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Research Papers

Absolute configuration and parasympathetic action: pharmacodynamics of enantiomorphic and diastereoisomeric esters of β-methylcholine

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The parasympathomimetic and atropine-like actions of enantiomorphic esters of β-methylcholine and various esters of choline or β-methylcholine containing an asymmetric centre in the acid moiety have been studied. The potent parasympathomimetic drugs have a configuration identical with that of muscarine, suggesting a close affinity with the receptor. There is a gradual change from agonist to antagonist with increasing molecular size of the acid moiety, antagonism beginning with the butyric ester. Optimal affinity as a mimetic in the lower homologues is obtained with the acetic ester. Maximum affinity as an antagonist is obtained with the ester of the bulky hexahydrobenzilic acid. The configuration of the choline part is irrelevant for high atropine-like potency in the compounds derived from β-methylcholine. Clear differences in affinity are found, however, between stereoisomers of potent antagonists containing an asymmetric centre in the acid moiety, provided the asymmetric carbon atom does not bear isosteric groups. It is concluded that the atropine-like agents, although being competitive antagonists of the parasympathomimetics at best cover the agonistic receptor area only partially.

THE optical isomers of muscarine and acetyl-β-methylcholine differ widely in potency (Major & Bonnet, 1935; Glick, 1938; Waser, 1961), and the configurations of the active isomers of both substances are identical at the asymmetric centre (Ellenbroek & van Rossum, 1960; Beckett, Harper, Clitherow & Lesser, 1961). Slight alterations in the structure of potent parasympathomimetic drugs lead to a loss in affinity (van Rossum & Ariëns, 1959; Waser, 1961). These results indicate that potent parasympathomimetic agents are highly complementary to their receptors.

For atropine-like drugs there is similarly a great difference in potency between optical isomers (Long, Luduena, Tullar & Lands, 1956); for instance, natural (−)-hyoscyamine is 50–100 times more potent than its (+)-isomer (Maffii, 1960). As the atropine-like drugs are always larger molecules than the parasympathomimetic drugs, it seems likely that the receptors for both are not identical (Ariëns & Simonis, 1960). It is therefore important to investigate whether the same configurational requirements hold for both parasympathomimetic and atropine-like drugs. To this end a series of esters of choline and β-methylcholine have been synthesised, several of which, notably those with atropine-like action, contain an asymmetric centre in the acid moiety. Data about their biological activity indicate that parasympathomimetics and their antagonists interact only partially with common receptors.

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Experimental

METHODS AND MATERIALS

The biological activity of parasympathomimetic compounds was assessed on the isolated rat jejunum by making cumulative dose-response curves (van Rossum, 1963). A typical record is given in Fig. 1. A

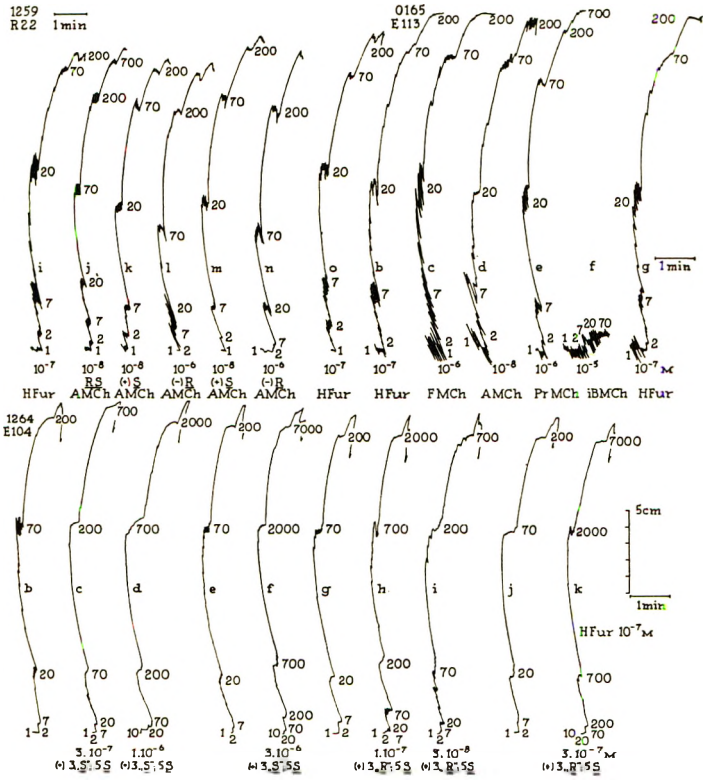


FIG. 1. Records of cumulative dose-response curves on the isolated rat jejunum. Experiment 1259 R 22: curves of the optical isomers [(+)*S* and (−)*R*] of acetyl-β-methylcholine (AMCh) and the racemic mixture (*RS*) compared with curves of the reference compound furtrethonium (HFur). Experiment 0165 E 113: curves of an homologous series of esters of racemic β-methylcholine. The esters of formic acid (FMCh), acetic acid (AMCh) and propionic acid (PrMCh) are full agonists whereas the ester of isobutyric acid (iBMCh) is practically inactive. Experiment 1264 E 104: dose-response curves of furtrethonium in the presence or absence of diastereoisomeric esters of hexahydrobenzilic acid. These diastereoisomers antagonize the action of furtrethonium but differ in potency. (The notation used is explained in the text.)

Tyrode solution of the following composition (g/litre distilled water) was used as bathing fluid: NaCl 8·0; KCl 0·2; $CaCl_2$ 0·2; $MgCl_2$ 0·1; $NaHCO_3$ 1; NaH_2PO_4 0·05; dextrose 1.

The potency of the parasympathomimetic agents has been expressed in terms of intrinsic activity and affinity (van Rossum, 1963; Ariëns, 1964). The intrinsic activity is the ratio of the maximum effect obtained with a

drug under study and the maximum effect of the reference compound furtrethonium. The pD_2-value was used as a measure of the affinity; this is the negative logarithm of the molar concentration that causes 50% of the maximum effect of the compound in question, and can be calculated from the dose-response curves of the parasympathomimetic compounds (see Fig. 2).

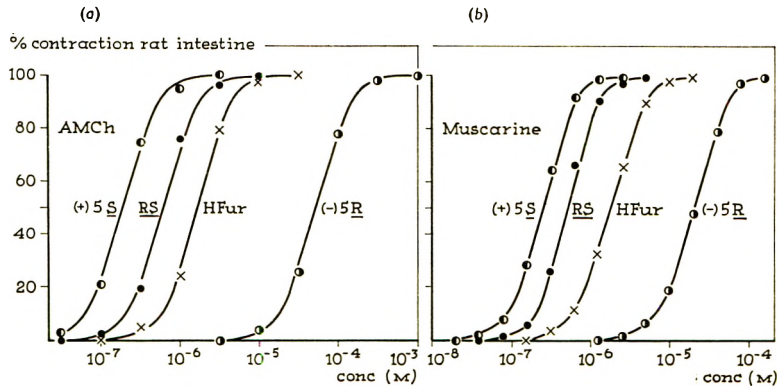


FIG. 2. Dose-response curves of optical isomers and racemic mixtures. (*a*) Curves of acetyl-β-methylcholine (AMCh) and the reference compound furtrethonium (HFur). (*b*) Curves of natural muscarine (5*S*) and the optical isomer (5*R*) compared with the reference compound furtrethonium. Note a large difference in the potency of the optical isomers as parasympathomimetic agents. The configuration of the potent isomers in position 5 is identical (see Table 5).

The atropine-like drugs which are antagonists of the mimetic drugs have an intrinsic activity equal to zero. They were therefore studied in combination with an agonist (see Fig. 1). The affinities of the antagonists are expressed as pA_2-values, this parameter being the negative logarithm of that molar concentration causing a shift by a factor of 2 in the dose-response curve of an agonist (see Figs. 4 and 5). A difference in the pD_2- or pA_2-value of 0·3 is significant at the 5% level.

The various isomers and racemic substances were synthesised in the department of organic chemistry (Ellenbroek, 1964). The notation for absolute configuration (*R* and *S*) is used according to the proposals of Cahn, Ingold & Prelog (1956).

Results

OPTICAL ISOMERS OF PARASYMPATHOMIMETIC AGENTS

(±)-Acetyl-β-methylcholine (methacholine, Mecholin, AMCh) is slightly less potent than acetylcholine as a parasympathomimetic drug in the isolated rat jejunum. Both drugs are agonists having intrinsic activities equal to that of the reference drug furtrethonium (HFur). The (+)-isomer (+)*S*-AMCh is about twice as potent as the racemate, but its potency exceeds that of the (−)-isomer (−)*R*-AMCh by about a factor of 300. Dose-response curves for these compounds calculated from similar experiments to those recorded in Fig. 1 are given in Fig. 2a. The curves

are identical in shape, but different in their position on the abscissa. This indicates that the differences in potency are due only to differences in affinity, while the intrinsic activities of the compounds are equal.

Similar results are obtained in an analogous manner with the optical isomers of natural muscarine and its racemic form (Fig. 2b). The average pD_2-values and intrinsic activities calculated from the dose-response curves are in Table 1. Here the affinity ratio S/R is about 320 in both cases.

TABLE 1. INTRINSIC ACTIVITIES (i.a.) AND AFFINITIES (pD_2 OR pA_2) OF ENANTIOMORPHS OF MUSCARINE AND ACETYL-β-METHYLCHOLINE AND SOME REFERENCE COMPOUNDS

Drug	Configuration*	i.a.	pD_2 (pA_2)	Relative affinity (acetylcholine = 100)	Affinity ratios
(+)-Muscarine	2*S*; 3*R*; 5*S*	1	7·1	160	$5S/5R = 320$
(−)-Muscarine	2*R*; 3*S*; 5*R*	1	4·6	0·5	
(+)-Acetyl-β-methylcholine	5*S*	1	6·9	80	$5S/5R = 320$
(−)-Acetyl-β-methylcholine	5*R*	1	4·4	0·25	
Acetylcholine	—	1	7·0	100	
Furtrethonium	—	1	5·9	8	
Atropine	racemate	0	(8·8)	6,400	
(−)-Hyoscyamine	3*S*	0	(9·1)	12,500	

* The numbering of the atoms has been explained in Table 5.

However, if only 0·3% of the *S*-compound were present as an impurity of the *R*-compound, the effect could be attributed to the impurity. Thus it is uncertain if the compounds with the *R*-configuration have any agonistic activity. Those with the *S*-configuration approach the potency of acetylcholine, suggesting that the β-methyl group in the *R*-configuration is an interfering factor in receptor occupation.

FROM PARASYMPATHOMIMETIC AGENTS TO ATROPINE-LIKE DRUGS

Slight alterations in the molecular structure of potent mimetic drugs cause a decrease in both intrinsic activity and affinity. Introduction of substituents with increasing molecular weight often results in a transformation of agonists into competitive antagonists, sometimes via intermediates or partial agonists (van Rossum & Ariëns, 1959; Ariëns, 1964).

A similar study was made beginning with (±)-acetyl-β-methylcholine. Dose-response curves of various esters from formic to butyric are given in Fig. 3*a*. It can be seen that the formic ester (FMCh), the acetic ester (AMCh) and the propionic ester (PrMCh) are pure agonists having the same intrinsic activity as the reference drug. The cyclopropanecarboxylic ester (CpMCh), which has a partially agonistic character, is noteworthy. There is an affinity optimum in acetyl-β-methylcholine in this series of agonists (see Table 2). This result agrees with that of the analogous series of choline esters.

The higher homologues are inactive. All these compounds, however, behave as antagonists of furtrethonium. They are atropine-like drugs, as can be seen for the isobutyric ester of β-methylcholine (iBMCh) in Fig. 3*b*. The dose-response curve of the standard of comparison is

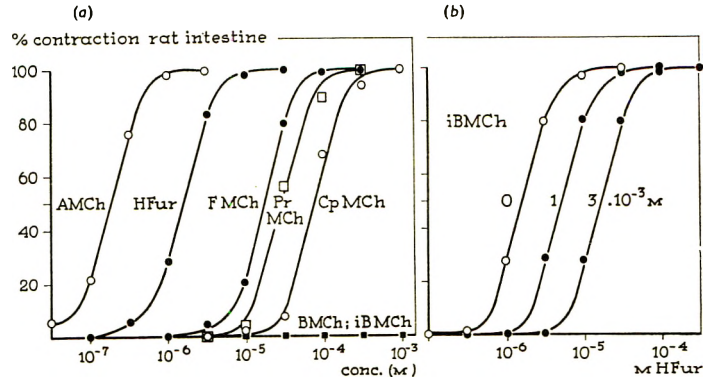
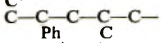
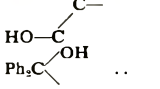
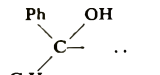


FIG. 3. (*a*) Dose-response curves of an homologous series of esters of aliphatic carboxylic acids and racemic β-methylcholine. The lower homologues FMCh, AMCh, PrMCh and in this particular experiment, the ester of cyclopropane carboxylic acid (CpMCh) are full agonists although they differ greatly in potency. The acetic ester has optimal affinity. The higher homologues, the butyric esters (BMCh and iBMCh), are inactive as parasympathomimetic agents. (*b*) Dose-response curves of furtrethonium in the presence of constant concentrations of the ester of isobutyric acid and (±)-β-methylcholine which shows that the inactive homologue of (*a*) acts as an atropine-like agent although having low affinity.

TABLE 2. INTRINSIC ACTIVITIES (i.a.) AND AFFINITIES (pD_2 OR pA_2) OF ESTERS OF (±)-β-METHYLCHOLINE AND VARIOUS CARBOXYLIC ACIDS

R in R—C(=O)—O—C(—C—$\overset{+}{N}Me_3$)—C	i.a.	pD_2	pA_2	Relative affinity (acetylcholine = 100)
H—	1	4·9	—	0·8
C—	1	6·7	—	50
C—C—	1	4·8	—	0·6
	0·6*	4·2	—	0·16
C—C—C—	0·1	—	3·8	0·06
	0	—	3·8	0·06
	0	—	4·6	0·4
	0	—	7·4	400
	0	—	8·3	2,000
	0	—	8·5	2,500

* Mean of values between 1 and 0·3.

shifted to higher concentrations by isobutyryl-β-methylcholine. Similar families of curves were obtained with the other antagonists. In Table 2 average pD_2- and pA_2-values, obtained from dose-response curves of various esters of β-methylcholine are given. There is a sharp rise in affinity of the antagonists if the acid moiety contains planar rings and polar groups.

ATROPINE-LIKE ESTERS FROM SYMMETRICAL BULKY ACIDS AND *R*- OR *S*-β-METHYLCHOLINE

Esters were synthesised from the optical isomers of β-methylcholine with diphenylacetic acid as well as with benzilic acid. In Fig. 4 various dose-response curves of furtrethonium in the presence of the two isomers of the benzilic esters are given. The average pA_2-values and relative affinities, calculated from these curves, are presented in Table 3.

In clear contrast to the results mentioned for the agonistic parent compound ($S/R = 320$), the difference in affinity of the enantiomorphs now appears to be small in both cases, the *R*-compounds having slightly greater affinities.

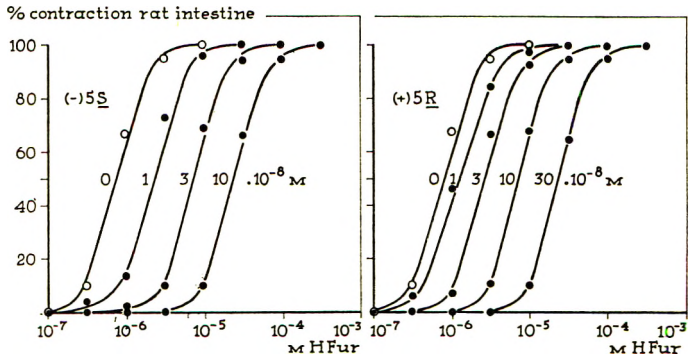


FIG. 4. Dose-response curves of furtrethonium in the presence of optical isomers of the esters of benzilic acid and β-methylcholine. Both esters are atropine-like drugs since they cause a shift in the dose-response curves and have affinities of similar magnitude.

TABLE 3. INTRINSIC ACTIVITIES (i.a.) AND AFFINITIES (pA_2) OF VARIOUS STEREOISOMERIC ESTERS

Configuration	[α]D in methanol	i.a.	pA_2	Relative affinity (atropine = 100)	Affinity ratios
Enantiomorphic esters of diphenylacetic acid and β-methylcholine					
5*S*	−12·4	0	5·0	0·016	5*S*/5*R* = 0·2
5*R*	+12·5	0	5·7	0·08	
Enantiomorphic esters of benzilic acid and β-methylcholine					
5*S*	+48·3	0	8·0	16	5*S*/5*R* = 0·8
5*R*	−48·1	0	8·1	20	
Diastereoisomeric esters of *O*-acetylmandelic acid and β-methylcholine					
Acid moiety / Alcohol moiety					
3*R* / 5*R*	−93·9	0	3·7	0·0008	3*R*: 5*S*/3*R*: 5*R* = 2
3*R* / 5*S*	−38·5	0	4·0	0·0016	3*S*: 5*S*/3*S*: 5*R* = 3
3*S* / 5*R*	+39·9	0	3·7	0·0008	3*S*: 5*R*/3*R*: 5*R* = 1
3*S* / 5*S*	+90·2	0	4·2	0·0025	3*S*: 5*S*/3*R*: 5*S* = 1·6
Enantiomorphic esters of phenyl-2-thienylglycollic acid* and choline					
(−)	−8·9	0	8·6	60	(−)/(+) = 5
(+)	+7·2	0	7·9	12·5	

* Configuration not known.

ATROPINE-LIKE ESTERS OF OPTICALLY ACTIVE BULKY ACIDS AND *R*- OR *S*-β-METHYLCHOLINE

A further step in the analysis of the stereochemical requirements for potent atropine-like action was the investigation of esters having an asymmetric centre in the acid moiety. For this purpose the four esters of *R*- and *S*-β-methylcholine and (+)- and (−)-α-methyltropic acid were prepared (Ellenbroek, 1964). The absolute configuration of the α-methyltropic acids is not known with certainty, but in view of the known configurations of the tropic acids (Fodor & Csepreghy, 1961) it seems likely that the (−)-isomer is the *S*-compound, the (+)-isomer having the *R*-configuration. In Fig. 5, dose-response curves of the four diastereoisomeric esters are presented.

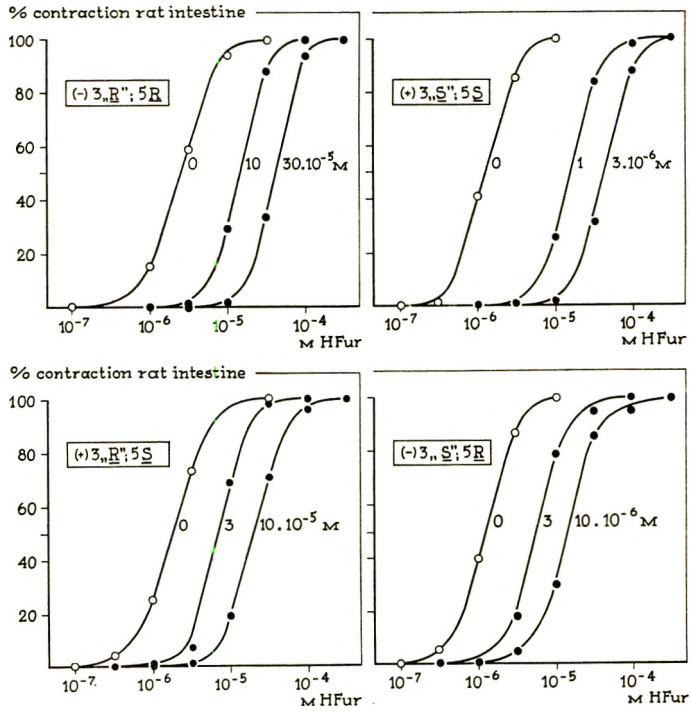


FIG. 5. Dose-response curves of furtrethonium in the presence of constant concentrations of the 4 possible isomeric esters of α-methyltropic acid and β-methylcholine. The optical isomers differ in potency. The configuration in the acid part (position 3) is of much greater importance than that in the choline moiety (position 5).

Both esters derived from the (−)-α-methyltropic acid are seen from the Figures to have much larger affinities than those from the isomeric acid. In Table 4, average pA_2-values and relative affinities are given. Diastereoisomeric esters, which are derived from the same acid isomer and differ only in the configuration of their β-methylcholine component exhibit only

a small difference in affinity. Their affinity ratios are about 1, as was found also with the β-methylcholine esters of diphenylacetic acid and benzilic acid. However, large ratios, of about 100, are found for the esters in which the configuration of the acid moiety differs. Similar measurements with the enantiomorphic esters of choline and α-methyltropic acid (see Table 4) reveal also that the ester of *S*-α-methyltropic acid is a much more potent atropine-like agent than is the corresponding *R*-acid ester.

TABLE 4. INTRINSIC ACTIVITIES (i.a.) AND AFFINITIES (pA_2) OF VARIOUS STEREOISOMERIC ESTERS

Configuration*						
Acid moiety	Alcohol moiety	$[\alpha]_D$ in methanol	i.a.	pA_2	Relative affinity (atropine = 100)	Affinity ratios
Diastereoisomeric esters of α-methyltropic acid and β-methylcholine						
3"*S*"	5*R*	−37·7	0	6·2	0·25	3*R*:5*S*/3*R*:5*R* = 1·5
3"*S*"	5*S*	+48·4	0	6·9	1·3	3*S*:5*S*/3*S*:5*R* = 5
3"*R*"	5*R*	−47·3	0	4·7	0·008	3*S*:5*R*/3*R*:5*R* = 30
3"*R*"	5*S*	+43·3	0	4·9	0·013	3*S*:5*S*/3*R*:5*S* = 100
Enantiomorphic esters of α-methyltropic acid and choline						
3"*S*"		−2·2	0	8·1	20	3*S*/3*R* = 300
3"*R*"		+1·8	0	5·6	0·06	
Diastereoisomeric esters of hexahydrobenzilic acid and β-methylcholine						
3"*R*"	5*R*	−39·7	0	8·9	125	3*R*:5*S*/3*R*:5*R* = 0·24
3"*R*"	5*S*	+32·0	0	8·3	30	3*S*:5*S*/3*S*:5*R* = 0·6
3"*S*"	5*R*	−35·1	0	6·9	1·3	3*R*:5*R*/3*S*:5*R* = 100
3"*S*"	5*S*	+37·9	0	6·7	0·8	3*R*:5*S*/3*S*:5*S* = 40
Enantiomorphic esters of hexahydrobenzilic acid and choline						
3"*R*"		−5·3	0	10·4	4,000	3*R*/3*S* = 100
3"*S*"		+6·1	0	8·4	40	

* The notation "*S*" and "*R*" for the configuration of the acids is only tentative. The numbering of the atoms has been explained in Table 5.

