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The Pharmaceutical Society of Great Britain

Volume 23 Number 9 September 1971

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Journal of Pharmacy and Pharmacology

Published by THE PHARMACEUTICAL SOCIETY OF GREAT BRITAIN
17 Bloomsbury Square, London, WC1A 2NN Telephone: 01-405 8967

Volume 23 Number 9 **September 1971**

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J. Pharm. Pharmac., 1971, **23**, 649–661 *Received March* 9, 1971

Structure-activity relations for anticholinergic 2[1-aryl(or cyclohexyl)-1-hydroxy-1-phenyl]methyl-1,3-dioxolans

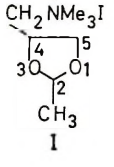
R. W. BRIMBLECOMBE, T. D. INCH, JANET WETHERELL AND NANCY WILLIAMS

Chemical Defence Establishment, Porton Down, Salisbury, Wiltshire, U.K.

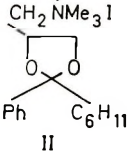
The syntheses and configurational assignments of some 4-dimethyl aminomethyl-2[1-aryl (or cyclohexyl)-1-hydroxy-1-phenyl]methyl-1,3-dioxolans are described. The anticholinergic potency of the 4-dimethylaminomethyl-2[1-cyclohexyl-1-hydroxy-1-phenyl]methyl-1,3-dioxolans, both in tertiary and quaternary form, depends principally on the configuration of the benzylic carbon atom, secondly on the C–2 configuration and thirdly, and to a much lesser extent, if at all, on the C-4 configuration. The dioxolans, which are derived formally from 4-dimethylaminomethyl-2-methyl-1,3-dioxolan methiodide (or its tertiary analogue) by replacement of the 2-methyl substituent by a 2[1-aryl (or cyclohexyl)-1-hydroxy-1-phenyl]methyl group and the glycollates which are derived formally from acetylcholine (or its tertiary analogue) by corresponding substitution of the acetoxymethyl group have closely similar anticholinergic potencies.

In an attempt to delineate the optimum steric requirements for high potency in anticholinergic drugs a number of chemically distinct types of anticholinergic drugs have been prepared for pharmacological evaluation. The design of the anticholinergic drugs has been facilitated by the observation that they may be formally derived from cholinergic drugs by the introduction of suitably placed bulky substituents (for relevant references see Brimblecombe & Inch, 1970). For example the glycollate*, diphenylhydroxyacetylcholine, may be considered to be the anticholinergic derivative of acetylcholine. Studies of anticholinergic drugs prepared on the basis of this model offer the additional advantage that stereochemical comparisons of related cholinergic and anticholinergic drugs can provide information about the relation of the cholinergic and anticholinergic receptor sites (Brimblecombe, Green & Inch, 1970).

Brimblecombe & Inch (1970) showed that replacement of both the substituents (hydrogen and methyl) on the acetal carbon (C-2) in the potent cholinergic drug 4-dimethylaminomethyl-2-methyl-1,3-dioxolan methiodide (I) by bulky substituents



I



II

afforded anticholinergic drugs (e.g., II) with peripheral potencies similar to atropine. This paper describes the synthesis and properties of some anticholinergic drugs that

* The term "glycollate" is used in this paper as a collected name for a variety of anticholinergic esters of both benzilic and glycollic acids (cf. Abood, 1968).

were derived formally from the cholinergic 1,3-dioxolan (I) by replacement of the 2-methyl substituent with (1,1-diphenyl-1-hydroxy)methyl (Fig. 1. Type A where $X = \overset{+}{N}Me_3I$) or with (1-cyclohexyl-1-hydroxy-1-phenyl)methyl (Fig. 1. Type C where $X = NMe_3I$). Additionally, for purposes of direct comparison with dioxolans of Type A, a third type of compound (Fig. 1. Type B where $X = \overset{+}{N}Me_3I$) has been prepared in which the 2- and 4-substituents in dioxolans of Type A are reversed. For the purpose of delineating the steric requirements for high anticholinergic potency emphasis has been placed as previously (Brimblecombe & Inch, 1970) on pharmacological comparisons of optically pure isomers of known absolute configuration.

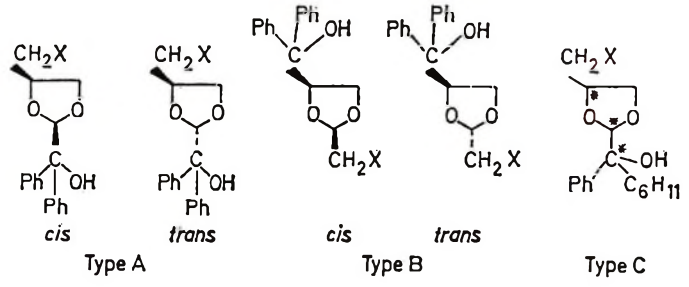


FIG. 1.

CHEMISTRY

Nomenclature

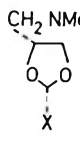
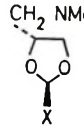
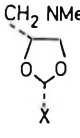
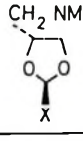
Racemic mixtures of Type A where for example X = Cl are, according to I.U.P.A.C. Tentative Rules for the Nomenclature of Organic Chemistry, Section E (*J. org. chem.*, 1970, *35*, 2899), designated *r*-4-chloromethyl-*cis*- and *trans*-2-(1,1-diphenyl-1-hydroxy)methyl-1,3-dioxolans. Similarly, compounds of Type B where for example $X = NMe_2$ may be most conveniently designated *r*-2-dimethylaminomethyl-*cis*- and *trans*-4-(1,1-diphenyl-1-hydroxy)methyl-1,3-dioxolans. The third type of compound, Type C, has three asymmetric centres (marked with asterisks) and all eight isomers have been prepared. The *cis*- and *trans*-isomers which were obtained by condensing *R* and *S*-2-cyclohexyl-2-hydroxy-2-phenylacetaldehyde with 1-*O*-toluene-*p*-sulphonyl-D- or L-glycerol, may be named unequivocally by *R* and *S* nomenclature (Cahn, Ingold & Prelog, 1966). However, for reasons which were outlined previously (Brimblecombe & Inch, 1970) a combination of *R* and *S* nomenclature is used in conjunction with D and L nomenclature and the compounds are designated *cis* when the 2- and 4-substituents are on the same side of the plane of the 1,3-dioxolan ring and *trans* when on opposite sides. The D or L refers to the configuration at C-4 and is the configuration of the 1-*O*-toluene-*p*-sulphonyl glycerol from which the dioxolan was prepared. For example, compound VIIT (Table 3) is named D-*cis*-4-*R*-toluene-*p*-sulphonyloxymethyl-2-*R*-(*R*-1-cyclohexyl-1-hydroxy-1-phenyl)methyl-1,3-dioxolan. For the Tables an abbreviated notation is used. Thus in Table 3, VIIT is the D-*cis* 4*R*,2*R*(R) isomer.

In this paper, for compounds of types A, B and C, where $X = NMe_2$, the compounds are given a simple number and derived hydrochlorides and methiodides are denoted by H and M (e.g., IV, IVH and IVM). Where X = Cl, a C notation is used (e.g., IIIC) and where X = OTs a T notation is used (e.g., XIVT).

DISCUSSION

The *cis*- and *trans*-4-dimethylaminomethyl-2(1,1-diphenyl-1-hydroxy)methyl-1,3-dioxolans (III and IV) were prepared by acid catalysed condensation of 1-chloropropan-2,3-diol with diphenylhydroxyacetaldehyde (or diphenylhydroxyacetaldehyde dimethylacetal), separation of the resulting *cis* and *trans*-4-chloromethyl derivatives (IIIC and IVC) by chromatography over silica, and subsequent treatment of IIIC and IVC with dimethylamine. The hydrochlorides and methiodides (Table 1) of

Table 1. *Pharmacological results for* 1,3-*dioxolans with a* 2-(1,1-*diphenyl*-1-*hydroxy*-*methyl substituent.*

No.	Compound	m.p. (solvent)	Guinea-pig ileum: Affinity constant (log K)	Antagonism of oxotremorine effects in mice: ED50 (μmol/kg with 95% limits) Salivation	Tremors
IIIM		206° (ethanol)	8·5 (1)	4·4 (2·7–7·1)	23·2 (15·0–36·0)
IVM		193° (ethanol)	8·35 (2)	2·7 (1·4–5·3)	4·6 (3·3–6·5)
IIIH		170° (from acetone)	6·7 (2)	23·4 (15·1–39·8)	11·4 (5·8–22·3)
IVH		228° (from ethanol)	6·65 (2)	27·3 (17·2–43·1)	15·3 (12·5–23·8)

X = $Ph_2C(OH)$–
These compounds were also tested for their mydriatic potency in mice. Potencies relative to atropine were IIIM, 0·2; IVM, 0·8; IIIH and IVH, <0·01.

the *cis*- and *trans*-4-dimethylaminomethyl compounds were prepared in the usual manner.

The *cis*- and *trans*-2-dimethylaminomethyl-4-(1,1-diphenyl-1-hydroxy)methyl-1,3-dioxolans were prepared by the reaction sequence illustrated in Fig. 2. The *cis*- and *trans*-2-dimethylaminomethyl-4-(1,1-diphenyl-1-hydroxy)methyl-1,3-dioxolans were separated chromatographically over silica and converted into their hydrochlorides and methiodides (Table 2) in the usual manner. Configurational assignments to V and VI were not made.

The eight isomers of 4-dimethylaminomethyl-2-(1-cyclohexyl-1-hydroxy-1-phenyl)-methyl-1,3-dioxolan (compounds VII to XIV) were prepared by separate acid catalysed condensations of 1-*O*-toluene-*p*-sulphonyl-D- or L-glycerol with *R* or *S*-2-cyclohexyl-2-hydroxy-2-phenylacetaldehyde, separation of the *cis*- and *trans*-isomers

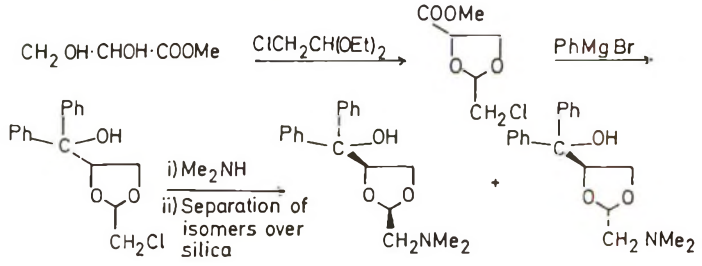
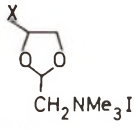
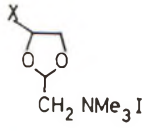
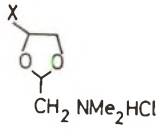
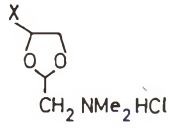


FIG. 2.

Table 2. *Pharmacological results for* 1,3-*dioxolans with a* 4-(1,1-*diphenyl*-1-*hydroxy*) *methyl substituent.*

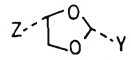
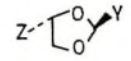
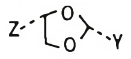
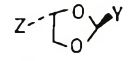
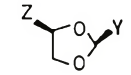
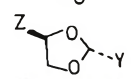
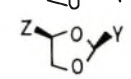
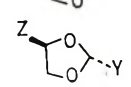
No.	Compound	m.p. (solvent)	Guinea-pig ileum: Affinity constant (log K)	Antagonism of oxotremorine induced salivation in mice: ED50 (μmol/kg with 95% limits)
VM			6·95 (1)	53·6 (38·2–75·2)
VIM		224–226° (ethanol)	6·42 (1)	77·9 (37·2–93·6)
VH		186–189° (ethanol)	6·89 (1)	35·35 (37·2–93·6)
VIH		176–179° (ethanol)	6·41 (1)	>100

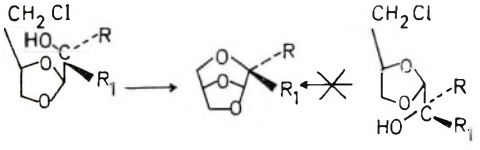
X = $Ph_2C(OH)$–

by chromatography over silica and replacement of the 4-*O*-toluene-*p*-sulphonyl group by dimethylamine. The physical constants of the eight isomeric 4-toluene-*p*-sulphonyloxymethyl-2(1-cyclohexyl-1-hydroxy-1-phenyl)methyl-1,3-dioxolans are listed in Table 3. The 4-dimethylaminomethyl-2-(1-cyclohexyl-1-hydroxy-1-phenyl)-methyl-1,3-dioxolans were converted in the usual manner into the corresponding hydrochlorides and methiodides.

The preparations of dioxolans, which involved the acid catalysed condensation of glycerol derivatives with diphenylhydroxyacetaldehyde or 2-cyclohexyl-2-hydroxy-2-phenylacetaldehyde, were complicated by the fact that under the reaction conditions used, the aldehydes underwent intramolecular rearrangement reactions. For example, diphenylhydroxyacetaldehyde rearranged to PhCHOHCOPh and

Table 3. *Melting points and specific rotations of* 4-*toluene*-p-*sulphonyloxymethyl*-2-(1-*cyclohexyl*-1-*hydroxy*-1-*phenyl*)*methyl*-1,3-*dioxolans.*

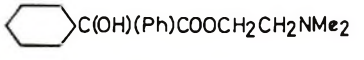
No.	Compound Z = CH_2OTs, Y = $C_6H_{11}C(OH)(Ph)$–	Configuration of Y	Configurational designation	m.p. from ethanol)	$[\alpha]_D^{20}$ (*1 in $CHCl_3$)
VIIT		*R*	D-*cis*, 4*R*,2*R*(*R*)	102–103°	−9·93
VIIIT		*R*	D-*trans*,4*R*,2*S*(*R*)	107–109°	+7·75
IXT		*S*	D-*cis*, 4*R*,2*R*(*S*)	113–116°	−3·88
XT		*S*	D-*trans*,4*R*,2*S*(*S*)	137–139°	+3·64
XIT		*R*	L-*cis*,4*S*,2*S*(*R*)	113°	+2·64
XIIT		*R*	L-*trans*,4*S*,2*R*(*R*)	139°	−4·40
XIIIT		*S*	L-*cis*,4*S*,2*S*(*S*)	104°	+9·10
XIVT		*S*	L-*trans*,4*S*,2*R*(*S*)	107–109°	−6·24°



IIIC, R = R_1 = Ph
XVIC, R = C_6H_{11}, R_1 = Ph
XVIIC, R = Ph, R_1 = C_6H_{11}

XV, R = R_1 = Ph
XX, R = C_6H_{11}, R_1 = Ph
XXI, R = Ph, R = C_6H_{11}

IVC, R = R_1 = Ph
XVIIIC, R = C_6H_{11}, R_1 = Ph
XIXC, R = Ph, R_1 = C_6H_{11}



XXII Dimethylaminoethyl *R*-2-cyclohexyl-2-hydroxy-2-phenylacetate
XXIII Dimethylaminoethyl *S*-2-cyclohexyl-2-hydroxy-2-phenylacetate

2-cyclohexyl-2-hydroxy-2-phenylacetaldehyde rearranged to $PhCOCHOHC_6H_{11}$, $PhCHOHCOC_6H_{11}$ and finally to cyclohexenylbenzyl ketone (Inch, Watts & Williams, 1971). Because these rearrangements were irreversible, and because the reactions between the aldehydes and glycerol derivatives were reversible, yields of the required dioxolans were often very low even where the reactions were carefully monitored and controlled. Consequently, to conserve pure materials for pharmacological evaluation, specific rotations of the methiodides and hydrochlorides (listed in Table 4) were not obtained since the rotational data of the parent toluene-*p*-sulphonates were considered sufficient proof of optical purity.

The geometrical relations between the 2- and 4-substituents in the dioxolans of Types A and C were established by the following procedures. The *cis*-configuration

Table 4. *Pharmacological results for* 1,3-*dioxolans with a* 2-(1-*cyclohexyl*-1-*hydroxy*-1-*phenyl*)*methyl substituent.*

Compound	Configuration	Guinea-pig ileum: Affinity constants (log K) Individual determinations	Antagonism of oxotremorine induced salivation in mice: ED50 (μmol/kg with 95% limits)
VIIH	D-*cis*, 4*S*,2*R*(*R*)	7·07, 7·06	40·6 (30·8–53·9)
VIIIH	D-*trans*, 4*S*,2*S*(*R*)	8.36, 8·41	1·5 (0·76–2·6)
IXH	D-*cis*, 4*S*,2*R*(*S*)	6·56	Inactive at 100 μmol/kg
XH	D-*trans*, 4*S*,2*S*(*S*)	<7	Inactive at 100 μmol/kg
XIH	L-*cis*, 4*R*,2*S*(*R*)	8·79, 8·89	1·7 (1·3–2·2)*
XIIH	L-*trans*, 4*R*,2*R*(*R*)	7·35, 7·40, 7·25	28·3 (16·2–49·3)
XIIIH	L-*cis*, 4*R*,2*S*(*S*)	<6·5	Inactive at 100 μmol/kg
XIVH	L-*trans*, 4*R*,2*R*(*S*)	6·24, 6·28	Inactive at 100 μmol/kg
VIIM	D-*cis*, 4*S*,2*R*(*R*)	7·28, 7·41	46·6 (33·2–65·5)
VIIIM	D-*trans*, 4*S*,2*S*(*R*)	9·37, 9·33	0·22 (0·124–0·393)
IXM	D-*cis*, 4*S*,2*R*(*S*)	6·56, 6·52	Inactive at 75 μmol/kg
XM	D-*trans*, 4*S*,2*S*(*S*)	—	16·2 (11·2–23·5)
XIM	L-*cis*, 4*R*,2*S*(*R*)	11·09, 11·08	0·07 (0·04–0·12)
XIIM	L-*trans*, 4*R*,2*R*(*R*)	7·60, 7·53	17·7 (9·9–31·4)
XIIIM	L-*cis*, 4*R*,2*S*(*S*)	—	Blocks 2/5 at 100 μmol/kg
XIVM	L-*trans*, 4*R*,2*R*(*S*)	6·77, 6·79	Blocks 2/5 at 100 μmol/kg

* Also blocked tremors ED50 = 62·4.

was assigned to the 4-chloromethyl-2(1,1-diphenyl-1-hydroxy)methyl-1,3-dioxolan, IIIC, which had a higher R_F value than the *trans*-isomer (IVC) and was eluted before the *trans*-isomer from silica, because on treatment with sodium hydride in *NN*-dimethylformamide at room temperature for 10 min it was converted rapidly into 2,2-diphenyl-3,7,8-trioxabicyclo[3,2,1]octane (XV). Only compounds in which the 4-chloromethyl group and the 2[1,1-diphenyl-1-hydroxy)methyl groups are on the same side of the dioxolan ring can undergo such a cyclization reaction and indeed the *trans*-isomer (IVC) was unchanged when treated under similar conditions.

To establish the configuration of the 2(1-cyclohexyl-1-hydroxy-1-phenyl)methyl-1,3-dioxolans in a similar manner, one compound (VIIT, VIIIT, IXT, and XT) from each of the four enantiomeric pairs of 4-toluene-*p*-sulphonyloxymethyl derivatives was converted first into the corresponding 4-chloromethyl derivatives by treatment with excess of lithium chloride in toluene and *NN*-dimethylformamide. Because only small quantities of these materials were available, the ring closure experiments were performed first with the corresponding racemic 4-chloromethyl derivatives which were prepared by direct condensation of 1-chloro-propan-2,3-diol with 2-cyclohexyl-2-hydroxy-2-phenylacetaldehyde. In order of elution the following enantiomeric pairs were obtained by chromatographic separation of the product over silica: (a) the *cis*-enantiomers XVIC, (b) the *cis*-enantiomers XVIIC, (c) a mixture of the *trans*-enantiomeric pairs XVIIIC and IXXC. The 4-chloromethyl derivative from VIIT was chromatographically and spectroscopically indistinguishable from XVIC and on treatment with sodium hydride gave a product which was chromatographically indistinguishable from the bicyclo derivative (XX) which was prepared by treatment of XVIC under similar conditions. Thus the toluene-*p*-sulphonate VIIT (and its enantiomer XIIIT) could be assigned the *cis*-configuration. Similarly, it was possible to convert the 4-chloromethyl derivate from IXT and the

related racemic 4-chloromethyl derivative XVIIIC into the bicyclic derivative (XXI), thus establishing that IXT (and its enantiomer XIT) also have the *cis*-configuration. The relations of the 4-chloromethyl derivatives from VIIIT and XT with the racemic *trans*-4-chloromethyl dioxolans XVIIIC and XIXC were established by chromatographic and spectroscopic methods and it was found that brief treatment of XVIIIC and XIXC with sodium hydride in *NN*-dimethylformamide failed to give bicyclic derivatives. These experiments indicated that VIIIT and XT and their enantiomers XIVT and XIIT respectively, had *trans*-configurations.

PREPARATIVE CHEMISTRY

All the compounds described had satisfactory analyses and/or nmr and infrared spectra. T.l.c. was performed with microscope slides coated with Merck silica gel G and column chromatography was performed with Merck silica gel of particle size 0·05–0·2 mm. The chromatoplates were developed with 50% sulphuric acid and/or iodine. All reactions were monitored by t.l.c. to establish optimum reaction conditions. The following general procedures for column chromatography were adopted. For the separation of compounds with similar R_F values, a solvent system was determined by t.l.c. in which the compounds had R_F values from 0·2–0·5. Without modification this solvent system was used for eluting compounds from the column. Light petroleum refers to the fraction b.p. 60–80°. Specific rotations were measured at 20° in 0·5 dm cells with a Hilger and Watts Standard Polarimeter Mk. III.

Hydrochlorides were prepared by addition of a hydrogen chloride saturated solution of ether to solutions of the dimethylaminomethyl compounds in ether and the methiodides were formed by addition of methyl iodide to ether solutions of the dimethylaminomethyl compounds. Where the dimethylaminomethyl compounds were previously purified by chromatography, no purification of the hydrochlorides and methiodides was necessary.

r-4-*Dimethylamino*-cis- *and* trans-2(1,1-*diphenyl*-1-*hydroxy methyl*-1,3-*dioxolan* (*III*) *and* (*IV*). A solution of diphenylhydroxyacetaldehyde dimethylacetal (10 g) (in some experiments diphenylhydroxyacetaldehyde was used) and 1-chloropropan-2,3-diol (5 g) in benzene (200 ml) containing toluene-*p*-sulphonic acid (0·5 g) was boiled under reflux for 2 h. The solution was neutralized [Amberlite IRA 400 resin (OH^- form)], concentrated and the residue resolved chromatographically over silica in ether–light petroleum (1:4) to yield *r*-4-chloromethyl-*cis*-2(1,1-diphenyl-1-hydroxy)-methyl-1,3-dioxolan (IIIC) (7·5 g, 54%), m.p. 95° (from light petroleum) R_F 0·35. (Found: C, 66·7; H, 5·8. $C_{17}H_{17}ClO_3$ requires C, 67·0; H, 5·6%), and *r*-4-chloromethyl-*trans*-2(1,1-diphenyl-1-hydroxy)methyl-1,3-dioxolan (IVC) (1·5 g, 11%), m.p. 96–97° (from light petroleum) R_F 0·2. (Found: C, 67·7; H, 5·9. $C_{17}H_{17}ClO_3$ requires C, 67·0; H, 5·6%). The *cis* and *trans*-4-chloromethyl derivatives (IIIC) and (IVC) were treated with 33% solutions of dimethylamine in ethanol at 100° in sealed tubes for 8 h, and the solutions concentrated to yield the corresponding *cis* and *trans*-4-dimethylaminomethyl derivatives (III) and (IV) as viscous syrups. Compounds (III) and (IV) were converted into their respective hydrochlorides (IIIC and IVC) and methiodides (IIIM and IVM) (Table 1) under usual conditions.

(*IIIC*) *and* (*IVC*) – *sodium hydride reactions.* Excess of sodium hydride was added to solutions of (IIIC) and (IVC) in *NN*-dimethylformamide and the solutions stored for 10 min at room temperature, poured into water and extracted with ether.

