catabolism of many other amines such as histamine.

To test the remote possibility that the therapeutic effect of iproniazid in patient C was connected with histaminase inhibition we gave aminoguanidine for 3 weeks, with no effect on the patient's mental depression. Later, iproniazid therapy was started again, with a good therapeutic result.

In order to evaluate the rate of formation and release of histamine in man *in vivo* it appears essential to develop methods for the determination of metabolic products of non-radioactive histamine. Imidazoleacetic acid is not a suitable metabolite for this purpose since the greater part of it is conjugated with ribose and hydrolysis of the riboside without carrier may cause losses in extraction. These losses are probably of no consequence with the method used in this paper since they should affect the carrier and the radioactive compound in equal proportions. Furthermore, imidazoleacetic acid may arise from

histidine by a pathway which does not involve histamine (Baldridge and Tourtellette, 1958; Lindell and Schayer, 1958b). Inferences about the histamine formation from analyses of urinary imidazoleacetic acid, free or conjugated, may therefore be entirely misleading. It would seem more promising to determine the methylated histamine derivatives. Of these, methylhistamine is probably more easily measurable. It is therefore of interest to know that after treatment with iproniazid a large percentage of injected histamine will be found in the urine as methylhistamine, and the determination of the urinary output of methylhistamine may be a measure of the total amount of histamine released or formed in such a patient.

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SUPERSENSITIVITY OF THE SUBMAXILLARY GLAND FOLLOWING EXCLUSION OF THE POSTGANGLIONIC PARASYMPATHETIC NEURONE

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The repeated subcutaneous injection of four different parasympatholytic agents into cats has been found to cause a supersensitivity of the submaxillary glands to adrenaline. The fact that the level of sensitivity reached is the same with all four drugs is taken as evidence that the supersensitivity is due to the absence of an action of acetylcholine on the gland cells. This acetylcholine can only in part be that released by the secretory impulse, for the supersensitivity produced is more marked than that caused by preganglionic parasympathetic denervation. It is assumed that the postganglionic cholinergic neurone exerts some action of its own on the gland cell. Sensitization above the level reached after decentralization ensues when such an action is abolished by parasympatholytic agents. Degenerative section of the postganglionic fibres would also exclude this action, but for anatomical reasons this cannot be made complete. The supersensitivity which follows this operation is greater than after decentralization but less than that produced by treatment with atropine-like drugs.

The supersensitivity to various secretory agents acquired by denervated submaxillary gland cells can to a great extent be attributed to the fact that the cells have been deprived of the secretory impulses from the central nervous system, which normally impinge upon them. Evidence for this view can be found in the following observations: supersensitivity develops, both when the flow of secretory agents is reduced by cutting some of the afferent fibres of the secretory reflex arc, and when it is abolished by treatment of the experimental animal for some time with ganglionic blocking or parasympatholytic agents; furthermore, the supersensitivity caused by denervation disappears when the gland is supplied with injected secretory agents such as pilocarpine, carbachol, or adrenaline (Emmelin and Muren, 1950, 1951, 1952; Emmelin, 1952, 1956, 1959).

Those experiments in which parasympatholytic agents were used to create a supersensitivity deserve special attention. When such a drug, Hoechst 9980 ($\alpha\alpha$ - diphenyl - γ - piperidinobutyramide), was given subcutaneously for some weeks to cats, the supersensitivity of the submaxillary gland to adrenaline which ensued was found to exceed that caused by cutting the preganglionic parasympathetic fibres of the chorda tympani (Emmelin and Strömblad, 1957). The hypothesis was put forward that the drug not only deprives the gland cells of secretory impulses but in addition abolishes an action on the cells of acetylcholine continuously leaking from the endings of the postganglionic neurone (Emmelin and Strömblad, 1957, 1958).

In order to test this hypothesis two types of experiments, described in the present paper, were carried out.

The effect of postganglionic parasympathetic denervation on sensitivity was studied. The ideal experiment would be to compare the level of sensitivity produced by a parasympatholytic drug with that attained after complete section of the postganglionic parasympathetic fibres. Unfortunately this is not feasible, since many of the synapses are situated within the gland and the postganglionic fibres are therefore not anatomically accessible. An attempt could only be made to remove those nerve cells which are found close to the hilum of the gland and to compare the effect of this operation with that caused by section of the preganglionic fibres or treatment with atropine-like drugs.