Results obtained with the four diastereoisomeric esters of β-methylcholine and hexahydrobenzilic acid substantiate these findings. Affinities calculated from dose-response curves are given in Table 4. As with the previously mentioned compounds the absolute configuration of the acids is unknown. However, the most potent antagonists in this series are the esters derived from the (−)-acid and it seems probable that this enantiomorph will have the same configuration as "*S*"-α-methyltropic acid. In the notation of Cahn & others (1956) (−)-hexahydrobenzilic acid would be the "*R*"-acid, a designation we use tentatively. Also the optical isomeric esters of choline and hexahydrobenzilic acid which have an asymmetric centre only in the acid moiety, differ widely in potency (see Table 4).

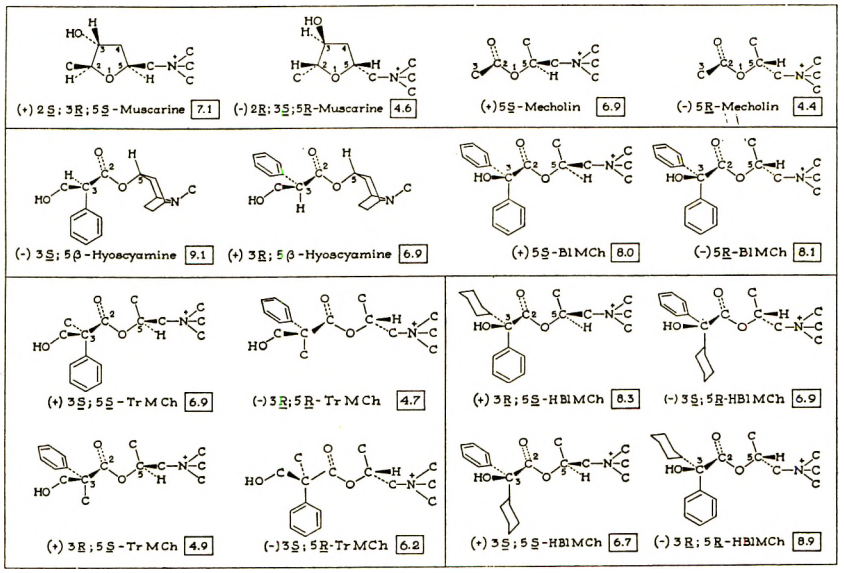
As the absolute configuration of the *O*-acetylmandelic acids is known, an attempt was made to obtain more precision about the required absolute configuration of the acid component in the asymmetric atropine-like esters by examination of the esters of *O*-acetylmandelic acid and β-methylcholine. However, the various isomers all appeared to be weak atropine-like drugs, which apparently do not fit the receptors very well. As a consequence their stereospecificity is small and the affinity ratios (3*S*/3*R* as well as 5*S*/5*R*) are both about 1 (Table 3).

Finally the isomers of the choline ester of phenyl-2-thienylglycollic acid have been investigated. Both are potent antagonist drugs and differ only slightly in affinity (Table 3). Apparently the receptor does not differentiate between the phenyl and thienyl groups.

Discussion

Pharmacologically active stereoisomers of the parasympathomimetic drugs muscarine and methacholine have identical absolute configurations at the asymmetric carbon, β to the ammonium group (position 5 in Table 5). In the notation of Cahn & others, this configuration must be indicated as *S* in both compounds. Recently it was shown that the most potent of the four diastereoisomers of (2-methyldioxolan-4-ylmethyl)trimethylammonium iodide (Methyldilvasène, F 2268) had the 2*S*:4*R*-configuration (Belleau & Puranen, 1963). The 4*R*-carbon in Methyldilvasène corresponds to the 5*S*-carbon of muscarine and acetyl-β-methylcholine. Thus,

TABLE 5. ABSOLUTE CONFIGURATION OF RELATED PARASYMPATHOMIMETIC AND ATROPINE-LIKE AGENTS (THE POTENCY AS pD_2- OR pA_2-VALUES IS GIVEN IN A BOX AFTER THE NAME OR THE ABBREVIATION OF THE VARIOUS COMPOUNDS)



all these compounds have an identical spatial arrangement around the asymmetrical carbon atom bearing the trimethylammonium-methyl group. These results indicate that all these compounds interact with a common cholinergic receptor and are highly complementary to it.

Exchange of the acetyl group in acetyl-β-methylcholine for other acyl residues causes a decrease in the intrinsic activity (see Table 2). The formic and propionic esters are still complete agonists like the parent compound, but with the esters of the four-carbon acids (cyclopropanecarboxylic acid, butyric acid and isobutyric acid) a lowering of the intrinsic

activity is observed; the cyclopropanecarboxylic ester is only a partial agonist, and the isobutyric ester is completely inactive as such. On introduction of still heavier groups the character of the compounds do not alter further; they remain "inactive". The inactive compounds are, however, inactive as agonists while still having affinity for the receptors. They therefore behave as competitive antagonists. The higher homologues are potent atropine-like agents approaching the affinity of atropine compounds.

The affinity of the β-methylcholine esters presents a more complex picture. The optimum in affinity is reached with the acetic ester. With increasing molecular size there is a gradual decrease in affinity. However, the esters of β-methylcholine and bulky acidic components, especially those containing large ring systems and polar groups, exhibit large affinities, which often exceed that of the parent mimetic compound. Similar findings have been reported for several series of related com-, pounds, for example, acetylcholine or Dilvasène (Schneider & Timms 1957; van Rossum & Ariëns, 1959; Koopman, 1960).

To explain the sharp increase in affinity as a consequence of the introduction of large acyl moieties, the presence of additional receptor areas located in the surroundings of the parasympathetic receptor has been postulated (Ariëns & Simonis, 1960). They would provide the corresponding drug-receptor complexes with a gain in binding energy. It even seems possible that the receptors for agonist and antagonist compounds, although neighbours, are completely different in location, but nevertheless identical in a functional sense.

In accordance with this hypothesis it appears that the asymmetric centre in the choline part, which is in a key position in parasympathomimetic esters of β-methylcholine, does not occupy this critical position in the homologous esters with atropine-like character; the enantiomorphic esters of diphenylacetic acid and benzilic acid (Table 3) showed only small differences in affinity.

The concept that atropine-like agents adhere to slightly different positions on the receptor surface in comparison with mimetics is corroborated by the data given in Table 4. From the results with choline esters of α-methyltropic acid and hexahydrobenzilic acid, it appears that the receptor strongly differentiates between isomeric compounds with an asymmetric centre in the acid component. Unfortunately, the absolute configurations of the stereoisomeric forms of the acids are not known with certainty. Considering the pharmacological measurements, it seems attractive to suppose that the most potent compounds of both pairs of isomers contain acids with identical configurations, tentatively referred to as "*S*"-α-methyltropic acid and "*R*"-hexahydrobenzilic acid.

A similar picture is obtained with the esters of the same acids with β-methylcholine as alcoholic component. Now four diastereoisomeric forms are possible. Higher affinities are found with the esters of "*S*"-α-methyltropic acid and "*R*"-hexahydrobenzilic acid. The configuration of the β-methylcholine is irrelevant. Clearly the stereochemical specificity

of the receptor is mainly directed to the asymmetric centre in the acid moiety of the atropine-like esters.

At first sight the measurements with the esters of *O*-acetylmandelic acid and phenyl-2-thienylglycollic acid, summarised in Table 3, seem to be in contradiction with this proposition, but it appears that the affinities of all diastereoisomeric esters of *O*-acetylmandelic acid and β-methylcholine are very low. Apparently none of the compounds fit very well to the receptor and therefore the stereospecificity is small. For the enantiomorphic esters of phenyl-2-thienylglycollic acid and choline, which are both very potent atropine-like agents, it might be suggested that the receptor does not differentiate between the isosteric thienyl and phenyl groups. Comparable biological activity of compounds which differ only by substitution of an ethylene group (−CH=CH−) in a ring system for a sulphur atom has also been found in other instances (Martin-Smith & Reid, 1959). The high affinity of the "*S*"-hexahydrobenzilic ester of choline, which is the less potent enantiomorph, may be ascribed also to the fact that phenyl and cyclohexyl rings can interchange their roles on the receptor surface to some extent. The extremely high affinity of the ester of choline and "*R*"-hexahydrobenzilic acid cannot be explained.

Pfeiffer (1956) has plotted potency ratios of many pairs of optical isomers against the effective doses used in clinical or animal experiments. He found the ratio to be larger if the effective dose was lower. This means that large affinity ratios between isomers can be anticipated when the affinity of a racemate or the most potent enantiomorph is high, whereas small ratios are to be expected with compounds of low affinity. From the experiments described in this paper, Pfeiffer's statement should be extended with two comments. Firstly, the rule holds good only if the asymmetric centre has a key position in the drug-receptor complex. Secondly, the rule fails when two groups at the asymmetric centre of potent enantiomorphs are bio-isosteric.

The results presented in the paper suggest the general conclusion that parasympathomimetics and their antagonists react with different or partially different receptors, although they are competitive antagonists of each other. It is therefore not justified to follow the procedure of Barlow, Scott & Stephenson (1963) who calculate relative efficacies by assuming that ethylation in a series of agonists results in the same affinity change as in a series of antagonists.

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Method for evaluating spasmolytic activity of drugs on the bile duct

A. CREMA, G. BENZI, G. M. FRIGO AND F. BERTÉ

A method has been described for examining the spasmolytic activity of drugs on the bile duct of the guinea-pig *in situ*. The terminal bile duct was pulled away from its insertion into the duodenum and perfused with Tyrode solution containing either carbachol or barium chloride to produce spasm. Injected intravenously, atropine methonitrate, oxyphencyclimine hydrochloride, tropenziline bromide, phenetamine, papaverine hydrochloride or diprophylline were spasmolytic.

DRUGS may affect flow through the terminal bile duct by acting on the bile duct itself or by acting on the musculature of the duodenum where it surrounds the orifice of the duct (Crema, Berté, Benzi & Frigo, 1963; Benzi, Berté, Crema & Frigo, 1964). To test a drug for its effect in relieving biliary spasm, the bile duct must be made to contract; morphine, codeine, bethanechol or barium chloride, given parenterally or infused through the duct, have been used to produce this contraction. However, these drugs also cause contraction of the duodenum, particularly in the dog or cat, because of the anatomical arrangement of the terminal portion of the duct within the duodenal wall (Boyden, 1937). In man, the pathological conditions of the biliary tract requiring the use of antispasmodic drugs are mainly restricted to the biliary tree without involving the duodenum. Therefore it is desirable to test drugs with potential therapeutic use in alleviating biliary spasm by a method in which alterations in duodenal tone do not interfere with flow through the bile duct. The guinea-pig provides a suitable preparation of the bile duct, which is described in this paper together with observations on the effects of drugs on flow through the duct.

Experimental

METHODS

Guinea-pigs were anaesthetized with a mixture of urethane (1·0 g/kg) and chloralose (20 mg/kg) injected intraperitoneally, and given artificial respiration. Blood pressure was measured through a cannula inserted into a carotid artery. 80 animals were used.

The anatomy of the terminal bile duct in the guinea-pig is shown in Fig. 1 P. The terminal portion of the duct expands into an oval pouch lying adjacent to the duodenum, and a small duct passes from this through the duodenal wall. The smooth muscle fibres of the bile duct are abundant and are arranged in 3 layers (Higgins, 1927). The experimental arrangement is shown diagrammatically in Fig. 1. The abdomen was opened and an inflow cannula was inserted through the gall bladder into the cystic duct and tied immediately below Lütken's sphincter. The hepatic ducts were ligated caudally and partially excised to allow leakage

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of bile which was removed by suction. The oval pouch of the terminal bile duct was dissected free from the peritoneum and the small duct was gently pulled out from the duodenum. The hole in the duodenum was ligated. The slight bleeding from the free end of the duct sometimes stopped in a few min, but occasionally continued throughout the experiment without affecting the results.

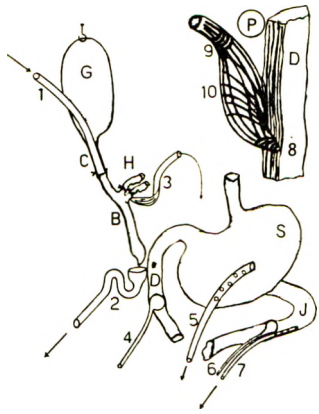

FIG. 1. Diagram of the experimental arrangement. B, bile duct. C, cystic duct. D, duodenum. G, gall bladder. H, hepatic ducts. J, jejunum. S, stomach. 1, inflow-tube. 2, collecting-tube. 3, collecting-tube connected with a suction pump. 4, 6, rubber balloon. 5, 7, drainage tubes. P is a sketch of terminal bile duct in the guinea-pig. 8, sphincter papillae. 9, sphincter choledochus. 10, ampulla's musculature.

Tyrode solution from a Mariotte bottle was perfused through the inflow cannula. The rate of flow was measured with a drop counter inserted between the bottle and the duct. The pressure necessary to produce a flow of about 0·1 ml/min was from 3 to 6 cm of water in the untreated duct and from 6 to 12 cm of water in the presence of drugs producing biliary spasm. The outflow from the duct was collected in a funnel and drained away outside the abdomen.

Movements of the duodenum and the jejunum were recorded by inserting water-filled rubber balloons into the lumen. A drainage tube was inserted into the stomach.

Spasm of the bile duct was produced by adding either barium chloride (30 μg/ml) in the presence of atropine (0·1 μg/ml) or carbachol (0·01 μg/ml) to the Tyrode solution perfusing the duct.

The following drugs were tested for their ability to relive biliary spasm: papaverine hydrochloride, diprophylline and 2-(α-cyclohexylbenzyl)-1,3-di(diethylamino)propane (phenetamine), each in M/100 solution, and atropine methonitrate, oxyphencyclimine hydrochloride, and *O*-benzil-oyl-7-methoxy-*N*-methyltropinium bromide (tropenziline bromide), each in M/1000 solution. The doses are expressed as ml of these solutions injected intravenously.

Results

PRODUCTION OF BILIARY SPASM

Barium chloride or carbachol perfused through the bile duct caused constriction and decreased the flow without changing the blood pressure or the motility of the intestine. Obviously, when these drugs were injected intravenously, they had effects on blood pressure and caused an increase in intestinal motility as well as constricting the bile duct.

In a typical experiment in which the bile duct was constricted by perfusion with 0·01 μg/ml of carbachol, the flow was reduced to about 25% of that occurring in the control period. There were no effects on the motility of the duodenum or the jejunum or on the blood pressure. An intravenous injection of 0·5 μg/kg of carbachol caused a reduction in the flow through the duct to about 30% of that in the control period, and caused increases in pressure and activity in the duodenum and jejunum, and a small fall in blood pressure.

The response of the bile duct to an intravenous injection also showed that the blood supply was not severely impaired, even though it had been pulled away from the duodenum.

RELIEF OF BILIARY SPASM

The effects of intravenous injection of papaverine, diprophylline and phenetamine were examined in relaxing bile ducts which had been put into spasm by perfusing with Tyrode solution containing barium chloride. Fig. 2A shows the log-dose response lines for three doses of each of these drugs. Phenetamine had almost twice the potency of papaverine, and diprophylline had one-tenth the potency of papaverine.

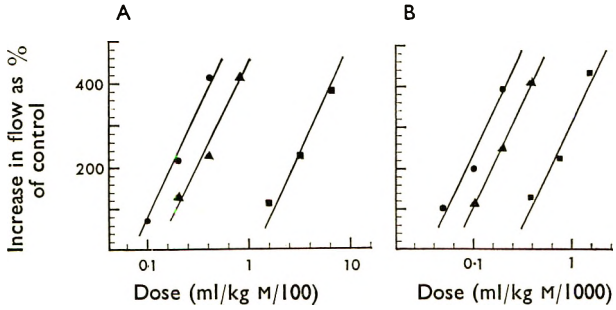

FIG. 2. Log dose-response curves for A, phenetamine (●), papaverine (▲) and diprophylline ■; B, atropine (●), oxyphencyclimine (▲) and tropenziline (■). Estimated relative potencies with 95% confidence limits: papaverine, 1, phenetamine 1·79 (1·49–2·16); diprophylline 0·10 (0·09–0·11); atropine, 1; oxyphencyclimine, 0·57 (0·56–0·58); tropenziline, 0·16 (0·15–0·17).

The effects of intravenous injection of atropine, tropenziline and oxyphencyclimine were examined in relaxing bile ducts which were constricted by perfusing with Tyrode solution containing carbachol.

The results are shown in Fig. 2B. Atropine was the most potent, oxyphencyclimine had slightly more than one-half its potency, and tropenziline had about one-sixth of the potency of atropine. In a typical experiment the injection of 0·1 ml/kg of a M/1000 solution of oxyphencyclimine caused an increase in the rate of flow through the duct to slightly more than double the previous rate. It should be noted that this dose of oxyphencyclimine had no effect on the spontaneous motility of the duodenum or jejunum. However, it was noticed in some experiments that if the fluid from the end of the bile duct was allowed to flow over the duodenum it caused increased motility, and then the antagonistic drugs reduced this in the same doses that relaxed the bile duct.

Discussion

The guinea-pig bile duct *in situ* provides a convenient preparation for the quantitative comparison of drugs useful in relieving biliary spasm. In these experiments, spasm was induced in the duct in two ways. Perfusion with carbachol was used for testing the effects of atropine-like drugs. Perfusion with barium chloride in the presence of atropine was used for testing papaverine-like spasmolytic drugs; atropine was present to prevent the cholinergic component in the response to barium chloride (Feldberg, 1951; Necheles, Scruggs, Kraft & Olson, 1953; Ambache & Lessin, 1955; Della Bella, Gandini & Teotino, 1963). Morphine and other morphine-like drugs have been used by others to induce spasm, but the mechanism of this action is unknown.

Atropine itself was the most potent of the drugs tested against carbachol-induced spasm; tropenziline was less active, as was also reported by Taeschler, Konzett & Cerletti (1960). Phenetamine was the most potent of the other group of spasmolytics, as reported by Levis, Preat & Beersaerts (1960).

It is interesting to note that the bile duct artificially constricted is much more sensitive to antispasmodic drugs than the normal duct and intestinal tract: in fact there is a range of doses for each antispasmodic agent able to relax the hypertonic biliary tract without acting on the intestine. By injecting the spasmogenic agent parenterally and thus producing a spasm in both the organs, previously separated, it is possible to compare the response of the two organs and to study the true selectivity of the drugs.

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Bacteriostatic actions of some tetracyclines

J. BENBOUGH AND G. A. MORRISON

6-Demethyl-6-deoxytetracycline, 6-methyleneoxytetracycline, 7-chlortetracycline and 7-chlor-6-demethyltetracycline inhibit *Aerobacter aerogenes* in glucose-mineral salt media, by two modes of action, I and II, shown by tetracycline and oxytetracycline (Jones & Morrison, 1962; 1963): Mode II by 6-demethyl-6-deoxytetracycline is not detected by inhibition of growth of the organism but by inhibition of the anaerobic consumption of pyruvate which is also a consequence of Mode II. The chlorine-containing tetracyclines also inhibit the growth of the organism by a third mode of action (III) which is probably an interference with the provision of D-glutamate during aerobic growth. The inhibitions produced by a tetracycline are linearly related to the amount of the antibiotic in the molecular form present in the cultures. All three modes of action so far described are probably interferences with hydrogen transfer reactions. The bacteriostatic activities of six tetracyclines are compared.

IT has been shown by Jones & Morrison (1962; 1963), using a quantitative assessment of inhibition of the rate of growth of bacteria (Harris & Morrison, 1961), that tetracycline and oxytetracycline interfere with the metabolism of *Aerobacter aerogenes* in two ways and that only one of these modes of action limits the rate of growth of the organism under any one set of conditions. Inhibition of the rate of growth by both modes is linearly dependent on the concentration in the culture of the molecular form of the antibiotic and not on the concentrations of its ionic forms or of its complex with magnesium ions. Mode I was identified as a derangement of protein synthesis; Mode II as an interference with the transfer of hydrogen or the production of a hydrogen acceptor required in unaerated cultures. The inhibition of the rate of consumption of pyruvate by non-growing cells under the same conditions was also accounted for as a consequence of Mode II. Equimolecular concentrations of the molecular forms of the two tetracyclines are equally effective by Mode I but not by Mode II.

The simpler tetracycline, 6-demethyl-6-deoxytetracycline, and also 6-methyleneoxytetracycline, 7-chlortetracycline and 6-demethyl-7-chlortetracycline have now been used to examine further the structure-action relationships in this group of antibiotics. The primary sites of inhibition corresponding to each mode have been identified more closely and a new mode discovered.

Experimental and results

ORGANISM AND MEDIA

The organism, media and temperature of incubation, 37° ± 0·1°, are the same as those used previously (Jones & Morrison, 1962). Since 7-chlortetracycline is less stable in aqueous solution than the tetracylines previously examined, instead of being introduced before inoculation the antibiotics were added to the growing cultures just before growth was adequate enough to be measured optically. Inocula were taken from the third of three cultures grown in rapid succession in media containing only sufficient glucose to permit two-thirds of the amount of growth obtained in

From the Edward Davies Chemical Laboratory, University College of Wales.

a normal culture. The slopes of the plot of Index Ratio (the mean generation time of the inhibited culture divided by the mean generation time of a non-inhibited culture: Harris & Morrison, 1961), against concentration of inhibitor in culture, in the "basic" medium at pH 7·0, have a maximum deviation of $\pm$ 5% from that of the ideal plot calculated from the results of fourteen experiments. As the concentrations of antibiotic needed were small and had to be obtained by serial dilution, in each subsequent experiment involving changes of conditions and a freshly made up solution of the antibiotic, one culture in "basic" medium (Jones & Morrison, 1962) at pH 7·0 was used as an additional control so that any deviation from the intended concentration could be determined from the ideal plot.

ANTIBIOTICS

The nomenclature used is essentially that of Jones & Morrison (1962); $TH_3^+Cl^-$ denotes the hydrochloride of a tetracycline, TH_2 the molecular form, TH^- and T^{2-} the other ionic forms and $MgTH^+$ the complex with magnesium ion, the particular tetracycline being declared in the text when necessary. The dissociation constants and the association constants for the complexes with magnesium ions of tetracycline and oxytetracycline have been listed previously (Jones & Morrison, 1962); those for the other tetracyclines, calculated from potentiometric data by standard methods, are given in Table 1. The potentiometric titration curves indicate that complex formation is between the singly charged TH^- and the Mg^{2+} ions.

EFFECT OF CONCENTRATION OF ANTIBIOTIC UPON GROWTH

In "basic" medium initially at pH 7·0, increasing concentrations of all the antibiotics caused a progressive increase of mean generation time of both aerated and unaerated cultures. The results of plotting the calculated Index Ratios against the concentrations of antibiotic are shown in Fig. 1. In the instances of the 7-chlor-substituted tetracyclines the mean generation times decreased sharply after about 300 min; measurements of rate of logarithmic growth before this change were reproducible.

Growth in aerated cultures. The results for aerated cultures in "basic" medium (in which the four new tetracyclines showed only a single mode of action) indicated how much less potent was the 6-demethyl-6-deoxytetracycline (Fig. 1). When the "basic" medium was enriched with a mixture of amino-acids however, the chlorine-containing tetracyclines showed diminished potency; an effect also obtained in medium enriched with D-glutamate only (L-glutamate had no effect). The inhibition of growth by the chlorine-containing tetracyclines in "basic" medium was not due to either Mode I or Mode II and hence a new mode, Mode III, concerned with the availability of D-glutamate, has to be postulated.