The ether solution from the *cis*-isomer (IIIC) was dried and concentrated to yield 2,2-diphenyl-3,7,8-trioxabicyclo-[3,2,1]octane (XV), m.p. 134–137° (from ether) R_F 0·38 [ether–light petroleum (1:1)]. (Found: C, 76·8; H, 6·18. $C_{17}H_{16}O_3$ requires C, 76·1; H, 6·01). The ether solution from the *trans*-isomer (IVC) contained unreacted (IVC). Under prolonged reaction conditions (>1 h), (IVC) was converted by sodium hydride in *NN*-dimethylformamide into diphenylhydroxyacetaldehyde.

r-2-*Dimethylaminomethyl*-cis- *and* trans-4-(1,1-*diphenyl*-1-*hydroxy methyl*-1,3-*dioxolans*. The product from the acid catalysed condensation of methyl glycerate (Inch & Williams, 1970) and chloroacetaldehyde diethylacetal was treated with an excess of phenylmagnesium bromide and the mixture boiled under reflux for 1 h. The crude product, which was isolated from the Grignard reaction mixture under usual conditions, was treated with dimethylamine in ethanol for 48 h at 100° in a sealed tube. The solution was concentrated and the residue chromatographed over silica in benzene–ether 1:1 to give isomer V. R_F 0·5, m.p. 108–110° (from ethanol) and isomer VI R_F 0·2, m.p. 68–72° (from ethanol). Compounds V and VI converted into the hydrochlorides (VH and VIH) and methiodides (VM and VIM) respectively.

Preparation of 4-*toluene*-p-*sulphonyloxymethyl*-2(1-*cyclohexyl*-1-*hydroxy*-1-*phenyl*)-*methyl*-1,3-*dioxolans*. Solutions of equimolar quantities of 1-*O*-toluene-*p*-sulphonyl-D- (or L)-glycerol (Brimblecombe & Inch, 1970) and *R* (or *S*)-2-cyclohexyl-2-hydroxy-2-phenylacetaldehyde (Inch, Ley & Rich, 1970) in toluene containing a catalytic amount of toluene-*p*-sulphonic acid, were boiled under reflux and water was removed azeotropically. The reaction was monitored by t.l.c. (ether–light petroleum 1:1). [When the D-glycerol derivative was condensed with the *R*-aldehyde or when the L-glycerol derivative was condensed with the *S*-aldehyde, the required *cis* and *trans*-1,3-dioxolan derivatives had R_F values of 0·3 and 0·2 respectively and were easily separated. When the D-glycerol derivative was condensed with the *S*-aldehyde or when the L-glycerol derivative was condensed with the *R*-aldehyde the required *cis*- and *trans*-1,3-dioxolans were not separated but appeared as an elongated spot at R_F 0·33. With all the reactions cyclohexenyl benzyl ketone (R_F 0·75) was also formed and under prolonged reaction conditions was the only product.] The solutions were neutralized with Amberlite IRA 400 resin (OH^- form), concentrated and chromatographed over silica in ether–light petroleum (3:7) where in all cases separations of the *cis-trans* pairs were achieved. Without exception the *cis*-isomers were eluted before the *trans*-isomers from the silica. The physical constants of the eight isomers are listed in Table 3.

Reaction of DL-1-*chloropropan*-2,3-*diol and* RS-2-*cyclohexyl*-2-*hydroxy*-2-*phenyl-acetaldehyde*. A solution of 1-chloropropan-2,3-diol (1·2 g) and 2-cyclohexyl-2-hydroxy-2-phenylacetaldehyde (2·1 g), prepared by oxidation of 2-cyclohexyl-2-phenylethane-1,2-diol with acetic anhydride in dimethylsulphoxide (Inch, Watts & Williams, 1971) in toluene containing toluene-*p*-sulphonic acid (0·2 g) was boiled under reflux for 0·75 h and water was removed azeotropically. The reaction was monitored by t.l.c. (ether–light petroleum, 3:7). The solution was neutralized (Amberlite IRA 400 resin (OH^-) form), concentrated and the residue chromatographed over silica to yield amongst other products the enantiomorphic mixtures (a) *r*-4-D (or L) chloromethyl-*cis*-2[*R* (or *S*)-1-cyclohexyl-1-hydroxy-1-phenyl]methyl-1,3-dioxolan (XV), m.p. 97° (from light petroleum); R_F 0·4. (Found: C, 66·1; H, 7·4. $C_{17}H_{23}O_3Cl$ requires C, 65·7; H, 7·6%). (b) *r*-4-D (or L) chloromethyl-*cis*-2[*S* (or *R*)-1-cyclohexyl-1-hydroxy-1-phenyl]methyl-1,3-dioxolan (XVI), m.p.

85–92° (from light petroleum), R_F 0·37. (Found: C, 66·0; H, 7·5. $C_{17}H_{23}O_3Cl$ requires C, 65·7; H, 7·6%. (c) a mixture of the enantiomorphic pairs *r*-4-D (or L)-chloromethyl-*trans*-2[*R* (or *S*)-1-cyclohexyl-1-hydroxy-1-phenyl]methyl-1,3-dioxolan (VIII) and *r*-4-D (or L)-chloromethyl-*trans*-2[*S* (or *R*)-1-cyclohexyl-1-hydroxy-1-phenyl]methyl-1,3-dioxolan (XVIII).

Conversion of 4-*toluene*-p-*sulphonyloxymethyl*-2(1-*cyclohexyl*-1-*hydroxy*-1-*phenyl*)-*methyl*-1,3-*dioxolans into the corresponding* 4-*chloromethyl derivatives.* The first listed enantiomers (*ca.* 0·05 g) from each of the pairs VIIT and XIIIT, VIIIT and XIVT, IXT and XIT, and XT and XIIT, were dissolved separately in solutions of equal parts of toluene and *NN*-dimethylformamide and excess of lithium chloride was added. The solutions were stirred vigorously, boiled under reflux for 1 h, poured into water, extracted with ether and the ether extracts dried ($MgSO_4$) and concentrated. The products were purified by chromatography over silica in ether–light petroleum (1:4). The 4-chloromethyl derivatives which were obtained from VIIT, VIIIT, IXT and XT were chromatographically and spectroscopically indistinguishable from the racemic 4-chloromethyl derivatives XV, XVI, XVII and XVIII respectively.

The 4-chloromethyl derivatives of VIIT and IXT were converted by treatment with sodium hydride in *NN*-dimethylformamide at room temperature for 5 min into products with chromatographic properties indistinguishable from the bicyclic compounds XX and XXI. The 4-chloromethyl derivatives from the *trans*-toluene-*p*-sulphates VIIIT and XT did not react with sodium hydride in *NN*-dimethylformamide under similar conditions.

Reactions of XV, XVI, XVII and XVIII with sodium hydride. (a) A solution of XV (0·1 g) and excess of sodium hydride in *NN*-dimethylformamide was stored at room temperature for 5 min, poured into water and extracted with ether. The ether extract was concentrated and the product purified over silica in ether–light petroleum (3:7) to yield the 2-cyclohexyl-2-phenyl-3,7,8-trioxabicyclo[3,2,1]octane isomer (XX), m.p. 134–138° (from light petroleum). (Found: C, 74·8; H, 8·0. $C_{17}H_{22}O_3$ requires C, 74·4; H, 8·1%). (b) Similar treatment of XVI, afforded the 1-cyclohexyl-2-phenyl-3,7,8-trioxabicyclo[3,2,1]octane isomer (XXI), m.p. 168–172° (from light petroleum). (Found: C, 74·3; H, 8·2. $C_{17}H_{22}O_3$ requires C, 74·4; H, 8·1%). (c) A mixture of XVII and XVIII and excess of sodium hydride in *NN*-dimethylformamide was stored at room temperature for 10 min, then a small portion was removed, diluted with water and extracted with ether. T.l.c. on the ether solution showed that XVII and XVIII were essentially unchanged. After storage at room temperature with sodium hydride and dimethylformamide for 1 h however XVII and XVIII decomposed to 2-cyclohexyl-2-hydroxy-2-phenylacetaldehyde.

PHARMACOLOGY

Methods

All the compounds were tested for anticholinergic activity using the following two procedures.

Antagonism of acetylcholine-induced contractions of the isolated guinea-pig ileum. The method used was essentially that described by Barlow, Scott & Stephenson (1963) which enabled affinity constants for the drugs to be measured. A segment of ileum was taken from a freshly-killed guinea-pig at a point about 5 cm from the ileo-caecal junction and suspended in a 5 ml organ bath containing Ringer-Tyrode

solution at 37°. A mixture of 5% carbon dioxide in oxygen was bubbled through the solution. Regular contractions were obtained in response to two doses of acetylcholine, then the anticholinergic drug was dissolved in the Ringer-Tyrode solution and the doses of acetylcholine increased to obtain comparable responses. Thus it was possible to obtain the dose ratio corresponding to a particular concentration of antagonist. (Dose ratio is equal to the dose of agonist required to produce a given response in the presence of an antagonist, divided by the dose required to produce the same response in the absence of the antagonist = A/a). The affinity constant of the antagonist can then be calculated from the equation $BK = A/a - 1$ (Gaddum, 1957) where B is the concentration of the antagonist and K its affinity constant.

Antagonism of oxotremorine effects in mice. A solution of the anticholinergic drug in normal saline was injected intraperitoneally to 18–25 g male albino mice 15 min before the intravenous injection into a tail vein of 100 μg/kg of oxotremorine. Animals were examined at 5, 10 and 15 min after the oxotremorine injection for the presence of salivation or tremors, or both. Four groups, each containing 5 mice, were used and ED50s for block of salivation and of tremors were calculated by probit analysis.

RESULTS

The pharmacological results are summarized in Tables 1, 2 and 4.

DISCUSSION

The structural and pharmacological similarities of acetylcholine (or more precisely acetyl-β-methylcholine) and *cis*-4-dimethylaminomethyl-2-methyl-1,3-dioxolan methiodide (I) are well documented (Bebbington & Brimblecombe, 1965; Belleau & Lavoie, 1968; Chothia, 1970). Accordingly, since replacement of the acetoxymethyl group in acetylcholine derivatives by bulky substituents such as (1,1-diphenyl-1-hydroxy)methyl or (1-cyclohexyl-1-hydroxy-1-phenyl)methyl affords potent anticholinergic drugs, it could be predicted by analogy that replacement of the 2-methyl group in I by similar bulky substituents would also afford anticholinergic drugs. Also, since the tertiary analogues of anticholinergic drugs formally derived from acetylcholine usually have appreciable anticholinergic potencies, it could be predicted that related tertiary 1,3-dioxolan derivatives would have good anticholinergic properties. To test these predictions, the anticholinergic potencies of DL-*r*-4-dimethylaminomethyl-*cis*-2(1,1-diphenyl-1-hydroxy)methyl-1,3-dioxolan hydrochloride (IIIH) and the methiodide (IIIM) and the corresponding *trans*-isomers IVH and IVM were measured by the methods described above. The results obtained (Table 1) showed that the quaternary derivatives IIIM and IVM had similar peripheral potencies to related anticholinergic esters which are formally derived from acetylcholine. For example, the affinity constants of IIIM and IVM (log K = 8·5 and 8·38) are similar to those measured by Abramson, Barber & others (1969) for diphenylhydroxyacetylcholine (log K = 8·5) and to the pA_2 values obtained by Ellenbroek, Nivard & others (1965) for the enantiomeric diphenylhydroxyacetyl-β-methylcholine (pA_2 = 8·0 and 8·1). However the peripheral anticholinergic potency of the tertiary derivatives IIIH and IVH was a little lower than potencies recorded for related tertiary dimethylaminoalkyl esters (Brimblecombe, Green & others, 1971).

The fact that the anticholinergic drugs IIIH, IIIM, IVH and IVM, formed by

replacement of the 2-methyl group in I (or its tertiary aminoalkyl analogue) had the expected order of anticholinergic potency encouraged a more detailed examination of compounds of this type. The choice was influenced by previous observations that the anticholinergic activity of 1,3-dioxolans depended on the absolute configuration of C-2 (and to a less extent on that at C-4) and not on the geometrical relation between the C-2 and C-4 substituents, and also that the potency of chiral anticholinergic glycollates varied widely with the configuration of the benzylic carbon atom. Thus, as optically pure *R* and *S*-2-cycloalkyl-2-hydroxy-2-phenylacetaldehydes were available (Inch, Ley & Rich, 1968) it was decided to prepare and evaluate the eight isomers of 4-dimethylaminomethyl-2-(1-cycloalkyl-1-hydroxy-1-phenyl)-methyl-1,3-dioxolan and the corresponding methiodides. We believe this to be the first report of the pharmacological examination of all the possible isomers of a compound that contains three asymmetric centres. Unfortunately, for some of the isomers, sufficient material was obtained only for measurements of affinity constants and of activity in antagonizing oxotremorine effects, whereas earlier assessments (e.g., Brimblecombe & Inch, 1970) included the mydriatic potency. The results (Table 4) in these two tests for the hydrochlorides and methiodides of the eight isomers showed a clear pattern. Firstly it was evident that, as in the glycollates, the activity depends critically on the configuration at the benzylic centre. Thus the four isomers in which the 2-(1-cyclohexyl-1-hydroxy-1-phenyl)-methyl substituent had the *S* configuration were much less potent than those where the X-substituent had the *R*-configuration. In the *R*-isomers the order of potency for the methiodides was L-*cis* > D-*trans* > L-*trans* > D-*cis*, showing that, as for the 2,2-disubstituted 1,3-dioxolans (Brimblecombe & Inch, 1970), the absolute configuration of the C-2 and C-4 asymmetric centres is more important than the geometrical relations between the C-2 and C-4 substituents. Also, the compounds with the highest activities, the L-*cis*- and D-*trans*-isomers, have the *S*-configuration at C-2. The configuration at C-4 was also important in that the L-*cis*-compounds were significantly more potent than the D-*trans*-compounds and the L-*trans*-compounds were noticeably more potent than the D-*cis*-compounds.

The pharmacological results make an interesting comparison with those for the hydrochlorides and methiodides of dimethylaminomethyl *R* and *S*-2-cyclohexyl-2-hydroxy-2-phenylacetate (XXII and XXIII) (Brimblecombe & others, 1971). For example, the affinity constant for XIM (log K = 11·08) is higher than that of XXIIM (log K = 9·66) although the potencies of the two compounds in antagonizing the effects of oxotremorine-induced salivation (0·07 and 0·06 μmol/kg) were similar. The *S*-ester (XXIIIM) was slightly more potent (8·34 μmol/kg) than the most active (*S*)-dioxolan (XM—16·2 μmol/kg) in this test. Also there were only small differences in the potencies of the L-*cis*-4*R*,2*S*(R)-dioxolan hydrochloride (XIM) (log K = 8·8, salivation 1·7 μmol/kg) and the dimethylaminoethyl ester hydrochloride, XXIIH (log K = 9·06, salivation 0·76 μmol/kg). The affinity constant of XIM (log K = 11·08) and the pA_2 values determined by Ellenbroek & others (1965) for the *R*-2-cyclohexyl-2-hydroxy-2-phenylacetic acid esters of *R* and *S*-1-dimethylaminopropan-2-ol methiodides (pA_2 = 8·9 and 8·3) may also be compared. From these examples, it is clear that related glycollates and dioxolans have similar potencies, in many respects. It is also clear that replacement of the 2-methyl substituent in I with a 2(1-cyclohexyl-1-hydroxy-1-phenyl)methyl group (Type C) afforded much more potent compounds than when the C-2 substituents in I were replaced by, e.g.,

phenyl and cyclohexyl groups (II) (Brimblecombe & Inch, 1970). For example, XIM (log K = 11·08) had a much higher affinity constant than L-*cis*-(2*S*,4*R*)-2-cyclohexyl-2-phenyl-4-dimethylaminomethyl-1,3-dioxolan methiodide (log K = 8·73) and these differences were reflected by the respective potencies in antagonizing the effects of oxotremorine-induced salivation (0·07 and 2·9 μmol/kg).

Because the anticholinergic potency for the series of compounds VII–XIVH and M, depended critically on the configuration at C-2, as well as on that of the benzylic centre, it is evident that the dioxolan ring itself makes a significant contribution to the affinity of the anticholinergic dioxolans for their receptor. If the dioxolan ring had acted simply as a "spacer" between the dimethylaminomethyl and the (1-cyclohexyl-1-hydroxy-1-phenyl)methyl groups the dependence of anticholinergic potency on the C-2 (and C-4) configuration would not have been so significant.

Although further evidence in support of this conclusion was provided by the results, which showed that the affinity constants of the quaternary 4-dimethylaminomethyl derivatives (Table 1) were much higher than those of the corresponding 2-dimethylaminomethyl derivatives (Table 2), there was conflicting evidence in that the tertiary 2-dimethylaminomethyl (Table 2) and 4-dimethylaminomethyl dioxolan derivatives (Table 1) had essentially similar affinity constants. Any interpretation of this result was impeded by a lack of understanding of the variable effects which quaternary groups have on the peripheral potency of anticholinergic drugs. For example, in the glycollate series it was observed that the affinity constants of tertiary drugs were increased by quaternization to a small (log K = 0·3) but uniform extent (Brimblecombe & others, 1971). In contrast, the increases in affinity constants caused by quaternization of Type C dioxolans varied appreciably [e.g., VIIH (log K = 7·07) → VIIM (log K = 7·3) and XIIH (log K = 8·8) to XIIM (log K = 11·9)].

The differences in stereochemical requirements for high biological activity between cholinergics related to acetylcholine and anticholinergics derived from acetylcholine have been considered to signify that the receptor sites for cholinergic and anticholinergic drugs are quite distinct (Brimblecombe, Green & Inch, 1970). The evidence from stereochemical comparisons of cholinergic and anticholinergic dioxolans is not so clear cut. Although in both series of quaternary compounds those with the L-*cis*-configuration are most active, there are small differences in the order of potencies relative to configuration within the two series. The order of activity within the cholinergic series is (Belleau & Lavoie, 1968), L-*cis* > L-*trans* > D-*trans* > D-*cis* and in the anticholinergic series it is L-*cis* > D-*trans* > L-*trans* > D-*cis*.

In the former series the relation between cholinergic potency and C-4 configuration is paramount and in the latter series, although anticholinergic potency depends more on the configuration at C-2 than at C-4, the configuration at C-4 still influences activity. Whereas the similar dependence of the activity on C-4 configuration of the quaternary cholinergic and anticholinergic dioxolans suggests that they both interact with similar receptor sites, the tertiary anticholinergic dixolans show little dependence on the configuration at C-4, a result which, as in the acetylcholine–glycollate series, points to differences in the cholinergic and anticholinergic receptors. The value of such comparisons may be questioned because of the inter-relation of the 2- and 4-substituents about the comparatively rigid dioxolan ring.

Some other more pharmacological points arise from these results. One of the

most striking is the fact that the quaternary ammonium salts IIIM and IVM blocked oxotremorine-induced tremors. It is generally accepted that such salts cannot penetrate into the central nervous system where the tremors originate. It seems almost certain, therefore, that this block of tremors is due to a peripheral action of these dioxolans and indeed Upshall (unpublished results) has confirmed by whole body autoradiography that a [^{14}C] labelled sample of IVM (25 mg/kg intravenously to mice) did not penetrate into the central nervous system. To date, attempts to find peripheral pharmacological actions of these drugs that might account for their ability to block tremors have been in vain.

Previous experience in this laboratory with a range of anticholinergic drugs has shown a reasonable degree of parallelism between *in vitro* estimates of their activity and their *in vivo* potency in blocking oxotremorine effects. With the compounds studied here, as with other series, the general trend was that those drugs with log K values of about 6·6 and above had ED50s of <100 μmol/kg for block of oxotremorine-induced salivation. The correlation between *in vitro* estimates of potency and doses required to block tremors is less good. For example, XIH with a log K of about 8·8 and an ED50 for block of salivation of 1·7 μmol/kg has an ED50 for block of tremors of 62·4 μmol/kg whereas compounds IIIH and IVH with log K values of about 6·7 and ED50s for block of salivation of about 25 μmol/kg have ED50s for block of tremors of 11 and 15 μmol/kg respectively. Presumably this reflects differences in the relative ability of the drugs to penetrate various biological barriers and gain access to the central nervous system.

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J. Pharm. Pharmac., 1971, **23**, 662–670 *Received March* 15, 1971

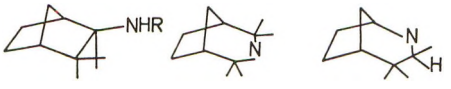
The ganglion blocking activity of diastereoisomeric dimethylaminobornyl acetates and their methiodides

G. H. COOPER, D. M. GREEN, R. L. RICKARD AND PAMELA B. THOMPSON

Chemical Defence Establishment, Porton Down, Salisbury, Wiltshire, U.K.

Some diastereoisomeric dimethylaminobornyl acetates and their methiodides have been prepared and tested for ganglion blocking activity. Included in these compounds was an enantiomeric pair and associated quaternary salts. These optical antipodes displayed virtually no difference between their actions at the ganglion. Differences between the activities of the least and most potent diastereoisomers was limited to a factor of about five. Assays were made upon the cat superior cervical ganglion and also the guinea-pig vas deferens preparation the successful quantitative use of which is described.

In 1956, Stone, Torchiana & others reported that mecamylamine (I, R = Me) possessed ganglion blocking activity comparable with hexamethonium. This finding suggested that secondary amines might prove to be classes of compounds with biological activities comparable with those of bis quaternary ammonium salts of the hexamethonium type. Further work (Lee, Wragg & others, 1958) on isomers II and III of mecamylamine and normecamylamine (I, R = H) led to investigations on simpler six membered structures where it was found that 1,2,2,6,6-pentamethyl piperidine (pempidine) exhibited ganglion blocking activity to a high degree (Corne & Edge, 1958; Spinks, Young & others, 1958; Spinks & Young, 1958).



I II III

With the activity of these amines established it was decided to compare the biological activities of three diastereoisomeric dimethylaminobornyl acetates (1–3) and their methiodides (1M–3M). To enlarge the scope of the present work the enantiomers (4 and 4M) of the *trans* isomers (3 and 3M) were also prepared and studied.

These compounds combine bridge-ring features associated with high affinity together with an acetoxy group, present in the transmitter substance acetylcholine. Possibly this would lead to compounds with high activity. From the affinities of the compounds it might, in addition, be possible to make deductions about how they are bound to receptors. The molecules are rigid and so changes in affinity are unlikely to arise from any change in the preferred conformation of the drug, only from differences in ability to fit groups in the receptor.

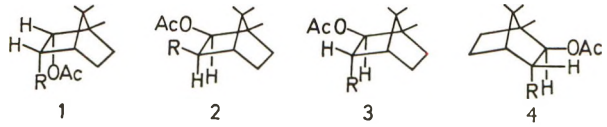
CHEMISTRY

The preparation of the three diastereoisomeric dimethylaminobornyl acetates (1–3) derived from 1*R*, 4*R*-(+)-camphor has been described elsewhere (Chittenden & Cooper, 1970).

Compound (4). Enantiomer of (3). This was prepared from 1*S*, 4*S*-(−)-camphor by an exactly similar procedure to that used in the preparation of (3). Hydrochloride m.p. 279–281° (put on at 270°). (Found: C, 61·0; H, 9·5; N, 5·0; $C_{14}H_{26}NO_2Cl$ requires C, 61·0; H, 9·5; N, 5·1%.) $[\alpha]_D$ − 9·0°.

Quaternary methiodides (1M–4M). The free, tertiary bases (1–4) were dissolved in acetone to which a little sodium bicarbonate had been added. A 3 molar excess of methyl iodide was added and the mixture refluxed for 5 h. The inorganic material was filtered off and ether added to precipitate the quaternary salts. These were crystallized from an ether–methanol mixture.

The formulae of the compounds studied are shown in Fig. 1 and the physical constants of the methiodides are listed in Table 1.



1 2 3 4

FIG. 1. Formulae of compounds.

Compound No. Compounds derived from 1*R*, 4*R*-(+)-camphor

1

1 R = NMe_2: *cis*, *endo*-2-acetoxy-3-dimethylaminobornane hydrochloride.
1M R = N^+Me_3: *cis*, *endo*-2-acetoxy-3-dimethylaminobornane methiodide.

2

2 R = NMe_2: *cis*, *exo*-2-acetoxy-3-dimethylaminobornane hydrochoride.
2M R = N^+Me_3: *cis*, *exo*-2-acetoxy-3-dimethylaminobornane methiodide.

3

3 R = NMe_2: 2-*exo*-acetoxy-3-*endo*-dimethylaminobornane hydrochloride.
3M R = N^+Me_3: 2-*exo*-acetoxy-3-*endo*-dimethylaminobornane methiodide.