In the second series of experiments a number of parasympatholytic agents were given to produce a supersensitivity. It was thought that if various different drugs of this pharmacological

group were found to create a supersensitivity surpassing that caused by section of the chorda this would support the view that the additional sensitization could be attributed to the abolition of the action of a cholinergic mechanism on the gland cells.

METHODS

Cats were used as experimental animals. The level of sensitivity of the submaxillary glands to secretory drugs was estimated in one and the same cat about once weekly over a period of several months; after initial ether the cats were anaesthetized with hexobarbitone, injected intracardially, and the two submaxillary ducts cannulated from the mouth (Emmelin and Muren, 1952). To elicit secretion, standard doses of adrenaline were injected intracardially. This was the only test drug used in cats treated with parasympatholytic agents. In the denervation experiments acetylcholine, methacholine, and synephrine were given as well. Care was taken not to inject acetylcholine and methacholine immediately after the administration of hexobarbitone because of the slight atropine-like effect of the latter drug (Emmelin, 1941).

Preganglionic denervation was made by cutting the chorda or chorda-lingual nerve. For partial postganglionic denervation the chorda fibres were traced along the salivary ducts, under the dissecting microscope, and cut as near the gland as possible. The distal end of the piece of nerve cut out was examined histologically for ganglion cells. This was kindly done by Dr. Dora Jacobsohn of Lund. The operation was done aseptically under hexobarbitone anaesthesia.

The following parasympatholytic drugs were used: lachesine chloride [(2-benziloyloxyethyl)ethyldimethylammonium chloride], Hoechst 9980, isopropamide iodide [Tyrimid; (3 - carbamoyl - 3,3 - diphenylpropyl)diisopropylmethylammonium iodide], and methscopolamine nitrate (hyoscine methonitrate). I am very grateful to Professor Burn, of Oxford, for a gift of lachesine. Hoechst 9980 was kindly supplied by A. B. Webass, Gothenburg, isopropamide by A. B. Leo, Hälsingborg, and methscopolamine by A. B. Pharmacia, Uppsala. The doses of the drugs used and further technical details are given below.

Some cats were finally studied in acute experiments under chloralose anaesthesia as described below.

RESULTS

Partial Postganglionic Denervation

After the dissection of the chorda towards the gland a supersensitivity developed which was more pronounced than that of a gland preganglionically denervated. This is illustrated in the experiment of Fig. 1, in which the chorda of the right submaxillary gland was dissected and the left chorda-lingual nerve cut at the same time. The supersensitivity resembled that seen after preganglionic section in the following respects: it developed with the same time course; the threshold dose was lowered, and the secretion time increased; it was unspecific, so that sensitization to adrenaline, synephrine, acetylcholine, and methacholine was seen. The difference was that the threshold was even lower than after preganglionic denervation, the secretion period for a certain dose longer, and the number of drops obtained after a moderate dose greater. With large doses the responses of the dissected and the contralateral, preganglionically denervated gland were about the same. In two cats the glands were cut out more than a month after the operations. They were found to weigh 0.705 (dissected) and 0.756 g. (chorda-lingual cut) in one animal, and 0.596 and 0.610 g. respectively in the other. The weight of a normal gland is about 1 g. (Emmelin, Jacobsohn, and Muren, 1951). Both glands had thus atrophied, and to about the same extent; this corresponds to the fact that the maximal secretory rate of the two glands was about the same.

In the dissection ganglion cells were removed. This was shown by the following observations:

(*a*) The distal part of the excised piece of nerve was often thicker than the rest of the chorda, and when squeezed in forceps gave the same feeling as, for instance, a superior cervical ganglion. Histological examination revealed the presence of numerous nerve cells.