The inhibiting powers of 6-demethyl-6-deoxytetracycline and of 6-methyleneoxytetracycline in aerated "basic" medium, and of the chlorine-containing tetracyclines in aerated "basic" medium enriched with casein hydrolysate, were unaffected by enrichment of the media with bacteriological peptone (Difco) or with yeast-extract (Difco) and thus corresponded to the inhibition produced by the Mode I of tetracycline and oxytetracycline (Jones & Morrison, 1962).

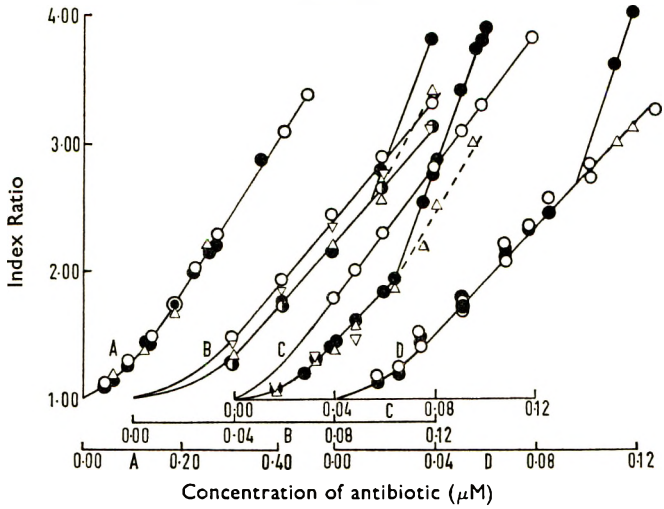


FIG. 1. Inhibition of cultures of *A. aerogenes* by some tetracyclines. Index Ratio (the mean generation time of the inhibited culture) is divided by the mean generation time of the uninhibited culture. A, 6-demethyl-6-deoxytetracycline; B, 7-chlortetracycline; C, 6-demethyl-7-chlortetracycline; D, 6-methyleneoxytetracycline. Unaerated cultures, addition: ●, none; △, 0·05% w/v yeast extract. Aerated cultures, addition: ○, none; ◑, 0·2% w/v casein hydrolysate; ▽, 1·1% w/v glutamate.

Growth in unaerated cultures. At all concentrations of 6-demethyl-6-deoxytetracycline and below concentrations (μM) of 0·093 for 7-chlortetracycline, 0·063 for 6-demethyl-7-chlorotetracycline and 0·096 for 6-methyleneoxytetracycline, enrichment of the medium with yeast-extract had no effect on inhibition. This indication that inhibition was by Mode I was confirmed by the fact that the ratio of the amount of nitrogen utilised to amount of cells produced was greater initially in the inhibited cultures; this is as expected if the utilisation of amino-acids was being inhibited (Jones & Morrison, 1962).

The chlorine- and methylene- substituted tetracyclines at higher concentrations than those in the preceding paragraph, inhibited the rate of growth of cultures in unaerated medium more severely than by Mode I, but inhibition was by Mode I in medium enriched with yeast-extract. Thus inhibition by these concentrations in unaerated cultures corresponded to that produced by the Mode II of tetracycline and oxytetracycline (Jones & Morrison, 1962).

pH OF THE MEDIUM AND INHIBITION

The inhibition of growing cultures caused by a particular *total* concentration—the sum of the concentrations of all the forms of the tetracycline present—of any of the four tetracyclines, irrespective of which mode was operative, was increased by lowering the pH of the medium and decreased by increasing the pH. This can be effected by varying the concentration of sodium ions in the medium. An example illustrating this general finding (Benbough, to be published) is given in Table 2. The severity of

TABLE 1. IONISATION CONSTANTS (K_1, K_2, K_3) OF SOME TETRACYCLINES AND STABILITY CONSTANTS (K_B) OF THEIR COMPLEXES WITH MAGNESIUM

	6-Demethyl-6-deoxytetracycline	6-Methylene-tetracycline	7-Chlor-tetracycline	6-Demethyl-7-chlortetracycline
pK_1	3·45	3·11	3·57	3·36
pK_2	7·87	7·61	7·57	7·37
pK_3	9·67	9·64	9·53	9·62
$\log_{10}K_B$	4·95	4·19	4·41	4·17

TABLE 2. INHIBITION BY 6-DEMETHYL-6-DEOXYTETRACYCLINE IN UNAERATED MEDIA INITIALLY AT pH 6.20, 7·00 AND 7·80

Concentration of antibiotic (μM)	0·044	0·062	0·089	0·124	0·133	0·178	0·187	0·220	0·249	0·306
Index ratio ± 0·08										
at pH 6·20	1·23		1·46		1·94	2·16		2·50		
at pH 7·00	1·11		1·27		1·51	1·75		2·03		
at pH 7·80		1·05		1·23			1·42		1·68	2·32

inhibition associated with a particular concentration of the *molecular* form of a tetracycline, calculated from the data in Table 1, was independent of the initial pH of the medium except with 7-chlortetracycline in aerated cultures at pH 7·80 and 7-chlor-6-demethyltetracycline in both aerated and unaerated cultures at pH 7·80, when the inhibitions were apparently more severe than in the corresponding cultures at pH 7·00. However, the pH of a culture decreases as growth takes place because growth is accompanied by the production of acid (Jones & Morrison, 1963); and the proportion of the molecular form of a tetracycline present in the total concentration should be calculated for the pH prevailing when the rates of growth were measured and not for that prevailing when the culture was inoculated. The errors due to using the initial pH are not significant for the tetracyclines so far examined except the chlorine-substituted ones when the pH is relatively high. If it is assumed that the pH of cultures initially at pH 7·80 had decreased to 7·55 or 7·60 when the measurements of growth rate were made, the results for cultures initially at pH 7·80 and pH 7·00 inhibited by one of the chlorine-substituted tetracyclines are concurrent.

Inhibitions by the four tetracyclines as a function of concentration of molecular form are compared with each other and with tetracycline and oxytetracycline in Figs 2 and 3. Inhibition by Mode I due to tetracycline, oxytetracycline and 6-demethyl-6-deoxytetracycline are not distinguishable; neither are inhibitions due to Mode I for 7-chlor-6-demethyltetracycline and 6-methyleneoxytetracycline, with which inhibition by Mode I due to 7-chlortetracycline is almost concurrent; but inhibitions by Mode II differ in threshold concentrations or rates of increase with concentration, or both, as do inhibitions by Mode III.

MAGNESIUM ION CONCENTRATION AND INHIBITION

As with tetracycline and oxytetracycline (Jones & Morrison, 1962), increasing the concentration of magnesium in the medium decreased the severity of the inhibition caused by a particular *total* concentration of one of the tetracyclines irrespective of which mode was operating, provided the

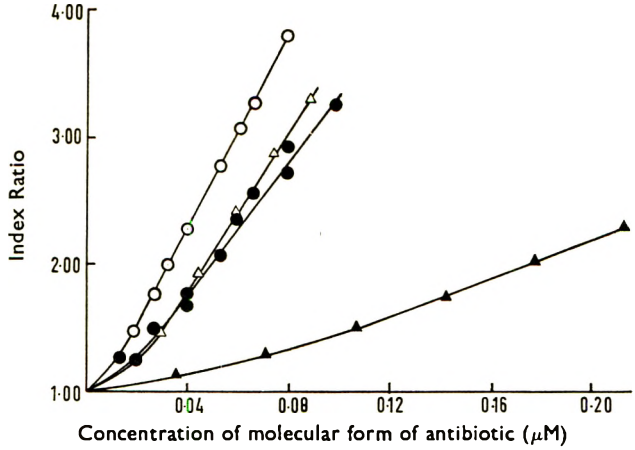


FIG. 2. Comparison of inhibitions of *A. aerogenes* by some tetracyclines in aerated cultures. Cultures inhibited by: 6-demethyl-7-chlortetracycline, ○; 7-chlortetracycline, △; 6-methyleneoxytetracycline, ●; 6-demethyl-6-deoxytetracycline, ▲. The corresponding plots for tetracycline and oxytetracycline (data from Jones & Morrison, 1962) are concurrent with that for 6-demethyl-6-deoxytetracycline (mode I).

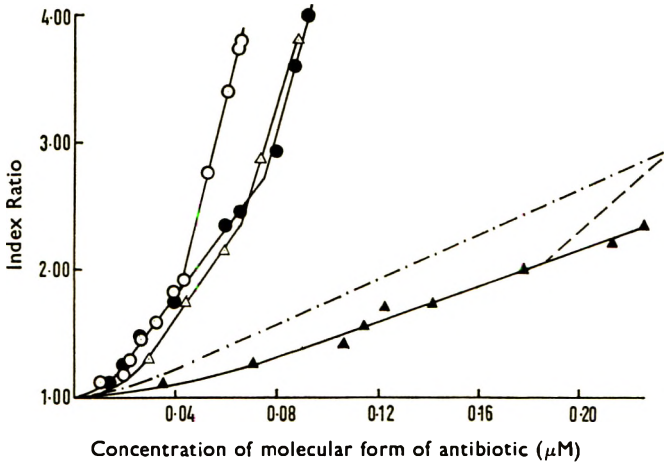


FIG. 3. Comparison of inhibitions of *A. aerogenes* by some tetracyclines in unaerated cultures. Cultures inhibited by: 6-demethyl-7-chlortetracycline, ○; 7-chlortetracycline, △; 6-methyleneoxytetracycline, ●; 6-demethyl-6-deoxytetracycline, ▲. The corresponding plots (data from Jones & Morrison, 1962) for: tetracycline (mode I), concurrent with that for 6-demethyl-6-deoxytetracycline; tetracycline (mode II), — —; oxytetracycline (mode II), — · —.

concentration of magnesium was sufficiently large. An example of this general finding (Benbough, to be published) is given in Table 3. When the measurements were plotted against the calculated concentrations of the molecular species, they were close to the corresponding plots in Figs 2 and 3, which have been drawn from the results obtained in medium initially at pH 7·0 and containing 82μM magnesium. The existence of an apparent

TABLE 3. CONCENTRATION OF MAGNESIUM AND INHIBITION OF UNAERATED CULTURES INITIALLY AT pH 7·0 BY 6-DEMETHYL-7-CHLORTETRACYCLINE

Magnesium concentration (μM)	82	164	820	1650	3300	6600
Index ratio ± 0·08 produced by (μM):						
0·025	1·22	1·22	1·22	1·15	1·09	1·04
0·040	1·47	1·49	1·47	1·36	1·25	1·12
0·050	1·55	1·58	1·68	1·50	1·37	1·16
0·075	2·68	2·68	2·66	2·16	1·84	1·39
0·100	3·49	3·57	3·42	3·00	2·50	1·67

of antibiotic.

threshold concentration of magnesium, below which there was no measureable effect on inhibition, was to be expected since low concentrations of magnesium have insignificant effects on the concentrations of the molecular species in the medium; the size of the threshold concentration depends on the values of K_2 and K_S which vary from one tetracycline to another (Table 1). Because of this threshold concentration, with the present limits of accuracy of measuring inhibition, it is impossible to determine whether or not a concentration of magnesium less than 10 μM is necessary for the tetracyclines to be inhibitors.

USE OF ADAPTED STRAINS OF THE ORGANISM

Present work, with that of Jones & Morrison (1962), indicates that the tetracyclines studied may inhibit the rate of growth of the organism because of at least three interferences with its metabolism: Modes I, II and III. This being so, strains of the test organism that are resistant to one tetracycline in that they are unaffected by one or more of its modes of inhibition should show the appropriate cross-resistance to other tetracyclines.

Adapted strains in unaerated medium—Modes I and II. The strain was grown twice in six separate volumes of "basic" medium at pH 7·0, each of which contained a sufficient concentration of one of the tetracyclines to cause inhibition of the rate of growth by Mode II for oxytetracycline and by Mode I for the others. The inhibitions of each growth are given in Table 4. The strain adapted more readily to the 7-chlor- and 6-methylene-

TABLE 4. EFFECT ON INHIBITION OF ONE GROWTH IN THE PRESENCE OF A TETRACYCLINE

Antibiotic	Concentration mμM	Mode	Index ratio ± 0·08	
			1st growth	2nd growth
Tetracycline	83·3	I	1·34	1·34
Oxytetracycline	80·7	II	1·60	1·50
6-Demethyl-6-deoxytetracycline	88·9	I	1·32	1·32
7-Chlortetracycline	77·8	I	2·12	1·58
6-Demethyl-7-chlortetracycline	45·0	I	1·60	1·32
6-Methyleneoxytetracycline	51·9	I	1·71	1·44

substituted tetracyclines than to the others. The two phases of growth in the presence of the 7-chlor- substituted tetracyclines, referred to earlier, is more likely to be due to the readiness of the organism to adapt to these antibiotics than to a loss of antibiotic by degradation in aqueous solution; degradation in aqueous solution is fairly rapid for 7-chlortetracycline but not for 7-chlor-6-demethyltetracycline (Goldberg, 1959; Jones, 1961).

In separate experiments, the organism was grown 10 times in rapid succession in medium containing sufficient of one of the antibiotics to produce an Index Ratio of 3·0 for the first growth (inhibition by Mode II except for 6-demethyl-6-deoxytetracycline which shows only Mode I in growth experiments). This procedure yielded a strain resistant to the tetracycline used at the concentrations that were normally required to produce the inhibitions in unaerated medium shown in Fig. 1. The strains resistant to tetracycline and to 7-chlortetracycline respectively were insensitive also to the presence of the other antibiotics over the ranges of concentration shown in Fig. 1; the cross-resistance to inhibitions by Mode I, or by Mode II, was complete.

Adapted strains in aerated medium—Modes I and III. A strain adapted to become resistant to the presence of sufficient 7-chlortetracycline to produce an Index Ratio of 3·0 in aerated medium by the procedure described in the previous paragraph, also was insensitive to the presence of other tetracyclines in aerated medium in the concentrations shown in Fig. 1. Thus the cross-resistance to inhibitions by Mode I and by Mode III, was complete.

PRIMARY SITES OF INHIBITION BY TETRACYCLINES

Use of redox systems. A strain of *Klebsiella cloacae* will grow readily in aerated basic medium in the presence of the redox dye brilliant cresyl blue, provided the culture is shielded from strong light. Under these conditions phenol, an inhibitor of the organism's normal hydrogen transfer systems, now becomes ineffective (Harris, 1956; Harris & Morrison, 1961). *Aerobacter aerogenes* also grows readily after 3–4 sub-cultures in basic medium containing 0·002% w/v of this dye, aerated cultures having a mean generation time of 30 min and unaerated ones 42 min. In the presence of the dye the organisms were unaffected by any of the six tetracyclines at concentrations which normally produce Index Ratios in excess of 3·0 under the same conditions. There are three possible explanations: (1) the dye caused a rapid alteration of the tetracyclines to inactive forms; (2) the dye itself was used as a by-pass to the parts of the system which are affected by tetracyclines; (3) growth in the presence of the dye resulted in the development of alternative metabolic routes which were not susceptible to the tetracyclines. However, measurements of the concentration of 7-chlortetracycline after incubation in "basic" medium with and without the presence of the dye by the method of Grove & Randall (1955), showed no loss of the antibiotic due to the presence of the dye, and, after growth in medium containing dye, the organisms grew readily in "basic" medium in the absence of dye and were still completely unaffected by normally bacteriostatic concentrations of the tetracyclines. Growth in the presence of the dye must have resulted in the production of a strain which was resistant because of an alteration of metabolism. Similar results were obtained with methylene blue. At concentrations of 7-chlortetracycline greater than 0·2 μM, inhibition reappears even in the presence of brilliant cresyl blue, the Index Ratios being 1·37 and 1·97 respectively at 0·353 and

0·588 μM. This would appear to be a Mode IV, but it has not been investigated further.

Since the redox potentials, E'_0 (V), of brilliant cresyl blue and methylene blue are 0·045 and 0·011 respectively, neither could accept hydrogen from, for example, reduced cytochrome *a* (E'_0, 0·262) but could well do so from reduced cytochrome *b* (E'_0, −0·04) or hydrogen-transferring systems with even more negative E'_0 values.

The effects on inhibitions by tetracyclines of the presence of other redox systems with E'_0 values more positive than that of brilliant cresyl blue, were determined. In aerated cultures, inhibition by 7-chlor-6-demethyltetracycline was unaffected by ascorbic acid (E'_0, 0·204) but was decreased by *o*-cresol-indo-2,6-dichlorophenol (E'_0, 0·188). The amount of the decrease depended on the concentration of the indophenol; 0·0568 μM antibiotic normally produces an Index Ratio of 2·20 by Mode III and in the presence of D-glutamate a value of 1·80 by Mode I, but 20 mg/litre of the redox indicator decreased the value to 1·78 and 60 (or more) mg/litre to 1·59. This indicated that Mode III was an interference with a hydrogen transfer system of E'_0 between 0·188 and 0·204 V, while Mode I was an interference with one of E'_0 approximately 0·188 V.

The effect of *o*-cresol-indo-2,6-dichlorophenol on inhibition of unaerated cultures by 0·25 μM tetracycline was determined. The inhibition was decreased from an Index Ratio of 2·84, expected for inhibition by Mode II, to 1·64, which also was less than would have been expected for Mode I. Thus Mode II was eliminated also, and Mode I was decreased, as found with 6-demethyl-7-chlortetracycline.

Consumption of oxygen. The effects of tetracycline and 7-chlortetracycline on the rates of uptake of oxygen at *p*H 7·0 by suspensions of non-growing cells oxidising glucose (in "basic" medium without the nitrogen source) were measured. Though tetracycline at higher concentrations decreased the rate to, for example, 65% of the normal at 4 μM, within the bacteriostatic range of concentration (0 to 0·4 μM) it stimulated the rate to 109% at 0·05 μM and 103% at 0·4 μM. 7-Chlortetracycline within its bacteriostatic range of concentration (0 to 0·12 μM) also increased this rate and even at 0·19 μM the rate was 104% of normal. Thus although the previous work indicated that Modes I and III were in the hydrogen transfer mechanism of the test organism they could not be direct inhibitions of steps of the mechanism normally used to transfer hydrogen to its ultimate acceptor, oxygen; they must be inhibitions of steps in pathways linking the main hydrogen transfer system to the growth mechanisms, which would not be operating fully when the measurements were made. The stimulations observed indicated that the use of such pathways was inhibited in the bacteriostatic ranges of concentration. If this were so, the efficiency with which glucose is utilised for the synthesis of new cellular material should be impaired by bacteriostatic concentrations.

When the concentration of glucose is limiting, there is a linear or almost linear relationship between the amount of growth and concentration of glucose (Dagley, Dawes & Morrison, 1951). If the efficiency of utilisation of glucose is impaired, the slope of the plot of amount of growth against

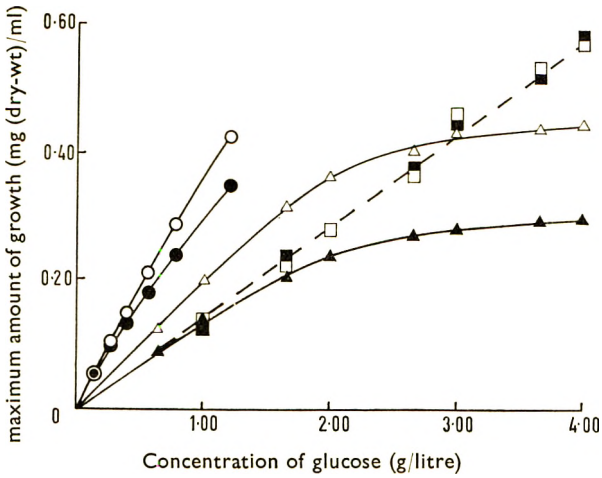


FIG. 4. Effect of 0·1781 μM tetracycline on the efficiency of utilisation of glucose for growth of *A. aerogenes*. Aerated basic media: uninhibited, ○; inhibited, ●. Unaerated basic media: uninhibited, △; inhibited, ▲. Aerated media containing 0·002% w/v brilliant cresyl blue: no tetracycline, □; tetracycline present, ■.

glucose utilised should be decreased. Fig. 4 shows that this is so for tetracycline inhibiting by Modes I and II, and that tetracycline has no effect on the efficiency of utilisation of glucose by a strain which is resistant to the antibiotic.

Consumption of inorganic phosphate. Side pathways linking the main pathway for transfer of hydrogen to oxygen, to the growth mechanism are likely to be associated with phosphorylation. Cells were suspended in aerated isotonic maleic buffer containing: (g/litre); glucose, 10, $MgSO_4.7H_2O$, 0·07: (μg/litre); NaH_2PO_4, 155; and various concentrations of tetracycline. The concentration of inorganic phosphate was determined at intervals by the method of Fiske & Subbarow (1927). The eventual amount of inorganic phosphate consumed was lessened, but the initial rate of consumption was stimulated—for example it was increased 13, 44, 94 and 119% by 0·208, 0·417, 2·080 and 4·170 μM tetracycline respectively. Clearly there was no direct inhibition of phosphorylation, and as the rate of uptake of oxygen was decreased by the higher concentrations of tetracycline, the phosphorylation must be associated with a side pathway rather than with the main pathway.

RELATION BETWEEN MODE II AND UTILISATION OF PYRUVATE

Tetracycline and oxytetracycline inhibit the utilisation of pyruvate by non-growing suspensions of cells under the same conditions as they inhibit the rate of growth by Mode II; though the utilisation of pyruvate cannot be the reaction primarily affected by Mode II, it also could be a consequence of Mode II (Jones & Morrison, 1963). This work has been extended to include the four other tetracyclines at pH 7·0. All inhibited the utilisation of pyruvate under unaerated conditions but not if aeration was used; 6-demethyl-6-deoxytetracycline, which does not inhibit rate of growth by

Mode II, inhibited the pyruvate utilisation much less severely. In each instance after a threshold concentration of the antibiotic, the plot of the reciprocal of the rate of consumption of pyruvate against concentration of antibiotic is linear, as it is for the previously studied tetracyclines (Jones & Morrison, 1963). The slopes of these linear plots, for all five tetracyclines inhibiting the rate of growth by Mode II, in turn plot linearly against the rates of increase of inhibition of rate of growth by Mode II (Fig. 5). Thus

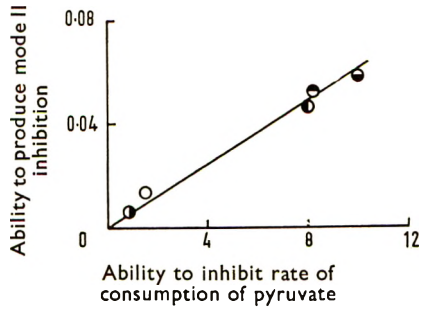


FIG. 5. Relation between mode II inhibition (change of index ratio per mμM antibiotic in basic media at pH 7·0) and inhibition of consumption of pyruvate [min/mM (pyruvate) per 0·1 g (dry-wt) cells/ml per mμM antibiotic]. Antibiotic: tetracycline, ○; oxytetracycline, ◑; 7-chlortetracycline, ◐; 6-demethyl-7-chlortetracycline, ◓; 6-methylene-oxytetracycline, ◒.

the ability of a tetracycline to inhibit the consumption of pyruvate in unaerated medium is quantitatively related to its ability to inhibit rate of growth by Mode II. The simplest explanation is that inhibition of pryuvate consumption is a consequence of Mode II. 6-Demethyl-6-deoxytetracycline, with an ability to affect pyruvate consumption of 0·6 in the units of Fig. 5, should have the ability to inhibit rate of growth by Mode II to an extent of 0·004 per mμM; its ability to inhibit by Mode I is greater than this and hence inhibition of rate of growth is by Mode I.