Compounds derived from 1*S*, 4*S*-(—)-camphor.

4

4 R = NMe_2: 2-*exo*-acetoxy-3-*endo*-dimethylaminobornane hydrochloride.
4M R = N^+Me_3: 2-*exo*-acetoxy-3-*endo*-dimethylaminobornane methiodide.

Table 1. *Physical constants of the methiodides of the dimethylaminobornyl acetates studied.*

Methiodide	M.p.	$[\alpha]_D$ (*c* in EtOH)	Microanalysis* C	H	N	Yield (%)
1M	259–261°	—17·9 (2·07)	47·55	7·1	3·8	70
2M	248–249°	+22·9 (1·88)	47·0	7·1	3·6	35
3M	185–187°	+ 7·6 (2·1)	47·3	7·15	3·8	82
4M	186–188°	— 5·1 (2·45)	47·6	7·5	3·6	45

* $C_{15}H_{28}NO_2I$ requires C, 47·25; H, 7·4; N, 3·7%.

PHARMACOLOGY

The compounds were tested on preparations containing parasympathetic ganglia (guinea pig isolatedileum) and sympathetic ganglia (stimulated guinea-pig vas deferens and cat superior cervical ganglion preparations) and for their ability to stimulate

receptors in the neuromuscular junction (the isolated semispinalis preparation of the chick).

Guinea-pig isolated ileum

Approximately 2 cm of ileum was taken from a freshly killed guinea-pig at a point 5 cm from the ileo-caecal junction. This was suspended in a 5 ml organ bath containing Ringer Tyrode solution at 37° gassed with 5% carbon dioxide in oxygen. Contractions of the ileum were recorded on a kymograph, using a frontal lever, the magnification being approximately 8:1. The compounds were tested for their ability to depress submaximal contractions produced by the following agonists added to the bath: acetylcholine, nicotine, 5-hydroxytryptamine and barium chloride. The test compound was added to the bath 1 min before the agonist.

Guinea-pig isolated vas deferens preparation, stimulated through the hypogastric nerve and transmurally

Male guinea-pigs, 400–800 g, were stunned by a blow on the head and bled. The vas deferens was dissected (Huković, 1961) and set up on a Perspex holder in a 50 ml bath, containing McEwan solution (1956) maintained at 29° and gassed with 5% carbon dioxide in oxygen. Conventional silver electrodes (unshielded) were used to stimulate the hypogastric nerve and parallel wire electrodes were used for transmural stimulation (Birmingham & Wilson, 1963). For both types of stimulation the stimulus parameters were identical at a frequency of 25 Hz for 10 s and a pulse width of 0·1 ms. Supramaximal voltage was usually 80–100 V for transmural stimulation and 25–40 V for the hypogastric nerve. Contractions were recorded by means of a Devices 2LDO1 linear displacement optical wedge transducer connected to a Smiths Servoscribe pen recorder. For quantitative studies the nerve was stimulated every 3 min, and the preparation stimulated transmurally once before, and 12 min after the addition of the drug to the bath (see Fig. 2). The reduction in response to nerve

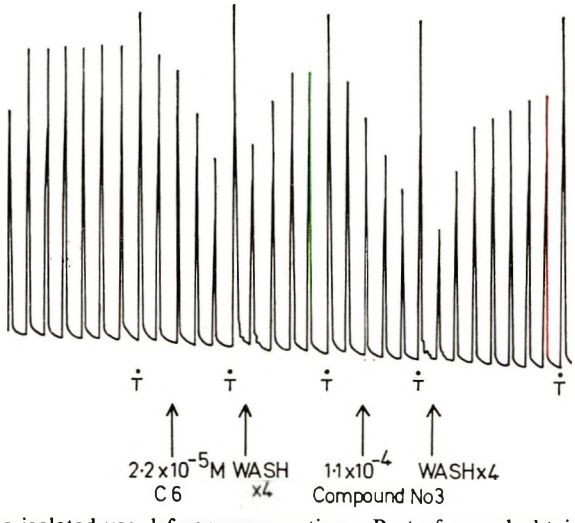


FIG. 2. Guinea-pig isolated vas deferens preparation. Part of record obtained during bioassay of 2-*exo*-acetoxy-3-*endo*-dimethylaminobornane HCl (Compound No. 3) against hexamethonium (C_6). All contractions were elicited by stimulation of the hypogastric nerve for a period of 10 s. T indicates contractions elicited by transmural stimulation. A time cycle of 3 min was used between each stimulation.

and transmural stimulation was measured 9 and 12 min respectively after contact with the drug, and plots of percentage reduction in response against log doses were made. In some cases reduction in response to nerve stimulation was also measured 30 min after contact with the drug. Two doses were chosen lying on the straight line portion of the log dose response curve, and a latin square 2 × 2 point assay was made using hexamethonium as standard drug. As full recovery did not always occur after washing out, it was not possible to complete the latin square (16 doses) on a single preparation; results from two or three preparations were combined to complete the procedure.

Cat nictitating membrane (superior cervical ganglion) preparation

13 cats, 2–2·5 kg, were anaesthetized with chloralose (0·1 g/kg intravenously) after induction by ether. The assay procedure for determining the ganglion blocking activity was essentially that described by Fakstorp & Pederson (1954). The trachea was intubated and a deep cervical well was prepared by forward reflection of the oesophagus and larynx. The cervical sympathetic trunk was dissected free from the vagus nerve and cut at a point approximately 2 cm caudal to the superior cervical ganglion. The cervical well was filled with liquid paraffin and for preganglionic stimulation the cut cervical sympathetic nerve was stimulated by means of bipolar silver electrodes. The nerve was stimulated once every 2 min with stimulus parameters of 25 Hz for 6 s and a pulse width of 0·25 ms. Voltage was adjusted to give a supramaximal contraction which was obtained between 3–5 V. The resting tension in the nictitating membrane was 4 g and contractions were recorded in a manner similar to those of the isolated vas deferens preparation. The compounds were injected intravenously into the femoral vein and the maximum reduction in height of the nictitating membrane was measured. Dose levels giving approximately 25 and 50% reduction in height were used in a latin square 2 × 2 point assay using hexamethonium as standard. The drugs were injected only when the nictitating membrane contractions had returned to their original height. One assay could be completed on one preparation as full recovery from doses of the aminobornylacetates and hexamethonium occurred within 20 and 30 min respectively. The results obtained from two preparations in the assay of mecamylamine against hexamethonium were combined owing to the lengthy recovery time for mecamylamine (80–100 min).

The effects of the compounds on contractions evoked by post-ganglionic stimulation were also studied at dose levels that reduced by 50% contractions evoked by preganglionic stimulation. The post-ganglionic trunk was exposed approximately 5 mm from the ganglion and a monopolar silver electrode was hooked under the nerve; the indifferent electrode was placed on one of the neck muscles. The stimulus parameters used were identical to those used for preganglionic stimulation.

Isolated semispinalis muscle of the chick

The semispinalis muscle was removed from 3–10 day old chicks anaesthetized with ether (Child & Zaimis, 1960). Recordings of the contractions of the muscle were made in the manner described for the guinea-pig ileum. Submaximal contractions were produced by 2 μM solution of nicotine and the test compounds were studied for their ability to produce contractions relative to nicotine.

Drugs

Mecamylamine hydrochloride was purchased from Merck Sharpe and Dohme Ltd., and hexamethonium bromide was purchased from May & Baker Ltd.

RESULTS

Effects on guinea-pig isolated ileum

At a relatively high bath concentration of 100 μM the eight aminobornyl acetate compounds did not produce contraction of the ileum, neither did they affect contractions produced by acetylcholine, 5-hydroxytryptamine and barium chloride. At a bath concentration of 10 μM they reduced the contraction produced by nicotine, demonstrating that all the aminobornyl acetate compounds possessed ganglion blocking activity. The guinea-pig ileum proved inadequate for precise quantitative estimates of ganglion blocking activity as the degree of reproducibility of assays was poor owing mainly to the preparation failing to contract consistently under stimulation by nicotine. It has been reported (Fakstorp & Pederson, 1954) that 1,1-dimethyl-4-phenylpiperazinium (DMPP) is a more suitable agonist than nicotine when assaying for ganglion blocking activity on the ileum. In our experience, however, DMPP has not shown any advantage over nicotine.

Effects on guinea-pig isolated vas deferens

Birmingham & Wilson (1963) published convincing pharmacological evidence for the presence of ganglia in the hypogastic nerve. In the present work compounds under investigation were assayed for their ganglion blocking activity by reducing the contractions of the vas deferens elicited by preganglionic stimulation of the hypogastric nerve. The results are shown in Table 2 where activities of the compounds

Table 2. *Ganglion blocking activity of compounds studied.*

Compound	Equipotent molar ratios relative to hexamethonium with 95% confidence limits Guinea-pig isolated vas deferens prepn 9 min Contact time	30 min Contact time	Cat superior cervical ganglion
1	3·57 (2·3 –4·17)	3·03 (2·17–3·84)	7·7 (4·54 –11·1)
1M	3·03 (2·27–4·17)	3·03 (2·7 –3·45)	7·7 (6·67 – 9·1)
2	4·00 (1·27–9·10)	—	12·5 (8·3 –20·0)
2M	1·37 (0·61–2·27)	—	4·0 (3·85 – 4·17)
3	5·00 (3·70–7·70)	—	20·0 (16·7 –25·0)
3M	2·33 (1·69–3·13)	—	6·3 (4·17 – 8·3)
4	5·56 (4·17–6·67)	—	16·7 (11·1 –25·0)
4M	2·70 (1·96–3·70)	—	4·76 (4·17 – 5·56)
Mecamylamine	0·31	—	1·17 (0·93 – 1·44)

are expressed as equipotent molar ratios relative to hexamethonium. Ganglion blocking activity was assayed against hexamethonium and not mecamylamine as standard because the latter drug proved very difficult to wash out of the preparation and has been classified as a non-competitive antagonist (Rossum & Ariens, 1959; Trendelenburg, 1961).

A portion of a record obtained in the assay procedure (Fig. 2) shows that after contact with the drug a reduction in contraction proceeds with time but the contractions showed no signs of reaching a plateau within 9 min. In any assay procedure

it is advisable to measure the reduction in response at the plateau where the drug has reached equilibrium with the receptor, since measurements at the plateau eliminate differences in activity caused by different diffusion rates. It was found that with the aminobornyl acetates a plateau of the contractions was reached after a 25–30 min contact time, and slopes of log dose response curves were reasonably parallel to those obtained with hexamethonium (a known competitive antagonist). These findings indicate that the aminobornyl acetate compounds appear to be acting as competitive antagonists. A comparison of the results obtained with compounds 1 and 1M assayed at contact times of 9 and 30 min showed no significant difference, and the use of a 9 min contact time for the assay of all the other compounds was therefore considered justified. The use of a 30 min contact time throughout would have been time consuming and results would have had to be combined from a number of preparations.

The vas deferens preparations was also used to study the degree of specificity of ganglion blockade by stimulating the preparation preganglionically through the hypogastric nerve and postganglionically by stimulation transmurally. Dose response curves for pre- and post-ganglionic stimulation obtained in the assay of compound 1 and 1M are shown typically in Fig. 3. The tertiary aminobornyl acetates were

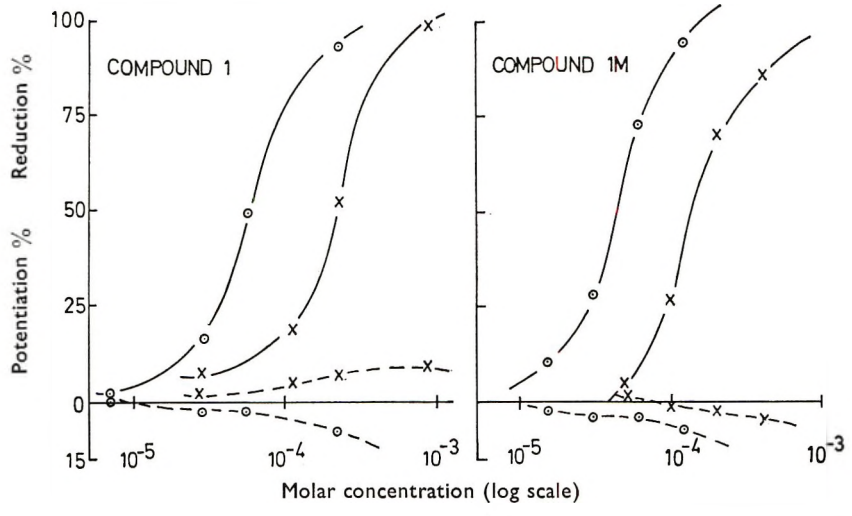


FIG. 3. Dose response curves obtained in bioassay of *cis-endo*-2-acetoxy-3-dimethylaminobornane HCl (Compound 1) and its methiodide (Compound 1M) against hexamethonium using guinea-pig isolated vas deferens preparation. (×——×) preganglionic stimulation with test compounds. (⊙——⊙) preganglionic stimulation with hexamethonium. (×— — —×) postganglionic (transmural) stimulation with test compounds. (⊙— — —⊙) postganglionic stimulation with hexamethonium.

slightly less specific in their ganglion blocking activity than were their respective methiodides. This was demonstrated by the tertiary compounds producing a slight depression of contractions elicited by post-ganglionic stimulation whereas no depression, but sometimes a slight potentiation, was observed with the quaternary compounds.

Effects on cat superior cervical ganglion

Table 2 shows the ganglion blocking activities measured using this type of assay. Experiments using post-ganglionic stimulation to test the specificity of the compounds corroborate the results obtained on the vas deferens preparation. Thus, no reduction in response to post-ganglionic stimulation was obtained with the methiodides whereas a 5–7% reduction in response was manifested by the tertiary bases.

Effects on chick isolated semispinalis muscle

None of the compounds produced a contracture when given at a 100 μM bath concentration, thus demonstrating the lack of any depolarizing action at the neuromuscular junction (Child & Zaimis, 1960).

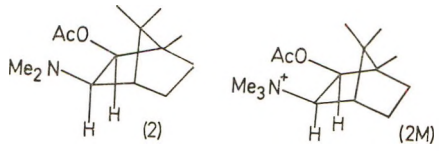
DISCUSSION

The results show that all of the eight compounds tested are weak, but specific, ganglion blocking agents and indicate a competitive type of action. When compared with those obtained from the vas deferens preparation, the individual equipotent molar ratios (EMR's) derived from the nictitating membrane vary by a factor of between two to four. However the EMR's obtained by the two techniques are significantly related by a correlation coefficient of 0·99 although the cat nictitating membrane preparation is less sensitive to these compounds than the guinea-pig vas deferens.

With the exception of the *cis*, *endo* compound (1), there is a significant increase in potency upon quaternizing each of the tertiary aminoacetates, the weakest quaternary salt being more potent than the strongest tertiary compound. By both assay techniques the most potent compound is the quaternary salt (2M); the least potent is compound (4) (from the vas deferens results) or compound (3) (from the nictitating membrane). The ratio between the least potent and most potent is 4·1 (vas) or 5·0 (nictitating membrane). The low values for this ratio (bearing in mind the biological variation shown by the 95% confidence limits) probably reflects the comparative unimportance of stereochemical variations in this group of compounds.

Similarly, Stone, Torchiana & others (1962) noted only small differences between the gangliolytic activity of the *exo* and *endo* epimers of (±)-mecamylamine (*ca* 1·5, based on antagonism to nicotine convulsions in mice). In addition they found no difference between the (+)- and (±)-forms of mecamylamine. Our observations upon the activities of the pairs of enantiomers (3) and (4), (3M) and (4M), confirm, as with mecamylamine, the lack of enantiomeric dependence of ganglion blocking activity in bicyclo (2.2.1) heptane compounds.

Larger differences in activity between isomers have been recorded for compounds whose actions at ganglia proceed via a depolarizing mechanism. For example, the enantiomers of nicotine (Barlow & Hamilton, 1965) display a difference of about fourteen between their equipotent molar ratios derived from the cat superior cervical ganglion. Acetylcholine itself has a depolarizing action at the ganglion and it could be argued that in converting the tertiary dimethylaminobornyl acetates to their methiodides, e.g. (2) to (2M), structures more closely aligned with acetylcholine



would be produced. In consequence a depolarizing action at the ganglion could well have been expected together with a definite stereochemical dependence of blocking activity. The results of the chick semispinalis muscle and guinea-pig ileum experiments suggest that the present compounds are not operating via a depolarizing mechanism as no contractions occurred, and the small stereochemical dependence of ganglion blockade is perhaps not surprising.

In the compounds tested there is only a small dependence of gangliolytic activity upon stereochemical variation. This small dependence of the cholinergic receptor in sympathetic ganglia upon the chiral properties of the antagonist is reflected in the small difference in the activities of the enantiomers of trimetaphan. The (+)-isomer is only about twice as active as its enantiomer (Randall, Peterson & Lehman, 1949). In view of such observations it is likely that the drug-receptor complex in such ganglia is not asymmetric to any observable extent. With molecules like pempidine and mecamylamine the presence of large groups close to the onium nitrogen atom appear to be important for high affinity at the ganglion receptor (Barlow 1964). The redistribution of the methyl groups from the 2- and 3-positions in mecamylamine to the 1- and 7-position in the present compounds, together with the introduction of the acetate function has resulted in an overall decrease in activity.

Early work on the Huković isolated nerve vas deferens preparation was based on the assumption that the hypogastric nerve contains mainly post-ganglionic nerve fibres. Sjöstrand (1962) and Birmingham & Wilson (1963) provided evidence which suggested that this assumption was invalid and concluded that contractions in the Huković preparation were initiated mainly by stimulation of preganglionic fibres. This strongly suggested the presence of ganglionic synapses in the hypogastric nerve. The significant correlation demonstrated for ganglion blocking activity by the two procedures used in the present study confirms pharmacologically the presence of such synapses in the hypogastric nerve of the guinea-pig. To date the guinea-pig vas deferens preparation has been used only qualitatively but the results presented in this paper show that this preparation may be used quantitatively.

Acknowledgement

The authors wish to thank Miss R. A. L. Power for her technical assistance.

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J. Pharm. Pharmac., 1971, **23**, 671–677 *Received March* 29, 1971

Correlation of the recovery of the granular uptake-storage mechanism and the nerve impulse induced release of [^{3}H]noradrenaline after reserpine

O. ALMGREN AND P. LUNDBORG

Department of Pharmacology, University of Göteborg, Göteborg, Sweden

Rats were treated intraperitoneally with 10 mg/kg reserpine. After various time intervals 1 μg/kg + [^{3}H]noradrenaline (^{3}H-NA) was administered intravenously, and the uptake into subcellular fractions of the submaxillary gland was measured or, in some of the rats, the sympathetic chain of the neck was electrically stimulated with 10 impulses/s for 30 min. The release of ^{3}H-NA and the contraction response of the lower eye-lid were measured. A striking parallel was observed between the recovery of the ^{3}H-NA uptake into the amine storage particles and the nerve impulse-induced release of ^{3}H-NA, and also the recovery of the functional response. The somewhat earlier recovery of the ^{3}H-NA uptake into the coarse fraction might reflect the existence of another type of amine storage granule or might represent granules present near the nerve cell membrane. A possible increase in the turnover of the adrenergic transmitter during the period of recovery after reserpine is discussed.

Treating animals with reserpine depletes the stores of noradrenaline in the tissues and also reduces the response of various organs to sympathetic nerve stimulation (Bertler, Carlsson & Rosengren, 1956; Muscholl & Vogt, 1958).

After a single dose of reserpine the nerve function recovers centrally (Häggendal & Lindqvist, 1963; 1964) and peripherally (Andén, Magnusson & Waldeck, 1964; Andén & Henning, 1966) within 2 or 3 days, while the monoamine concentrations are still low. At the time of functional recovery there is a sudden rise in the ability of the amine granules to take up and retain amines; in the adrenal medulla the uptake of monoamines increases to almost normal values during this time (Lundborg, 1963; Carlsson, Jonason & Rosengren, 1963) but there is only partial recovery in the sympathetic nerves (Lundborg & Stitzel, 1968). It would, however, be of value to relate the recovery of the ability of the neuron to take up and to retain noradrenaline to the release of the amine from the neuron after electrical stimulation of the nerve.

In the present investigation we have compared in the same organ (salivary gland of the rat) the uptake of tritiated noradrenaline (^{3}H-NA) into the catecholamine-containing granules and the release of ^{3}H-NA elicited from the gland by sympathetic nerve stimulation. These studies were conducted from 4 to 72 h after reserpine administration. The effect of nerve stimulation on the tension of the lower eye lid was also investigated.

MATERIALS AND METHODS

Male Sprague-Dawley rats, 175–225 g, were used.

In one series of experiments, rats were given ^{3}H-NA, 1 μg/kg (7·45 Ci/mmol), intravenously into a tail vein. Atropine (1 mg/kg) was given 30 min before the

labelled amine to reduce the variations in ^{3}H-NA uptake into the salivary glands (Almgren, 1970).

In some experiments reserpine (10 mg/kg) was given intraperitoneally at various intervals before the ^{3}H-NA.

The animals were killed 30 min after the administration of ^{3}H-NA. The submaxillary plus sublingual glands were removed, weighed and homogenized in an ice bath with a Teflon pestle in 0·25M sucrose containing 0·005M phosphate buffer, pH 7·4 and 0·001M $MgCl_2$. The coarse particles were removed by centrifuging the homogenate at 4° at 2000 *g* for 10 min. The resulting supernatant was then centrifuged at 100 000 *g* for 60 min in a Spinco Model L ultracentrifuge at 4° to give the particulate fraction and the high speed supernatant used. After protein precipitation, extracts of the various fractions were passed through cation-exchange resin columns. The catecholamines were eluted with hydrochloric acid and the tritium content of the eluate was determined by liquid scintillation counting (Stitzel & Lundborg, 1967).

In other experiments rats were given reserpine, atropine and ^{3}H-NA as described above. The rats were anaesthetized with urethane (1 g/kg) intraperitoneally and the right cervical sympathetic trunk was dissected free for stimulation (Almgren, Lundborg & Stitzel, 1969). The sympathetic trunk of the contralateral side was cut proximal to the superior cervical ganglion. Thirty min after the injection of ^{3}H-NA, the sympathetic chain was stimulated preganglionically with a Grass S4 Stimulator giving supramaximal pulses (3–5 V, 2 ms) at a frequency of 10 impulses/s. The contraction of the lower eye-lid was recorded according to Obianwu (1967). The stimulation was continued for 30 min. Immediately after the stimulation period the submaxillary plus sublingual glands from each side were removed, weighed and homogenized in ice-cold 0·4N perchloric acid. The ^{3}H-NA content was determined as described above.

Substances used: (±)-Noradrenaline-1-^{3}H (New England Nuclear Chemicals). Commercially available atropine sulphate was used. Reserpine (Serpasil) was generously supplied by the Swedish Ciba, Ltd, Vällingby.

RESULTS

Effect of reserpine on the uptake of ^{3}H-NA into subcellular fractions of salivary glands

The absolute values are given in Table 1, and in Fig. 1 the recovery of the coarse and particulate fractions as well as the total ^{3}H-NA content of the glands are expressed as a percentage of controls.

Six and 12 h after pretreatment with reserpine, there was an almost complete blockade of the uptake-retention of ^{3}H-NA into the salivary glands (Fig. 1), the value obtained being about 6% of the control values. A slight recovery was observed 24 h after the injection of reserpine, reflecting an increased amount of ^{3}H-NA recovered from the coarse and supernatant fractions at this time. The particulate fraction was unchanged. Thirty-six h after reserpine, there was an increase in ^{3}H-NA content of the particulate fraction to about 10% of the control values, which had increased to about 40% at 48 h, while in the coarse and supernatant fractions about 65% of the noradrenaline content, as compared to the control values, had been recovered.

Effect of reserpine on the release of ^{3}H-NA from rat salivary glands by nerve stimulation

In untreated animals, stimulation of the cervical sympathetic chain for 30 min caused about a 56% reduction in the ^{3}H-NA content of the stimulated gland (Fig. 2a).

Table 1. *Uptake and retention of ^{3}H-NA in subcellular fractions of rat salivary glands at various time intervals after reserpine.* The animals except the controls received an i.p. injection of 10 mg/kg reserpine. At various time intervals after the reserpine injection 1 mg/kg atropine was administered i.p. and 30 min thereafter 1 μg/kg ($\pm$) ^{3}H-NA. 30 min after the administration of the labelled amine the rats were killed and the submaxillary and sublingual glands were removed. The analytical procedure is given in the text. The values are in ng/g and represent mean $\pm$ s.e. C = coarse fraction, P = particulate fraction, S = supernatant fraction.