(*b*) Eserine, injected through the duct towards the gland, had less secretory effect on the dissected

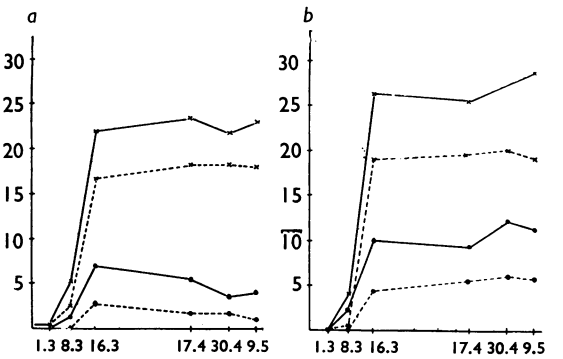

FIG. 1.—Supersensitivity following dissection and removal of the chorda towards the gland (solid lines) and simultaneous section of the contralateral chorda-lingual nerve (broken lines). Abscissa: dates (day and month) when the sensitivity was tested; ordinate: drops of saliva. *a*, Responses to 5 μg./kg. adrenaline (crosses) and 5 μg./kg. acetylcholine (rings); *b*, responses to 2 mg./kg. synephrine (crosses) and 2 μg./kg. methacholine (rings).

side than on the side of preganglionic section, in spite of the fact that the gland of the former side was more sensitive to acetylcholine than that of the other side. Eserine is assumed to act by preservation of acetylcholine leaking from the endings of the postganglionic neurone (Emmelin and Strömblad, 1958). Such an experiment is shown in Fig. 2. The smaller dose of eserine given

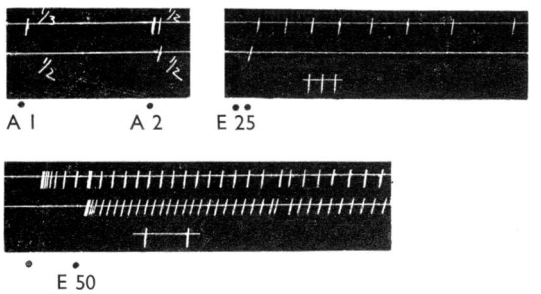

FIG. 2.—Chloralose anaesthesia. Secretory effects of acetylcholine (A 1=1 μg./kg., A 2=2 μg./kg.) and eserine (E 25=25 μg., E 50=50 μg.) 3 weeks after dissection of the chorda (upper line of the tracing) and section of the contralateral chorda-lingual nerve (lower line). Acetylcholine was given intravenously. Eserine, dissolved in 0.1 ml. saline solution, was injected during 5 sec. through a rubber connexion attached to the salivary cannula. The rubber tube was clamped distally during the injection and the following 5 sec. The dots mark the time of injection of the drugs. For eserine, the first dot corresponds to the injection into the duct of the dissected gland, the second into the other duct. Time in min. (in the lower tracing the drum was run at a higher speed).

in this experiment caused a secretion from the gland on the dissected side only (the single drop from the other side is probably part of the fluid injected which was expelled); this corresponds to the greater sensitivity of that side towards acetylcholine shown in the first part of the tracing. When the dose of eserine was raised, both glands secreted. The flow was now more rapid from the gland subjected to preganglionic denervation only. It could be objected that the higher dose of eserine could have a paralytic effect on the more sensitive gland; this was, however, not the case, for 100 and 200 μg. of eserine were found to cause a similar secretion to 50 μg. both on this gland and on the contralateral one. The conclusion from these experiments is that some postganglionic cholinergic fibres had degenerated on the dissected side.

(*c*) Nicotine, injected intravenously, was found to have less secretory effect on the dissected side than after mere preganglionic denervation. Dihydroergotamine was given in advance to abolish a secretory action of sympathin released by the nicotine from the adrenergic neurone or the adrenal medulla, and the experiments were carried out immediately after the operations in order to avoid the complication caused by sensitization.

That the postganglionic denervation was incomplete, as expected, was evident from the fact that eserine and nicotine still caused a marked secretion on the dissected side.