Discussion

A summary of the relevant literature up to 1960 (Snell & Cheng, 1962) shows the diversity of the interferences with the metabolisms of bacterial cells that can be due to tetracyclines. Tetracyclines have since been found to affect the permeability of the cell walls to amino-acids (Okamoto & Mizuno, 1964) and the transfer of amino-acid to the ribosomal protein in both animal and bacterial systems (e.g. using leucine; Franklin, 1963); the fraction of the ribosomal protein concerned is insoluble in cold trichloracetic acid and the transfer process is dependent on the presence of phospho-enol-pyruvate and another fraction of the ribosome (Franklin, 1964). "The problem becomes one of sorting out their various contributions to inhibition and the order in which they apply (as a function of increasing concentration) to various biological systems" (Snell & Cheng, 1962).

Because they found that 2·1 μM tetracycline (1·0 μg/ml of the hydrochloride), a bactericidal concentration, arrested cell division of *Staphylococcus aureus H* in a medium of undefined pH different to that used in this laboratory, Hash, Wishnik & Miller (1964) suggest erroneously that the optical measurements made on cultures of *Aerobacter aerogenes* in the glucose-salt medium containing 0–0·35 μM tetracycline, have "nothing to do with mean generation times" [*sic*]. Direct measurements of the dry weight of bacterial substance per ml in fully grown cultures confirm that the concentrations of antibiotic used by Jones & Morrison (1962) and by the present authors do not alter significantly the amount of growth produced, and this is correctly obtained from the optical measurements. The mean generation time properly required for the calculation of Index Ratios is the time for the doubling of bacterial mass, viz., log 2 {dt/dlog (dry-wt cells/ml)} and not log 2 {dt/dlog (number of cells/ml)} as was inferred; it was appreciated that any change in size of the bacteria would introduce an error in the latter, which was avoided. In fact, the error would not have been serious; direct measurements with a Coulter medical counter Model A show that all the cultures had an approximately hundred-fold increase in the number of cells per ml and that a change of size could not be detected unless the concentration of antibiotic is near the limit of that required. Hash & others (1964) also do not distinguish between the effects on rate of growth and on amount of growth.

The present use of a method which measures inhibition of growing cells and identifies the mechanism of inhibition, has confirmed with four more tetracyclines the conclusion of Jones & Morrison (1962) that inhibition by a tetracycline is quantitatively related to the concentration of its molecular form in the medium. The two originally described modes of inhibition have again been found to operate but, in addition, a third mode shown by tetracyclines containing a chlorine atom in the 7-position has been identified. The slopes of the plots of Index Ratio against concentration of molecular form provide a measure of the relative intrinsic activities of the various tetracyclines from which the actual activity at a particular pH and concentration of free Mg^{2+}ions, can be calculated (Table 5). The ability

TABLE 5. POTENTIAL ACTIVITIES OF SOME TETRACYCLINES

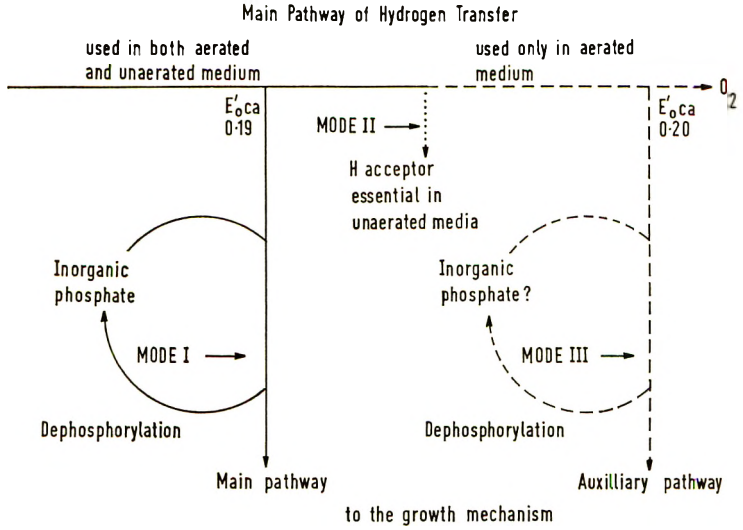
Antibiotic	Change of index ratio per mμM antibiotic in the molecular form					
	Mode I		Mode II		Mode III	
	actual	relative	actual	relative	actual	relative
6-Demethyl-6-deoxytetracycline	0·007	1·0	0·004*	0·5*	?	?
Oxytetracycline	0·007	1·0	0·009	1·2	?	?
Tetracycline	0·007	1·0	0·017	2·2	?	?
6-Methyleneoxytetracycline	0·026	3·6	0·072	9·9	?	?
7-Chlortetracycline	0·028	3·8	0·063	8·6	0·031	4·2
6-Demethyl-7-chlortetracycline	0·026	3·6	0·080	11·0	0·038	5·2

*Calculated from inhibition of consumption of pyruvate.

to inhibit by Mode III may be common to all the tetracyclines but, if so, the intrinsic activity as inhibitors by Mode III of the tetracyclines not containing chlorine at the 7-position, is less than 0·007 per mμM, and inhibition by

Mode I prevents inhibition of rate of growth of *Aerobacter aerogenes* by Mode III. The intrinsic activity to inhibit by Mode I is unaltered by substitution of —OH for —H at the 5-position, and by simultaneous demethylation and replacement of —OH by —H (and probably to demethylation alone) at the 6-position, but it is increased markedly by a methylene group at the 6-position or a chlorine atom at the 7-position. The intrinsic activity as an inhibitor by Mode II is decreased by —OH at the 5-, and by —H replacing —OH at the 6-, but is increased by methylene at the 6- or chlorine at the 7-position. There is no obvious relationship between the intrinsic activities as inhibitors by Modes I and II, and the sizes of the dissociation constants or of the association constants for the complexes with Mg^{2+}. It should be pointed out that these constants do affect the degree of inhibition in practice as that is dependent on the environmental conditions so that, e.g. in our "basic" test medium at pH 7·0, 6-methyleneoxytetracycline is the most potent by Mode II.

Modes I and III, according to the results with added redox systems, measurements of oxygen uptake, and measurements of the initial rates of uptake of inorganic phosphate, are interferences with reaction sequences linking the main pathway of hydrogen transfer from glucose to oxygen, to the growth mechanism. A possible relationship between these two modes and Mode II is:



Reversibility of the reaction sequence in which Mode I occurs, coupled with a release of inorganic phosphate by dephosphorylation of intermediates occurring further down the same reaction sequence, could account for the temporary stimulation of uptake of phosphate that was observed in the presence of tetracycline.

Correlating the present findings with the inhibitions found by other workers is complicated. (*a*) The quantitative relationship of bacteriostatic action to inhibitions of isolated parts of various metabolic systems

in media of differing composition and pH, cannot be assessed without knowing the relationships between internal and external concentrations of materials which are present within the cells and in the surrounding media. (*b*) It may not be possible to associate a published measure of bacteriostatic ability with a definite mode of action. For example, if the present results for chlortetracycline had been expressed as the concentration needed to inhibit the rate of growth by 50% (corresponding to an Index Ratio of 2·0 in Fig. 1) the inhibition could have been by any of the three modes according to the conditions. In many instances inhibitions are expressed as '% inhibition of growth' with no indication of the nature of the actual measurements. (*c*) In the media only the concentrations of the molecular forms of six different tetracyclines are related to the bacteriostatic action and it is impossible to calculate these concentrations unless information about the interrelations of materials is available.

Qualitatively, however, connections may be suggested. Indeed with inhibitions of reductases, dehydrogenases, oxidases, oxidation or reduction systems, and energy requiring systems, possible connections are obvious. The inhibitions in Franklin's experiments with systems isolated from *Escherichia coli* and rat liver cells (1963, 1964) may also be connected with Mode I or Mode II. He used 400 μM chlortetracycline at pH 7·80 in the presence of 10 mM magnesium which, if all the magnesium is assumed to be free, provides 1 μM of the molecular form. Such a concentration is ten times that needed to produce an Index Ratio of 4 by Mode II and eight times that needed to produce the same degree of inhibition by Mode I for *Aerobacter aerogenes*; but the inhibition of the isolated systems may well occur at lower concentrations of antibiotic, and a higher internal pH than that of the medium may result in a higher internal concentration of antibiotic within the intact cells of *Aerobacter aerogenes*. Mode I in *Aerobacter aerogenes* is a derangement of protein synthesis, and though Mode II does not operate in this strain in aerated medium, Franklin's experiments used only part of the whole cell mechanism and he found that phosphoenol-pyruvate was required: there is a connection between Mode II and inhibition of the consumption of pyruvate.

The inhibition of the utilisation of D-glutamate by *Escherichia coli*, which is possibly due to competition between the A-ring of (oxy)tetracycline and D-glutamate for the same enzyme (Snell & Cheng, 1962), might be identified with Mode III which is certainly concerned with D-glutamate. This in turn, as D-glutamate is a constituent of cell walls, could affect the permeability of the walls of *Escherichia coli* as reported for tetracycline (Okamoto & Mizuno, 1964) and chlortetracycline (Alexander, 1960).

Since the kinetics of the investigated inhibitions of intact cells that we report could be dominated by the external concentration of the molecular form of the inhibitor, if that were the only form able to penetrate the cells, it is not possible to rule out mechanisms of inhibition which depend on the power of tetracyclines to form complexes with certain cations. If the kinetics are so dominated, and other forms are inhibitory, the intrinsic activities in Table 5 would each have to be amended by the appropriate factor calculated from the constants in Table 1 and the internal pH

pertaining when the experimental measurements were made. This possibility will be examined later.

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Antagonism of 5-hydroxytryptamine-induced bronchospasm in guinea-pigs by 8β-carbobenzyloxy-aminomethyl-1-methyl-10α-ergoline

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The drug 8β-carbobenzyloxyaminomethyl-1-methyl-10α-ergoline (MCE) has been shown to have a potent and prolonged antagonism to 5-hydroxytryptamine-induced bronchospasm in guinea-pigs. 4–5 hr after 2 μg/kg of MCE, given intraveneously, the effects of the 5-HT were still markedly inhibited (dose ratio more than 1 : 300). A subcutaneous dose of 150 μg/kg of the drug partially counteracted the effects of 5-HT for 4–7 days. Comparison between MCE and 1-methyl-(+)-lysergic acid butanolamide tartrate (methysergide, UML 491) revealed that the antagonism to 5-HT by MCE developed more slowly but lasted longer than that elicited by methysergide. The results show that MCE is a specific antagonist of 5-HT-induced bronchospasm in guinea-pigs as it does not antagonise the bronchospasm-inducing effects of acetylcholine, histamine and eledoisin.

IN a previous paper Beretta, Ferrini & Glässer (1965) reported the strong antagonism of 5-hydroxytryptamine (5-HT) by a new 6-methylergoline derivative synthetised in our laboratories (Bernardi, Camerino, Patelli & Redaelli, 1964), namely 8β-carbobenzyloxyaminomethyl-1-methyl-10α-ergoline (MCE).

The new compound showed a remarkable antagonism to 5-HT *in vitro* and reduced local 5-HT-induced oedema in the rat paw for a long time. This prolonged action is a characteristic of the compound and to provide further confirmation of this property, another test on a different animal species was used. As preliminary experiments had shown that MCE exerted a potent antagonism towards guinea-pig bronchospasm evoked by 5-HT, it was decided to investigate fully its inhibitory effect in this test.

We compared the anti-5-HT action of MCE with that of 1-methyl-lysergic acid butanolamide tartrate (methysergide, UML 491), one of the most potent of 5-HT antagonists (Fanchamps, Doepfner, Weidmann & Cerletti, 1960).

Experimental

METHOD

The method is based on that of Konzett & Rössler (1940) with a water manometer as recording system. Guinea-pigs of 500 to 700 g were anaesthetised with urethane, 1 g/kg i.p. and 1·5 g/kg s.c.

The trachea was cannulated and the lungs inflated by a Starling pump at 36 strokes/min. To suppress the spontaneous respiratory movements, guinea-pigs were injected intravenously with a single dose of 20 mg of gallamine triethiodide (Sincurarina Farmitalia). Optimal stroke volume (varying from 5 to 15 ml) was chosen for each animal.

At the end of each experiment the trachea was clamped and the maximal excursion of the water manometer was measured in mm and recorded as

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"total bronchospasm" (i.e. the maximal resistance to inflation of the air). The effects of doses of bronchoconstrictor agents were expressed as percentage of total bronchospasm. Solutions of drugs in saline were injected at 4 to 8 min intervals through a cannula in the external jugular vein, the volume of each injection (0·5 ml washed in 0·5 ml of saline) being constant. After the resistance to inflation had been increased by a drug, it was returned to the original level by clipping the tube leading to the recording apparatus briefly, thus forcing one full stroke volume of the pump into the lungs.

Two types of experiments were made. In the first, the animals were prepared as described above and drugs (both agonists and antagonists) were administered intravenously. The action of MCE against 5-HT and other bronchoconstrictor drugs was compared with that of other antagonists. 5-Hydroxytryptamine creatinine sulphate, histamine hydrochloride, synthetic eledoisin, synthetic bradykinin and acetylcholine bromide were used as agonists. MCE, methysergide, mepyramine, atropine sulphate, phenoxybenzamine, dihydroergotamine and morphine were used as antagonists.

The second set of experiments was made on groups of guinea-pigs (5 animals in each) treated subcutaneously with different doses (1·5–15 and 150 μg/kg) of MCE or with 150 or 1500 μg/kg of methysergide.

At intervals of 6, 24 and 72 hr after pretreatment, the animals of each group were surgically prepared as described above and dosed intravenously with increasing doses of 5-HT. Three increasing doses of histamine were also administered intravenously to control the sensitivity of the animal preparation.

Injection of these agonists always followed the same regimen: 5-HT (2·25, 4·50 and 9 μg/kg), histamine (2·5, 5 and 10 μg/kg) and then 5-HT again (22·5, 45, 90 and 450 μg/kg).

Thus for each experimental animal a dose-response curve was obtained. For each interval and for each dose of the antagonists a new group of guinea-pigs was used to test the antagonistic effect.

Results

The threshold dose of 5-HT producing bronchospasm in guinea-pigs varied from 2·25 to 4·50 μg/kg i.v. of 5-HT base. Moreover, doses of up to 9 μg/kg i.v. gave a good dose-response relationship. Nearly 90% of total bronchospasm was obtained with 22·5 μg/kg i.v. of 5-HT. No tachyphylaxis was observed. Evaluation of 5-HT effects as a percentage of total bronchospasm seems to be a useful modification of the original method of Konzett & Rössler (1940).

MCE, at a dose of 1 μg/kg i.v., showed little or no antagonism to 5-HT, but 2 μg/kg i.v. had a pronounced inhibitory effect. Inhibition was slow to develop, increased gradually and lasted for a long time (see Fig. 1); the effects of 5-HT induced bronchospasm being still markedly inhibited 4–5 hr after administration of the MCE (dose ratio more than 1 : 300).

Methysergide was also seen to be a potent antagonist of the bronchospasm produced in guinea-pigs by 5-HT. Its action developed more quickly but declined more rapidly than that of MCE. Methysergide, 1 μg/kg i.v.,

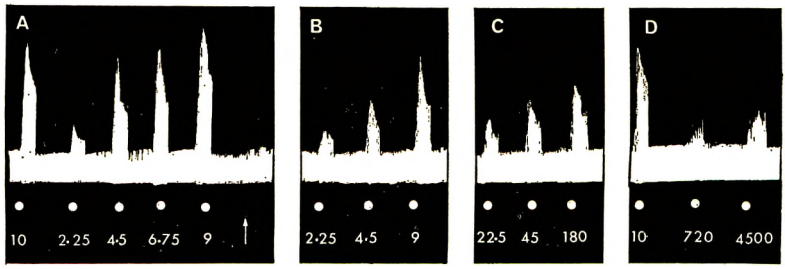

FIG. 1. Bronchospasm induced by 5-hydroxytryptamine 2·25–4500 μg) and by histamine (10 μg) in an anaesthetised guinea-pig) (A) before, (B) 20 min, (C) 1½ hr. and (D) 4 hr after MCE (2 μg) injected at the arrow. All drugs were injected intravenously.

showed a strong and relatively short-lasting inhibitory effect; 2 μg/kg i.v. elicited a stronger longer-lasting inhibition. However 4 hr after the 2 μg/kg dose, the initial effect produced by 5-HT was obtained with doses of 5-HT only 30–40 times higher than those used initially (dose ratio 1 : 30; 1 : 40).

The anti-5-HT action of MCE is specific. At high doses (250–500 times higher than those active against 5-HT) it did not affect the bronchospasm provoked by histamine hydrochloride (10–15 μg/kg i.v.), acetylcholine bromide (5–10 μg/kg i.v.) or synthetic eledoisin (0·2–0·3 μg/kg i.v.). At a dose of 1 mg/kg i.v., MCE exerted a weak and short-lasting anti-bradykinin action (about 5 min) and sometimes itself produced a broncho-spasm.

Morphine, phenoxybenzamine and dihydroergotamine, which are known to be powerful antagonists of 5-HT in other tests, were inactive or only slightly active against 5-HT bronchospasm in the guinea-pig, even at high doses (0·5–1·0 mg/kg i.v.). Atropine showed an inhibitory

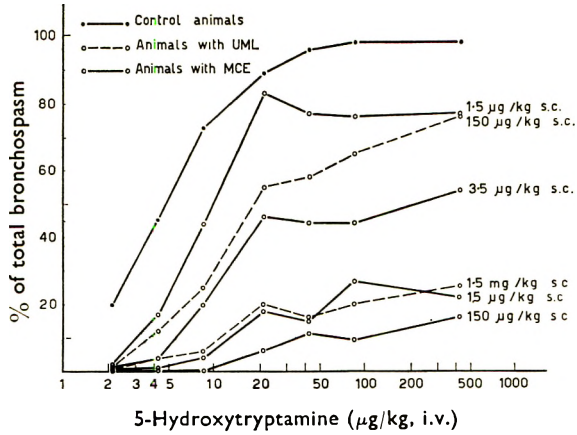

FIG. 2. Bronchospasm-inducing effects of increasing doses of 5-hydroxytryptamine i.v. 6 hr after pretreatment (s.c.) with saline alone (control animals) or with different doses of MCE or of methysergide (UML). Each curve represents the mean of five animals.

effect towards 5-HT at doses of 0·5–1·0 mg/kg i.v., however, the antagonism was not specific as the effect of acetylcholine on guinea-pig bronchospasm was also inhibited by atropine at far smaller doses (1·5 μg/kg i.v.).

The antihistamine drug mepyramine, which specifically antagonised the bronchospasm-inducing effects of histamine when given in small amounts (2–10 μg/kg i.v.), did not modify the response to 5-HT, but at higher doses (0·5–1·0 mg/kg i.v.) it did diminish the 5-HT effects.

The results from the second set of experiments are in Figs 2–4, from which it can be seen that the curves for the control animals have a linear dose response relation for the increasing doses of the agonist (5-HT) up to 9 μg/kg i.v. Higher doses of 5-HT elicited maximal or nearly maximal effects.

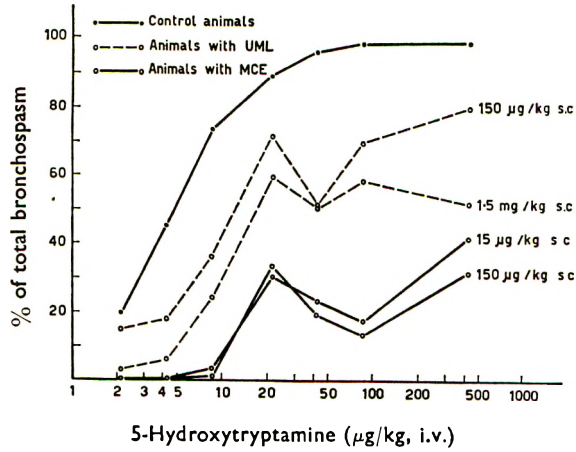

FIG. 3. As Fig. 2 except that the time interval between antagonists and 5-hydroxytryptamine was 24 hr.

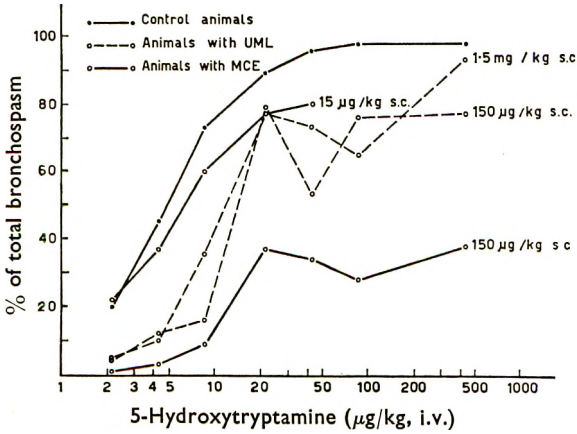

FIG. 4. As Fig. 2 except that the time interval between antagonists and 5-hydroxytryptamine was 3 days.

The curves for the animals subcutaneously pretreated with antagonists showed that both MCE and methysergide were potent anti-5-HT agents. By increasing the doses, 5-HT inhibition increased and persisted longer. From Fig 2–4 it can also be seen that the shapes of the curves for the treated animals differ from those of the control animals, and do not show a simple shift on the log-dose axis (see Fig. 5). Comparison between

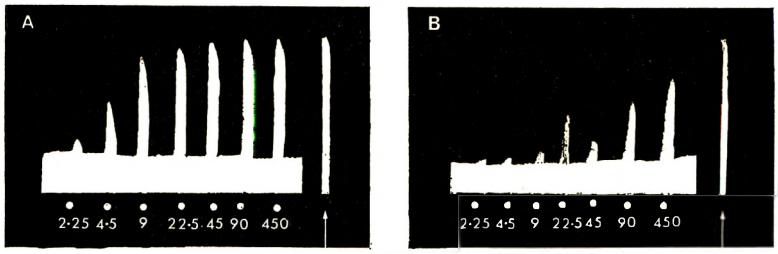

FIG. 5. Bronchospasm-inducing effects of increasing doses of 5-hydroxytryptamine (μg/kg, i.v.) in two anaesthetised guinea-pigs s.c. pretreated 4 days before (A) with saline alone and (B) with 150 μg/kg of MCE. At arrow, total bronchospasm for definition see text).

MCE and methysergide 6 hr after dosing shows that MCE is about 100 times more potent than methysergide, the threshold inhibitory dose of MCE being 1·5 μg/kg s.c. After 24 hr, the inhibitory action of methysergide diminished markedly, while that of MCE at doses even 100 times smaller was still very noticeable.