Time interval after reserpine (h)	No. of exp.	^{3}H-NA content ng/g		
		C	P	S
Controls	14	0·81 ± 0·07	0·24 ± 0·02	0·33 ± 0·03
6	4	0·03 ± 0·00	0·01 ± 0·00	0·05 ± 0·01
12	6	0·03 ± 0·00	0·01 ± 0·00	0·05 ± 0·00
24	10	0·15 ± 0·05	0·01 ± 0·00	0·11 ± 0·03
36	12	0·30 ± 0·04	0·03 ± 0·00	0·11 ± 0·01
48	6	0·54 ± 0·05	0·10 ± 0·00	0·23 ± 0·02

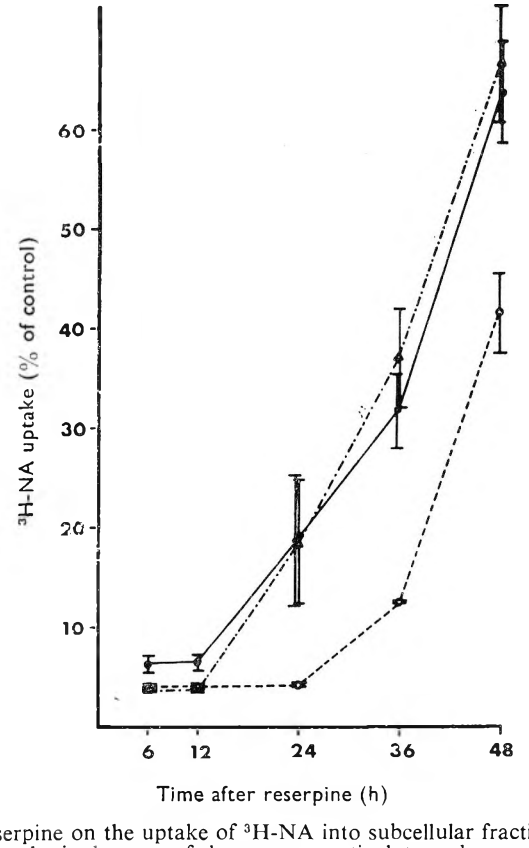

FIG. 1. Effect of reserpine on the uptake of ^{3}H-NA into subcellular fractions of the rat salivary glands. The total uptake is the sum of the coarse, particulate and supernatant fractions. The ^{3}H-NA values are given as a percentage of controls (n = 14). The absolute values, corresponding to 100% are given in Table 1. Vertical lines indicate standard errors of the means, and the number of experiments were 4, 6, 10, 12 and 6 at 6, 12, 24, 36 and 48 h respectively.

Four and 24 h after reserpine, nerve stimulation caused no further reduction in the already low ^{3}H-NA content of the gland. At 36 and 48 h after reserpine the ^{3}H-NA content of the stimulated gland was reduced by 53 and 59% respectively.

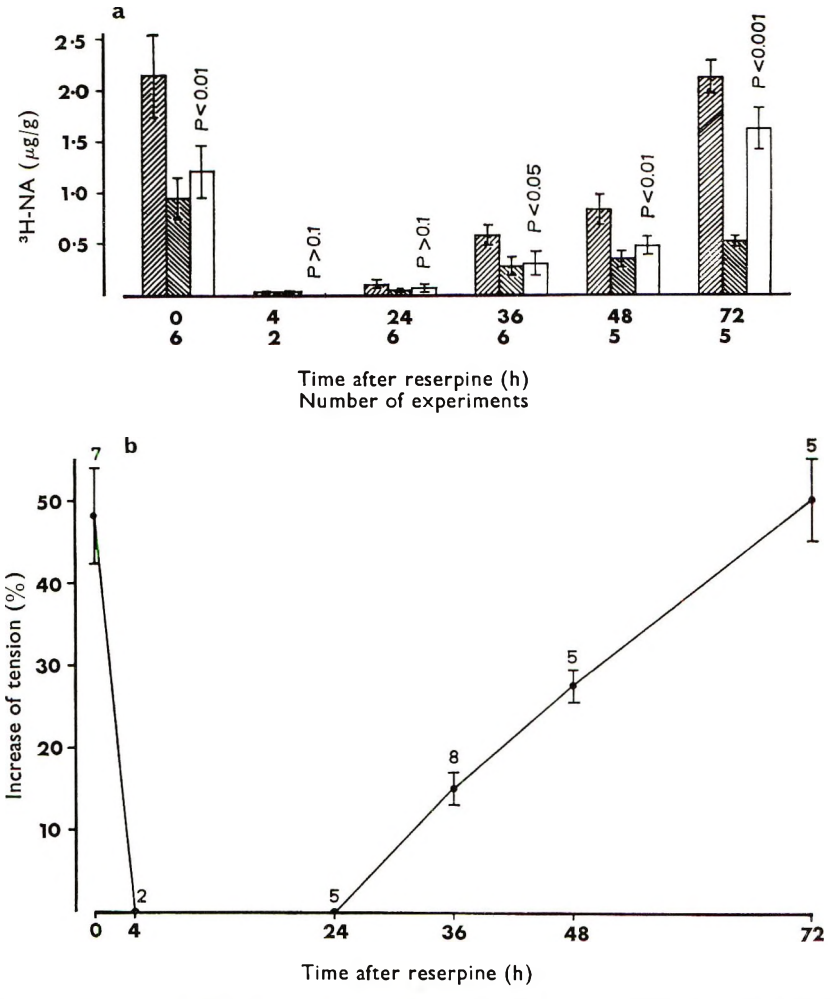

FIG. 2. a. Release of ^{3}H-NA from rat salivary glands by sympathetic nerve stimulation. The experiments were made at various time intervals after the i.p. injection of 10 mg/kg of reserpine (RES). Thirty min after the i.v. injection of 1 μg/kg of ^{3}H-NA, the right sympathetic chain was stimulated with supramaximal pulses at a frequency of 10/s for 30 min. The left (control) side was decentralized. The vertical bars indicate the standard errors of the means. The P values given were calculated by a t-test on the difference between matched pairs.

b. The contraction response of the lower eye-lid of rats, induced by sympathetic nerve stimulation at various intervals after the administration of 10 mg/kg reserpine. The eye-lid was given a base tension at rest of 0·5 g. The increase of tension was recorded on a Grass polygraph, and is given in the figure as per cent of the base tension. Supramaximal pulses at a frequency of 10/s were given over 30 min. Vertical bars indicate the standard errors of the means and the figures the number of experiments.

Seventy-two h after reserpine the uptake of ^{3}H-NA into the unstimulated (decentralized) glands had returned to normal. Nerve stimulation reduced the ^{3}H-NA content by about 75% (which was a significantly greater reduction than in the controls ($P < 0{\cdot}05$).

Effect of reserpine on the response of the lower eye-lid of the rat to nerve stimulation

In untreated rats stimulation of the sympathetic chain resulted in an increase in the tension of the lower eye-lid by about 0·25 g (Fig. 2b). Four and 24 h after reserpine the response of the lower eye-lid to stimulation was completely abolished. Thereafter a gradual recovery in response was observed until at 72 h it was normal.

DISCUSSION

Considerable evidence indicates that reserpine acts on particle-bound stores.

In vitro, reserpine blocks the ATP-Mg $^+$-dependent uptake of catecholamines into isolated adrenal medullary (Carlsson, Hillarp & Waldeck, 1962; Kirshner, 1962) and nerve (Euler and Lishajko, 1963) granules. Also, after *in vivo* administration of reserpine the *in vitro* uptake of catecholamines is blocked (Lundborg, 1963).

Histochemical studies have shown that after pretreatment with reserpine and an inhibitor of the monoamine oxidase, exogenous noradrenaline can accumulate in the sympathetic nerves (cf. Malmfors, 1965) thus indicating that reserpine is not interfering with the uptake at the level of the nerve cell membrane.

^{3}H-NA given to reserpine-treated animals is not accumulated in sympathetic nerves. Such an accumulation can however be demonstrated with [^{3}H]α-methylnoradrenaline, an amine resistant to monoamine oxidase, which is accumulated in the nerves after reserpine treatment. However, there is a pronounced blockade of the uptake of this amine into the particle-bound stores (particulate fraction) with its subsequent extra-particular accumulation (Lundborg & Stitzel, 1967).

Thus much evidence has accumulated in support of the hypothesis that reserpine exerts its effect by interfering with the particle-bound stores. The immediate conclusion could be drawn that the data obtained in the present study are not in favour of this concept. However, accepting the evidence that reserpine acts on particle-bound stores, factors influencing the recovery of noradrenaline uptake-retention after reserpine and the isolation of amine-containing particles will be discussed.

A clearcut difference was revealed between the coarse and the particulate fractions for recovery of ^{3}H-NA uptake-retention after reserpine. Already at 24 h there was a slight but significant increase in ^{3}H-NA content of the coarse fraction while the particulate fraction was still unchanged. This discrepancy between the two fractions remained at 48 h. The earlier recovery in the ^{3}H-NA content of the coarse fraction might indicate that that fraction contains not only "pinched off nerve-endings" but also amine storage particles which differ in uptake properties from those of the particulate fraction.

The noradrenaline content of the supernatant fraction, although not reduced by reserpine to the same extremely low values as the coarse and particulate fractions, may in part be an artifact representing amines released during the homogenization procedure (Lundborg, 1967). Therefore, in the present study, this fraction appeared to be less important in providing information on the recovery of noradrenaline uptake-retention after reserpine.

The possible existence of two different types of granules has been pointed out. By a continuous gradient centrifugation technique Roth, Stjärne & others (1969) have been able to differentiate between "light" and "heavy" granules. In electron microscopical studies, dense-cored vesicles of different sizes have been observed in adrenergic neurons (for ref. see Hökfelt, 1968). The small type (around 50 nm) is very

common in the nerve terminals. The large type is also found in the nerve terminals, but there are more in the cell bodies and in the axons (Hökfelt, 1969). Häggendal & Dahlström (1970, 1971) have suggested the possibility that the large dense-cored vesicles correspond to the young amine granules and that with the gradual loss of chromogranin (see Geffen, Livett & Rusk, 1969), the large vesicles shrink and develop into the smaller type. The small type would then possibly represent the old amine granules. According to the suggestion discussed above, it is possible, that in the present subcellular distribution studies, the young large vesicles have properties causing them to be "trapped" and spun down with the low speed sediment (coarse fraction). The suggested (see above) dominance of young granules in the recovery phase after reserpine could thus account for the early recovery in ^{3}H-NA content of the coarse fraction. It is also possible that, during the first period of recovery, all functioning granules will be localized close to the nerve cell membrane. During the fractionation procedure these granules might stick to membrane fragments and be spun down with the low speed sediment.

In experiments in which the cervical sympathetic chain was stimulated, observations were run up to 72 h after injection of reserpine. At this time the ^{3}H-NA content of the unstimulated glands was back to normal, a result that would appear to be inconsistent with previous findings (e.g. Andén, Magnusson & Waldeck, 1964; Andén & Henning, 1966) since in these related studies the ^{3}H-NA content of various organs at 72 h was respectively 40 and 25%. A possible explanation for the discrepancy is that in the present study the glands were preganglionically denervated before stimulation was started (see Methods). Thus the ^{3}H-NA content of these glands was not influenced by the physiological impulse flow during a period of 45 out of 60 min. It might be speculated that, during the recovery period after reserpine, there is an increased turnover rate of the transmitter (Iggo & Vogt, 1960). The ^{3}H-NA should then be released at a higher rate from intact (not decentralized) sympathetic nerves. In the studies of Andén & others quoted above, the content of ^{3}H-NA of various organs was measured 30 min after the administration of the labelled amine and, in the reserpine-treated animals, more of the ^{3}H-NA initially taken up might have been released by them than in untreated animals. The recovery of this granular uptake function might thus have been partly masked by an increased turnover and release of ^{3}H-NA. By using decentralized organs, this experimental error can be circumvented. The observation (see Fig. 2a) that about 75% of the ^{3}H-NA content was released by nerve stimulation 72 h after reserpine than in the untreated animals (about 55%) could be taken as support for such a view.

In the present study a striking correlation in time was observed between the reappearance in (i) granular uptake-storage function, (ii) the ability of nerve stimulation to release ^{3}H-NA and (iii) functional response, thus demonstrating the importance of functioning storage granules for normal neuronal function such as release of transmitter onto specific receptor sites.

Acknowledgements

The research reported has been supported by the Swedish State Medical Research Council (B71-14X-2464-04 and B71-14P-3266-01) and Läkemedelsindustriföreningen. For skilful technical assistance we are indebted to Miss Lena Ramstedt and Mrs Marianne Olofsson.

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J. Pharm. Pharmac., 1971, **23**, 678–686 *Received February* 19, 1971

The reversal of experimental cardiac arrhythmias by indoramin (Wy 21901)

B. J. ALPS, MADELINE HILL, K. FIDLER, E. S. JOHNSON AND A. B. WILSON

Department of Pharmacology, Wyeth Institute of Medical Research, Taplow, Bucks, U.K

Indoramin was approximately equipotent with (±)-propranolol but more potent than lignocaine in reversing adrenaline-induced multiple ventricular ectopic beats and ventricular tachycardia in halothane-anaesthetized cats. Indoramin was more potent than either propranolol or lignocaine in reversing ouabain-induced ventricular tachycardia or fibrillation to sinus rhythm. The local anaesthetic activity of indoramin was three times greater than that of procaine and lignocaine and twice that of (±)-propranol and quinidine when assessed by the guinea-pig weal test. Experiments on the guinea-pig isolated ileum supporting a local anaesthetic action of indoramin showed that nicotine contractions were reduced by indoramin whereas acetylcholine responses were unaffected. Based on the evidence presented and that published elsewhere, it is considered likely that indoramin abolishes adrenaline-induced arrhythmias by a combination of membrane stabilization and blockade of myocardial α-adrenoceptors whereas ouabain arrhythmias may be reversed by membrane stabilization alone.

Indoramin (Wy 21901) is a potent hypotensive drug which possesses α-adrenoceptor blocking, myocardial inhibitory and anti-arrhythmic properties (Alps, Hill & others, 1970; Alps, Johnson & Wilson, 1970). Since these authors have shown the cardiac actions of indoramin not to be due to β-adrenoceptor blockade, experiments have been made to determine the mechanism of the anti-arrhythmic action of the drug.

METHODS

Local anaesthetic evaluation

Local anaesthesia was measured by the guinea-pig weal test (Bülbring & Wajda, 1945) and from the selective depression of contractions of the guinea-pig isolated ileum induced by nicotine.

A mean percentage response, with standard errors, for each concentration of drug was obtained from the results of 4–6 injections of 0·25 ml of test solution given intradermally into the anterior and posterior areas of the shaved flanks of guinea-pigs (>550 g). Saline was used as a control. A log dose-response curve was then plotted to give an estimate of the degree of local anaesthesia produced over the 30 min experimental period as assessed every 5 min by pricking the weal.

Test compounds, in 0·9% saline, were examined over the following concentration ranges: indoramin hydrochloride, $2{\cdot}5 \times 10^{-4}$ to 5×10^{-3}M (its solubility limit in saline); lignocaine hydrochloride, 10^{-3} to 2×10^{-2}M; (±)-propranolol hydrochloride, 10^{-3} to 2×10^{-2}M; procaine, $2{\cdot}65 \times 10^{-3}$ to $8{\cdot}45 \times 10^{-2}$M; quinidine sulphate, 10^{-3} to 10^{-2}M.

Nicotine contractions of the guinea-pig ileum. Guinea-pig ileum, in 2 cm segments, was set up as described by Brownlee & Johnson (1963). Agonist-antagonist experi-

ments were made and agonist dose-response curves plotted to nicotine and acetylcholine, alone and in the presence of indoramin hydrochloride (10^{-7} to 10^{-5}M). The recovery of the responses was followed after washing in antagonist-free Krebs solution.

Anti-arrhythmic evaluation

Adrenaline-induced arrhythmias. Cats of either sex (2–4 kg) were anaesthetized with 1–2% halothane in oxygen. Aortic, carotid or left ventricular blood pressure was measured by means of a Statham P23 pressure transducer via a catheter inserted through the left carotid artery. E.c.g. recordings were made from bipolar and augmented unipolar limb leads, although throughout the experiment lead II was the standard reference lead; the other leads were used to detect the earliest return from arrhythmias to sinus rhythm when lead II records were equivocal. Most e.c.g.'s were recorded on a Mingograf Cardirex 24 ink spray oscillograph, a few were recorded on a Grass model 7 polygraph.

Adrenaline was infused continually from a motor-driven syringe through a catheter in the right femoral vein. The rate of infusion was generally 3 μg/kg min^{-1} or less but in some cases higher rates were used to induce severe arrhythmias.

The anti-arrhythmic test compounds were injected through a catheter in the right cephalic vein.

Ouabain-induced arrhythmias. Cats were anaesthetized with pentobarbitone sodium (30 mg/kg intrapleurally). Blood pressure and e.c.g. were recorded as before. Glycoside arrhythmias were induced by the intravenous injection of ouabain, 60 μg/kg initially, followed 30 min later by doses of 10 μg/kg every 10 min until the required severity of arrhythmia was produced.

Anti-arrhythmic test substances were injected intravenously either during the development of severe arrhythmias or immediately after the onset of ventricular fibrillation.

RESULTS

Local anaesthesia

Guinea-pig weal. Log dose-response curves for indoramin, (±)-propranolol and procaine for the 30 min experimental period were parallel and linear over the intermediate range (Fig. 1). The points for lignocaine fell either side of the curve for procaine and the

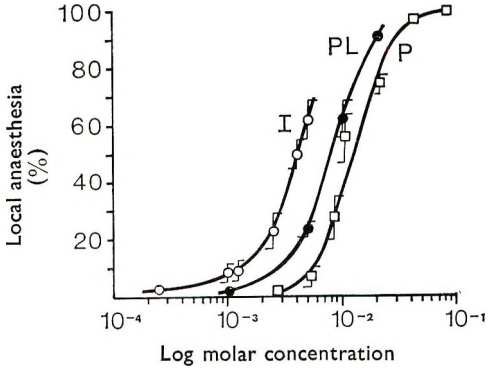

FIG. 1. Log dose-response curves to show % local anaesthesia produced by indoramin hydrochloride (I), propranolol hydrochloride (PL) and procaine (P), as assessed over a 30 min period by the guinea-pig weal method. The points for lignocaine hydrochloride fell either side of the curve for procaine; the points for quinidine sulphate were almost identical with those for propranolol.

points for quinidine sulphate were almost identical with those for propranolol. The relative potencies estimated at the ED50 level of these curves were:—

Indoramin > propranolol = quinidine > lignocaine = procaine
3·1 : 1·6 : 1·7 : 1 : 1

The degree of local anaesthesia estimated over the 30 min for indoramin at its maximum solubility in 0·9% saline at room temperature (5×10^{-3}M) was only 62%. The remaining drugs were sufficiently soluble to enable the use of higher concentrations and the production of complete anaesthesia throughout the 30 min observation period.

Guinea-pig isolated ileum. Nicotine dose-response curves were unaffected by concentrations of indoramin hydrochloride of 10^{-6}M and less. However, concentrations of 5×10^{-6} and 10^{-5}M reduced the nicotine responses, so that the maximal response was only 20% of the control maximal response to acetylcholine (Fig. 2A, B).

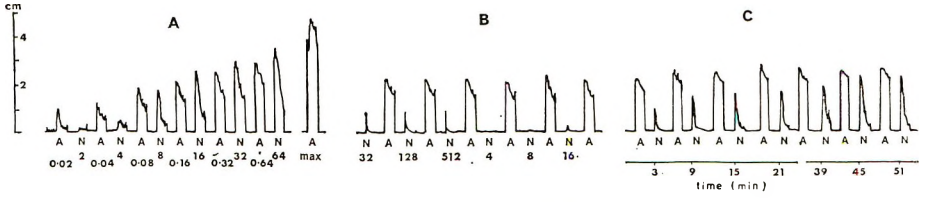

FIG. 2. Contractions of the guinea-pig isolated ileum to alternate doses of acetylcholine (A) and nicotine (N); the doses are μg base added to a 12 ml bath. (A) Control dose-response curves. (B) Dose-response curve for nicotine and responses to constant doses of acetylcholine (0·16 μg) after 60 min treatment with 10^{-5}M indoramin hydrochloride. (C) Responses to constant doses of acetylcholine (0·16 μg) and nicotine (16 μg) showing the progressive recovery of the nicotine response in antagonist-free Krebs solution. In C the recorder was stopped for 12 min before the 39 min point.

The block produced by 5×10^{-6} and 10^{-5}M indoramin hydrochloride was completely reversed within 16 and 45 min respectively by washing in antagonist-free Krebs solution (Fig. 2C). Acetylcholine dose-response curves were unaffected by these concentrations of indoramin.

Thus, on this preparation indoramin antagonized responses to nicotine in concentrations that had no effect on responses to acetylcholine.

Anti-arrhythmic tests

Adrenaline arrhythmias were easy to maintain and would generally reverse within a few minutes of stopping the infusion. The induction dose varied; rates as low as 0·6 μg/kg min^{-1} were occasionally successful. The normal dose used (3 μg/kg min^{-1}) caused multiple atrial or ventricular extrasystoles, or ventricular tachycardia; ventricular fibrillation was induced on only a few occasions. After effective doses of some of the anti-arrhythmic drugs the animal could withstand increases in the adrenaline infusion to 60 μg/kg min^{-1} without cardiac failure.

Ouabain-induced arrhythmias were much more severe than those caused by adrenaline and were of sudden onset. They usually resulted in ventricular fibrillation. The usual total cumulative dose before arrhythmias were seen was between 80–90 μg/kg. Usually the anti-arrhythmic drugs were tested against arrhythmias caused by low doses of ouabain and then again against ventricular fibrillation induced by

larger doses. Sometimes the glycoside induced fibrillation early in the experiment, then the drugs were tested against fibrillation only.

Adrenaline arrhythmias

Indoramin. Indoramin reversed multiple ventricular extrasystoles caused by adrenaline 3 μg/kg min^{-1} and at doses lower than those of lignocaine. For example, in one cat indoramin, 28 μg/kg, abolished the extrasystoles for 200 s (Fig. 3) and in

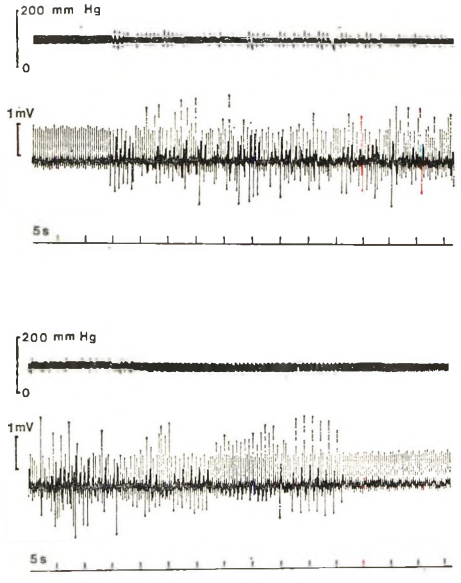

FIG. 3. Records of the left carotid blood pressure and lead II of the e.c.g. in a halothane-anaesthetized cat. In the Lh panel multiple ventricular extrasystoles have been induced by a constant intravenous infusion of 3 μg/kg min^{-1} of adrenaline. In the Rh panel 28 μg/kg indoramin hydrochloride re-established sinus rhythm within 58 s. Normal rhythm was maintained for 200 s before multiple ventricular extrasystoles recurred in response to the continuous adrenaline infusion. The interval between the panels was 100 s.

most animals 0·1 mg/kg reversed the gross ectopic beats caused by 6 μg/kg min^{-1} adrenaline. Indoramin, 1 mg/kg, reversed adrenaline-induced arrhythmias in all five animals tested.

Propranolol. The anti-arrhythmic activity of propranolol was similar to that of indoramin. In one experiment, multiple ventricular extrasystoles caused by adrenaline (3 μg/kg min^{-1}) were reversed to sinus rhythm by 10 μg/kg propranolol. In two other cats, 0·1 mg/kg propranolol successfully reversed the multiple ventricular extrasystoles (Fig. 4); 1 mg/kg propranolol reversed these arrhythmias in all animals and a marked increase in the adrenaline infusion rate ($\times 5$) was required to re-initiate the abnormal rhythm.