Postganglionic sympathetic fibres are known to enter the gland with the artery at the hilum. Section of these fibres is followed by a supersensitivity which can be superimposed upon that caused by cutting the chorda. Although care was taken not to touch the tissue surrounding the artery it seemed desirable to ascertain that the sensitization above the chorda level was not merely due to damage to sympathetic fibres. Two types of control experiments were carried out. (1) In some cats the sympathetic trunk was exposed in the neck but not cut. The trunk was stimulated electrically and the secretory response noted. The chorda was then immediately dissected towards the gland and stimulation of the sympathetic trunk repeated. The second secretory response was found to be as big as the first one. The wounds were then sutured. A supersensitivity was found to develop which surpassed that produced by section of the chorda-lingual nerve. This could not be due to injury of the preganglionic sympathetic fibres during the stimulation, for section of the sympathetic trunk does not sensitize the submaxillary gland cells (Emmelin and Engström, 1960). (2) In some cats the superior cervical ganglion was extirpated bilaterally when the level of supersensitivity had reached a plateau about three weeks after the usual parasympathetic operations. The sensitivity was found to increase on both sides, and the gland on the dissected side still showed a more pronounced supersensitivity than the gland deprived of the preganglionic parasympathetic (and postganglionic sympathetic) innervation.

Treatment with Parasympatholytic Agents

The chorda on one side was cut, and when the supersensitivity had reached a maximum, treatment with the parasympatholytic drugs started. The drugs were given subcutaneously. Hoechst 9980 was injected once a day in a dose of 1 mg./kg. It was found to cause a higher supersensitivity to adrenaline than that produced by preganglionic parasympathetic denervation, as

described earlier (Emmelin and Strömblad, 1957). A similar result was obtained with isopropamide iodide, 1 mg./kg. a day. When the same dose of lachesine chloride was administered, the sensitivity of both glands started to rise, but after two weeks it was found to be lower than after one week. Obviously a tolerance to the drug had developed. The dose had to be raised successively in order to prevent a fall in sensitivity, and doses as high as 10 mg./kg., given twice a day, were eventually used in some cats. Similar results were obtained when atropine was used (Emmelin and Muren, 1950a, 1951). The experiment shown in Fig. 3 demonstrates the decline of the sensitivity

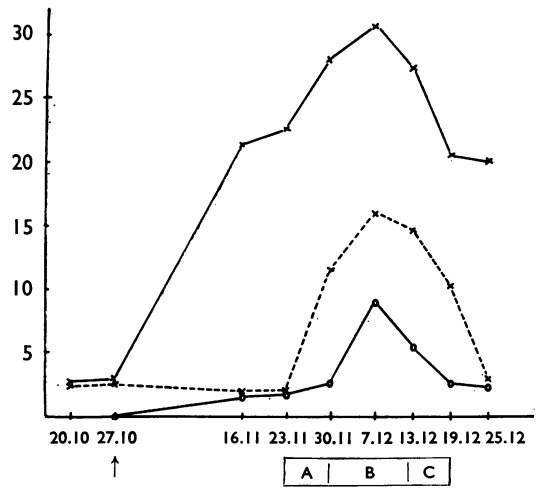

FIG. 3.—Abscissa; date (day and month) of estimation of the sensitivity, ordinate: drops of saliva secreted in response to 5 μg./kg. (crosses) and 1 μg./kg. (rings) of adrenaline for the right gland (solid lines) and for the left gland (broken line; no response to 1 μg./kg.). At the arrow the right chorda tympani was cut. Treatment with lachesine chloride, A: 1 mg./kg. once a day, B: 1 mg./kg. twice a day, C: 2 mg./kg. twice a day.

curves in spite of a moderate increase in the dose of lachesine. Nevertheless, it is apparent from the Fig. that a higher supersensitivity can be reached by treatment with this drug than that caused by preganglionic denervation. Methscopolamine nitrate was initially given daily in doses of 1 mg./kg. Since there seemed to be a slight tendency for a tolerance to develop, it was often found suitable to raise the dose to 2 mg./kg. a day after 1 or 2 weeks. A supersensitivity above that caused by section of the chorda was found to ensue.

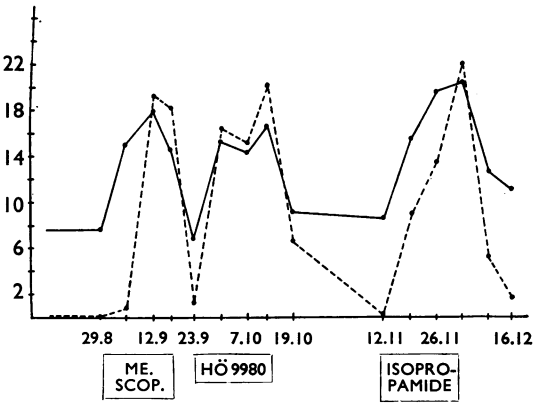

FIG. 4.—Secretory responses from the right (solid line) and the left gland (broken line) to a standard dose of 10 μg./kg. adrenaline. The right chorda had been cut 3 weeks before the experiment started. The dose of the three drugs was 1 mg./kg. a day.