Three days after treatment (Fig. 4) the actions of methysergide 150 and 1500 μg/kg s.c. and of MCE, 15 μg/kg s.c. were practically undetectable. At this time 150 μg/kg s.c. of MCE was still very active and the activity was still present 4–7 days after dosing (see Fig. 5).

Discussion

MCE has a strong and specific antagonistic action towards 5-HT-induced bronchospasm in guinea-pigs. Its specificity of action was proved when it was tested against other bronchoconstrictor drugs. No inhibition was found against histamine, acetylcholine, and eledoisin even at doses 250–500 times higher than those active against 5-HT. The anti-bradykinin action detectable with 1 mg/kg i.v. of MCE is too slight and short-lasting to be considered important. The action of MCE is characterised by a very slow and gradual onset and by an extraordinary long duration. These results are in agreement with those obtained in other tests both *in vitro* and *in vivo* (Beretta, Ferrini, & Glässer, 1965).

We suggest that these particular properties of the compound may be due to a tenacious fixation of MCE, or of an active metabolite, to the 5-HT receptors.

Phenoxybenzamine or morphine at high doses (1 mg/kg i.v.) failed to antagonise 5-HT bronchospasm. At this dose phenoxybenzamine was found to be very active in blocking the pressor effects of adrenaline in

guinea-pigs (unpublished data). Atropine and mepyramine partly antagonised the action of 5-HT but the effect was not specific as they diminished the bronchoconstrictor action of acetylcholine and histamine respectively at far smaller doses, and their 5-HT antagonism was at least 250–500 times less than that of MCE.

The well known anti-5-HT drug, methysergide was very active in the Konzett-Rössler test, but, compared with MCE, its action developed more rapidly and did not last as long. The shape of the curves for inhibition of 5-HT bronchospasm following s.c. injection of inhibitors, is unusual, e.g. Fig. 3. This might be explained by assuming that when inhibitors are present, doses of 5-HT greater than 20 μg/kg exert both stimulant and tachyphylactic actions, although these are not seen with 5-HT alone.

The last suggestion seems to be supported by the anti-5-HT action exerted by 5-HT itself on guinea-pig bronchospasm as previously described by Courvoisier & Lean (1959). Our results show that the use of 5-HT bronchospasm, as modified by us, is suitable for studying the anti-5-HT drugs with long-lasting action.

Acknowledgement. We thank Sandoz, Ltd. (Switzerland) for generous supplies of methysergide and of synthetic bradykinin. We are also grateful to Dr. C. D. Bianchi for his help in translating the text.

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Estimation of dopamine, noradrenaline, adrenaline and 5-hydroxytryptamine from single rat brains

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A solvent extraction method was used to extract dopamine, noradrenaline, adrenaline and 5-hydroxytryptamine from single rat brains. Spectrophotofluorimetric estimations of dopamine, noradrenaline, adrenaline and 5-hydroxytryptamine were completed 5½ hr after beginning the extraction procedure. The method was applied to the routine estimation of brain amines.

PUBLISHED methods for the extraction and estimation of dopamine from tissue extracts usually involve the use of chromatographic columns and are time consuming. This disadvantage is avoided by applying the solvent extraction method devised by Shore & Olin (1958) for noradrenaline, to the extraction of dopamine. Since in addition to noradrenaline and dopamine, adrenaline and 5-hydroxytryptamine (5-HT) are also extracted by this method, they may be estimated in the same extract from a single rat brain. This paper describes the method and establishes its authenticity and reliability.

Experimental

METHODS

Male Wistar rats, 180–260 g, were killed by stretching the neck; the brains were dissected, the meninges and the cerebellum removed and the remainder stored at −10°.

Extraction of brain amines. A single rat brain was dropped into liquid nitrogen or liquid air, shattered (Callingham & Cass, 1963) and dispersed in 30 ml of butanol reagent to which was added 2 ml 0·01N hydrochloric acid and 5 g sodium chloride. The suspension was shaken for 60 min in a 70 ml stoppered bottle to extract the brain amines into the butanol phase. After centrifugation at 2,000 rev/min for 5 min, 25 ml of the butanol layer was withdrawn and added to 50 ml n-heptane + 7 ml 0·01N hydrochloric acid. Shaking for 5 min sufficed to pass the amines from the organic phase into the aqueous phase, and after centrifugation at 2,000 rev/min for 2 min, of the aqueous phase recovered, 6·5 ml was distributed for assay as follows: 2·5 ml for the dopamine assay, 1·5 ml for the noradrenaline assay, 1·5 ml for the adrenaline assay, and 1·0 ml for the 5-HT assay.

Assay of dopamine. (Derived from the method of Carlsson & Waldeck, 1958). To 2·5 ml of the 0·01N hydrochloric acid extract is added 0·5 ml of 0·1M phosphate buffer (pH 6·5). The pH is then adjusted to 6·46–6·54 by dropwise addition of 0·5M and 0·1M potassium carbonate whilst bubbling with nitrogen. Adjustment of the pH is critical (pH was measured with a pH meter using micro-electrodes). Aliquots of 2 × 1·2 ml are placed in 4 inch × ⅝ inch test tubes (one for test, one for blank) and to each is added 0·05 ml of the iodine-potassium iodide solution.

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After 5 min, 0·5 ml alkaline sulphite solution is added to the test and 0·5 ml 2·5N sodium hydroxide is added to the blank. After a further 5 min, 0·6 ml of 2·5N acetic is added to both test and blank and these are then irradiated vertically under an ultraviolet lamp (Phillips TL20 W/8) for 20 min, when 0·05 ml water is added to the test and 0·05 ml M Na_2SO_3 solution is added to the blank. The fluorescence is measured in a spectrophotofluorimeter, with an excitation wavelength of 335 mμ and a fluorescence wavelength of 378 mμ (uncorrected values).

Assay of noradrenaline, adrenaline and 5-HT. Noradrenaline and adrenaline were estimated by the method of Shore & Olin (1958) but using one quarter of the volume of all reagents specified in their text.

5-HT was estimated by the method of Udenfriend, Weissbach & Bogdanski (1955).

Drugs. Dopamine hydrochloride; adrenaline hydrochloride; noradrenaline bitartrate; 5-hydroxytryptamine creatinine sulphate; phenelzine sulphate; amitriptyline hydrochloride; reserpine.

Reagents. A.R. grade reagents were used unless noted. The water used throughout was triple glass-distilled.

Butanol reagent: 500 g of sodium chloride was shaken with a mixture of 4 litres of n-butanol (D.C.L. Biscol) and 0·7 litres 0·01N hydrochloric acid. Excess acid was removed and the saturated butanol allowed to stand for 24 hr when any further acid was removed. The reagent was stored over sodium chloride. *n-Heptane* (Phillipps, 99 mol %, or B.D.H.). 0·1M *Phosphate buffer* pH 6·5: 1·075 g $Na_2HPO_4.12H_2O$ + 0·952 g KH_2PO_4 was made up to 100 ml with water. *Iodine-potassium iodide solution:* 0·085 g iodine + 1·67 g potassium iodide were dissolved in 5 ml water and diluted to 100 ml with water. *Alkaline sulphite solution:* 2·52 g $Na_2SO_3.7H_2O$ was dissolved in 10 ml of water, 10 g sodium hydroxide was added and water to 100 ml. 2·5N *Acetic acid:* 14·25 ml glacial acetic acid was made up to 100 ml with water.

Results

The relation between the concentration and the fluorescence intensity of dopamine developed by this method is linear, the points falling on a straight line within the range 0·01 and 1·28 μg/ml (Fig. 1). That dopamine is extracted quantitatively by the extraction procedure is shown in Fig. 2. When pure dopamine solutions are extracted, 75% of the dopamine is recovered in the final acid aliquot.

When dopamine is added to rat brain tissue before extraction, 72% $\pm$3·8% s.e. (14 experiments) of that added is recovered in the final acid aliquot. Similarly, the recovery from rat brain tissue of added noradrenaline is 59% $\pm$1·6% s.e. (14 experiments) and of added 5-HT is 87% $\pm$3·2% s.e. (14 experiments).

Noradrenaline, adrenaline and 5-HT do not contribute significantly to the fluorescence developed by an equal weight of dopamine. The fluorescence intensities developed by equal weights of base are: dopamine 100·0; dopa 74·0; noradrenaline $<$0·1; adrenaline 0·2; 5-HT $<$0·1. Although dopa fluoresces under these conditions, its interference in

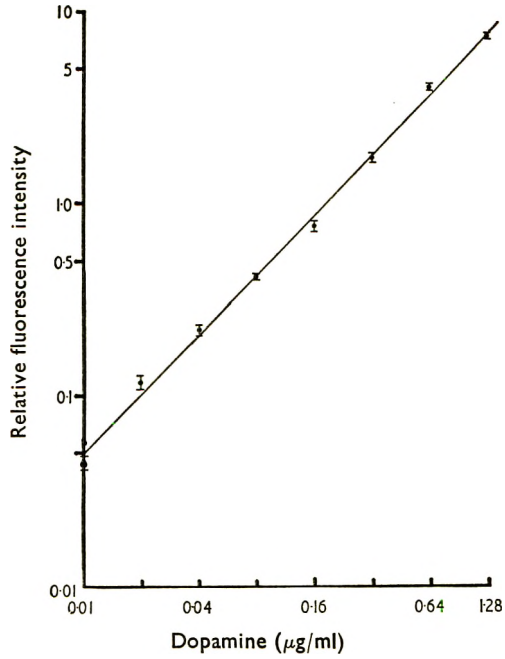

FIG. 1. The relation between fluorescence intensity developed and concentration of dopamine. Each point is the mean from 6 experiments, and the vertical lines represent the s.e. of the mean.

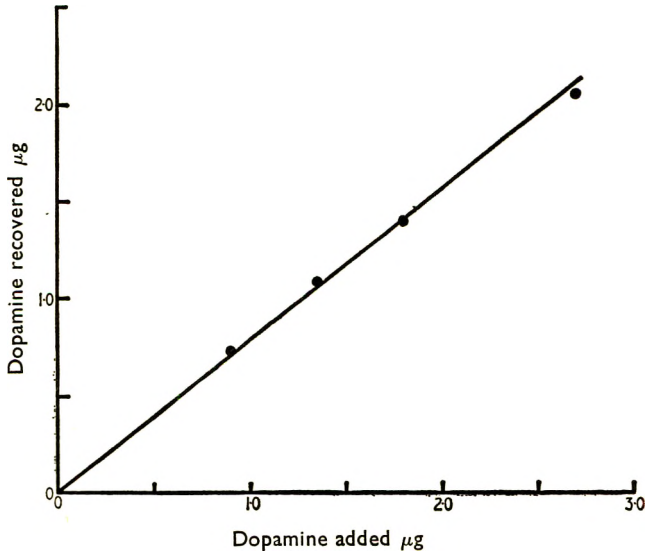

FIG. 2. Graph showing the quantitative extraction of dopamine by this method. Each point is the mean from three experiments.

tissue extracts is negligible, since the amount present in most tissues is small (less than 0·04 μg/g, Anton & Sayre, 1964).

The excitation and fluorescence spectra of rat brain extracts which have been subjected to the dopamine assay procedure are shown in Fig. 3.

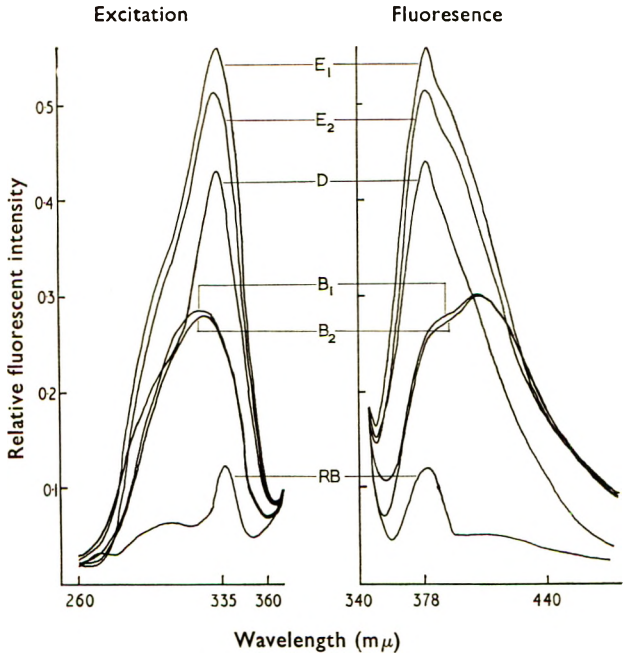

FIG. 3. Excitation and fluorescence spectra of extracts from rat brain (E_1 and E_2), tissue blanks (B_1 and B_2), pure dopamine 0·1 μg/ml (D) and a reagent blank (RB).

The spectra peaks from the brain extracts correspond to the spectra peaks of pure dopamine. The scatter peaks for all samples are similar and the small peaks in the reagent blank spectra coincide with the wavelengths calculated for Raman scatter.

The fluorescence intensity of blanks from brain extracts is reproducible and consistent; the peaks are larger than those of a reagent blank but are broad and non-specific compared with the dopamine peaks. With an extract from a single rat brain there is sufficient difference between the test and the blank readings to allow precise estimates of the dopamine content. The values obtained for the absolute content of dopamine, noradrenaline, adrenaline and 5-HT in rat brain are in agreement with values previously published in the literature (Table 1).

APPLICATION OF THE METHOD

The application of the method was challenged by examining the changes of brain catecholamine levels in the rat after treatment with drugs known to influence these substances. Thus after treatment with the monoamine oxidase inhibitor, phenelzine, 30 mg/kg intraperitoneally, the levels of

TABLE 1. COMPARISON OF VALUES FOR AMINE CONTENTS OF RAT BRAIN OBTAINED BY DIFFERENT WORKERS

Method	Amine content as μg/g fresh brain				Authors
	Dopamine	Noradrenaline	Adrenaline	5-HT	
Column separation methods	0·62 ± 0·03*	0·39 ± 0·01	—	0·51 ± 0·01	Gey & Pletscher (1961)
	0·60	0·49	—	—	Carlsson (1959)
	0·68	0·32	0·04	—	Anton & Sayre (1964)
	0·54 ± 0·04	0·42 ± 0·01	—	—	Moore & Lariviere (1963)
Present method	0·68 ± 0·05	0·40 ± 0·02	<0·01	0·59 ± 0·01	Brownlee & Spriggs

* Denotes s.e. of mean. The sign — means not reported.

dopamine, noradrenaline and 5-HT were all increased 4 hr after injection. After reserpine, 1 or 2 mg/kg, the characteristic reduction in all three amines was found; in addition the effects were dose dependent. Amitriptyline, however, did not alter the dopamine, noradrenaline or 5-HT content of the rat brain. These results are shown in Fig. 4 in which each histogram represents the mean from three rat brains.

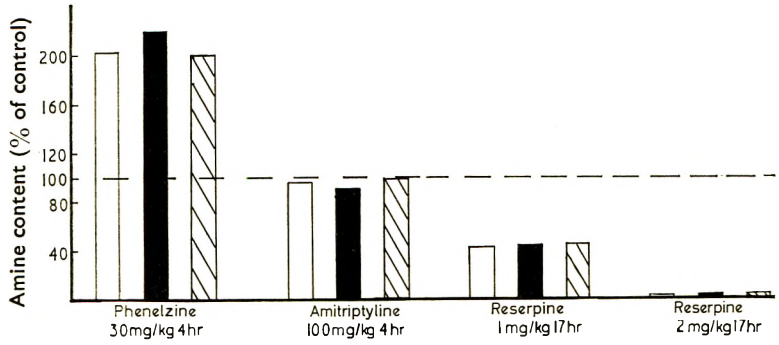

FIG. 4. The effects of phenelzine, reserpine or amitriptyline on the amine content of rat brain. Dopamine (solid columns), noradrenaline (open columns) and 5-HT (hatched columns) levels are increased after phenelzine, reduced after reserpine and unmodified after amitriptyline. Each histogram represents the mean from three rat brains.

Because of the fluorescence arising from non-specific substances extracted from tissues, the method, when applied to dopamine, is unsuitable for the estimation of the absolute amounts in tissues, when these fall below about 0·15 μg/g.

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Observations on the pharmacology of halquinol

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Halquinol non-specifically depresses tone and motility in isolated intestinal smooth muscle and reduces intestinal motility in intact animals. These properties may be involved in its therapeutic effects.

HALQUINOL (chlorhydroxyquinoline) is prepared by controlled chlorination of 8-hydroxyquinoline. It is a mixture of 5-monochlor-8-hydroxyquinoline (34·27%), 5,7-dichlor-8-hydroxyquinoline (64·22%) and 7-monochlor-8-hydroxyquinoline (2·24%). It is effective *in vitro* against a wide range of micro-organisms including bacteria, yeasts and fungi (Heseltine & Freeman, 1959). Studies in rats indicated only slight absorption after oral administration, but later investigation in man and other species showed that substantial portions of oral doses of the drug could be found in the urines (Heseltine & Freeman, 1959; Heseltine & Campbell, 1960; Freeman & Heseltine, 1963). This investigation was undertaken to obtain more information on the actions of halquinol on smooth muscle-containing tissues and organs.

Experimental

Solutions for use with isolated tissue preparations were prepared by dissolving the drug or any one of its constituents in the minimum quantity of 2N sodium hydroxide solution, and adjusting to pH 11 with 2N hydrochloric acid. Lower pH values caused precipitation. Matching control solutions were prepared in an identical fashion. Since the amount of drug solution added to the bath was small compared with the bath volume there was no precipitation and little effect on pH.

The effects of halquinol, 5-monochlor-8-hydroxyquinoline, 5,7-dichlor-8-hydroxyquinoline and 7-monochlor-8-hydroxyquinoline have been assessed using the isolated frog rectus abdominis muscle, the isolated guinea-pig ileum, the rat diaphragm-phrenic nerve and the gastrocnemius muscle-sciatic nerve preparation, blood pressure and nictitating membrane of the cat.

To investigate gastrointestinal actions, the effect of halquinol upon the peristaltic reflex, using the Trendelenburg preparation, was examined. Certain experiments were made only upon intact animals. These were:

(1) Groups of ten male albino mice, 18–25 g, were fasted for 36 hr. The drug was then given by intraperitoneal injection and 1 hr later the animals were allowed to feed from a "charcoal" diet (see below) for ½ or 3 hr. After a further 3 hr, they were killed and the intestines removed and examined to find the distance travelled by the charcoal diet. Since in the controls, the distance travelled by the charcoal varied from animal to animal, the colour of the appendix was taken as the criterion (Janssen &

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Jageneau, 1957). After giving the drug, blackening of the appendix with charcoal was taken to indicate no slowing of the passage of the gut contents.

(2) Groups of 20 mice [as (1) above] and Wistar strain male rats, weighing 200–250 g, were fasted for 36 hr. Ten animals were given 5 g of ordinary diet, and 10 given 5 g of halquinol diet (see below). Each animal was kept in a separate cage during the experiment and allowed free access to water. After each hour, the faecal pellets from each cage were collected and counted. Separate experiments were made in which groups of 5 rats or mice were kept similarly but without food to see if fasting influenced the output of faecal pellets. A similar series of experiments was made in which the drug in suspension in 0·25% tragacanth mucilage was given by stomach tube at either 50 or 100 mg/100 g body weight. In these experiments, the animals were fasted for only 18 hr. The number of faecal pellets was used as a qualitative index of gastrointestinal motility but as the pellets varied in size and weight, in one experiment the weights as well as the numbers were recorded.

Because of the great variations in numbers of faecal pellets from animal to animal, animals were selected which gave fairly similar numbers of pellets over hourly intervals, but, even in selected animals, the numbers were not reproducible from day to day.

(3) Inhibition of intestinal motility was measured using the method of Bryant, Felton & Krantz (1957). Male Wistar rats weighing 150–250 g were fasted for 36 hr. A suspension of halquinol in 1% sodium carboxymethylcellulose was given by stomach tube (100 mg/100 g weight). 30 min later an aqueous suspension of 10% charcoal in 5% gum acacia mucilage was given. The control animals received an equal volume of 1% sodium carboxymethylcellulose suspension. After 15 min the animals were killed, the intestines removed from the pylorus to the appendix and the most advanced point of travel of the charcoal measured. The distance was calculated as a percentage of the total pylorus-appendix distance (Table 3).

In all instances a statistical analysis was made using Students' "*t*" test. Because of the great variations in the output of faecal pellets per rat, it was decided to take as a measure the increase in the numbers of pellets from the second to the seventh hr after drug-administration.

Charcoal diet. Diet No. 41 was powdered; 10% by weight of powdered animal charcoal and 5% by weight of powdered gum acacia was added and the whole made into a paste with water. The mass was formed into suitably shaped pieces and dried in an oven.

Halquinol diet. This was made similarly but instead of charcoal and acacia, 10% by weight of drug was incorporated. No heat was used during drying.

Results

There was no direct effect on the frog rectus (50 μg to 1 mg; 10 ml bath). No graded inhibition of acetylcholine-induced contractures was observed but 1 mg or more irreversibly depressed the ability to respond to acetylcholine. No effects were seen on neuromuscular transmission in the rat

diaphragm (0·5 to 8·0 mg; 80 ml bath) or the cat gastrocnemius-sciatic preparation (1 to 3 mg/kg).

Variable effects were seen on the guinea-pig ileum. There was no direct effect (10 to 40 μg; 10 ml bath); the responses to acetylcholine and histamine were slightly depressed while antagonism was neither graded nor specific and incompletely reversible even after repeated washings.

All the compounds tested (0·5 to 2·0 mg; 62 ml bath) produced a marked inhibition of peristalsis (Fig. 1). The effects were similar to those

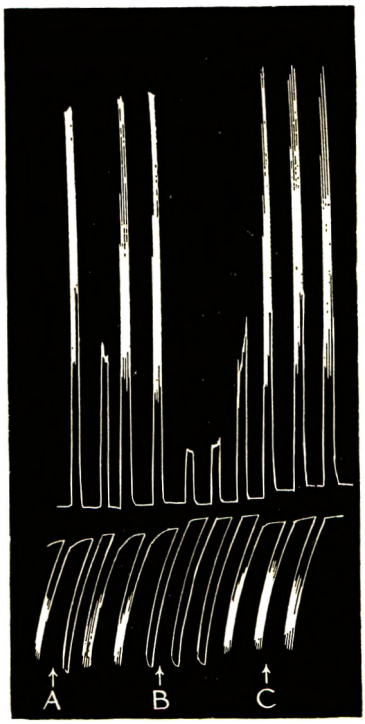

FIG. 1. Effect of halquinol upon contractions of the longitudinal muscle (upper trace) and peristalsis (lower trace) in the guinea-pig ileum. At A, 1 mg hexamethonium. At B, 1 mg halquinol. At C, 1 ml control solution. 5-Monochlor-8-hydroxyquinoline, 5,7-dichlor-8-hydroxyquinoline and 7-monochlor-8-hydroxyquinoline had similar effects to halquinol on this tissue.

of hexamethonium (Fig. 1). No reduction in the height of contraction of the nictitating membrane of the anaesthetised cat was caused by 1 to 5 mg/kg. 1 to 5 mg/kg caused a marked but short-lived fall in blood pressure in the anaesthetised cat which was not prevented by atropine or mepyramine.