High doses of propranolol (>3 mg/kg) complicated the experiment by inducing conduction changes, and in one cat 3·6 mg/kg propranolol caused complete electrical silence. In contrast, indoramin failed to cause similar effects when given in repeated doses up to 15 mg/kg.

The occurrence of adrenaline-induced ventricular fibrillation is relatively uncommon and was reversed by propranolol in one out of 2 cases.

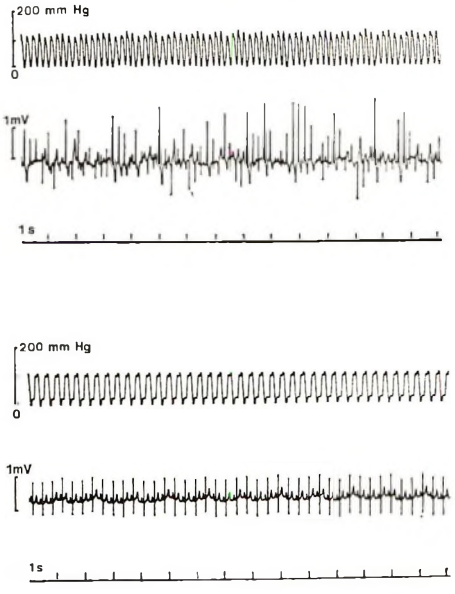

FIG. 4. Records of left intraventricular pressure and lead II of the e.c.g. in a halothane-anaesthetized cat. In the Lh panel a ventricular arrhythmia, characterized by multiple extrasystoles, was induced by a constant intravenous infusion of 3 μg/kg min^{-1} of adrenaline. In the Rh panel 0·1 mg/kg propranolol re-established sinus rhythm within 20 s (recorded 44 s after the start of injection). Normal rhythm was maintained for 15 min before the ventricular arrhythmia recurred in response to the continuous adrenaline infusion. The interval between the panels was 100 s.

Lignocaine. The dose of lignocaine required to reverse adrenaline-induced multiple ventricular extrasystoles varied widely (0·4–5 mg/kg) from animal to animal and the arrhythmia reverted to sinus rhythm in only 2 out of 3 experiments. However, the gross arrhythmia in the third animal was partially reversed by 3·2 mg/kg indicating considerable stabilization of the myocardium.

Ouabain arrhythmias

Indoramin. Ouabain-induced ventricular tachycardia (ouabain dose 80–140 μg/kg) was readily reversed to sinus rhythm by indoramin (0·25–2 mg/kg) in all 4 experiments made (Fig. 5). Ouabain-induced ventricular fibrillation was also reversed by indoramin (dose 1–2 mg/kg) in 4 out of 4 experiments (Fig. 6).

Propranolol. Ouabain-induced coupling, multiple extrasystoles and ventricular tachycardia (ouabain dose 76–160 μg/kg) were converted to sinus rhythm by propranolol (mean 5 mg/kg) in 2 out of 3 animals. Fig. 7 shows a ventricular tachycardia caused by 120 μg/kg ouabain which was reversed to sinus rhythm by propranolol (1·1 mg/kg). In the third animal grossly abnormal ventricular complexes were not reversed to sinus rhythm by 7 mg/kg propranolol but, as judged from the blood pressure response, the contractions of the myocardium were more co-ordinated and mechanically effective.

Ouabain-induced ventricular fibrillation (1 experiment) was not reversed by propranolol (4 mg/kg).

Lignocaine. Ouabain-induced coupling and ventricular extrasystoles (ouabain dose 90–156 μg/kg) were reversed by lignocaine in doses of 1–2 mg/kg. Lignocaine

(4 mg/kg) failed to reverse ouabain-induced ventricular fibrillation in the 2 experiments in which this arrhythmia was studied.

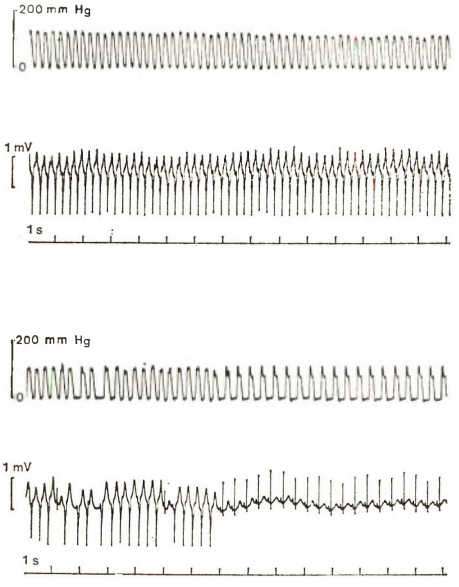

FIG. 5. Records of left intraventricular pressure and lead II of the e.c.g. in a pentobarbitone-anaesthetized cat. The Lh panel shows a ventricular tachycardia induced by a cumulative dose of 100 μg/kg of ouabain. In the Rh panel 2 mg/kg indoramin hydrochloride re-established sinus rhythm within 50 s (recorded 40 s after the start of injection). Normal rhythm was maintained for 12 min before ventricular tachycardia recurred. The interval between the upper and lower records was 60 s.

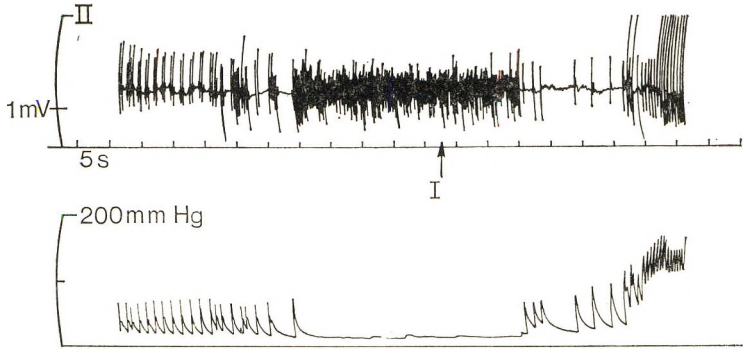

FIG. 6. Lh tracing is lead II of the e.c.g. after a cumulative dose of 80 μg/kg of ouabain, showing characteristic coupling and ventricular ectopic beats leading on to ventricular fibrillation. The Rh tracing shows the aortic blood pressure recorded simultaneously with the e.c.g. It can be seen that an effective myocardial output was restored within 15 s of the intravenous injection of indoramin (1 mg/kg) accompanied by partial reversal to normal rhythm.

DISCUSSION

Indoramin (Wy 21901) has been shown to be an effective anti-arrhythmic agent active against both adrenaline-induced and ouabain-induced arrhythmias. Indoramin was approximately equipotent with propranolol but more potent than lignocaine in reversing adrenaline-induced multiple ventricular ectopic beats and ventricular

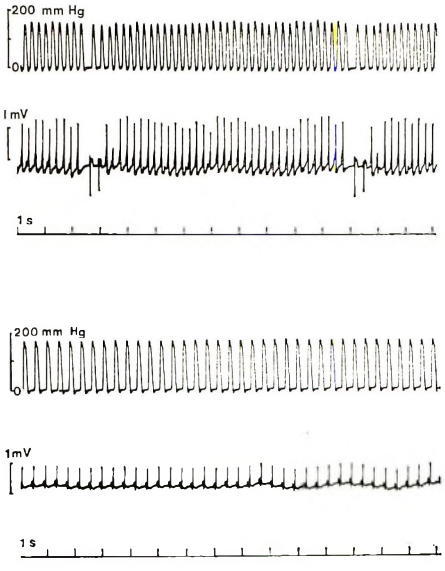

FIG. 7. Records of left intraventricular pressure and lead II of the e.c.g. in a pentobarbitone-anaesthetized cat. The Lh panel shows a ventricular tachycardia induced by a cumulative dose of 120 μg/kg of ouabain. In the Rh panel 1·1 mg/kg propranolol re-established sinus rhythm within 40 s (recorded 65 s after the start of injection). Normal rhythm was maintained for 10 min with occasional ectopic beats occurring up to 16 min before ventricular tachycardia recurred. The interval between records was 90 s.

tachycardia in halothane-anaesthetized cats. In contrast, indoramin was superior to both propranolol and lignocaine in opposing ouabain-induced ectopic beats, ventricular tachycardia or ventricular fibrillation in pentobarbitone-anaesthetized cats.

Although indoramin and the β-adrenoceptor blocking agent propranolol were equally effective in reversing adrenaline-induced arrhythmias, blockade of cardiac β-adrenoceptors cannot explain the anti-arrhythmic properties of indoramin. Evidence for this conclusion is given by the work of Alps & others (1970) who showed that even in high concentrations, indoramin was without β-adrenoceptor blocking activity against the relaxation of the guinea-pig tracheal spiral induced by noradrenaline and isoprenaline, or the increase in the force and rate of contraction of the rabbit isolated heart caused by isoprenaline.

It has been known for some 20 years that α-adrenoceptor blocking agents effectively inhibit cardiac arrhythmias induced by adrenergic stimuli (Acheson, Farah & French, 1949; Nickerson & Nomaguchi, 1951), but only recently has interest in this area been renewed (Brkic & Stern, 1965; Garvey, 1969). Garvey (1969) and Nickerson & Hollenberg (1967) have provided evidence that the cardiac adrenergic receptors responsible for these arrhythmias are α-adrenoceptors that are selectively blocked by α-adrenoceptor blocking drugs. It seems probable that at least part of the anti-arrhythmic action of indoramin is due to its potent α-blocking action ($pA_2 = 7{\cdot}4$; Alps & others, 1970) on the α-adrenoceptors in the myocardium.

Blockade of α-adrenoceptors in the peripheral vasculature may also contribute to the cardiac anti-arrhythmic actions of indoramin. Lees & Tavernor (1970), Dresel (1962), Riker, Depierre & others (1955), Roberts, Standaert & others (1956) have shown that in the horse, cat and dog, adrenaline induces arrhythmias both by an

adrenergic action on the myocardium and also indirectly by an increase in vagal tone. This vagal effect is probably a reflex response to the pressor action of adrenaline and can be expected to be antagonized by an α-adrenoceptor blocking drug such as indoramin, which produces a significant lowering of systemic blood pressure (Alps, Johnson & Wilson, 1970).

Because of the known local anaesthetic activity of propranolol (Davis, 1970), and because of the similarity in the cardio-inhibitory actions of propranolol and indoramin in the rabbit isolated heart (Alps & others, 1970) and the anaesthetized cat (Alps, Johnson & Wilson, 1970), experiments were made to determine the local anaesthetic potency of indoramin. By the guinea-pig weal method, its action was found to be three times greater than that of procaine and lignocaine and twice that of propranolol and quinidine. Confirmation of this property of indoramin was supplied by experiments with the guinea-pig isolated ileum in which responses to nicotine were antagonized by the drug while those to acetylcholine were unaffected. Since nicotine contracts the ileum by an indirect action on the cholinergic innervation and acetylcholine acts directly on the smooth muscle, the most likely site of action of indoramin is on the nerve fibres of the intramural plexus.

Local anaesthetic drugs are extremely effective in stabilizing the myocardial membrane, causing reduction of depolarization and depression of irritable foci. Because local anaesthetic membrane stabilization is non-specific, all irritable foci will be modified regardless of their aetiology. Thus the local anaesthetic drug, lignocaine, was approximately equiactive in reversing both adrenaline- and ouabain-induced arrhythmias. The greater potencies of propranolol and indoramin in antagonizing adrenaline-induced arrhythmias would appear to be explained by their additional properties of adrenoceptor-blockade. In contrast, ouabain-induced arrhythmias are not known to be associated with myocardial adrenoceptors and it is to be anticipated that anti-arrhythmic activity against ouabain will be related to membrane stabilizing or local anaesthetic potency of the anti-arrhythmic drugs. This relation is borne out by the present experiments which showed indoramin to be superior either to propranolol or lignocaine in opposing ouabain-induced arrhythmias, the order of anti-arrhythmic activity being the same as the local anaesthetic order of potency of indoramin, 3·1, (±)-propranolol, 1·6 and lignocaine, 1·0, as assessed on the guinea-pig skin by the Bülbring and Wajda technique.

In conclusion it seems likely that indoramin abolishes adrenaline-induced arrhythmias by virtue of its α-adrenoceptor blocking and membrane stabilizing actions, whereas ouabain-induced arrhythmias may be antagonized by membrane stabilization alone.

Acknowledgements

The authors are indebted to Mrs Susan Molesworth for her technical support and to Miss Eileen Isitt for her secretarial assistance.

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J. Pharm. Pharmac., 1971, **23**, 687–694 *Received March* 18, 1971

The polymorphism of sulphathiazole

R. J. MESLEY

*Laboratory of the Government Chemist, Cornwall House, Stamford Street, London, S.E.*1, *U.K.*

The existence of two species of the low temperature form of sulphathiazole, postulated by Moustafa & Carless (1969) and by Shenouda (1970), has been confirmed. Contrary to the opinions of these workers it is concluded that these are two distinct forms having different lattice structures which give rise to distinguishable X-ray diffraction patterns and infrared spectra. The fact that commercial samples frequently contain both forms casts doubt on the validity of previous solubility measurements and on the use of differential scanning calorimetry as an assay procedure for the low temperature form.

The polymorphism of sulphathiazole has been reported by numerous workers but the relationship between the two generally recognized forms has continued to present problems. Grove & Keenan (1941) obtained two distinct forms, hexagonal prisms which melted on rapid heating at 173–175° and prismatic rods melting at 200–202°. On slow heating the hexagonal form was largely transformed to the rod form below 173°, and little melting was observed below 200°. Miyazaki (1947) reported a third form, making the distinction between the α form, which underwent a solid–solid conversion at 173–175° to the β form, and the α' form which melted at 173–175° without conversion. Milosovich (1964), Guillory (1967) and Higuchi, Bernardo & Mehta (1967) all described two forms, the latter workers publishing X-ray diffractograms for the high and low temperature modifications. Kuhnert-Brandstätter & Martinek (1965) compared solubilities of forms I and II, but in a subsequent paper Kuhnert-Brandstätter & Wunsch (1969) mentioned that commercial sulphathiazole sometimes contains a hydrated form, which is converted on heating to the high temperature form. Two additional forms were also noted, which are stabilized by isomorphism in mixed crystals with sulphapyridine.

Carless & Foster (1966) reported that the B.P.C. Authentic Specimen of sulphathiazole contained two or more polymorphs, though it was subsequently suggested (Moustafa & Carless, 1969) that this conclusion, based on differential scanning calorimetry, was in error. Meanwhile, however, Mesley & Houghton (1967) also examined the B.P.C. material and found infrared evidence for the presence of two forms, but this was disputed by Moustafa & Carless (1969). Nevertheless, the latter workers obtained the low temperature form in two varieties having different transition temperatures. This work was repeated by Shenouda (1970), who found that crystallization of sulphathiazole from certain solvents yielded two species, one of which melted at 170–176° while the other underwent a solid–solid transition at 150–166°. This author concluded that it was misleading to call the melting species a third physical form but followed this statement by saying that "it is probable that a third form does exist, although highly unstable on grinding, and this is probably identical to the melting species reported in this investigation". Evidence is here presented for the existence of a second low temperature form which may be distinguished by differences in its infrared absorption spectrum and more noticeably in its

X-ray diffraction pattern. These features clearly indicate a difference in its lattice structure, and therefore justify its description as a separate polymorphic form. The fact that both low temperature forms are present in many commercial samples has an obvious bearing on the various solubility studies which have been carried out, and on the infrared identification test of the B.P.C.

MATERIALS AND METHODS

Samples of sulphathiazole used were the B.P.C. Authentic Specimen and a commercial sample (Evans Medical Co.) which had been stored in a screw-top jar for five years after first being opened. Other commercial samples were also examined. Infrared spectroscopy showed that all of these samples were mixtures of two forms, though the proportions varied between individual samples.

Infrared spectra were recorded as mulls in Nujol using Grubb Parsons GS2 and Spectromaster grating spectrometers. Spectra at elevated temperatures were recorded with a Beckman-RIIC VLT-2 cell and TEM-1 temperature controller.

X-ray powder diffraction patterns were recorded photographically using a 9 cm camera and a Philips PW1009 generator with vanadium-filtered chromium Kα radiation. The traces shown in Fig. 3 were obtained with a Joyce recording microdensitometer.

Differential scanning calorimetry was carried out with a Perkin-Elmer DSC-1B apparatus using open pans with aluminium covers (not crimped), nitrogen at 20 ml/min as carrier gas and a heating rate of 8°/min.

RESULTS AND DISCUSSION

Preparation of forms

It was previously stated (Mesley & Houghton, 1967) that the two forms present in the B.P.C. specimen could be isolated by recrystallization from a mixture of chloroform and acetone and from dilute ammonia solution respectively. Shenouda (1970) has since stated that both of these treatments yield a mixture of two species distinguishable by hot-stage microscopy. Moustafa & Carless (1969) also found differences in recrystallization behaviour from Mesley & Houghton, and this variability has now been confirmed. Recrystallization from alcohols containing three or more carbon atoms yields the high temperature form, here designated form I to conform with Kuhnert-Brandstätter & Wunsch (1969)*. Recrystallization from aqueous ammonia usually, but not invariably, produces the more common of the two low temperature forms (here designated form IIA) and in fact this is also given by recrystallization from water. All other treatments tend to give mixtures of form IIA with varying proportions of the other low temperature form, IIB. All attempts to isolate form IIB during the present investigation, including recrystallization from chloroform-acetone mixtures, have proved unsuccessful.

Both form IIA and the mixture (conveniently called "form II") can be converted wholly to form I by heating in an oven above 180°, from which it must be assumed

* In most studies of polymorphic systems in which the thermal relationship between the forms is known it is conventional to use Roman numerals to designate individual polymorphs in descending order of melting point, so that additional metastable forms can be added without the necessity of renumbering the stable form. An alternative convention has been used for some enantiotropic dimorphic systems, including sulphathiazole, in which the form stable at room temperature is designated I and the high temperature form II, but this makes no allowance for additional metastable forms.

that any material which melts at 175° subsequently crystallizes as form I. Kuhnert-Brandstätter & Wunsch (1969) stated that form I was enantiotropic with form II; in fact form I normally reverts on long standing to the mixture of forms IIA and IIB, as apparently does form IIA over a period of months. On the other hand, a commercial sample containing the usual mixture was found after five years to have a surface layer containing predominantly form IIB. There seems to be little doubt, however, that the most stable form of sulphathiazole at room temperature is the "form II" mixture.

Characterization of forms

The infrared spectra of forms IIA and IIB are remarkably similar, and out of about 40 absorption bands all but seven are indistinguishable. The following distinctions may be noted:

1. The two NH_2 stretching bands occur at 3310 and 3270 cm^{-1} in form IIA and at 3345 and 3280 cm^{-1} in form IIB (compared with 3460 and 3355 cm^{-1} in form I). The 3345 and 3310 cm^{-1} bands are sufficiently separated to be resolved by a grating spectrometer, and the mixture therefore shows three bands between 3350 and 3250 cm^{-1}.

2. Form IIA has a well-resolved doublet at 1279 and 1265 cm^{-1}, the former being stronger; in form IIB the intensities are reversed and the peaks tend to merge together.

3. Form IIB has a broad band of medium intensity at 886 cm^{-1} and a very weak peak at 866 cm^{-1}, both of which are absent from the spectrum of form IIA (see Fig. 1).

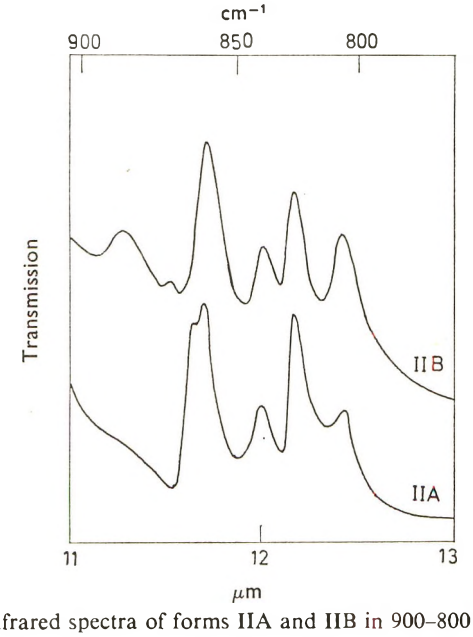

FIG. 1. Infrared spectra of forms IIA and IIB in 900–800 cm^{-1} region.

4. A single peak at 852 cm^{-1} in form IIB is replaced by a close doublet at 858 and 854 cm^{-1} in form IIA, but this difference is detectable only with pure material and a high resolution spectrometer.

5. A well-resolved peak at 805 cm^{-1} in form IIB is reduced to a shoulder in form IIA, due to filling in of the trough at 810 cm^{-1}.

6. A weak peak occurs at 699 cm^{-1} in form IIA and at 703 cm^{-1} in form IIB. With a grating spectrometer linear in wavelength the mixture gives rise to a doublet (see Fig. 2), but with small instruments having linear wavenumber scales this difference is not detectable.

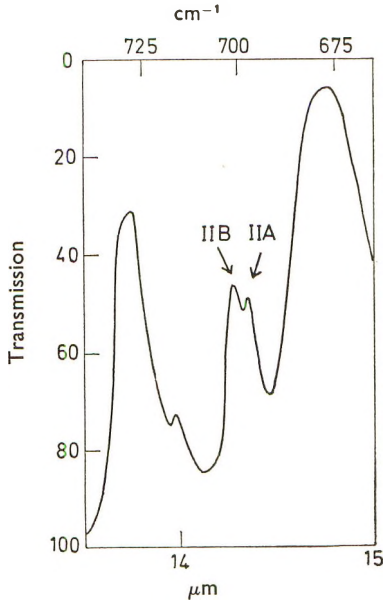

FIG. 2. Infrared spectrum of "form II" mixture, showing doublet near 700 cm^{-1}.

7. Beyond the rock salt region, a weak band at 518 cm^{-1} in form IIA is replaced by a stronger absorption at 524 cm^{-1} in form IIB.

From the criteria above, it appears that the spectrum published by Moustafa & Carless (1969), described as form I, is that of a mixture in which form IIA predominates. Similarly the partial spectrum of form II given by Kuhnert-Brandstätter & Bachleitner-Hofmann (1971) is clearly that of the mixture, although the frequencies quoted are higher than those given here.

X-ray diffraction patterns of the two forms were reproduced by Higuchi & others (1967). Their form I, crystallized from ethanol, appears to correspond to form IIA; this pattern is significantly simpler than that of the B.P.C. specimen (see Fig. 3), and the additional lines in the latter are therefore attributed to form IIB. Unfortunately it has not been possible to prepare form IIB of sufficient purity to record its diffraction pattern. Tabulation of the lines (Table 1) shows three lines, at 4·48, 4·13 and 3·56 Å, which are not present in either form IIA or form I.

The results of differential scanning calorimetry need careful interpretation to distinguish between forms IIA and IIB. Moustafa & Carless (1969) observed transitions around 160° and 176° (peak temperatures) for sulphathiazole crystallized from different solvents, and on mixing the two varieties obtained a thermogram showing both transitions. Shenouda (1970) also observed differences depending on the solvent used, and attributed peaks at 150–166° and at 170–176° to solid–solid

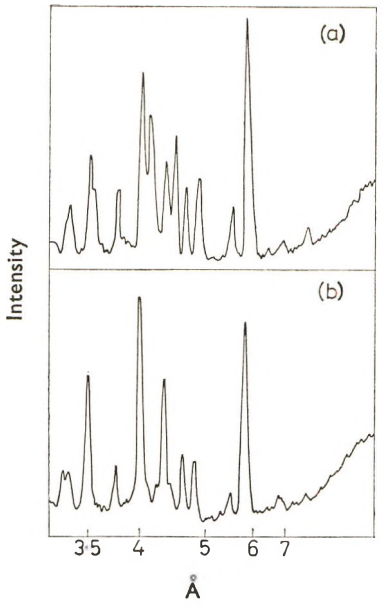

FIG. 3. X-ray diffraction patterns of (a) "form II" mixture (B.P.C. Authentic Specimen), (b) form IIA.

Table 1. *X-ray diffraction patterns of sulphathiazole polymorphs.* (d-spacings in Å).