In some cats it was possible to try several parasympatholytic drugs in the same animal. These experiments allow the conclusion to be drawn that all the drugs produce about the same high level of supersensitivity. Such an experiment is shown in Fig. 4, which demonstrates that methscopolamine, Hoechst 9980 and isopropamide sensitize both glands far above the level caused by the section of the chorda and that all three drugs create about the same degree of supersensitivity. There might be a slight difference

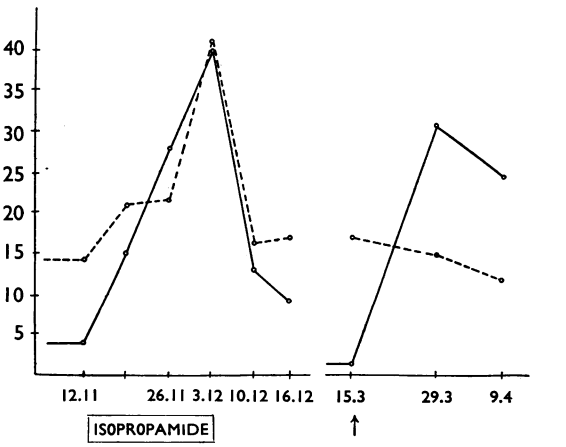

FIG. 5.—Secretion from the right (solid line) and left gland (broken line) caused by 5 μg./kg. adrenaline. The left chorda had been cut 3 weeks before the experiment started. Isopropamide iodide was given in a dose of 1 mg./kg. once a day. At the arrow the right chorda was dissected towards the gland.

between the responses of the right and left gland treated with blocking agents in this cat; this could be due to the fact that section of the chorda causes some atrophy of the gland, an effect which treatment with parasympatholytic agents does not seem to give (Emmelin, Jacobsohn, and Muren, 1951).

Comparisons were made between the supersensitivity caused by treatment with parasympatholytic agents and that produced by dissecting the chorda towards the gland. Fig. 5 shows that the operation produced a supersensitivity which was more pronounced than that following section of the chorda. It was, on the other hand, less marked than that caused by treatment with the parasympatholytic agent, which was to be expected since the postganglionic denervation was incomplete.

Discussion

The finding that four different parasympatholytic agents sensitize the submaxillary gland to the same level suggests that they act by virtue of their parasympatholytic properties only. Since the level of supersensitivity reached by far exceeds that attained by disconnecting the gland from the central nervous system via the chorda tympani it must be assumed that the drugs deprive the gland not only of acetylcholine released by the secretory impulse but in addition of some other influence exerted by a cholinergic mechanism. It is reasonable to focus interest on the postganglionic parasympathetic neurone. This neurone must then be assumed to have some action on the glandular cell apart from that due to the secretory impulse from the central nervous system, an action which persists after section of the preganglionic fibres. This postulated action of the neurone on the gland cell must be mediated by acetylcholine released in subthreshold concentration as regards secretion, since the gland does not secrete under ordinary conditions after section of the chorda. The fact that small amounts of acetylcholine can be detected in a perfusate of an eserinized gland even when the chorda is not stimulated (Emmelin and Muren, 1950b) is of interest in this connexion. Recent experiments with eserine (Emmelin and Strömblad, 1958) support the view that there is a continuous leakage of acetylcholine from the endings of the postganglionic neurone. The sensitization above the chorda level caused by parasympatholytic agents suggests that the leaking acetylcholine, although insufficient to cause secretion, exerts some action on the gland cell, the removal of which manifests itself in supersensitivity. The gland can be deprived of this action by treatment with atropine-like drugs. Another way of removing this influence would be to cause the postganglionic fibres to degenerate. Unfortunately this can only be done with some fibres; correspondingly, the sensitivity is then only raised to a level below that reached after treatment with parasympatholytic drugs.

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