No inhibitory effect on the passage of the charcoal diet in mice was shown when halquinol was given by intraperitoneal injection. When halquinol diet was given, there was no significant difference from control in the numbers of faecal pellets in rats (Table 1); when groups of selected

TABLE 1. HOURLY OUTPUT OF FAECAL PELLETS IN MICE AND RATS, FED A DIET CONTAINING 10% HALQUINOL. ALL VALUES ARE MEANS ± (S.E.) OF DETERMINATIONS ON 10 ANIMALS

	Time (hr) after feeding						
	Mice						
	1	2	3	4	5	6	7
Control	1·00 ± 0·28	3·00 ± 0·64	6·00 ± 1·02	7·40 ± 0·85	12·10 ± 1·42	13·00 ± 1·43	16·10 ± 2·34
Drug	0·90 ± 0·41	2·80 ± 0·42	4·30 ± 0·50	4·60 ± 0·58 *	4·90 ± 0·75 **	5·20 ± 0·73 **	5·30 ± 0·73 **
	Rats						
Control	2·60 ± 0·88	3·90 ± 0·83	6·20 ± 1·25	7·00 ± 1·29	8·00 ± 1·31	10·10 ± 1·53	11·0 ± 2·22
Drug	1·60 ± 0·65	2·30 ± 0·80	4·20 ± 1·29	5·40 ± 1·07	6·00 ± 0·85	7·10 ± 1·02	7·20 ± 1·07

Significance of difference from control * $0{\cdot}05 > P > 0{\cdot}01$ ** $P > 0{\cdot}001$.

TABLE 2. MEAN DECREASE (± S.E.) IN OUTPUT OF FAECAL PELLETS FROM 2ND TO 7TH HR AFTER HALQUINOL ADMINISTRATION COMPARED TO CONTROLS

Series	Species	Drug treatment	Mean ± s.e.
1	Rats randomly selected	Control Suspension (50 mg/100 g)	7·40 ± 1·37 5·20 ± 1·00
2	Rats randomly selected	Control Suspension (100 mg/100 g)	11·30 ± 1·17 6·88 ± 1·31*
3	Rats randomly selected	Control 10% diet	7·10 ± 2·07 4·90 ± 0·86
4	Mice randomly selected	Control 10% diet	21·77 ± 3·61 4·88 ± 0·88**
5	Rats selected	Control 10% diet	7·80 ± 1·68 2·80 ± 0·53*
6	Mice selected	Control 10% diet	12·55 ± 2·01 1·80 ± 0·81**

Significance of difference from control, * $0{\cdot}05 > P > 0{\cdot}01$; ** $P > 0{\cdot}001$.

rats were used and the average increase in numbers of pellets from the second to the seventh hr taken, there was a significant reduction in the number of pellets in the drug-treated animals (Table 2). In mice, however, a significant decrease in the number of pellets was observed in both selected and non-selected animals (Tables 1 and 2). The control animals consumed all the diet given, but the animals on the halquinol diet all left some. The lower numbers of pellets expelled by the latter may therefore be due to the smaller intake of food. In control experiments, mice and rats fasted for the same period of time expelled a higher number of pellets than the halquinol-treated animals (Figs 2 and 3). The results thus indicate some slowing of the movement of the gut contents by the drug.

When a suspension of the drug was given by mouth to rats, no significant difference was noted between drug and control when the numbers of pellets were compared at hourly intervals. When the increase from the second to the seventh hr was compared, 100 mg/100 g body weight caused a significant decrease (Table 2).

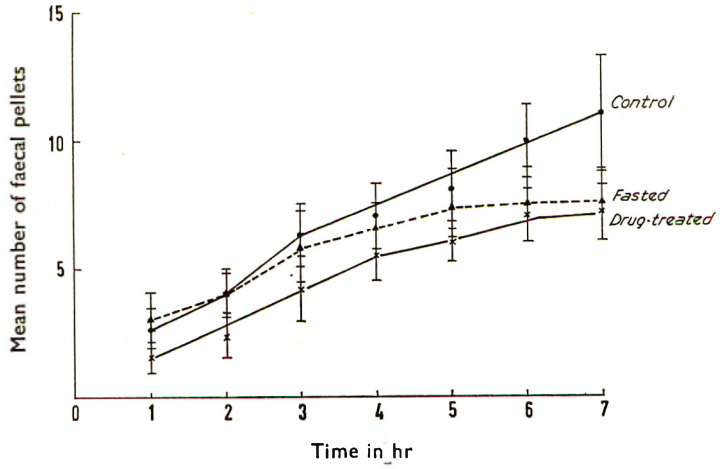

FIG. 2. Effect of 10% halquinol diet on hourly output of faecal pellets in the rat.

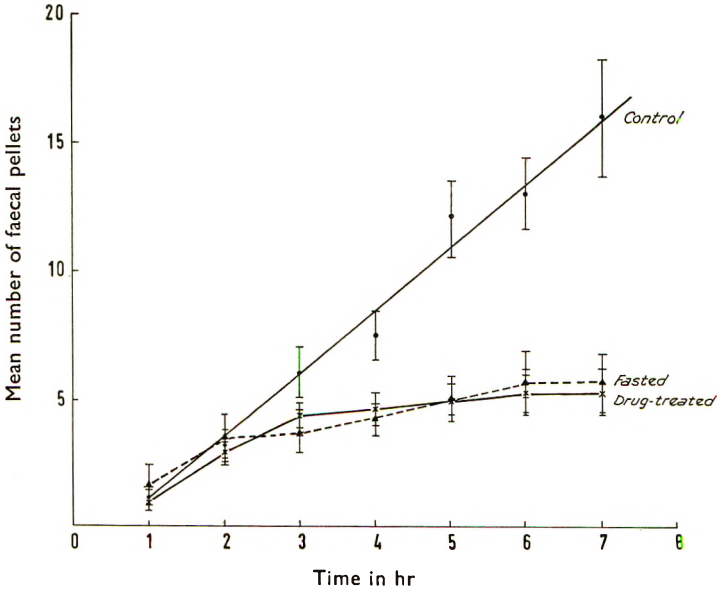

FIG. 3. Effect of 10% halquinol diet on hourly output of faecal pellets in mice.

When the faecal pellets were weighed the total mass expelled from drug-treated animals was not significantly different from the control.

The effect of halquinol on the movement of the charcoal meal in rats is shown in Table 3, from which it can be seen that there was a highly significant inhibition in the drug-treated animals. There was however no correlation between the actual length of intestinal segment and the distance traversed by the charcoal.

TABLE 3. THE EFFECT, IN RATS, OF ORAL HALQUINOL (100 MG/100 G) ON THE PROGRESS OF A STANDARD CHARCOAL MEAL THROUGH THE INTESTINES
All values are means ± (s.e.)

No. of animals	Length of intestine (a) (cm)	Distance traversed (b)	(a)—(b)	Inhibition %
20 (control)	101·3 ± 1·46	51 ± 2·00	50·3 ± 2·14	49·58 ± 1·88
22 (CHQ)	104·09 ± 1·49	36·68 ± 4·68	67·4 ± 4·96	64·55 ± 4·56*

Significance of difference from control * $P > 0.001$.

Discussion

The results on isolated intestinal muscle indicate a non-specific depression of tone and movement and there is some evidence of ganglion blockade which requires further analysis in view of the failure to reduce the contractions of the nictitating membrane in the cat. The prolonged effects on the guinea-pig ileum which are more pronounced after washing, indicate that the drug is firmly fixed to the tissue. This is of interest because Freeman & Heseltine (1963) have already shown that the gastrointestinal tract of the guinea-pig is highly susceptible to irritation by high concentrations of halquinol. It appears that the drug has no atropine-like activity, so that its effects upon intestinal motility are probably due to a direct action on the smooth muscle.

Acknowledgements. We thank Mr. W. W. Heseltine, Mr. Ridgeway and Mr. Bisknell for supplies of the drug and 5-monochlor-8-hydroxyquinoline and 5,7-dichlor-8-hydroxyquinoline, for preparing the solutions and for help in other ways; Doctor R. A. Robb of the Department of Mathematics, for much helpful discussion of the results; Mr. R. Callender for drawing the figures and Miss G. Marren and Mr. P. Leitch, for technical assistance.

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Some physical properties of lactose and magnesia

T. M. JONES AND N. PILPEL

Anomalies in some of the physical properties of spray dried lactose and granulated magnesium oxide have been traced to the presence of fines. These are strongly adsorbed onto particles of about 1,000 μ diameter, being held in position by van der Waals' and electrostatic forces. Although they are not displaced during sieving, they can be removed by washing the powders with organic solvents.

THERE has been relatively little systematic work done on the effect of particle size on the physical properties of powders. The equations for angle of friction (Cremer, Conrad & Kraus, 1952; Fowler & Chodziesner, 1959), angle of repose (Fowler & Wyatt 1960; Brown 1961a), density and rate of flow through orifices (Fowler & Glastonbury, 1959; Rose & Tanaka, 1959; Brown & Richards, 1960) have been derived from data relating to a large number of different materials. This has been necessary in order to cover a reasonable range of particle sizes, but the materials involved have often differed considerably in the shape, density and roughness of the particles, all of which would be expected to affect the results.

With the availability of granulated powders in which the shape, density and roughness of the particles remains sensibly constant over a wide range of sizes, it has become possible to eliminate the effects of some of these variables. Nevertheless there remain certain anomalies in the graphs obtained by plotting properties such as angle of repose, density and flow rate against particle size.

The purpose of the present work has been to investigate these anomalies for two materials, magnesia and lactose, in some detail and to attempt to explain their occurrence.

Experimental

Materials

Granulated magnesium oxide from the Washington Chemical Co. was dried at 800° for 2 hr. Spray dried lactose from McKesson & Robbins Limited was granulated in a dish granulator and dried at 120°. Both materials were sieved into narrow fractions on an Alpine Airjet Sieve, using 20 g portions, and were stored in dry screw-capped bottles.

It should be emphasised that the properties of both materials varied from batch to batch. For this reason it was necessary to obtain a large stock of each material and make all the measurements on the one batch.

Measurements

The sizes of particles in the range 40 to 2,000 μ were obtained by sieving. For material below 40 μ diameter the Andreasen pipette method was employed, using 500 ml of a 2% dispersion in toluene or carbon

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tetrachloride (Martin, 1960) and withdrawing eight 10 ml samples over a period of 2 hr and calculating the particle diameter from Stoke's Law.

Particle densities over the size range 50 to 1500 μ were measured at 22° in toluene or benzene by the specific gravity bottle method using the equation:

$$\text{Specific gravity} = \frac{S(W_2 - W_1)}{SV - (W_3 - W_2)} \quad .. \quad .. \quad .. \quad (1)$$

where S = specific gravity of toluene; W_1 = weight of the bottle; W_2 = weight of bottle plus powder; W_3 = weight of bottle plus powder plus toluene; V = volume of bottle.

The bulk density of each sieve fraction was obtained in the Heywood bulk density apparatus.

Tap densities were determined as described in British Standard (1948). The densities of compacts of the magnesia were determined in the apparatus shown in Fig. 1 by filling it with 25 g of the powder and then compressing at 168 kg cm^{-2} between the platens of an Avery Tensile Tester, and measuring the resulting thickness of the compact on the dial micrometer.

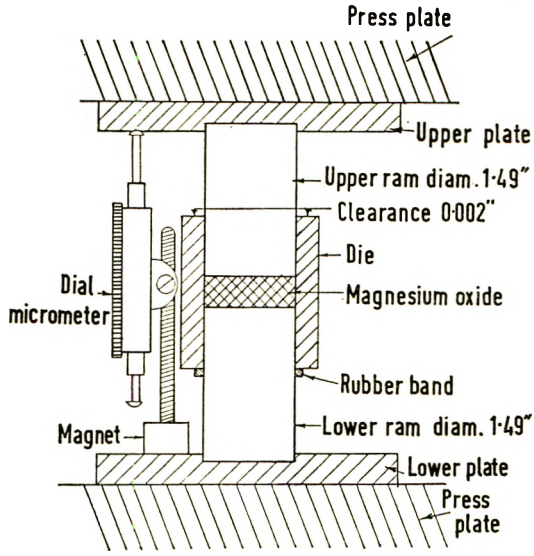

FIG. 1. Apparatus for compaction of magnesia.

A few granules from each sieve fraction were placed on a clean glass slide, one end of which was slowly raised until the granules started to slide. The elevation of the slide then yielded the angle of friction (Fowler & Chodziesner, 1959). Angles of repose of the different fractions were measured by a static method (Pilpel, 1964).

Rates of flow were measured from a 60° tapered glass separating funnel of maximum diameter 5 cm which was fitted with a sliding shutter to give interchangeable orifices having diameters between 0·47 and 1·70 cm.

Finally a representative number of particles from each sieve fraction were viewed under the microscope at magnifications of 200x and 60x to determine approximately the size and amount of fine material that was adhering to their surfaces.

A batch of the 1,000 μ material was then freed as completely as possible from fines by washing it with chloroform, and its angle of friction and angle of repose were measured. The size range of the displaced fines was also determined by sedimentation.

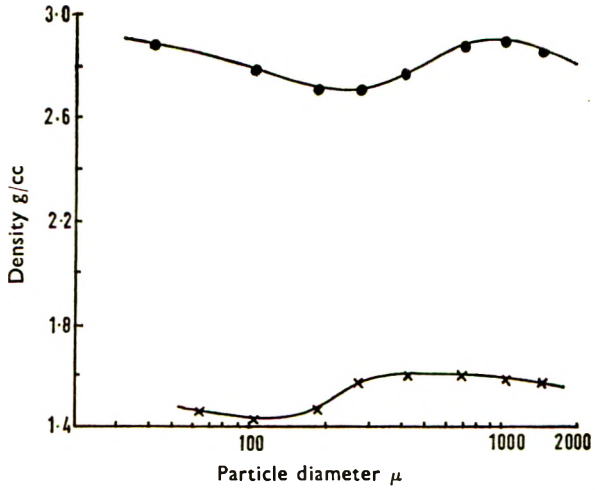

FIG. 2. Effect of particle diameter on particle density at 22° C. ● = Magnesia. × = Lactose.

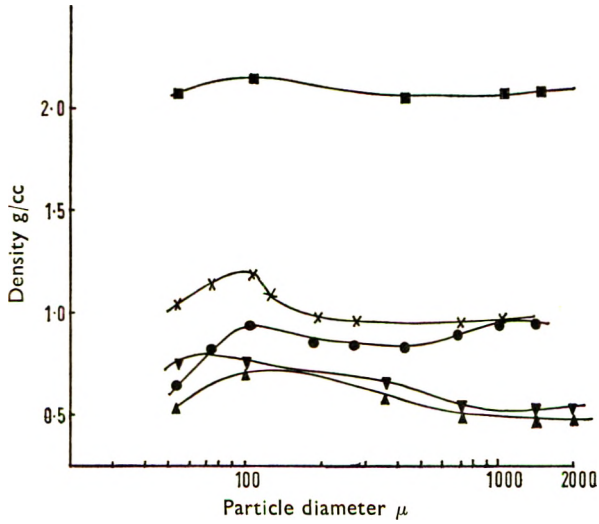

FIG. 3. Effect of particle diameter on powder density. ● = Bulk density of magnesia. × = Tap density of magnesia. ■ = Compacted density of magnesia at 160 kg/cm². ▲ = Bulk density of lactose. ▼ = Tap density of lactose.

Results

The graph relating the particle density to the particle size is given in Fig. 2. Fig. 3 shows the variation of bulk, tap and compacted densities with particle size. Fig. 4 relates the sizes of the particles to the elevation of the glass slide when sliding commenced.

The graphs of angle of repose versus particle size for narrow sieve cuts are given in Fig. 5, which also includes some literature results on sulphathiazole (Nelson, 1955) and silica sand (Brown, 1961a). Rates of flow

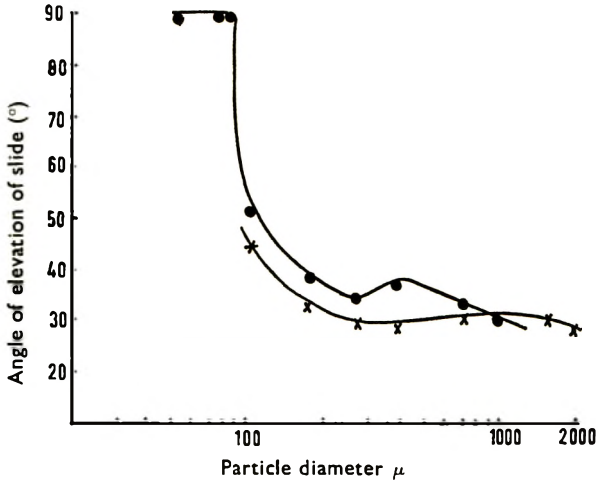

FIG. 4. Effect of particle diameter on angle of friction. ● = Magnesia. × = Lactose.

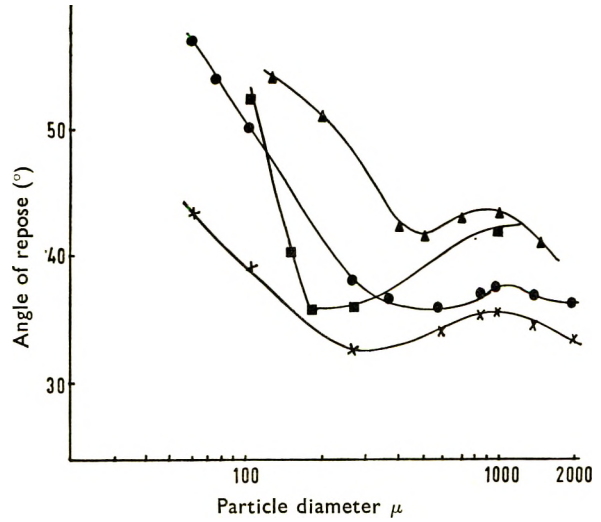

FIG. 5. Effect of particle diameter on angle of repose. ● = Magnesia. × = Lactose. ■ = Silica sand. ▲ = Sulphathiazole.

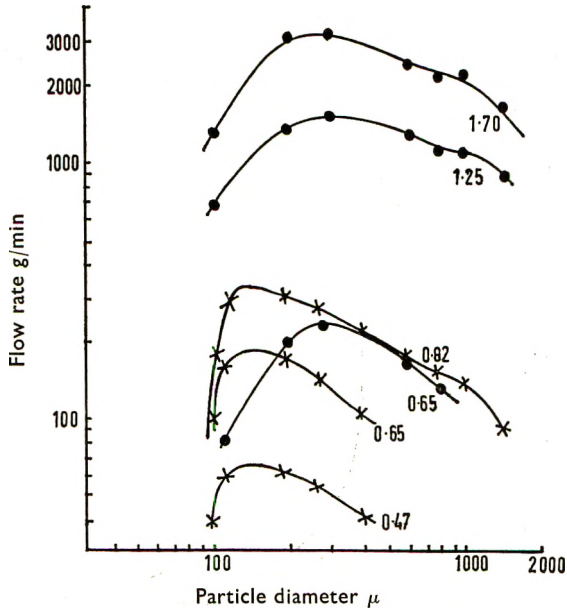

FIG. 6. Effect of orifice and particle diameter on rate of flow. ● = Magnesia. × = Lactose. Figures on the curves are the orifice diameters in cm.

through five different sized orifices are plotted in Fig. 6. All measurements were made in duplicate or triplicate and the limits of reproducibility were as follows: particle, bulk, tap and compacted densities $\pm 0{\cdot}02$ g/cc; angles of friction and repose, $\pm 0{\cdot}3°$; rates of flow, $\pm 7\%$.

Figs 2–6 show that over the size range 600–1,200 μ the physical properties of all the powders under investigation exhibit anomalies. The granule densities of magnesia and lactose pass through slight maxima, the bulk, tap and compaction densities rise to maxima at about 100 μ (when cohesion between particles becomes negligible) and fall to minimal values near 800 μ.

Regarding the angle of friction and the angle of repose, Figs 4 and 5 both show maxima and minima between 300 and 1,000 μ. The flow curves in Fig. 6 have the general shape expected (Rose & Tanaka, 1959) though between 700 and 1,000 μ some of the curves exhibit points of inflexion. The graphs of log flow rate versus log orifice diameter are practically linear for each size of particle and their slopes lie between 2·5 and 3·2 (see Franklin & Johanson, 1955).

As a result of washing the powders in chloroform, the angles of friction and of repose of the 1,000 μ magnesia and lactose were reduced as were kinks in the flow curves. Typical data before and after washing are given in Table 1. However, more fines were formed during handling of the powders; it was difficult to check the effect that their removal had had on the other physical properties. Sedimentation showed that about 80% of the displaced fines were in the size range 10–50 μ.

TABLE 1. EFFECT OF FINES BEFORE AND AFTER WASHING

Material	Angle of friction°		Angle of repose°		Flow rate g/min	
	Before	After	Before	After	Before	After
Magnesia 400 μ	37·5	36·0	36·5	36·5	—	—
" 1,000 μ	31·0	31·5	37·5	36·5	1,150	1,050
" 1,400 μ	—	—	37·0	36·5	—	—
Lactose 500 μ	—	—	34·0	34·0	—	—
" 1,000 μ	31·5	30·5	35·5	34·5	150	130

Discussion

It has been noted by many workers that fine particles in powders profoundly affect their properties. This is due to an enhancement of the van der Waals cohesive forces which operate between neighbouring particles. For example, an added weight fraction P_1, of fines with diameter D_1, increases the angle of repose θ of coarser magnesia (denoted by a subscript$_2$), the relevant equation being (Pilpel, 1964):

$$\left(\frac{\text{Sin } \theta}{\tan \theta_{\text{lim}}} - \text{Cos } \theta\right) = \frac{k}{g} \frac{D_1^{n-2}}{\delta_2 X_2} \left[\frac{P_1}{(\delta/\delta_2 - P_2)}\right]^{2/3} \quad .. \quad .. \quad (2)$$

where θ_{lim} is the limiting angle of repose of the coarse particles, k, n and X_2 are geometrical constants and δ is the particle density.

For magnesia and spray dried lactose, the cohesive forces become comparable to the weights of the particles when they are about 50 μ in diameter.

Particles of this size or below tend to be adsorbed on the surfaces of coarser particles forming a coating which may or may not be continuous. While cement particles apparently form a continuous coating on the balls used for grinding cement clinker (Bond, 1940), with the present materials, microscopic examination shows that particles 1,000 μ in diameter are only partially covered by adsorbed fines. These adhere sufficiently tenaciously to the surface not to be displaced during sieving. They can, however, be partially removed by washing the powders with chloroform.

By measuring their sedimentation rate in the Andreasen pipette apparatus, it has been found that about 80% of the fines fall in the size range 10–50 μ. There appear to be about 0·1 g of these fine particles associated with each gram of the 1,000 μ material.