Form I		Form IIA		B.P.C. Specimen "Form II" mixture	
10·16	w	7·81	w	7·81	w
8·18	m	6·81	w	6·90	w
7·59	vw	5·77	s	5·77	vs
6·94	w	5·47	w	5·47	w–m
6·68	w–m	4·79	m	4·83	m
6·17	w	4·62	m	4·62	m
5·59	s	4·33	m–s	4·48	m–s
5·06	s	4·03	s	4·33	m
4·75	s	3·76	w–m	4·13	s
4·29	m–s	3·51	m–s	4·03	s
4·07	s	3·35	w–m	3·79	m
3·91	w	3·31	w–m	3·56 ⎫	m
3·80	w			3·52 ⎭	m–s
3·65	m			3·35 ⎫	m
3·47	vw			3·31 ⎭	w–m
3·30	w				
3·18	m				

transition and to melting respectively. These peaks were highly susceptible to grinding of the sample and to crimping of the sample pans. In the present work form IIA consistently gave a broad endotherm with onset at 157°, while a mixture in which form IIB predominated gave a sharper peak with onset at 170° (results corrected where necessary to give an onset temperature of 202° for the final melting transition). Commercial mixtures such as the B.P.C. Authentic Specimen resembled form IIA, the onset of the broad endotherm being approximately 159°, with a barely detectable peak at about 175° in some thermograms. The absence of the first

transition in the mainly IIB mixture suggests that the behaviour of mixtures tends to be dominated by the major constituent.

Whether the 170° transition of form IIB represented melting, as suggested by Shenouda, was not apparent, as the bulk of the material remained solid. If this endotherm were due to partial melting, it would imply that the remainder of the material had undergone a solid–solid transition without showing any evidence of this in the thermogram. Experience with other polymorphic systems, such as cortisone acetate (Mesley, 1968) and phenobarbitone (Mesley, Clements & others, 1968), suggests that this may be the normal behaviour with organic compounds, and that polymorphic transitions are usually detectable by differential scanning calorimetry only when accompanied by tautomeric changes in molecular structure. However, the apparent constancy of the heat of transition, as mentioned by Moustafa & Carless (1969), would seem to support their statement that both peaks were caused by solid–solid transition. Against this, the higher results obtained by Shenouda (1970) in those instances where both transitions were observed would support the melting hypothesis, since the heat of fusion of a given form must be greater than the heat of transition to another solid form. The discrepancy is possibly explained by the fast heating rate (40°/min) used by Shenouda, which would favour melting before the solid–solid transition had time to occur.

Microscopic examination of behaviour on heating, using a Kofler block, showed about 60% of crystals melting at 170–173° with all samples examined, and the molten portion showed no tendency to recrystallize on further heating. The remaining crystals melted at around 200°. From this observation it appears that both forms IIA and IIB show some tendency to melt, and that the melting point is not diagnostic of either form (though this may not be true of pure form IIB).

Relationship between forms

Kuhnert-Brandstätter & Wunsch (1969) state that some commercial samples of sulphathiazole contain a hydrate, but they do not indicate how this was identified. In a previous paper (Kuhnert-Brandstätter & Grimm, 1968) solvation was detected by the appearance of bubbles when the solid material was heated on a Kofler block while immersed in silicone oil. When this technique was used with sulphathiazole recrystallized from water (form IIA) and with a mixture in which form IIB predominated, in each case some evolution of bubbles was observed as the material partially melted near 170°, and a more copious evolution accompanied the final melting at about 200°. Since there is no reason to suppose that form I is solvated, the second evolution can only be due to air trapped in the solid particles, and this seems an equally probable explanation for the first evolution. This experiment therefore gave no indication that either form is hydrated. Comparison of the infrared spectra of forms IIA and IIB again shows no evidence for the presence of water in either form, though admittedly there is some interference from NH_2 absorptions in the two regions (near 3300 and 1650 cm^{-1}) where water absorptions might be expected. Nevertheless any water present would have to be in less than molar proportion to escape detection.

Assuming the distinction between the two forms is not one of hydration, then the infrared spectra may indicate the nature of the structural differences. The positions of the N–H stretching absorptions indicate the formation of strong intermolecular hydrogen bonds. Comparison with the spectra of deuterated sulphathiazole (forms

I and IIA) shows that the sulphonamide NH group gives rise to a very broad absorption centred near 2900 cm^{-1}, the corresponding ND band being near 2200 cm^{-1}. This broad feature is common to all three forms, and is attributed to strong hydrogen bonding to a second nitrogen atom, probably that in the thiazole ring.

The NH_2 bands in form I occur at 3460 and 3355 cm^{-1}, corresponding to some degree of hydrogen bonding, probably to the oxygen atoms of the SO_2 group. Using the technique of Novak, Lascombe & Josien (1966) it can be shown from examination of partially deuterated form I that the two N–H bonds are not equivalent, so it appears that only one of the hydrogens is bonded to the SO_2 group.

The NH_2 absorptions in forms IIA and IIB have very much lower frequencies, and are indeed lower than those of all the 44 other sulphonamide forms examined by Mesley & Houghton (1967). The very low frequency again suggests bonding to nitrogen, rather than to the SO_2 group. There is no evidence to support the suggestion by Kuhnert-Brandstätter & Bachleitner-Hofmann (1971) that the difference between form I and "form II" is due to amide–imide tautomerism; this conclusion is apparently based on the erroneous assignment of the NH_2 absorptions in "form II" to the two highest frequency bands, which in fact arise from the two different forms IIA and IIB, and of the sulphonamide NH absorption to that at 3280 cm^{-1} instead of the broad band near 2900 cm^{-1}. Form IIB is the only one of the three forms in which the NH_2 frequencies conform to the relationship suggested by Bellamy & Williams (1957) as a test for equivalence of the two hydrogens, so in this form it appears that both hydrogens are strongly bound to nitrogen. In form IIA the frequencies are even lower, suggesting that one of the hydrogens is bound more strongly to nitrogen while the other may form a weaker hydrogen bond. Deuteration of form IIA has shown that the distinctive bands at 1266, 810 and 518 cm^{-1} are all associated with NH or NH_2 groups, so it may be supposed that virtually all the differences between forms IIA and IIB are attributable to the relative strengths of the hydrogen bonds.

Clearly the very strong intermolecular hydrogen bonds in both components of "form II" are sufficient to account for the lower solubility of the normal material relative to that of form I, as noted by Kuhnert-Brandstätter & Martinek (1965) and by Higuchi & others (1967). The energy required to break these bonds on conversion to form I explains the appearance of the endothermic peak in the 150–175° region in DSC and DTA thermograms. It seems probable that the transitions with onset temperatures of 157° and 170°, which apparently correspond to those of Moustafa & Carless (1969), are both attributable to solid–solid transitions, in which case the apparent constancy of the heats of transition noted by these workers implies a marked similarity in structure. The melting transition, when it occurs, is probably very close to the 170° transition of form IIB and would not normally be resolved from it. From the microscopic results it appears that the melting transition is not restricted to form IIB, and at least one thermogram of pure form IIA shows a very small endothermic peak at 175°. The similarity in melting point suggests that neither of these forms corresponds to form III or form IV, for which melting points of 162° and 158° were given by Kuhnert-Brandstätter & Wunsch (1969). Infrared spectra of the B.P.C. specimen recorded at elevated temperatures show no apparent tendency for interconversion between forms IIA and IIB; up to 120° the proportions were apparently unchanged, the only spectral difference being due to the appearance of some form I, and after 15 min at 160° the material was entirely form I.

CONCLUSIONS

Moustafa & Carless (1969) claimed that the heat of transition could be used to assay the low temperature form in sulphathiazole mixtures. The work of Shenouda (1970) has shown that this heat of transition may vary considerably unless all samples are ground and crimped. In view of the fact that commercial samples are frequently mixtures, this assay method is of doubtful validity (the effect of grinding a mixture of form I and "form II" could well alter the relative proportions).

The solubility measurements of Higuchi & others (1967), apparently carried out on pure form IIA, may also be invalid for normal commercial samples for the same reason. There is obviously a need for solubility data on both of the low temperature forms, though there is little doubt that both are significantly less soluble than the high temperature form. It is probable that the crystal growth studies of Carless & Foster (1966) resulted in different forms under different conditions, though the possibility of polymorphism was admitted by these authors.

The differences in infrared spectra, though slight, could affect the infrared identification test of the B.P.C. Conversion to the high temperature form (form I), either by heating as recommended by Moustafa & Carless (1969) or by recrystallization from n-propanol (Mesley & Houghton, 1967), is therefore recommended for this purpose.

Acknowledgement

Thanks are due to Mr. D. J. Barnfield, Dr. B. Flaherty and Miss S. A. Langston for practical assistance.

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J. Pharm. Pharmac., 1971, **23**, 695–698 *Received February* 9, 1971

Methods for the preparation of lysophosphatidylcholine

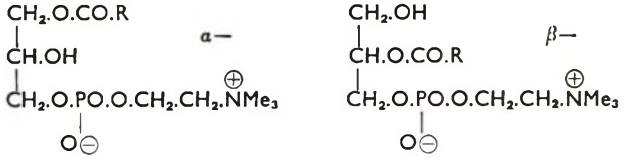
DANUTA S. MERRYFIELD AND J. J. WREN*

Lyons Central Laboratories, 149, Hammersmith Road, London, W.14, U.K.

The surfactant and solubilizing properties of lysophosphatidylcholine (LPC) could have industrial uses if it became available in technical quantity and purity. The two most promising routes for obtaining it are isolation from cereal starches and mono-deacylation of egg phosphatidylcholine (PC). Baker's yeast autolysed by Letters's method yielded up to 0·3% of LPC. The phospholipase A activity of pancreatin powder gave 80% of the theoretical yield of LPC under optimum conditions; modifications designed to facilitate scale-up reduced the yield. Base-catalysed glycerolysis at 75° gave 23% of the theoretical yield of LPC, and a reaction between PC and ethanolamine up to 45%. The crude product from ethanolamine after acetone precipitation gave an emulsifier containing LPC, PC and long-chain *N*-2-hydroxyethylamide.

Lysophosphatidylcholine (LPC) has many properties (Robinson, 1961) that could be of use in pharmaceutical and food preparations. It forms clear, micellar solutions in water and in numerous organic solvents, it solubilizes enzymes and other proteins, it forms complexes with some proteins and polysaccharides, and it is a powerful surfactant and emulsifying agent. Unlike all industrial ionic surfactants except soap and phosphatidylcholine (PC) it is a normal metabolite (see *e.g.* Eisenberg, Stein & Stein, 1967). Until recently LPC was known mainly for its haemolytic effect, and its isolation from tissues was often attributed to autolysis or chemical degradation. Its relative abundance in intestinal contents (Nilsson & Borgström, 1967) and in foods such as the cereal starches (Acker & Schmitz, 1967; Wren & Merryfield, 1970) indicate that it must be a relatively harmless substance for oral administration. Unfortunately, it is much more costly than other surfactants.

The term LPC is used in this paper to denote one or both of the α- and β-isomers (1- and 2-acyl, respectively) in which the fatty acid composition is unspecified:



Either isomer, with a single acyl group, can be prepared in a high state of purity *via* costly synthetic intermediates (see *e.g.* Slotboom, Haas & van Deenen, 1967). A more attractive route to a technical grade of LPC would be through direct acylation of α-glycerylphosphorylcholine, but this is not readily available as a starting material.

Two other routes seem more feasible for LPC on a technical scale, namely isolation from cereal starches (Wren & Merryfield, 1970) and mono-deacylation of PC. Table 1 shows the most promising deacylation methods reported. In present work

* Present address: Watney Mann Ltd., Mortlake, London, S.W.14, U.K.

Table 1. *Published methods for the preparation of LPC by deacylation of PC.*

PC	Method	Yield, % of theoretical	Reference
Egg (purified)	Enzymic (phospholipase A activity in pancreatin)	60	Ansell & Hawthorne (1964)
	Controlled methanolysis	35–40	Marinetti (1962)
	$AlLiH_4$ in ether	?	Urakami & Yamaguchi (1963)
Soya (purified)	Enzymic (snake or bee venom)	51	Klenk & Debuch (1960, 1962)
Synthetic (dipalmitoyl)	Controlled methanolysis	$\geqslant 38$	Pries (1965)

modifications of the pancreatin method were attempted, and also autolysis of baker's yeast (Letters, 1967), base-catalysed glycerolysis, and a novel use of ethanolamine.

MATERIALS AND METHODS

Analar solvents were used. Pancreatin powder was obtained from Hopkin and Williams. PC was isolated from fresh hen eggs (Wren & Merryfield, 1965) using 0·005% of the antioxidant butylated hydroxytoluene (Wren & Szczepanowska, 1964) in the solvents.

Solvents were removed in a rotary evaporator at $<50°$. Lipid fractions were identified by thin-layer chromatography and infrared spectrophotometry.

LPC was isolated from reaction products by chromatography on a silicic acid column (usually 20 g): product ($\geqslant 1$ g) was developed with 200 ml each of 10% methanol, 50% methanol (to recover PC) and 100% methanol (to elute LPC). In some experiments 100 g of product was chromatographed on 750 g of silicic acid.

Acetone precipitation was effected by adding just enough chloroform to the product to make it pourable and then stirring into acetone at 5°. The precipitate was recovered by centrifuging and washed with a little acetone.

RESULTS AND DISCUSSION

When purified egg PC was subjected to the full pancreatin method (Ansell & Hawthorne, 1964) LPC was isolated in 80% of theoretical yield by silicic acid chromatography. When the concentration of PC was raised, when pancreatin powder was used without the removal of insoluble matter, or when impure sources of PC (egg yolk, whole egg or commercial soya PC) were used, the yield fell to less than 50% of theoretical.

Baker's yeast, after autolysis for 20 h while mixed with an equal weight of acetone–water (7:3, v/v), and subsequent extraction with *n*-butanol (Letters, 1967), gave up to 0·3% of LPC. No extra LPC could be obtained by adding egg yolk or purified egg lecithin at the beginning of the autolysis period.

Base-catalysed glycerolysis is applied to triglyceride fats in the production of monoglycerides; if applied to PCs (Mattikow, 1942) at moderate temperature it should give a useful emulsifying mixture containing LPC and monoglyceride. When purified egg PC was heated at 75° for 7 days *in vacuo* with one-quarter of its weight of 2% NaOH in glycerol, the product yielded: LPC (23% of theoretical) 15%;

PC 28%; mono-, di-, triglycerides 45%. When commercial soya PC was used the reaction was slower, but it could be speeded by using ethanolamine as catalyst in place of NaOH.

Fatty acids (Roe, Scanlan & Swern, 1949) and triglycerides (Roe, Stutzman & others, 1952; Naudet, Sambuc & others, 1952) react with ethanolamine to give long-chain *N*-2-hydroxyethylamides (ethanolamides). PC has now been found to react similarly, and under suitable conditions can be converted to PC and ethanolamide. The latter is a non-ionic surfactant and, like LPC, occurs naturally in animal tissues (Bachur, Masek & others, 1965), in bean leaves, wheat and soya beans (Wren & Merryfield, 1965) and in peas (Quarles, Clarke & Dawson, 1968). No toxic effect has been found in any of several biological tests made with ethanolamides (Macht & D'Alelio, 1936; Merck, 1958; Coburn & Rich, 1960; Coburn, 1961). Hence the reaction products of PC and ethanolamine merit consideration for use in pharmaceutical and food preparations.

Table 2. *Yields of LPC obtained from PC and ethanolamine under various conditions (expressed as % of starting material; theoretical yield, 66%).*

Conditions	Molar ratio, PC: ethanolamine	Egg PC (purified)	Egg PC (BDH)	Soya PC (commercial)
Stirred under N_2 for 30–50 min at 140°	1:1	17, 22	18	4
	1:3	30		
	1:6		22	
Refluxed with chloroform (1:1, w/v) for 3 h	1:1	9	18	
	1:2		20	
	1:3	24, 28	22	
Kept over silica gel at room temp. for 18 h (or 2–3 days*)	1:1	20, 23*		4
	1:2	25*		6
	1:3	30		

Table 2 shows the yields of LPC obtained under various conditions and isolated quantitatively by silicic acid chromatography. Acetone precipitation of the reaction products gave 'technical-quality' emulsifiers: the composition of the product obtained at 140° from purified egg PC was: LPC 40%; PC 49%; ethanolamide 11%. Traces of ethanolamine contaminating these preparations were removed *in vacuo* over P_2O_5; however, preparations from soya PC retained an unpleasant odour derived from the starting material.

Table 3. *Fatty acid compositions of egg PC and LPC made from it.*

	C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$	$C_{18:2}$
PC (total)	34·0	2·2	17·6	31·6	14·4
PC (α-position*)	61		25	9·5	2·4
PC (β-position*)	4·8	1·7	2·4	59	26
LPC (enzymic, pancreatin)	67·7	2·3	24·1	4·6	
LPC (ethanolamine, 140°)	36·4	6·1	18·5	30·2	8·7
LPC (ethanolamine, chloroform)	33·0	1·6	15·6	31·3	17·7

* Menzel & Olcott (1964).

Fatty acid compositions (Table 3) show that, unlike the enzyme but like other basic catalysts (Marinetti, 1962), ethanolamine deacylates the PC molecule non-specifically.

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J. Pharm. Pharmac., 1971, **23**, 699–704 *Received May* 19, 1971

The effect of suspensions on the bactericidal activity of *m*-cresol and benzalkonium chloride

H. S. BEAN AND G. DEMPSEY

Department of Pharmacy, Chelsea College, University of London, Manresa Road, London, S.W.3, U.K.

The bactericidal activity of experimental aqueous suspensions of light kaolin with *m*-cresol as preservative agreed closely with the activity of aqueous *m*-cresol solutions not brought into contact with kaolin (i.e. aqueous reference solutions). In contrast the activity of light kaolin suspensions with benzalkonium chloride was lower than that of aqueous benzalkonium chloride solutions because of adsorption of preservative by the kaolin. Suspensions containing benzalkonium chloride possessed a greater activity than the corresponding supernatant solutions removed from contact with the kaolin, owing to some of the bactericide adsorbed on the kaolin becoming available to the bacteria. Suspensions of procaine penicillin with benzalkonium chloride gave similar results to those for kaolin.

The report to the Swedish National Board of Health by Kallings, Ringertz & others (1966) on microbiological contamination of medicinal preparations, focussed attention on the possible need for preservatives in oral mixtures. Kaolinite and bentonite in suspension are capable of stimulating the respiration of some bacterial species (Stotsky & Rem, 1966) suggesting that under suitable conditions the presence of certain suspended solids can provide ideal environments for growth of spoilage organisms.

Loss in antibacterial activity of some antibiotics owing to their adsorption on suspended solid matter has been shown by Pinck (1962) and El-Nakeeb & Yousef (1968), and McCarthy (1969) has pointed to a possible loss in activity of preservatives arising from their adsorption onto solids commonly used as medicaments. The spoilage of the B.P.C. mixture of sulphadimidine for infants is attributed to the adsorption of the preservative, benzoic acid, by the suspended sulphonamide (Beveridge & Hope, 1967).

We have examined the effect of light kaolin on the preservative activity of *m*-cresol and benzalkonium chloride. We chose *m*-cresol since preliminary studies established that it was not apparently adsorbed by the kaolin; hence with this system we looked for the effect, if any, of the presence of suspended solid on bacterial death and particularly for a possible protective effect of the solid to parallel the stimulating effect of some solids on respiration of some organisms shown by Stotsky & Rem (1966) Benzalkonium chloride, in common with other quaternary ammonium compounds, was known to be adsorbed by kaolin (Batuyios & Brecht, 1957) and so the system benzalkonium chloride-light kaolin was chosen to illustrate the effect of preservative adsorption on antibacterial activity and this was compared with the system procaine penicillin-benzalkonium chloride where again adsorption of the quaternary ammonium compound occurred. We were particularly interested in these experimental suspension systems containing benzalkonium chloride to see if they possessed greater antibacterial activity than that found in the suspending medium after removal of the

suspended particles, an effect noted by Batuyios & Brecht (1957) for aqueous suspensions of talc with cetyl pyridinium chloride as preservative. A parallel effect to this was reported by the Conference on the Control of Antibiotics (Ministry of Health, 1957); suspensions of procaine penicillin preserved by cetrimide showed 80% adsorption of preservative and the suspension was more bactericidal than the supernatant from the suspension.

MATERIALS AND METHODS

Materials

Kaolin. Light kaolin B.P. was sterilized by dry heat at 160° for 1 h and stored in an air-tight container. *Procaine penicillin* (Glaxo Laboratories Ltd), potency: 1000 i.u./mg. No microbial contamination was detected. m-*Cresol*, laboratory grade, was redistilled and the fraction boiling at 201–203° was used. *Benzalkonium chloride* (BAK) was a 50% w/v solution (Koch-Light Laboratories). *Polysorbate 80* (Honeywill-Atlas Ltd). *Calcium chloride* was the Analar anhydrous salt. *Penicillinase* (Boots Pure Drug Co Ltd) 1 ml inactivated 800 000 i.u. penicillin in 60 min at room temperature.

Methods

Adsorption of preservatives by solids. The procedure was basically as described previously for the adsorption of phenol on carbon (Bean & Dempsey, 1967). For the adsorption of BAK on procaine penicillin 3 h was sufficient for equilibrium to be established; for the light Kaolin-BAK system, equilibrium was attained in 6 h but for convenience the system was left overnight before assay, as was the *m*-cresol-kaolin system. Supernatant concentrations of BAK were determined using the colorimetric method of van Steveninck & Maas (1965); *m*-cresol was assayed by measuring the absorbance at 271 nm.

Bactericidal activities. The extinction times of the reaction mixtures were determined against *Escherichia coli* NCTC 5933 (20×10^6 organisms/ml) according to Bean & Dempsey (1967) for the *m*-cresol systems. For the BAK-procaine penicillin suspensions a 3 h equilibration period was allowed before commencing the bactericidal reactions. A single tube of indicator broth (Bean & Dempsey, 1967) containing 0·1% w/v Lubrol W non-ionic surfactant was used for the inactivation of each BAK sample, since preliminary experiments showed 0·1% Lubrol to be an effective inactivator for this purpose; in addition, for procaine penicillin-BAK systems, 0·1 ml of penicillinase was added to each tube of recovery medium. For systems containing BAK, 1 ml of reactant sample transferred to 24 ml of recovery medium resulted in a concentration of BAK below the minimum inhibitory concentration (M.I.C.) in the Lubrol indicator broth (M.I.C. 0·014% w/v compared to a value of 0·002% w/v in indicator broth without Lubrol).

The suspension systems tested were essentially experimental and for the light kaolin systems concentrations of suspended solid greater than 5·0 g/100 ml were not investigated since at concentrations greater than this considerable sampling difficulties arose.

RESULTS

The removal of BAK from solution by light kaolin (Batuyios & Brecht, 1957; McCarthy, 1969) followed the L-type isotherm described by Giles, MacEwan &

others (1960); with 2·5 g kaolin/100 ml solution and over an initial BAK concentration range of 37·5 to 56·0 $\times 10^{-3}$% w/v there was approximately 80% adsorption which fell within the plateau region of the isotherm. The adsorption was reversible, at least in part. In contrast, there was no detectable adsorption of *m*-cresol by light kaolin (2·5 g/100 ml) over the range of concentration used (i.e. 0·315 to 0·40% w/v).

The BAK concentrations used for the antibacterial investigations in the presence of light kaolin resulted in flocculated suspensions as shown by sedimentation volume studies (Martin, 1961). Aqueous *m*-cresol appeared to have no flocculating effect upon kaolin and it was decided to produce flocculation by addition of 0·1% w/v calcium chloride to the *m*-cresol systems (Anderson & Fitzgerald, 1967) so that subsequent bactericidal investigations were on suspensions in a similar state of aggregation for both *m*-cresol and BAK systems.

The bactericidal activities of *m*-cresol in the presence of flocculated light kaolin suspensions and the activities of the corresponding supernatants are shown in Table 1.

Table 1. *The bactericidal activity of* m-*cresol against* E. coli *at* 25° *for aqueous suspensions and the corresponding supernatants of light kaolin flocculated by addition of* 0·1% (w/v) *calcium chloride.*

Initial concn *m*-cresol (% w/v)	2·5 g kaolin/100 ml Extinction times (min)		5·0 g kaolin/100 ml Extinction times (min)		Extinction times (min) for aqueous control soln (*m*-cresol/0·1% calcium chloride)
	Suspension	Supernatant	Suspension	Supernatant	
0·315	200	216	220	220	200
0·350	90	92·5	88	85	91·25
0·375	44	43·75	47	51·25	40·0
0·400	30	25	30	29	27·5
0·435	—	—	15	11·75	17·0

Little or no difference existed between suspension and supernatant activity for both 2·5 and the 5·0 g/100 ml kaolin systems; the activities of the aqueous control and the suspension systems were almost identical as was to be expected since there was no obvious adsorption of preservative by the suspended solid.