Let it be assumed that the average radius of the adsorbed particles is r and that, on average, they cover a fraction, ϕ, of the surface of the larger particles, radius R. If the small particles are close packed in an hexagonal arrangement, then the number present on the surface of each large particle is $\frac{4\pi (R + r)^2 \phi}{2r^2\sqrt{3}}$. Since the densities of the large and small particles are similar (Fig. 2), it follows that $\frac{2\pi r(R + r)^2 \phi}{R^3\sqrt{3}}$ g of fines are associated with each g of the larger sized particles. Thus

$$\frac{2\pi r(R + r)^2 \phi}{R^3\sqrt{3}} = 0{\cdot}1 \quad .. \quad .. \quad .. \quad (3)$$

and substituting R = 500 μ and r = 15 μ, ϕ works out at approximately 0·4. This accords satisfactorily with the visual observation of the 1,000 μ particles under the microscope: a representative sample of 200 of these had approximately one-third of their total surface area covered by fines.

The presence of adsorbed fines is not restricted to particles of 1,000 μ. Both coarser (>2,000 μ) and finer (200–300 μ) particles exhibit some adsorption. But on these sizes, the fines seem to adhere less tenaciously and the majority are therefore displaced during the preliminary sieving.

The marked adsorption of 10–50 μ material onto approximately 1,000 μ particles suggests that these larger particles have surface irregularities 10–50 μ in diameter into which the smaller particles can fit. They are assumed to be held in position both by adhesive forces and by electrostatic forces of attraction (Harper, 1961). Considering first the adhesive forces F, if it is assumed that there is perfect contact between particles (Krupp & Sperling, 1964)

$$F = 2\pi r\Gamma\left(1 + \frac{\Gamma}{ZY}\right) \text{ dynes} \qquad .. \qquad .. \quad (4)$$

where Γ, the free adhesional energy is 1·5 times the free surface energy of the material, Y is its yield pressure and Z is approximately 4 Å. For the granulated magnesia, the yield pressure, derived from the Vickers hardness tester (Bowden & Tabor, 1964) is of the order of 5 kg mm^{-2} and the surface energy is about 10^3 erg cm^{-2} (Gregg 1961). Taking r as 15 μ, F works out at about 2×10^3 dynes.

This figure is based on the assumption that the particles are perfectly smooth and that the real area of contact is of the order of magnitude of r^2. In fact, the *real* area of contact between the particles at normal pressures probably does not exceed $r^2 \times 10^{-6}$ (Bowden & Tabor, 1958) and the force between the particles is therefore substantially less, i.e. about 10^{-3} dynes (Kitchener, 1961). This force is of the same order of magnitude as the weights of the particles involved.

Now the fact that the fines are displaced when the 1,000 μ material is immersed in an organic solvent, indicates that electrostatic attractive forces must also be playing some part, because the free surface energy of magnesia in chloroform is less than its free surface energy in air by only 27 ergs cm^{-2} (the surface tension of chloroform) and such a reduction would not significantly alter the value of F. Although it has not been possible to measure the magnitude of the electrostatic forces in the present work, one estimate (Harper, 1961) suggests that they might be as high as 10^{-3} dynes/particle. They are reduced in the presence of chloroform by a factor of 4·8, the dielectric constant of chloroform, and this results in partial detachment of the fines.

It has been considered of interest to compare some of the results in Fig. 6 with those predicted for powders flowing from a 60° funnel, by the minimum energy theorem (Brown, 1961b). The dimensionless quantity, γ, given by

$$\gamma = \frac{4Q}{\rho\pi(D_o - k)^2\{g(D_o - k)\}^{1/2}} \qquad .. \qquad .. \quad (5)$$

where Q is the flow rate in g/sec, ρ is the bulk density of the powder, D_0 is the orifice diameter and k is a measure of the *vena contracta*, has been calculated from the measured values of Q and D_0. Allowing for the fact that the different orifices unavoidably had slightly different geometries, the values, given in Table 2, are seen to agree very satisfactorily with the predicted value of 0·62.

TABLE 2. FLOW OF LACTOSE AND MAGNESIA

Material	Bulk density g/cc	k cm	Orifice diameter cm	Observed
Magnesia 550 μ	0·85	0·10	1·70	0·65
" "	"	"	1·25	0·75
" "	"	"	0·65	0·65
Lactose 550 μ	0·55	"	0·82	0·54
" 400 μ	"	0·09*	0·65	0·58
" "	"	"	0·47	0·58
			Mean	0·62

*Estimated.

Conclusion

The hypothesis that adsorbed fines contribute in part to the anomalous behaviour of lactose, magnesia and other powders is consistent with the data in Figs. 2–6. Their presence increases the diameters of particles in the region of 1,000 μ and therefore cause displacement of the points along the abscissae. The fines also affect the frictional, cohesive and packing properties of the particles, causing displacements of the experimental points on the ordinates. The hypothesis is consistent with the observation that when the fines are displaced by washing the powder with chloroform, or alternatively, when there are relatively few fines present, as for example in a partially sintered grade of magnesia with a Vickers hardness of about 10 kg mm^{-2}, the anomalies are diminished.

The same hypothesis explains the fact that in many powders there are fewer fines than would be expected on the basis of the Rosin Rammler distribution law (Herdan, 1960).

viz: $$\log\left(\log\frac{100}{R}\right) = \text{Constant} + n \log x \quad .. \quad .. \quad (6)$$

where R is the percentage by weight of particles bigger than a particular size, x, and n is a constant. While some fines are undoubtedly lost by air entrainment during the milling of powders, the present findings indicate that a significant fraction of material, less than about 50 μ in diameter, can also appear to be lost by adsorption on the surface of coarser particles.

Acknowledgement. The authors wish to thank Mr. E. Segovia for providing some of the data on spray dried lactose.

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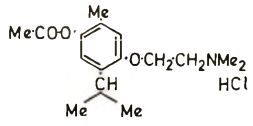
Competitive blockade of adrenergic α-receptors and histamine receptors by thymoxamine

A. T. BIRMINGHAM AND J. SZOLCSÁNYI

The adrenergic α-receptor blocking activity of 4-(2-dimethylaminoethoxy)-5-isopropyl-2-methylphenyl acetate, thymoxamine, was quantitatively investigated on the vas deferens of the guinea-pig and on arterial strips from guinea-pigs, rabbits, cats and dogs. The blockade fulfilled the established criteria for competitive antagonism of noradrenaline. On these various tissues the pA_2 was about 7·0; thymoxamine was more potent than piperoxan and less potent than dihydroergotamine. There was no evidence of β-receptor or 5-hydroxytryptamine receptor blocking activity. Against histamine as the agonist on the guinea-pig ileum, thymoxamine was a competitive antagonist with a pA_2 of about 6·5.

THE benzodioxans and phenoxyalkylamines have been extensively studied as agents blocking adrenergic α-receptors. From the thymyl ether of an alkylamine the first useful antihistamine drugs were developed but the α-blocking activity of thymoxyalkylamines does not seem to have been the subject of quantitative investigation of mechanism of blockade (see Barlow, 1964).

Greef & Schümann (1953) described 4-(2-dimethylaminoethoxy)-5-isopropyl-2-methylphenyl acetate (I)* as a sympathicolytic and histaminolytic agent. The compound has since been used clinically for a range of vascular disorders.



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This paper reports the results of quantitative experiments on isolated tissues which suggest that thymoxamine is a competitive antagonist at adrenergic α-receptors and at histamine receptors.

Experimental

METHODS

The isolated tissues were suspended in jacketed organ baths containing 20 ml of Krebs solution bubbled with 95% oxygen and 5% carbon dioxide. Contractions were recorded on smoked paper with isotonic frontal writing levers.

Guinea-pig vas deferens (Leach, 1956). Each vas deferens was removed without the hypogastric nerve and set up in Krebs solution at 32°; the load on the tissue was 0·5 g; the magnification was 4 times. Agonist drugs were added to the bath for 1 min in each 5 min period.

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* Proposed name Thymoxamine ("Opilon" Wm. R. Warner and Co. Ltd.).

Arterial strips (Furchgott & Bhadrakom, 1953). Spirally-cut strips were prepared from the thoracic or abdominal aorta of rabbits, guinea-pigs or cats and from the carotid artery of dogs. The Krebs solution was maintained at 37° for rabbit, cat and dog strips and at 32° for strips from guinea-pigs; the load was 0·25 g; the magnification was 10 times for rabbit, cat and dog and 20 times for guinea-pig. Agonist drugs were added to the bath for 3 min in each 15 min period (rabbits, guinea-pigs, dogs) or 2 min in each 10 min period (cats).

Guinea-pig ileum. Middle ileum in 3 cm lengths was used at 37° loaded at 0·5 g with a magnification of 4 times. The agonist, histamine, was added for a contact time of 1 min during each 4 min period.

Rat fundus (Vane, 1957). Longitudinal strips were kept at 37°, loaded at 0·5 g, with a magnification of 10 times. Dose response curves to 5-hydroxytryptamine (5-HT) were obtained in the absence of and in the presence of thymoxamine.

Guinea-pig auricles (Giotti, 1954). The isolated auricles were kept at 37° and spontaneous beats recorded by a spring-loaded side-writing lever on smoked paper. Adrenaline was added to the bath for a 1 min contact time. Responses to adrenaline were recorded in the absence of antagonist and after 2 or 15 min exposure to antagonist.

DRUGS

Drugs used were: acetylcholine chloride, (−)-adrenaline bitartrate, angiotensin II (Ciba), dihydroergotamine methane sulphonate, histamine acid phosphate, 5-hydroxytryptamine creatinine sulphate, (−)-noradrenaline bitartrate, piperoxan hydrochloride, propranolol hydrochloride, thymoxamine hydrochloride.

Concentrations are expressed as molar final bath concentration or in terms of the base.

Results

VAS DEFERENS

The threshold dose of noradrenaline was 2 or 4 $\times$ 10^{-6} M and the response increased with twofold increases in concentration up to maximal responses at 1·28 or 5·12 $\times$ 10^{-4} M. Initial dose response curves were repeated (usually three times) until the response of the vas was constant. Dose response curves to noradrenaline were repeated in the presence of a range of concentrations of thymoxamine which was added to the bath 2 min before each dose of noradrenaline. Concentrations of thymoxamine from 1 $\times$ 10^{-8} to 1·8 $\times$ 10^{-6} M moved the log dose response curves for noradrenaline to the right of the curves for noradrenaline alone. The curves in the presence of antagonist were parallel to the curves in the absence of antagonist. The heights of the maximal responses were not reduced by thymoxamine.

The horizontal distance between the curves obtained in the absence of and presence of thymoxamine was measured at 10% intervals from 10 to 80% of maximum response (Fig. 1). This distance was expressed as a dose ratio (Gaddum, Hameed, Hathway & Stephens, 1955) and the means of these dose ratios for four different concentrations of thymoxamine were plotted

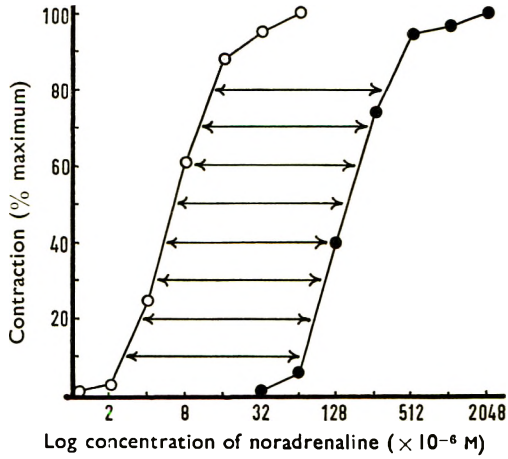

FIG. 1. Log dose response curves from a single experiment with a guinea-pig isolated vas deferens preparation. The curve for noradrenaline alone (○——○) and the curve for noradrenaline in the presence of $1{\cdot}8 \times 10^{-6}$M thymoxamine (●——●) for which the antagonist was added to the bath 2 min before each dose of noradrenaline. The arrows indicate the horizontal distances measured at each 10% of maximum interval (from 10% of maximum to 80% of maximum inclusive). These distances were converted to dose ratios for noradrenaline.

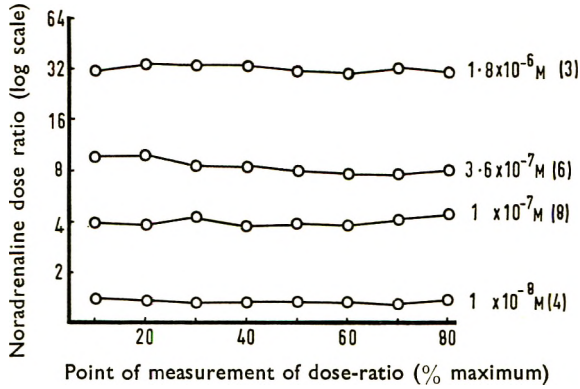

FIG. 2. The means of the noradrenaline dose-ratios determined on the guinea-pig vas deferens (measured as shown in Fig. 1) for four different concentrations of thymoxamine. The concentration of thymoxamine used is shown against each curve. The numbers in brackets indicate the number of vasa used to determine the means.

over the 10 to 80% range of response (Fig. 2). The mean dose ratios for each concentration of thymoxamine seem to lie on straight lines which are parallel to the abscissa.

The relation between dose ratio and antagonist concentration was analysed by the method of Arunlakshana & Schild (1959). The logarithm of (x — 1), where x equals the noradrenaline dose ratio, was plotted against the negative logarithm of B, where B is the molar concentration of thymoxamine (Fig. 3). The points for the four concentrations of

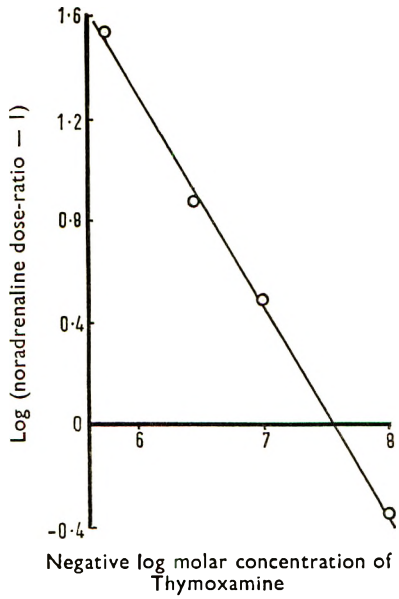

FIG. 3. Results from guinea-pig isolated vas deferens plotted by the method of Arunlakshana & Schild (1959). A calculated regression line is fitted to the four points. Each point is a mean, derived from the data shown in Fig. 2, for each concentration of thymoxamine. The line intercepts the abscissa at the pA_2 value of 7·57. Where log (x-1) equals 0·95 a perpendicular dropped from the regression line to the abscissa gives the pA_{10} value of 6·42.

thymoxamine lay on a straight line. The calculated regression line intercepted the abscissa at the pA_2 value 7·57. When measured directly in four experiments, by the method of Schild (1947), the pA_2 was 7·56. The pA_2–pA_{10} value determined from Fig. 3 was 1·15.

From the formula $\log (x - 1)/B = \log K_2$, values of $\log K_2$ were calculated for each dose ratio at each of the four molar concentrations of thymoxamine. The mean of these four values for $\log K_2$ was 7·44, which was close to the pA_2 value.

In 5 experiments the course of the reversibility of the blockade was estimated. With the thymoxamine added before the noradrenaline the rate of return of pre-antagonist sensitivity to noradrenaline ranged from 6 washes in 8 min for $1{\cdot}25 \times 10^{-8}$ M thymoxamine, to 45 washes in 75 min for 1×10^{-5} M thymoxamine. After 20 min exposure to 1×10^{-7} M thymoxamine 15 washes in 30 min restored sensitivity.

The pA_2 for dihydroergotamine was also measured, with the antagonist continuously present in the Krebs solution for a period of 30 min. Under these conditions the pA_2 for dihydroergotamine against noradrenaline was 8·25.

ARTERIAL STRIPS

Rabbit aorta. Measurements of pA_2 for thymoxamine against noradrenaline contractions were made after 10, 25 and 40 min contact

with thymoxamine on four rabbits (Fig. 4). The mean pA_2 values were 6·80, 6·88 and 6·90 respectively. pA_2 values for piperoxan similarly determined were 6·28, 6·39 and 6·39.

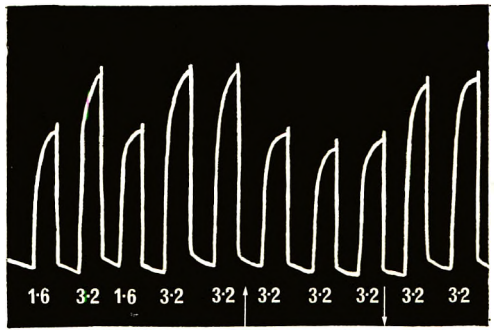

FIG. 4. Contractions of a rabbit aortic strip to noradrenaline. The concentration of noradrenaline used is shown by the figure beneath each contraction ($\times 10^{-7}$ molar). Contact time 3 min; 15 min cycle; three changes of bath fluid after each contraction. At the first arrow thymoxamine was added to the Krebs, to a final concentration of 8×10^{-8} M 7 min before the next contraction to noradrenaline. This concentration of thymoxamine was present in the Krebs until washed out at the second arrow, and it reduced the size of the response to the double dose of noradrenaline (3·2) to about that of the single dose (1·6). The rapidity of onset of the blockade and its ease of reversal by washing can be judged from this record.

Using aortic strips from three rabbits the effect of thymoxamine on contractions produced by angiotensin was observed. Concentrations from 1×10^{-7} to $2{\cdot}5 \times 10^{-5}$ M did not reduce the response of the aorta to angiotensin.

Guinea-pig aorta. Concentrations of 1 or 2×10^{-6} M thymoxamine were used on aortic strips from three guinea-pigs; in each experiment there was a parallel shift of the noradrenaline dose response curve to the right.

The pA_2 was measured on aortic strips from five guinea-pigs; the mean value was 7·20 for a 5 min antagonist contact period.

Cat aorta. Dose response curves to noradrenaline were made on aortic strips from three cats in the absence of and in the presence of thymoxamine concentrations from 1×10^{-7} to $6{\cdot}4 \times 10^{-6}$ M. The sensitivity of the strips and the slopes of the control dose response curves varied during an experiment. Thymoxamine moved the dose response curve to the right but the shift was not always parallel, the dose ratios being smaller nearer the maximum. The height of the maximum was not reduced. Estimates of the pA_2 from the dose ratio at the 50% of maximum point gave a mean value of 6·10.

Dog carotid. On carotid strips taken from four dogs, the pA_2 for thymoxamine against noradrenaline was measured by the method of Schild (1947). The mean value was 6·99 for a 13 min antagonist contact and 7·01 for 28 min contact.

GUINEA-PIG ILEUM

On sections of ileum from five guinea-pigs, dose response curves to histamine were made in the absence of and in the presence of thymoxamine in concentrations from 1×10^{-7} to $2{\cdot}56 \times 10^{-5}$ M. The curves in the

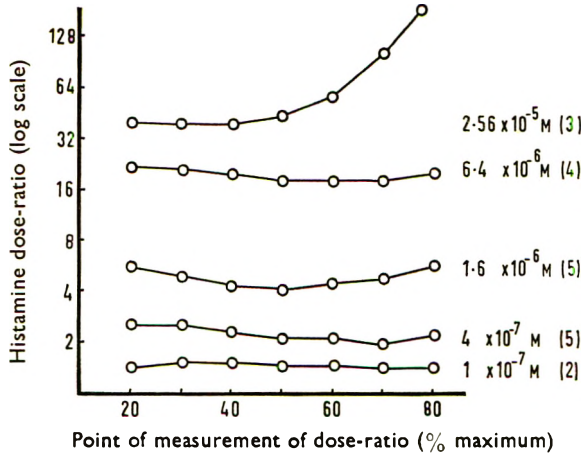

FIG. 5. The means of the histamine dose-ratios determined on the guinea-pig ileum for five different concentrations of thymoxamine. The concentration of thymoxamine used is shown against each curve. The numbers in brackets indicate the number of guinea-pigs used to determine the means.

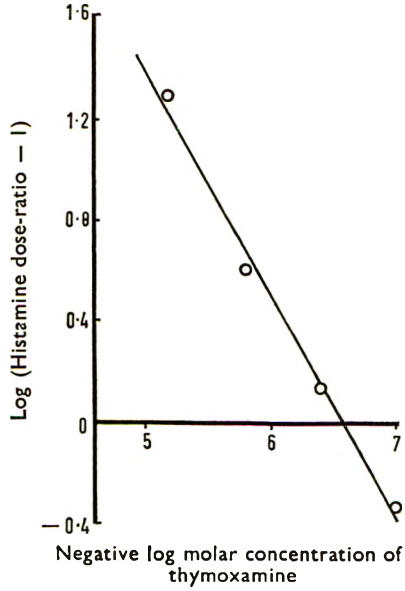

FIG. 6. Results from guinea-pig isolated ileum plotted by the method of Arunlakshana & Schild (1959). A calculated regression line is fitted to the four points. Each point is a mean, derived from the data shown in Fig. 5, for each concentration of thymoxamine. The line intercepts the abscissa at the pA_2 value of 6·57. Where log (x-1) equals 0·95 the pA_{10} on the abscissa equals 5·50.

presence of antagonist were to the right of those in the absence of antagonist, all the curves were parallel except for the highest concentration of thymoxamine, $2{\cdot}56 \times 10^{-5}$ M (Fig. 5). Plots of log (x − 1) against B, where x equalled the histamine dose ratio and B the molar concentration of thymoxamine, were made (Fig. 6). The calculated regression line

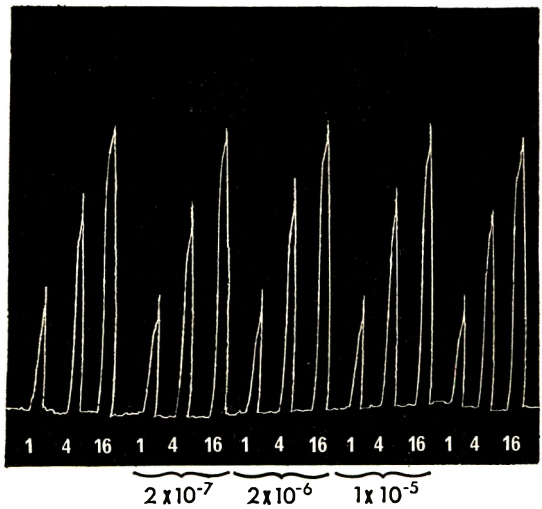

FIG. 7. Contractions of a rat isolated stomach fundus strip to 5-hydroxytryptamine. The concentration of 5-HT used is shown by the figure beneath each contraction ($\times 10^{-9}$ g/ml). Contact time one min; 5 min cycle; 5 changes of bath fluid after each contraction. The first three responses were in the absence of thymoxamine, the following three groups of three responses were in the presence of the concentrations of thymoxamine shown beneath the tracing, added 2 min before each 5-HT dose. The last group of three responses was again in the absence of thymoxamine.

intercepted the abscissa at the pA_2 value of 6·57. The pA_{10} from the curve was 5·5, so that pA_2–pA_{10} was 1·07. Log K_2 values were calculated for each of the four concentrations of thymoxamine used. The mean of these four log K_2 values was 6·52, which was close to the pA_2.

Wash recovery was measured in three guinea-pigs. Time of recovery to original sensitivity with repeated washing varied from 32 min with 4×10^{-7} M thymoxamine to 100 min with $6{\cdot}4 \times 10^{-6}$ M.

RAT FUNDUS

On fundus strips from three rats, concentrations of thymoxamine up to 1×10^{-5} M, added to the bath 2 min before the addition of the 5-HT agonist, did not alter the position of the 5-HT dose response curves (Fig. 7).

GUINEA-PIG AURICLES

On isolated auricles from three guinea-pigs concentrations of thymoxamine up to 1×10^{-5} M acting for 15 min did not change the effect of adrenaline (Fig. 8) whereas propranolol, 1×10^{-7} M, completely abolished the response to adrenaline.

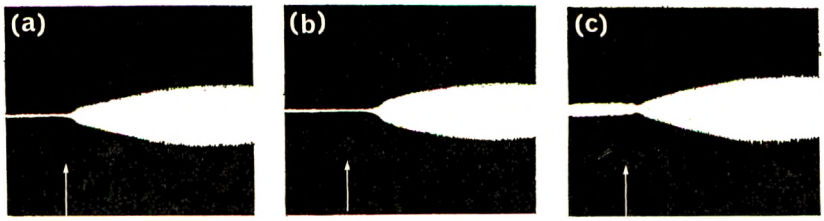

FIG. 8. Spontaneous contractions of guinea-pig isolated auricles. In panel (a) the response to a bath concentration of 8×10^{-8} g/ml adrenaline, acting for 1 min. Between (a) and (b) thymoxamine was added to a concentration of 1×10^{-6} molar 2 min before repeating the dose of adrenaline. Between (b) and (c) the auricles were exposed to a concentration of 1×10^{-5}M thymoxamine for 15 min before repeating the adrenaline dose. The adrenaline doses were added at the arrows.

TABLE 1. pA_2 VALUES

Tissue	Antagonist	pA_2	Remarks
Adrenergic α-receptor (agonist: noradrenaline)			
Guinea-pig vas deferens	Thymoxamine	7·57	Calculated from Fig. 3
	Dihydroergotamine	8·25	Antagonist contact time 30 min
	Piperoxan	6·47	Calculated from results of Leach (1956). Antagonist added 5 min before each agonist dose
Rabbit aortic strip ..	Thymoxamine	6·80	Antagonist contact time: 10 min
		6·88	Antagonist contact time: 25 min
		6·90	Antagonist contact time: 40 min
	Piperoxan	6·28	Antagonist contact time: 10 min
		6·39	Antagonist contact time: 25 min
		6·39	Antagonist contact time: 40 min
Guinea-pig aortic strip	Thymoxamine	7·20	Antagonist contact time: 5 min
Dog carotid strip ..	Thymoxamine	6·99	Antagonist contact time: 13 min
		7·01	Antagonist contact time: 28 min
Cat aortic strip ..	Thymoxamine	6·10	Calculated from dose response curves
Histamine receptor (agonist: histamine)			
Guinea-pig ileum ..	Thymoxamine	6·57	Calculated from Fig. 6
	Mepyramine	9·3	Arunlakshana & Schild (1959). Antagonist 14 min before agonist

Discussion

Some qualitative aspects of the pharmacology of thymoxamine were reported by Greef & Schümann (1953). They showed that thymoxamine abolished the response of the rabbit uterus and the guinea-pig seminal vesicle to adrenaline. Thymoxamine reduced but did not abolish adrenaline inhibition of the rabbit small intestine. Intravenous injections of thymoxamine lowered the blood pressure of the rabbit, cat or dog and reduced or blocked the hypertensive effect of injections of adrenaline or noradrenaline. Adrenaline reversal was seen in the dog but not in the cat or rabbit. The rise in blood pressure produced in the rabbit by

stimulation of the central end of the sciatic nerve, or in the cat by the carotid occlusion reflex, was reduced by thymoxamine. Contraction of the cat nictitating membrane by adrenaline was reduced by thymoxamine. On the guinea-pig ileum, thymoxamine slightly reduced the response to acetylcholine, blocked the response to histamine and did not reduce the response to barium chloride. Greef & Schümann concluded that thymoxamine had what they called sympathicolytic and histaminolytic properties but drew no conclusions about the mode of action of the drug.

The experiments now reported were made in an attempt to characterise the nature of the blockade of adrenergic or histamine receptors produced by thymoxamine. A second object was to measure the potency of thymoxamine.

In differentiating between competitive and non-competitive antagonism, a number of criteria for competitive antagonism have been proposed in the past (Gaddum, 1937, 1957; Schild, 1947, 1957; Furchgott, 1955; Arunlakshana & Schild, 1959). As an antagonist of noradrenaline at α-receptors on the vas deferens and on vascular smooth muscle and as an antagonist of histamine on the guinea-pig ileum, thymoxamine was found to fulfil these requirements of competitive antagonism.

(i) For both noradrenaline and histamine the log dose response curves in the presence of various concentrations of thymoxamine were to the right of and parallel to those in the absence of the antagonist. The antagonism was surmountable, maximal responses being of the same height in the presence of antagonist as in its absence.

(ii) The quantitative criteria were found to be satisfied. Over a 180-fold dose range for thymoxamine on the vas deferens there was a linear relation between dose-ratio of noradrenaline minus one, and concentration of antagonist. The pA_2 taken from this relation was 7·57 and was the same as that found by a separate direct determination (7·56). The value of pA_2–pA_{10} at 1·15 was in close agreement with the theoretical value of 0·95 for competitive antagonism. Furthermore, the mean of the four calculated log K_2 values at 7·44 was near to the pA_2 value of 7·57 which is indicative of the competitive nature of the antagonism.

With histamine as the agonist on the guinea-pig ileum there was a parallel shift of the dose response curves to the right over a 64-fold range of thymoxamine concentration. From the regression line fitted to the plot of dose ratio minus one against antagonist concentration, the pA_2–pA_{10} value of 1·07 was near to the theoretically required value of 0·95 for competitive antagonsim and the mean log K_2 value of 6·52 was close to the pA_2 of 6·57.

(iii) The antagonism of noradrenaline at α-receptors or of histamine at histamine receptors was completely reversible by repeated washing of the tissue with fresh Krebs solution. The amount of washing and the period of time needed to restore the original sensitivity to the agonist depended on the antagonist concentration used.

(iv) For the α-receptor, the pA_2 values measured on tissues from different species and different tissues from the same species were, except for the cat aorta, closely similar (Table 1). The pA_2 value in the cat

was measured from dose response curves which varied more than those from the other species. The cat aortic strips were thicker than aortic strips from rabbits or guinea-pigs or the carotid strips from dogs; this is the most noticeable difference and may account for the differences in pA_2 values.

The potency of thymoxamine may be judged from Table 1. As an antagonist at α-receptors thymoxamine is more potent than piperoxan and less potent than dihydroergotamine. At the histamine receptor thymoxamine is a relatively weak antagonist when compared with an established antihistamine drug such as mepyramine.

The relative specificity of thymoxamine for α-sympathetic receptors was indicated by its lack of effect on the response of the guinea-pig auricles to adrenaline, on which tissue thymoxamine appeared to have no β-receptor blocking activity. The specificity was further suggested by the lack of blocking action against contractions induced by angiotensin on the rabbit aorta. Again, the inability of thymoxamine to reduce the response of the rat fundal strip to 5-HT was evidence for the lack of blocking action at 5-HT receptors.

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Letters to the Editor

3-Epimerisation of digoxigenin in the rat

SIR,—It has been demonstrated in several species, including the rat and man, that the metabolism of certain cardiac glycosides involves the stepwise removal of the attached sugar molecules (Lauterbach & Repke, 1960; Wright, 1962). It has also been observed (Herrmann & Repke, 1964) that the resulting genins undergo further metabolism when incubated with liver slices and among the metabolites was a component which could not be separated from the corresponding 3-epi-genin when chromatographed on several systems of paper and thin-layer chromatography. However, Herrmann & Repke (1964) were unable to separate 3-epi-digitoxigenin from 17βH-digitoxigenin and since the corresponding 17βH-isomers were not used as reference materials in the examination of the metabolites of the other genins studied, further evidence in support of metabolic 3-epimerisation has been sought.

Digoxigenin-[12-^{3}H] (2·19 μc/mg) was administered by the peritoneal route to 6 rats and the bile collected through the cannulated bile ducts for 10 hr. The dose received by each animal was equivalent to 3 μg/g body weight. Extraction of the bile with chloroform removed 15·4% of the total radioactivity present in the bile. The radioactivity not extracted by chloroform was shown to be present only in compounds of greater polarity than either digoxigenin or 3-epi-digoxigenin. The chloroform extract was separated by thin-layer chromatography into 3 components. Two of these components behaved on several systems of chromatography as digoxigenin and 3-epidigoxigenin. The ratio of these 2 components was approximately 1 : 10 respectively.

The area corresponding to 3-epi-digoxigenin was eluted from a thin-layer chromatogram of the chloroform extract. The activity of the eluate was $4{\cdot}07 \times 10^5$ dpm. To this was added unlabelled 3-epi-digoxigenin (88 mg) The recovered 3-epi-digoxigenin was recrystallised 5 times from methanol-ethyl acetate. The specific activities of the 5 yields were 4·68, 4·56, 4·35, 4·46, $4{\cdot}40 \times 10^3$ dpm./mg. The calculated activity assuming no separation was $4{\cdot}64 \times 10^3$ dpm/mg. The possibility was considered that the recovered activity was due to the presence of labelled digoxigenin which may have failed to separate from the 3-epi-digoxigenin during the course of the above re-crystallisation. In a control experiment a mixture of ^{3}H-digoxigenin (1·07 mg) and 3-epi-digoxigenin (128 mg) was repeatedly crystallised from methanol-ethyl acetate. The activity of the recovered material fell steadily, 73% of the calculated activity being lost after six re-crystallisations.

It may be concluded that in the whole rat digoxigenin undergoes rapid metabolism comparable with that observed with rat liver slices and that 3-epimerisation constitutes a major pathway in the detoxification of digoxigenin as postulated by Herrmann & Repke (1964).

Acknowledgement. The digoxigenin-[12-^{3}H] was prepared by hydrolysis of a sample of digoxin-[12-^{3}H] kindly supplied by Sandoz Ltd., Switzerland.

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May 7, 1965

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Inhibition of the acetylcholine-destroying activity of the guinea-pig vas deferens by eserine or by dyflos

SIR,—The increase produced by eserine in the height of the responses of the guinea-pig isolated vas deferens to stimulation of the hypogastric nerve (Huković, 1961) has been reported by several workers (Boyd, Chang & Rand, 1960; Burn & Weetman, 1963; Ohlin & Strömblad, 1963; Della Bella, Benelli & Gandini, 1964), but measurements of the inhibition of cholinesterase activity by the concentrations of eserine used appear to have been made only by Ohlin & Strömblad (1963), who used the Warburg manometric method with acetylcholine as substrate.

When the vas deferens is contracted by transmural stimulation of post-ganglionic nerves (Birmingham & Wilson, 1963) the height of the response is increased by eserine (10^{-6} g/ml) or by di-isopropylphosphorofluoridate (dyflos, 10^{-5} g/ml) (Birmingham, 1964, communication to British Pharmacological Society).

Experiments have now been made to measure the degree of inhibition of the acetylcholine-destroying activity of the vas deferens produced by 20 min exposure to 10^{-6} g/ml eserine or to 10^{-5} g/ml dyflos. The method used was similar to that

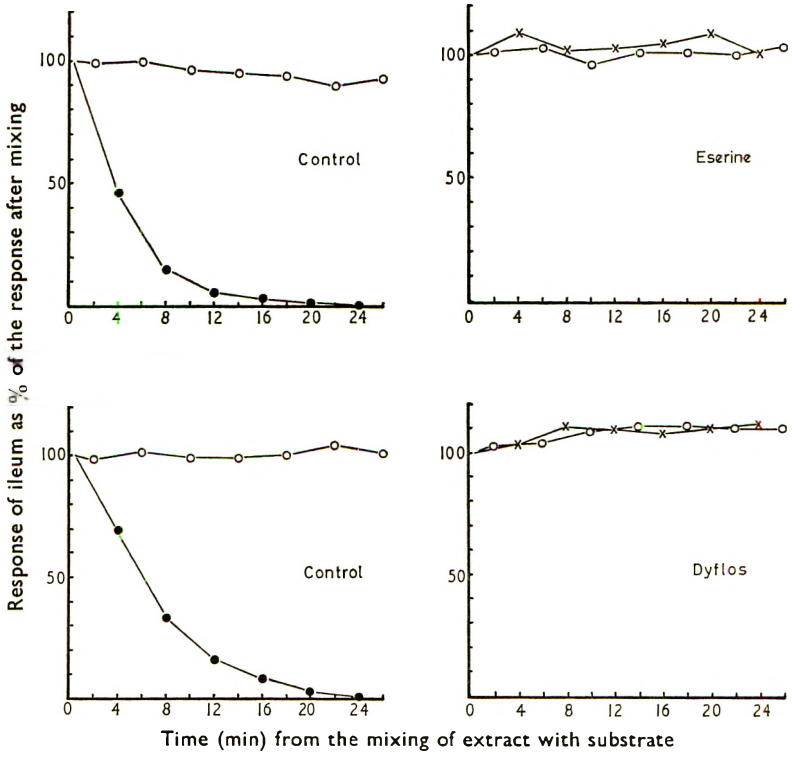

FIG. 1. Graphs of the mean responses of the guinea-pig ileum assay preparations to 0·15 ml samples from the reaction mixtures of the untreated controls —●—, the drug-treated extracts —x—, and the standard acetylcholine solution in Krebs —o—. The assay preparation was 3 cm of guinea-pig proximal ileum in 15 ml of Krebs solution at 37° bubbled with 95% oxygen, 5% carbon dioxide.

described by Boyd & others (1960) for their investigation of anticholinesterase activity in some anti-adrenaline drugs, and was chosen instead of the manometric method because it measures more directly the effect of the inhibiting drug and much lower substrate concentrations can be used. Drug concentrations are expressed in terms of the base.

For the four determinations made for each drug the enzyme mixture was obtained by grinding the vasa deferentia from two guinea-pigs with sand in a mortar containing 16 ml of Krebs solution. The supernatant part of this extract was decanted into a test tube kept in iced water. Aliquots of 2 ml were used as a source of acetylcholine-destroying enzyme and were either untreated, as controls, or treated with eserine (10^{-6} g/ml for 20 min) or dyflos (10^{-5} g/ml for 20 min) before being mixed with the 2 ml of Krebs solution containing the acetylcholine substrate. The final concentration of acetylcholine in the 4 ml of reaction mixture was 1×10^{-6} g/ml.

The reaction mixture was kept at 32° and the acetylcholine content of 0·15 ml samples was assayed at 4 min intervals on a preparation of guinea-pig ileum in 15 ml of Krebs solution at 37°. Samples from the reaction mixture were alternated with similar volumes from a standard acetylcholine solution in Krebs kept in the 32° water-bath.

The results are shown graphically in Fig. 1. Each point on the graphs is the mean of four determinations. The control graphs on the left show that the untreated enzymes mixtures gradually destroyed the acetylcholine substrate until at 24 min the samples from the reaction mixture failed to elicit any contraction from the ileum. The responses of the ileum to the standard doses of acetylcholine in Krebs kept under the same conditions were not reduced during the same period. The graphs on the right show the results obtained with eserine (upper graph) and with dyflos (lower graph). The acetylcholine concentration in the reaction mixtures did not diminish during the period of examination.

It is concluded from these results that a concentration of 1×10^{-6} g/ml of eserine or 1×10^{-5} g/ml of dyflos acting for 20 min inhibits completely the acetylcholine-destroying property of the guinea-pig vas deferens; this inhibition may account for the ability of eserine or dyflos to increase the height of the contractions of the vas deferens stimulated preganglionically or postganglionically.

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Solubilisation of preservatives by non-ionic detergents

SIR,—Donbrow & Rhodes (1965) suggest that my potentiometric method of determining solubilisation constants (Evans, 1959; 1964) differs from their method (Donbrow & Rhodes, 1963; Rhodes, 1964) in some important respects that involve fundamental points of theory and interpretation. On the contrary both methods are based on the same principle that changes in hydrogen ion concentrations which occur with mixtures of weak acids (or bases) and surfactants are due to the solubilisation of the unionised species by the detergent micelles. Assuming this to be so, the main difference lies in the method of calculating the distribution constants from the observed changes in hydrogen ion concentration, which for the sake of brevity was not given in the original papers (Evans, 1959; 1964).

From measured pH, $$-\log (H^+) = pH - 0{\cdot}5\sqrt{I} \quad .. \quad .. \quad (1)$$

From electroneutrality, $$(A^-) = (H^+) + (Na^+) \quad .. \quad .. \quad (2)$$

From dissociation Constant of acid K_a, $$\log (HA)_w = \frac{\log (H^+)(A^-)}{K_a} - \sqrt{I} \quad .. \quad (3)$$

By difference, $$(HA)_m = (HA)_T - (HA)_w - (A^-) \quad .. \quad (4$$

where I is the ionic strength, and $(HA)_T$, $(HA)_w$, $(HA)_m$ and (A^-) are total acid concentration, the concentration of unionised acid in the water phase, the concentration of unionised acid in the micellar phase, and the concentration of ionised acid in the water phase respectively (moles/litre). Thus although the ionic strength varies during the titration, it is low and is allowed for in the calculations. As an alternative procedure, the free acid can be titrated (Evans & Dunbar, 1964) either in the presence of a swamping concentration of electrolyte (e.g. 0·1M sodium chloride) to keep ionic strength constant, or in some other base medium which simulates conditions in a product or conditions in biological tests.

Since Evans & Dunbar (1964) have shown that data from their recent biological studies are in good agreement with those calculated from the potentiometric method, it would seem that the potentiometric method gives results of sufficient accuracy for bactericidal or pharmacodynamical studies. Furthermore the distribution constants obtained by my method have been used to calculate the concentration of "free" methyl *p*-hydroxybenzoate in polysorbate (Tween) 80 systems and have been shown by Evans & Dunbar (1964) to be in excellent agreement with those obtained by dialysis (Pisano & Kostenbauder, 1959).

This potentiometric method measures the activity of the acid in the water phase, and by difference the concentration of the acid in the micellar phase can be obtained. It is realised of course that the pseudophase model is an approximation, and arguments for and against the model have been given by Hutchinson, Inaba & Bailey (1955), Pethica (1960), Mukerjee (1962) and Elworthy & Macfarlane (1965). In view of the constancy of the calculated distribution constants under the conditions studied (Evans, 1964) the results do not conflict with the pseudophase model—that is, the micelles behave as a separate solvent phase and the solute distribution can be treated quantitatively. That the presence of micelles as a separate phase in the more concentrated solutions (Evans, 1964) should decrease the pH of hydrochloric acid by 0·1 unit (Donbrow & Rhodes, 1965) is debatable, but if accepted, the decrease in the most concentrated solution studied (Evans, 1964) should be no more than 0·04 pH unit. Since in the original work (Evans, 1959; 1964) the pH value was estimated in the second decimal place, the quoted pH values are accurate to $\pm 0{\cdot}03$ pH units. A decrease of less

than 0·04 pH units in 0·01M hydrochloric acid in the presence of a detergent would therefore barely be detectable, and would certainly be insignificant compared to the changes of up to 2 pH units observed with acids which are solubilised.

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The effect of monoamine oxidase inhibition on guanethidine-induced noradrenaline release and sympathetic blockade

SIR,—We have measured the noradrenaline content of the hearts of rats injected with iproniazid alone or followed by guanethidine and have related the noradrenaline depletion to the monoamine oxidase inhibition.

The noradrenaline in individual rat hearts was measured after butanol extraction by fluorimetry (Fielden & Green, 1965). Monoamine oxidase activity was assayed by the dinitrophenylhydrazine method (Green & Haughton, 1961). The hearts from groups of 4 rats were homogenised in 5 or 6 volumes of 0·1 M phosphate buffer (pH 7·4) and 3·2 ml samples of the homogenates were shaken in air at 25° with 0·125 M semicarbazide (0·4 ml) and 0·1 M tyramine (0·4 ml). After 30 min., the reaction was terminated with 0·5 N acetic acid (1 ml); the remaining steps in the assay were then as previously described.

Table 1 summarises the results of experiments in which various doses of iproniazid phosphate were injected subcutaneously into rats 20 hr before subcutaneous injection of guanethidine sulphate (10 mg/kg). The rats were killed after a further 4 hr for assay of the noradrenaline and monoamine oxidase in their hearts. Sympathetic blockade at this time was estimated from the extent of ptosis, which was recorded on a 0–8 scale (Rubin, Malone, Waugh & Burke, 1957). The 20 hr interval was chosen to minimise interference by shorter-lasting effects of iproniazid unconnected with monoamine oxidase inhibition, but very similar results were obtained in a few experiments in which the iproniazid was given only 2 hr instead of 20 hr before the guanethidine, as was done by Gessa, Cuenca & Costa (1963). It is clear from Table 1 that no significant protection would be afforded against guanethidine-induced noradrenaline

depletion by doses of iproniazid which did not inhibit monoamine oxidase, and that 50% or more inhibition is needed before protection becomes appreciable. This is in complete contrast to the results of Gessa & others (1963), who reported that iproniazid caused only 5% inhibition of monoamine oxidase at a dose (12·5 mg/kg), which reduced the extent of noradrenaline depletion due to guanethidine (10 mg/kg intraperitoneally) from 90% to 32%.

TABLE 1. MONOAMINE OXIDASE INHIBITION, HEART NORADRENALINE LEVELS AND PTOSIS IN RATS TREATED WITH IPRONIAZID AND GUANETHIDINE

The mean noradrenaline content is given, together with the range, in parentheses, and the number of rats, in brackets

Dose of iproniazid phosphate (mg/kg)	Monoamine oxidase inhibition (%)	Heart noradrenaline content (μg/g)		Ptosis iproniazid + guanethidine
		Iproniazid alone	Iproniazid + guanethidine	
0	0	0·97 (0·66–1·29) [18]	0·20 (0·14–0·31) [8]	5·8
10	35	—	0·21 [2]	6·0
20	55	1·01 (0·69–1·24) [4]	0·45 (0·34–0·60) [4]	4·6
40	75	0·95 (0·78–1·18) [4]	0·69 (0·60–0·85) [5]	5·2
80	90	1·11 (0·78–1·41) [4]	0·88 (0·75–1·02) [4]	3·6

Our results suggest that the protection afforded by iproniazid against guanethidine-induced noradrenaline depletion is related to the amount of monoamine oxidase inhibition, and that there may be no need to invoke a bretylium-like adrenergic neurone blocking effect (Gessa & others, 1963) to account for this protection. In addition, they lend further support to the conclusion reached by Kopin & Gordon (1963) from metabolic studies, that the bulk of the noradrenaline released from the tissue stores by guanethidine is metabolised intraneuronally by monoamine oxidase. It may also be noted from Table 1 that diminution by iproniazid of guanethidine-induced noradrenaline depletion is accompanied by no more than a slight lessening in the amount of ptosis. This may be contrasted with the well-known ability of iproniazid to prevent the ptosis caused by reserpine as well as reducing the extent of tissue depletion of noradrenaline (Carlsson, Rosengren, Bertler & Nilsson, 1957).

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Letters to the Editor

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