The suspension system BAK-light kaolin (2·5 g/100 ml) was more active than the corresponding supernatant from the suspension (Table 2). Because BAK flocculated

Table 2. *Bactericidal activity of benzalkonium chloride against* E. coli *at* 25° *in aqueous suspensions and the corresponding supernatants of light kaolin* (2·5 *g*/100 ml).

Initial concn BAK (% (w/v) $\times 10^{-3}$)	Aqueous phase (i.e. equilibrium) concnBAK (% (w/v) $\times 10^{-3}$)	Extinction time (min)		Extinction times (min) of aqueous control BAK at concentrations equal to aqueous phase of suspensions
		Suspension	Supernatant	
38·0	3·75	100·0	>120	83·0
40·0	5·20	79·5	>120	53·7
44·0	8·10	35·06	115	28·8
47·5	10·70	17·6	57·4	19·5
50·0	12·50	10·67	50	15·9
52·5	14·35	—	38·4	12·9
56·0	16·90	<1·0	14	10·2

the light kaolin, its activity was compared with that of a BAK-light kaolin system (2·5 g/100 ml) deflocculated by addition of 0·1% w/v Tween 80. The discrepancy between the suspension and supernatant activities persisted even in the deflocculated system; hence flocculation and therefore possible entrainment of the bacteria within the floccules was discounted as a possible reason for the discrepancy.

The effect of different concentrations of light kaolin suspension on activity is illustrated in Fig. 1 where the logarithm of aqueous phase concentration of BAK, found from adsorption data, is plotted against logarithm of extinction time.

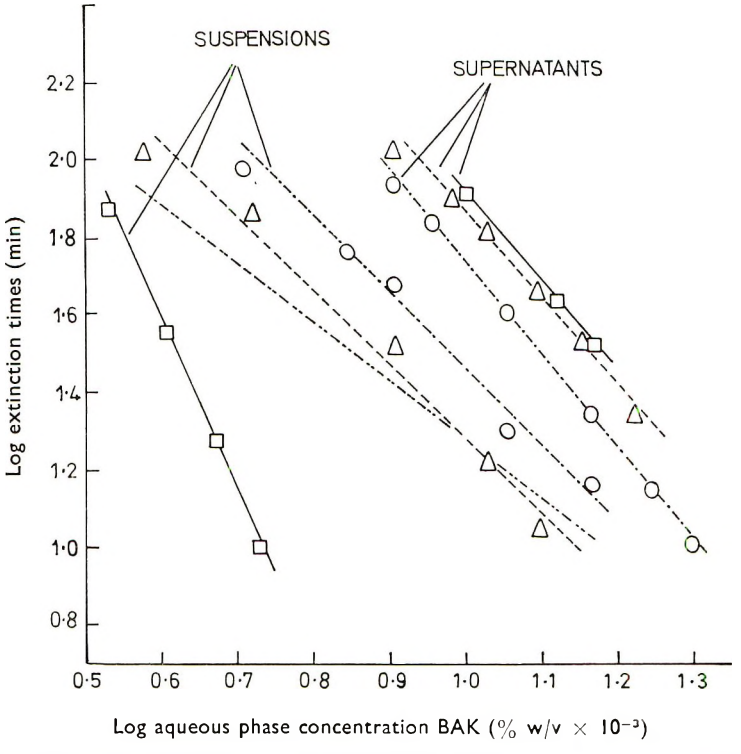

FIG. 1. Activities of suspensions and corresponding supernatants of system BAK/light kaolin against *E. coli* NCTC 5933 (20 × 10⁶/ml) at 25°. Aqueous reference: —··—··— Light kaolin concentrations (g/100 ml): —·—○—·—, 0·5; — — —△— — —, 2·5; ——□——, 5·0.

For a fixed aqueous phase concentration of BAK, the antimicrobial activity of the suspension systems increased with increase in amount of suspended solid (i.e. increase in amount of adsorbed BAK), whereas the activity of the corresponding supernatant solutions from the suspensions decreased. The decrease of activity of the supernatants was presumably due to the inactivating effect of cations on quaternary ammonium compounds (Ridenour & Armbruster, 1948), the cations arising by cation exchange when the BAK was adsorbed on the kaolin (van Olphen, 1966); thus the greater the concentration of suspended solid the greater the adsorption of BAK and the greater the number of inorganic cations liberated into solution.

Two different suspended solid concentrations of procaine penicillin were investigated, viz. 1 and 5 g/100 ml. At 5 g/100 ml, the adsorption of BAK averaged 40% over an initial concentration range of 7 to 25 × 10^{-3}% w/v BAK; the adsorption was

in part reversible. An increase in suspended solid concentration resulted in an increase in bactericidal activity for any fixed aqueous phase concentration of BAK. Also, for a fixed aqueous phase concentration of BAK, the activities of the supernatants from the suspensions were less than those of the corresponding suspensions. The supernatant solutions removed from contact with the procaine penicillin had approximately the same activity for a fixed aqueous phase concentration of BAK, irrespective of the initial procaine penicillin disperse phase concentration.

Procaine penicillin in solution had an inactivating effect upon BAK; for a fixed aqueous phase concentration of BAK, the activity of aqueous BAK solution not brought into contact with procaine penicillin was approximately two to two and a half times greater than that of the procaine-BAK supernatant solutions withdrawn from the suspended solid (Fig. 2).

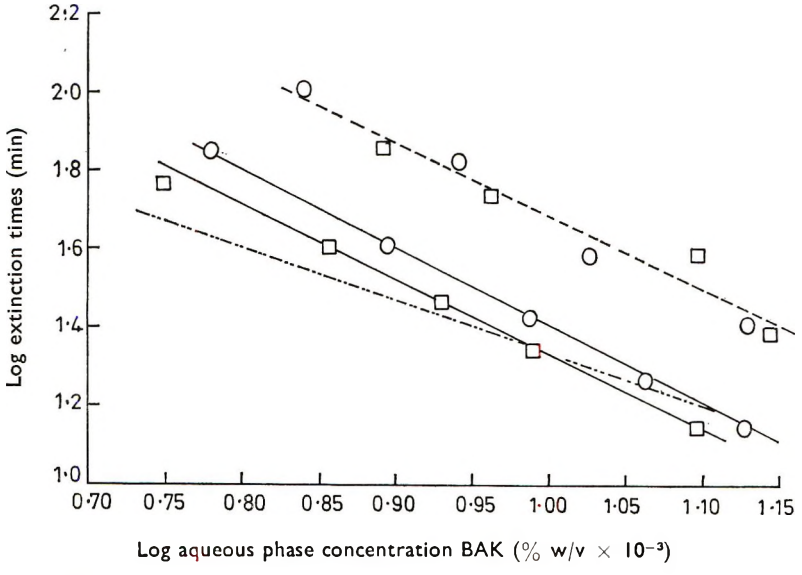

FIG. 2. Activities of suspensions and corresponding supernatants of system BAK/procaine penicillin against *E. coli* NCTC 5933 (20×10^6/ml) at 25°. Aqueous reference: —·—··—. Suspensions: ———. Supernatants: — — — —. Procaine penicillin concentrations (g/100 ml): ○, 1·0; □, 5·0.

DISCUSSION

The *m*-cresol-light kaolin system showed no significant difference between the activity of the suspension and simple aqueous solutions of *m*-cresol, indicating the lack of effect upon preservation of the suspended solid (cf. Stotsky & Rem, 1966) when no adsorption of the preservative was apparent. Where adsorption did occur, as with BAK on both light kaolin and procaine penicillin, the preservative activity of the system was less than that of an aqueous solution of the same total concentration since at least part of the adsorbed BAK was not available to the bacteria. The resultant antibacterial activity of the suspension systems was composed of a contribution from the aqueous residue of BAK plus a contribution from the adsorbed phase. If the activity of the BAK systems had been solely determined by the aqueous phase concentration of the preservative, then the activity of the suspension systems should have agreed with that of the supernatant solutions from them, but in fact, the

suspensions had a greater activity than the supernatants. This phenomenon could be attributed to a release of some of the adsorbed quaternary ammonium compound from the suspended solid since we have found that the inoculum of *E. coli* adsorbs BAK and, since the adsorption of BAK by the solids was in part reversible, the concentration of BAK in the aqueous phase might be partially restored by release from the solid following uptake by the bacteria. In this way the adsorbed phase of BAK on the solids would be acting as a reservoir of preservative. However, the results in Fig. 1, where an increase in suspension activity is shown for increase in suspended solid concentration at fixed equilibrium concentration of BAK, infer that there may have been a direct contribution to activity by the adsorbed phase of BAK quite apart from any activity in the aqueous phase.

It is difficult to predict whether adsorption of preservatives on suspensions will occur, but the surface activity of the preservative may be a guide in this respect; thus BAK is highly surface-active and was highly adsorbed by kaolin whereas the non-surface-active *m*-cresol was not adsorbed.

Acknowledgements

We are indebted to Glaxo Laboratories, Greenford, for the sample of procaine penicillin.

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J. Pharm. Pharmac., 1971, **23**, 705

LETTERS TO THE EDITOR

Interference of chemoluminescence with [^{3}H]scintillation counting*†

Tracing the passage of [^{3}H]chlorpromazine‡ into the tissues and excreta of various mammals, several scintillators were evaluated for the counting of solvent extracts of the labelled drug content from the biological materials, or of the unextracted homogenates of the specimens. When quaternary amine solubilizers, such as Hyamine (Beckman) or Soluene (Packard) were employed to dissolve tissues, especially keratinous material such as wool, fur or nails, high spurious counts were obtained for the unextracted solutions with toluene-type scintillation cocktails. These keratinous tissues had previously been found to accumulate relatively large amounts of chlorpromazine metabolites (Forrest, Forrest & Roizin, 1963). These drug derivatives were of unknown structure, could not be extracted from the alkaline tissue homogenates by solvents, and could be assayed as a group only by destructive methods. Hence, scintillation counting appeared to be the method of choice to establish quantitative criteria for this apparently significant auxiliary drug detoxication mechanism. However, control specimens from animals not having received any labelled drug yielded false positive counts approximating 10 000 to 26 000 counts/min for 50 to 100 mg samples of wool or fur, completely obscuring the small amount of radioactivity expected in the experimental samples. Obviously, the spurious counts were due to persistent chemoluminescence, which could be decreased only moderately at the rate of 10 to 30% by prolonged storage of the samples, lowering of the temperature or neutralizing some of the excess alkalinity, short of precipitating the dissolved keratinous material.

The problem was solved by using the Packard Model 300 Tri-Carb Sample Oxidizer for combustion of the wool or fur samples. This procedure yielded background counts of 25 to 37 counts/min for the controls, while the experimental specimens produced low but unequivocal counts of 10 to 20 times background. Simultaneously, quenching due to the yellow to brownish colours of the dissolved keratinous samples was avoided.

Similar chemoluminescence phenomena were encountered in the scintillation counting of unextracted biological materials such as serum, urine, faeces and tissues of experimental animals. No such interference was observed, when solvent extracts from the alkalinized materials were subjected to scintillation counting, using extraction and sample preparation procedures previously reported with any type of scintillator, even in the presence of Soluene (Bolt, Forrest & Serra, 1966; Bolt & Forrest, 1967; Forrest, Bolt & Serra, 1968; Brookes & Forrest, 1969). As new extraction procedures were to be evaluated, it was essential to establish completeness of extraction of the labelled drug fraction by radioquantitation of the various solvent extracts obtained at different pH's as compared to homogenates of the unextracted material. It was then established that unextracted faeces and tissues or their homogenates showed chemoluminescence comparable to wool or fur, and that treatment in the Packard Oxidizer should precede normal scintillation counting.

The spurious results obtained with various unextracted mammalian urines in the

* Supported in part by USPH grant MH 18190–02.

† Part of the data were presented at the Pacific Conference on Chemistry and Spectroscopy, 6th Western Regional Meeting, American Chemical Society, San Francisco, October 1970.

‡ We thank Dr. Harry Green of Smith Kline & French Laboratories for a generous gift of chlorpromazine tritiated at position 9 of the nucleus.

Table 1. *Effect of chemoluminescence on scintillation counting in two cocktails.*

Species	Material Unextracted 100 λ	Scintillator I (counts/min) Cycle 1	Scintillator I (counts/min) Cycle 2	Scintillator II (counts/min) Cycle 1	Scintillator II (counts/min) Cycle 2
Rabbit Dutch Belt male	Urine, control	108 963	97 194	37	37
Guinea-pig pigmented, short-haired female	Urine, control	5062	4639	36	34
	Urine, after ^{3}H-CP$^+$, 100 μCi				
	Day 1	7355	5281	4206	4160
	Day 10	5388	4010	236	248
	Day 16	3174	3091	35	36
	Day 62	946	824	33	35
Sheep Strong-Ramble A female	Urine, control	65 342	58 914	37	35
	Urine, after ^{3}H-CP, 2 mCi				
	Day 1	85 222	80 082	11 083	11 072
Rhesus monkey male	Urine, control	26 520	25 696	35	34
	Urine, after ^{3}H-CP, 64 μCi				
	(3-day pool)	15 225	13 361	1,014	977
female	Urine, control	5566	4467	32	33
	Urine, after ^{3}H-CP, 64 μCi				
	(3-day pool)	9997	8537	1164	1156
male	Serum, control	57	54	36	36
Human male	Urine, control	28 786	26 337	33	32
female	Urine, control	6168	5323	32	33
male, neonate	Urine, control	13 996	11 684	37	36

Counted in Packard Tri-Carb Scintillation System, efficiency 33–39%
Scintillator I: PPO 5 g; dimethyl POPOP 0·3 g; Soluene (Packard) 40 ml; toluene to 1000 ml.
Scintillator II: naphthalene 100 g; PPO 5 g; dimethyl POPOP 0·3 g; dioxane 730 ml; toluene 135 ml; methanol from 35 to 100 ml.
$^+$ ^{3}H-CP = tritiated chlorpromazine.

presence of a quaternary amine solubilizer (Soluene) in one of the scintillation cocktails (Scintillator I) are listed in Table 1. This indicates that the use of Scintillator II—which tolerates the presence of 3 to 12% water, according to methanol content of 35 to 100 ml—eliminates all false positive counts. Spurious counts ranging from 10 000 to 100 000 counts/min routinely occurred in control urines, when Scintillator I was employed, but were reduced to background values either by acidification or by substitution of Scintillator II. These measures were mandatory, since Scintillator I also yielded exaggerated counts in experimental samples, and simulated excessively prolonged excretion of the label in e.g. guinea-pig urine.

Persistent chemoluminescence in an alkaline scintillation medium as an interfering phenomenon in scintillation counting has also been reported in different contexts by other laboratories. Kahlbehn (1967) attributed the spurious counts to the interaction of Hyamine 10-X with such tissue components as proteins and polysaccharides. More recently, Woods & O'Bar (1970) described a new type of chemoluminescence arising from the combination of trichloroacetic acid and bathophenanthroline, as distinct from the analogous phenomenon due to reaction of quaternary amines with peroxides.

Department of Psychiatry,
Stanford University,
School of Medicine,
and
Veterans Administration Hospital,
Palo Alto, California 94304,
U.S.A.

I. S. FORREST
L. G. BROOKES
G. FUKAYAMA
M. T. SERRA

May 28, 1971

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A comparison of adrenergic α-receptors by the use of *NN*-dimethyl-2-bromo-2-phenylethylamine (DMPEA)

There has been much discussion of the possible subclassification of the α- and β-categories of adrenergic receptors (cf. *inter alia*, van Rossum, 1965; Lands, Arnold & others, 1967; Patil, 1969; Bristow, Sherrod & Green, 1970; Furchgott, 1970; Brittain, Jack & Ritchie, 1970; Patil, Patil & Krell, 1971; Triggle, 1971). Evidence in support of such subclassification comes from the discovery of tissue selective agonists and antagonists, the comparative sequences of activities of agonists and antagonists in various tissues and by the use of isomer activity ratios (Patil, 1969; Patil & others, 1971) in which the activities of *R* and *S* isomers of catecholamines are compared in a number of tissue systems experimentally controlled to minimize the influence of non-receptor catecholamine processes. This latter method has the theoretical advantage also in that problems of diffusion and access to the receptor, which may complicate comparisons between structurally dissimilar molecules, should be minimized. According to this procedure, the α-receptors of six tissues, including rabbit aorta and rat vas deferens, are not distinguishably different.

This finding agrees with our previous limited report (Moran, Triggle & Triggle, 1969) that the kinetics of recovery of response of the rabbit aorta and rat vas deferens to noradrenaline from irreversible antagonism by *NN*-dimethyl-2-bromo-2-phenylethylamine (DMPEA) were identical. This procedure would appear to afford another potential probe of comparative α-receptor structure, sharing the advantages of the isomer ratio technique, since recovery from an established blockade is measured and the product of the presumed receptor hydrolysis ($Ph{\cdot}CHOH{\cdot}CH_2{\cdot}NMe_2$) is inactive.

Table 1 presents our data, together with brief experimental details, for four α-receptor containing tissues. The results with rabbit aorta and rat vas deferens confirm our previous findings (Moran & others, 1969) and those with the guinea-pig and rabbit vas deferens extend them and show differences in the kinetics of recovery of α-receptor response.

It has been argued elsewhere (Belleau, 1958; Triggle, 1965, 1971) that the recovery of response from alkylation by DMPEA is consistent with a spontaneous intramolecular (direct nucleophilic or general base catalysed) hydrolysis of a β-dialkylaminocarboxylate ester. For such a reaction a decreased rate would be anticipated on shifting to a less polar environment since this would hinder charge production.

Table 1. *Recovery of response to noradrenaline after α-receptor blockade by DMPEA.*

Tissue	DMPEA (concn M)	% Blockade (initial)	Time (min) for 50% recovery of resp. (mean ±s.e.)	*n*
Rabbit aorta[a,b]	10^{-5}M/5′	95	23·8 ± 2·4	10
Rat vas deferens[b,c,d]	10^{-5}M/5′	96	23·0 ± 0·9	14
Guinea-pig vas deferens[b,e] ..	10^{-5}M/5′	95	75·0 ± 6·0	6
Rabbit vas deferens[c,e]	2×10^{-5}M/5′	86	124 ± 11·2	6

[a] Responses were recorded isometrically in Krebs-bicarbonate solution with Grass FT. 03C force-displacement transducers and Grass polygraph (Model 5D).
[b] Responses at various times were determined with a maximum challenging concentration of noradrenaline. Cumulative dose-response curves with the rat vas gave the same kinetics.
[c] Tissues were set up for recording isotonic contractions in Krebs bicarbonate by the method described previously (Moran & others, 1969). First order kinetics of recovery were observed for at least 70% of the recovery of response. Hukovič's solution (Hukovič, 1961) was used with the guinea-pig vas to minimize spontaneous contractions.
[d] Isotonic and isometric recording yielded identical data which have been pooled.
[e] Responses were followed by cumulative dose-response curves with noradrenaline.

The comparatively small decreases in rate shown in Table 1 may, therefore, represent an increasingly hydrophobic binding site for DMPEA.

Extension of this conclusion to the noradrenaline binding site of the α-receptor hinges upon the question of identity of binding sites for the agonist and antagonist. For the series of antagonists related to DMPEA several lines of evidence (Chapman & Graham, 1967; Moran & Triggle, 1970; Triggle, 1971) suggest at least a partial identity. Hence, the structural differences reflected in the data of Table 1 may reflect differences at or very close to the noradrenaline binding site of the α-receptor.

This work was supported by a grant from the National Institutes of Health (NS 09573).

Department of Biochemical Pharmacology,
School of Pharmacy, State University of
New York at Buffalo, Buffalo, N.Y., 14214, U.S.A.

R. A. JANIS
D. J. TRIGGLE

June 16, 1971

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Nikethamide and doxapram effects on pentazocine- and morphine-induced respiratory depression

Hunter & Major (1970) recently described nikethamide reversal of pentazocine- and morphine-induced respiratory depression in rabbits. These authors were concerned with pentazocine depression because it is not antagonized by nalorphine but is reversed by naloxone, a narcotic antagonist not yet available in the United Kingdom.

We now report a comparison of doxapram* and nikethamide in dogs anaesthetized with phenobarbitone sodium, 125 mg/kg. All drugs were administered intravenously. Respiratory parameters were recorded with a pneumotachograph, a volumetric pressure transducer, a unit integrator, and a polygraph. The respiratory stimulants were given 10 min after the analgesics, when respiratory depression was near its maximum. Changes (mean differences) produced by this treatment were considered significant when $P<0.05$ (Students' *t*-test).

Table 1. *Effects*[1] *of nikethamide and doxapram on pentazocine- and morphine-induced respiratory depression in anaesthetized dogs*[2,3].

Group	After i.v. injection of	minute volume Mean	s.e.	*P*	Respiratory rate Mean	s.e.	*P*	amplitude Mean	s.e.	*P*
1	morphine	43·8	6·0	>0·05	92·5	16·8	>0·5	67·7	14·0	>0·2
	nikethamide	60·8	5·0		79·8	9·2		88·7	15·5	
2	pentazocine	45·2	2·3	>0·5	51·3	4·3	>0·5	126·2	13·8	0·5
	nikethamide	47·7	3·8		45·7	7·1		140·8	15·6	
3	morphine	38·2	4·8	<0·005	54·5	8·9	<0·01	80·8	9·6	>0·05
	doxapram	87·3	12·4		93·7	7·3		111·8	13·0	
4	pentazocine	48·8	1·0	<0·001	52·5	6·2	<0·001	93·2	14·8	>0·2
	doxapram	138·7	15·1		117·8	11·3		117·5	16·1	

[1] expressed as % of control values.
[2] six dogs at each of the four test combinations.
[3] doses: nikethamide, 25 mg/kg; doxapram, 5 mg/kg; pentazocine, 8 mg/kg; morphine, 2 mg/kg (except one dog that received 4 mg/kg).

The results are summarized in Table 1. Respiratory minute volume was decreased by both pentazocine and morphine to about 45% of control values. Subsequent administration of nikethamide did not significantly increase minute volume that had been decreased by either analgesic. In contrast, doxapram significantly improved the respiratory depression that was induced by both analgesics as observed by the increase in minute volume. Respiratory amplitude was not reduced by pentazocine or morphine and it was not enhanced by either nikethamide or doxapram. Nikethamide had no effect on respiratory rate, but doxapram significantly antagonized both pentazocine- and morphine-induced bradypynoea.

Doxapram holds an important advantage over narcotic antagonists also in that it can maintain adequate respiratory function without detectably decreasing the pain-relieving action of narcotic analgesics Newell, Watson & others (1969).

A. H. Robins Research Laboratories,
1211 Sherwood Avenue,
Richmond, Virginia, U.S.A., 23220.

BERNARD V. FRANKO
JOHN W. WARD

April 12, 1971

* Doxapram hydrochloride monohydrate (Dopram, A. H. Robins Co.): to be available in the United Kingdom.

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Antagonism of (+)-amphetamine-induced hyperthermia in rats by pimozide

Whether hyperthermia induced by amphetamine in rats is due to a central or peripheral site of action is still unanswered. Hessa, Clay & Brodie (1969) stated that amphetamine-induced hyperthermia in rats is due to a peripheral site, whereas Hill & Horita (1970) attributed the hyperthermia in rabbits to a central site. The peripheral action was attributed to increased concentrations of plasma free fatty acid. However, we have demonstrated that increased plasma free fatty acid concentrations are not an integral part of hyperthermia observed after amphetamine administration in rats (Matsumoto & Shaw, 1971). We now present evidence for a central component of amphetamine-induced hyperthermia.

Male Wistar rats (Harlan Industries, Indianapolis, Indiana), approximately 175 g, were housed five per cage of 25 × 25 × 15 cm. After rectal temperatures were measured with a thermister probe (TRI-R), pimozide (10 mg/kg i.p, salt) was administered; 1 h later, (+)-amphetamine (5·52 mg/kg salt) was administered. Rectal temperatures were read at 30, 60, 120, 180 and 240 min after amphetamine,

Amphetamine increased body temperature from 36·8° by ~1° from 30 min to 2 h, and with a maximum at 1 h of 38·4°. At 3 h the temperature had fallen to 37·5° and was normal at 4 h and both saline and pimozide did not alter body temperature. However, pimozide effectively antagonized the hyperthermia due to amphetamine. According to Andén, Butcher & others (1970), pimozide antagonizes the action of dopamine in the cns. Moreover, Janssen, Niemegeers & others (1968) reported that pimozide is an effective antagonist of amphetamine's behavioral effects. Also, Costa & Groppetti (1971) reported that amphetamine increases the turnover of dopamine in the cns. Thus, the antagonism of amphetamine-induced hyperthermia by pimozide would be consistent with a central site of amphetamine's action and may involve a dopaminergic system.

The Lilly Research Laboratories,
Eli Lilly and Company,
Indianapolis, Indiana 46206, U.S.A.

CHARLES MATSUMOTO
WILLIAM GRIFFIN

May 26, 1971

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Variation in hydroxytryptamine metabolism in the rat: effects on the neurochemical response to phencyclidine

Lysergide reduces the turnover of 5-hydroxytryptamine (5-HT) in central neurons (Schubert, Nyback & Sedvall, 1970; Aghajanian, Warren & Sheard, 1968; Lin, Ngai & Costa, 1969; Sheard & Aghajanian, 1968). Lysergide was shown to produce a statistically significant increase in cerebral 5-HT concentrations in rats (Freedman & Giarman, 1962), and this increase was accompanied by decreased 5-hydroxyindoleacetic acid (5-HIAA) concentrations (Rosecrans, Lovell & Freedman, 1967). Tonge & Leonard (1969) demonstrated that four structurally dissimilar hallucinogenic drugs (phencyclidine, Ditran, lysergide and mescaline) all increased 5-HT and decreased 5-HIAA concentrations, though possibly by different mechanisms (Tonge & Leonard, 1970). But, some investigators failed to detect any increase in 5-HT concentrations after lysergide (Andén, Corrodi & others, 1968). We now report strain variation in the effects of at least one hallucinogen on 5-HT concentrations.

Drugs were administered by the intraperitoneal route to Wistar rats, 90–100 g, obtained from two sources. Animals were decapitated and 5-HT and 5-HIAA were determined in whole brain (less cerebellum). 5-HT was determined by the method of Snyder, Axelrod & Zweig (1965), 5-HIAA by that of Giacolone & Valzelli (1966) as modified by Tonge & Leonard (1969). In the presence of *p*-chlorophenylalanine, 5-HT was determined by the method of Bogdanski, Pletscher & others (1956).

The effects of phencyclidine (10 mg/kg) on brain 5-HT and 5-HIAA concentrations 30 min after injection are shown in Table 1.

Table 1. *The effects of phencyclidine* (10 mg/kg) *on brain 5-HT and 5-HIAA concentrations in Wistar rats from two sources.*

Time after injection (min)	Source A		Source B	
	5-HT	5-HIAA	5-HT	5-HIAA
	(n mol/g)		(n mol/g)	
0	2·68 ± 0·03	1·78 ± 0·01	2·38 ± 0·04	0·68 ± 0·02
30	***3·81 ± 0·06	**1·31 ± 0·02	**1·94 ± 0·05	**1·00 ± 0·01

Results are the means of 15 estimations ± s.e. Significance (Student's *t* test) is shown as: $*P = <0{\cdot}05$, $**P = <0{\cdot}01$, $***P = <0{\cdot}001$.

Table 1 shows that a dose of 10 mg/kg of phencyclidine caused an increase in 5-HT and decrease in 5-HIAA concentrations in rats from source A; directly opposite results were obtained from animals of source B.

These results suggested that there might be differences in the normal intraneuronal metabolism of 5-HT in the two sources. Both the absolute concentrations of 5-HIAA and the ratio of 5-HIAA to 5-HT are different: in source A, 5-HIAA : 5-HT = 0·66; in source B, 5-HIAA : 5-HT = 0·29.

The effects on brain 5-HT concentrations of *p*-chlorophenylalanine (PCPA) (400 mg/kg) in animals from the two sources at 0 and 16 min are (nmol/g): 2·95 ± 0·08 and ***0·89 ± 0·11 for source A and 2·93 ± 0·06 and **2·05 ± 0·07 for source B animals (significance as in Table 1; means of five estimations ± s.e.).

The difference in the rates of depletion of 5-HT after synthesis block suggests a possible explanation of the substrain variation in the response to phencyclidine.

SALLY R. TONGE

School of Pharmacy,
Liverpool Polytechnic,
Byrom Street,
Liverpool, U.K.

Pharmacology Section,
ICI Ltd.,
Pharmaceuticals Division,
Alderley Park, Macclesfield, Cheshire, U.K.

B. E. LEONARD

April 15, 1971

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On the mechanism of papaverine action on the control of vascular smooth muscle contractile activity by extracellular calcium

It has been suggested recently that the relaxing effects of papaverine on smooth muscle might be exerted through cyclic phosphodiesterase (PDE) inhibition and consecutive accumulation of cyclic-3′,5′-AMP (Triner, Vulliemoz & others, 1970). Since relaxation probably follows the decrease of intracellular free Ca^{2+} ions, it still remains to be explained how cyclic AMP influences calcium movements (Stoclet, Peguet & Waeldele, 1971). Kukovetz & Pöch (1970) suggested that cyclic AMP probably increases Ca^{2+} uptake by the membranes and might enhance active Na-exclusion in vascular smooth muscle.

We now report the influence of papaverine and exogenous cyclic AMP (*N*-2′-*O*-dibutyryl adenosine-3′,5′-monophosphate) on noradrenaline-induced isometric responses of isolated aortic strips from the rat in different extracellular calcium concentration salines.

The thoracic aortae of 10 to 12 weeks old male rats (EOPS OFA) were removed from the left carotid to the diaphragm and prepared (Godfraind & Kaba, 1969). Isometric responses elicited by noradrenaline ($7{\cdot}5 \times 1^{-7}$M) were recorded under an initial tension of 1 g. Papaverine or cyclic AMP were added to the bath 10 min before noradrenaline. The reference response of each aortic strip was obtained in "normal" Krebs-bicarbonate medium (Ca content 2·5 mM). Except when $CaCl_2$ was added during the record of noradrenaline effect, any change in calcium concentration of the saline was followed by a period of equilibration to obtain a constant response to noradrenaline. With calcium concentrations lower than normal, the aortae were previously depleted by incubations for 1 h in calcium-free Krebs bicarbonate containing ethylene glycol bis amino-2-ethylether-*NN*′-tetra-acetic acid (EGTA, 1 mM).

The response elicited by noradrenaline included a phasic component which was not directly influenced by extracellular calcium concentration, and a tonic component which was sustained for 20 min or more and varied with extracellular calcium concentration. It increased with calcium concentration rising from 0 to 2·5 mM and decreased slightly when calcium concentration further rose.

Papaverine inhibition of noradrenaline-induced vasoconstriction was characterized by the decrease with time of the tonic component. This effect appeared at a concentration of papaverine (5×10^{-6}M) which decreased only moderately both phasic and tonic components during the first minutes of noradrenaline action. Both these components were more completely and readily depressed by higher concentrations of papaverine, but for 5×10^{-6}M the tension stayed at a level which was approximately constant for some time. Both this plateau duration and the slope of the later relaxation varied with extracellular calcium concentration. In 1·25 mM Ca^{2+} Krebs bicarbonate, the tonic component was more rapidly abolished than in normal or 5 mM Ca^{2+} saline. A sudden increase of calcium concentration up to 10 mM just after the plateau strongly increased the rate of relaxation caused by papaverine.

Exogenous cyclic AMP mimicked papaverine's inhibitory effects, but induced rhythmic activity during relaxation and reduced to a lesser extent the maximal tension during the tonic component of the response. The rate of relaxation was strongly increased and the rhythmic activity abolished by raising extracellular calcium up to 10 mM.

Although the direct proof (actual determination of cyclic AMP) is still lacking, these results bring further evidence that papaverine exerts its inhibitory effects on vascular smooth muscle through the increase of cyclic AMP concentration. The qualitative differences between papaverine and dibutyryl-cyclic AMP action might correspond to differences of cellular repartition of cyclic AMP from endogenous or exogenous origin.

Both papaverine and cyclic AMP enhanced the inhibitory effect of calcium on contractile activity. Since this inhibitory effect is probably the consequence of membrane "stabilization", a tentative hypothesis is that cyclic AMP decreased or abolished the coupling between excitation and contraction by increasing the calcium uptake by the membrane of vascular smooth muscle. This membrane action of cyclic AMP might play a role in the control of contractile activity by extracellular calcium and occur simultaneously with the modification of actomyosin responses reported by Uchida & Mommaerts (1963).

Laboratoire de Pharmacodynamie,
U.E.R. de Sciences pharmaceutiques,
Université Louis Pasteur,
67-*Strasbourg, France.*

F. DEMESY
J. C. STOCLET

April 14, 1971

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Physical performance of mice treated with propranolol, sotalol and INPEA

I have made a comparative study of the behavioural effects of three adrenergic β-receptor blocking drugs frequently used in animal experiments.

Adult NMRI mice of either sex were allowed free access to a standard diet (Altromin R) and tap water and were housed at 25°. They were treated intraperitoneally with (±)-propranolol hydrochloride (±)-sotalol [4-(isopropylamino-1-hydroxyethyl) methane sulphonanilide HCl] or D-(−)-INPEA [1-(4′-nitrophenyl)-2-isopropylamino-ethanol HCl].

Spontaneous motor activity of single mice was recorded for 2 h in circular activity cages (Estler & Ammon, 1969) immediately after application of the drugs. Spontaneous orientational hypermotility of single mice was measured in the Basile activity cage (Estler & Ammon, 1969) for 15 min starting 30 min after the application of the drugs. Sedation or ataxia was tested on a sloping plane. The principle of this test was modified in such a way that quantitative data could be obtained. For this purpose the animals were placed on a small board which could be turned slowly from a horizontal to a vertical plane. The angle at which the animals could no longer cling to the board was registered, This test was made 45 min after administration of the drugs. The traction test (Julou, 1956), which also gives a measure for sedation or ataxia was used 45 min after injection of the drugs.

Mean values from 20–24 single determinations showed that propranolol (1, 5 and 20 μg/g) and sotalol (1·5 and 25 μg/g) did not change the spontaneous motor activity of mice, when compared with saline treated controls INPEA) 5 and 25 μg/g), was likewise ineffective, bnt at 100 μg/g it increased the motility by 60–230%. The maximum effect was seen 60 min after drug administration.

Orientational hypermotility was depressed by 1,5 and 20 μg/g of propranolol ($P < 0{\cdot}05$). Lower doses were ineffective. Sotalol had a biphasic effect: 0·05 and 0·2 μg/g slightly increased and 25 μg/g reduced the hypermotility. INPEA was ineffective at all doses (0·01–100 μg/g). (Results were from 20 single determinations).

Propranolol (0·01–20 μg/g) and sotalol (0·01–25 μg/g) did not affect the behaviour of the animals on the sloping plane. INPEA at the highest dose (100 μg/g) slightly but significantly ($P < 0{\cdot}05$) impaired the performance of the animals in this test (68° instead of 76° in the control group). In the traction test 0·01–20 μg/g propranolol and 0·01–25 μg/g sotalol were ineffective. After 100 μg/g of INEPA 90% negative results were recorded as compared with 7% (confidence limits 1–29%) in the control groups. This effect of INPEA must be ascribed to impaired muscular coordination of the exited and hyperactive animals. The lower doses of INPEA (0·01–25 μg/g) were ineffective.

The test for orientational hypermotility, which I found to be the most sensitive test for detecting central depressant properties, showed propranolol, in doses that are known to exert β-adrenergic blocking effects, to have some sedative properties. This agrees with the observations of others: (Leszkovsky & Tardos, 1965, Murmann, Almirante & Saccani-Guelfi, 1966; Estler & Ammon, 1969). INPEA in high doses, on the other hand, shows distinct central stimulating properties, as was also suggested by Murmann & others (1966). In contrast to Lish, Weikel & Dungan (1965) a sedative effect could be seen after 25 μg/g of sotalol, whereas lower doses of this drug appeared to increase orientational hypermotility, an effect just above the significance level ($P = 0{\cdot}05$).

Propranolol was kindly supplied by Dr. H. P. Kuemmerle, Rhein Pharma GmbH, Heidelberg, Germany, INPEA by Dr. W. Murmann, Selvi e C., Milan, Italy, and sotalol by Dr. P. M. Lish, Mead Johnson Res. Ctr., Evansville, Ind., U.S.A.

Pharmakologisches Institut C.-J. ESTLER
der Universität Erlangen-Nürnberg,
Universitätsstrasse, 22,
D-8520 Erlangen, Germany.

April 26, 1971

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Inhibition of (+)-amphetamine hyperthermia by blockade of dopamine receptors in rabbits

The hyperthermia produced in rabbits by (+)-amphetamine is apparently due to an action of the drug on the CNS (Hill, 1971). Indications for this central action include the findings that (+)-amphetamine hyperthermia in this species is markedly reduced by prior curarization (Belenky & Vitolina, 1962) or spinal section (Hill & Horita, 1970) but is not diminished by blockade of β- or peripheral α-adrenergic receptors (Hill & Horita, 1970). Other evidence suggests that (+)-amphetamine might produce hyperthermia by influencing a central dopaminergic system. For example, several of the neuroleptic drugs antagonize (+)-amphetamine hyperthermia in rats (Morpurgo & Theobald, 1967). These and other neuroleptics were later found to be potent inhibitors of central dopaminergic function (Andén, Butcher & others, 1970). That low doses of (+)-amphetamine can elevate both body temperature and the turnover rate of brain dopamine without altering the turnover rate of brain noradrenaline in the rat further implicates dopamine as the neurochemical concerned in (+)-amphetamine-induced hyperthermia (Costa & Groppetti, 1970).

However, it is difficult to determine from such information whether dopamine receptor activation is necessary for production of hyperthermia by (+)-amphetamine. The neuroleptics employed by Morpurgo & Theobald (1967) are known to also block central and peripheral α-adrenergic receptors (Janssen, Niemegeers & others, 1968; Andén & others, 1970). Further, an increased turnover rate of dopamine does not necessarily imply increased dopamine receptor activation. A more direct means of evaluating the possible involvement of central dopamine receptors in the production of hyperthermia by (+)-amphetamine is to assess the effect of specific dopamine antagonists on this response. Since pimozide* had been shown to selectively inactivate dopamine receptors in the CNS (Andén & others, 1970), the ability of this drug to antagonize (+)-amphetamine hyperthermia was investigated.

Male New Zealand rabbits (1·8–2·0 kg) received an injection of either pimozide or the pimozide solvent (dilute tartaric acid) intraperitoneally 3 h before intravenous injection of (+)-amphetamine or saline. Sedation and catalepsy were evident 30 min after pimozide administration, reached maximal intensity at about 2 h and persisted for more than 12 h in rabbits receiving no (+)-amphetamine. In addition, these animals exhibited marked and continuous miosis. Administration of (+)-amphetamine 3 h after pimozide resulted in a transient increase in pupillary size and motor activity. Sedation, catalepsy, and miosis were again evident 15 min later and persisted for more than 12 h.

* 1-{1-[4,4-bis(*p*-fluorophenyl)butyl]-4-piperidyl}-2-benzimidazolinone.

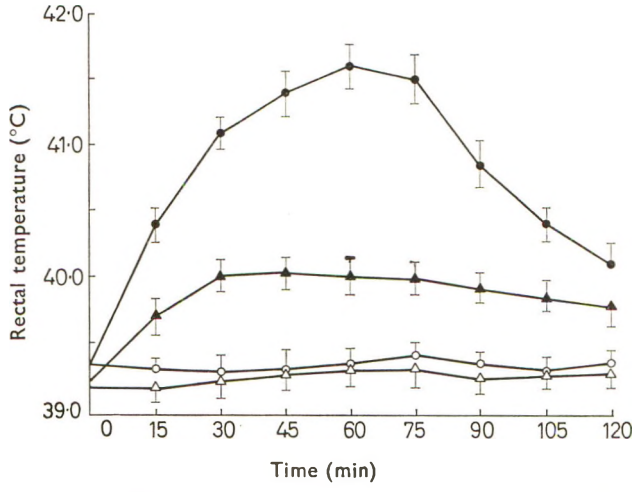

FIG. 1. The effect of pimozide on the time course of (+)-amphetamine-induced hyperthermia. Rabbits were pretreated with pimozide solvent (dilute tartaric acid) or pimozide solution (4 mg/kg pimozide i.p.) 3 h before to injection of (+)-amphetamine (5 mg/kg, i.v.) or saline. Each curve represents the mean rectal temperatures of 6 rabbits receiving: solvent before saline (○), pimozide before saline (△), solvent before (+)-amphetamine (●), and pimozide before (+)-amphetamine (▲). The vertical lines indicate s.e.

The effect of (+)-amphetamine on rectal temperature was examined in rabbits treated 3 h previously with pimozide (Fig. 1). (+)-Amphetamine produced significantly less elevation of rectal temperature ($P < 0{\cdot}01$) in pimozide-treated compared to solvent-treated rabbits at all but the last observation time (120 min). Peak hyperthermia was reduced by about 70% by pimozide pretreatment ($P < 0{\cdot}005$). Although the time required to reach maximal temperature after (+)-amphetamine injection was not significantly different in pimozide- or solvent-treated rabbits, the *rate* of temperature increase was decreased by pimozide. Saline injection had no effect on rectal temperature of rabbits with either pretreatment. These observations suggest that pimozide inhibits (+)-amphetamine hyperthermia by preventing activation of heat-generating mechanisms and not by enhancing heat-dissipation.

Pimozide has been shown to be a persistent antagonist of many apparently central dopaminergic functions (Janssen & others, 1968; Andén & others, 1970; Janssen, 1970; Fuxe, 1970). Whether the antagonism by pimozide of (+)-amphetamine hyperthermia was similarly persistent, was investigated in rabbits pretreated with saline or pimozide and then injected with (+)-amphetamine (5 mg/kg) at three 4 hourly intervals. The results indicate that a single injection of pimozide can inhibit (+)-amphetamine hyperthermia for at least 16 h. Cns excitatory effects of (+)-amphetamine (increased motor activity and chewing) were also inhibited throughout the experiment. No tolerance to (+)-amphetamine hyperthermia developed with the dosage regimen employed. This observation contrasts with the results of Gessa, Clay & Brodie (1968) which showed marked tolerance development in the rat with similar doses (5 mg/kg at 6 h intervals). Since development of tolerance to (+)-amphetamine appears to be related to neuronal accumulation of a *para*-hydroxylated "catabolite" in the rat (Brodie, Cho & Gessa, 1970), the fact that the rabbit produces very little *p*-hydroxyamphetamine from a given dose of (+)-amphetamine (Axelrod, 1954; Dring, Smith & Williams, 1966) might be the basis for this species difference in tolerance development.

Throughout these experiments, we noted that pimozide produced miosis which was only transiently reversed by (+)-amphetamine. This raised the possibility that pimozide

was acting as a competitive antagonist at peripheral α-receptors. Although we had previously reported that such receptors were probably not involved in the hyperthermic response to (+)-amphetamine, it was desirable to determine whether pimozide possessed α-adrenergic blocking activity in this species. Rabbits were anaesthetized with pentobarbitone and mechanically ventilated through a tracheal cannula. Blood pressure was monitored via a cannula in the left femoral artery. Solvent or pimozide (4 mg/kg, i.p.) was administered 2 h before intravenous injection of 5 μg adrenaline. Drug effects on blood pressure and the pressor response were as follows: saline, 82 and 33 mmHg; pimozide, 77 and 35 mmHg; phentolamine, 5 mg/kg, 65 and 8 mmHg. Pimozide was found to have perhaps a slight hypotensive effect. However, this drug did not inhibit the pressor response to noradrenaline. As usual, phentolamine inhibited the pressor response. Therefore, it appears that blockade of peripheral α-receptors is not the basis of the antagonism of (+)-amphetamine hyperthermia by pimozide.

These preliminary observations indicate that pimozide, a potent and selective inhibitor of central dopaminergic functions, is a potent antagonist of (+)-amphetamine hyperthermia in rabbits. Since pimozide is believed to exert its inhibitory effect at the receptor level (Andén & others, 1970), our results suggest that operational dopamine receptors are necessary for the production of hyperthermia by (+)-amphetamine.

This work was supported by grant MH02435 of the Institute of Mental Health, National Institutes of Health, U.S. Public Health Service.

We wish to thank Dr. Paul A. J. Janssen (Janssen Pharmaceutica, Beerse) for his generous gift of pimozide.

Department of Pharmacology,
School of Medicine,
University of Washington,
Seattle, Washington, 98105, U.S.A.

H. F. HILL
A. HORITA

May 27, 1971

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Reversal by sotalol of the respiratory depression induced in mice by ethanol

Smith & Hayashida (1970) reported that treatment with a β-adrenoceptive antagonist inhibited respiratory depression in mice subsequently given graded doses of ethanol, and correlated with this effect was a shortening of the sleeping time (Smith, Hayashida & Kim, 1970). While prevention of ethanol intoxication is of interest, reversal of the central depression would imply a therapeutic potential.

Swiss-Webster female mice, 18–22 g, had food and water available up to the time of the experiment. Groups of at least 10 mice for each dose were then injected with ethanol as a 25% solution (w/v) in normal saline, in doses ranging from 1 to 5 g/kg, The groups receiving only ethanol solution were tested 45 min after injection. An incision was then made in the ventral surface of the proximal third of the tail and the capillary blood collected into 125 μl heparinized tubes without significant air exposure. The contents of the tube were then transferred to a Radiometer microelectrode system for determination of the pH and pCO_2.

A dose-related fall in pH with a concomitant rise in pCO_2 was found (Fig. 1). The horizontal bars indicate the standard error. The curves are plotted in a linear fashion to allow comparison with normal values and the influence of the drug on control pH or pCO_2. Sotalol, (MJ 1999) 10 mg/kg, was given in aqueous solution 15 min after ethanol, at a time when many mice injected with ethanol were already asleep. Thirty min later capillary blood was obtained from the tails. Mice develop maximal respiratory depression about 30 min after parenteral injection of a moderate dose of ethanol. The pH and pCO_2 changes begin within minutes after ethanol injection. In the mice treated with sotalol, capillary blood pH did not fall except at the highest doses of ethanol, while pCO_2 rose only slightly.

The fact that mice treated with large doses of ethanol were already narcotized at the time of sotalol injection suggests that the drug acts not by preventing entry of ethanol into the brain but by antagonizing some action of ethanol or possibly acetaldehyde. Since the respiratory depressive effect of ethanol is closely related to dose, whereas acetaldehyde concentration is probably constant, it would seem that ethanol alone depresses respiration.

The sleeping time was slightly attenuated in mice treated with ethanol (4 g/kg) and subsequently with sotalol (40 $\pm$ 3·9 min versus 30 $\pm$ 3·6 min). There was however, no shortening of sleeping time for mice given the larger dose of 5 g/kg despite relief of the respiratory depression and acidosis. Evidently respiratory depression and narcosis are not equivalents.

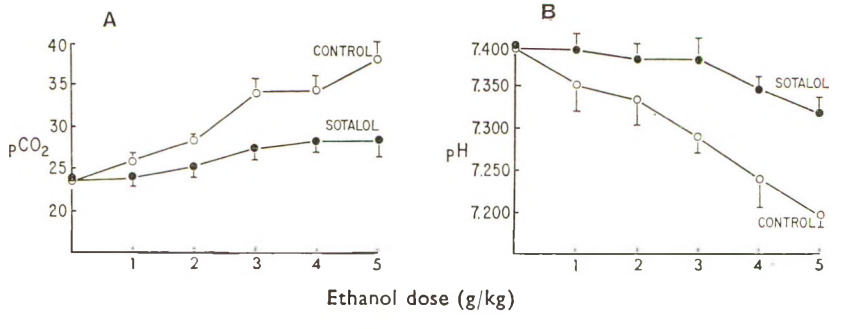

FIG. 1. Capillary blood pH and pCO_2 (mm) of mice 45 min after injection of graded doses of ethanol. Sotalol (MJ 1999) was given 15 min after ethanol. Horizontal bar represents s.e.

Departments of Anesthesiology and Pharmacology, New York Medical College, New York 10029, U.S.A.

KIKUE HAYASHIDA
ALFRED A. SMITH

June 8, 1971

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The fluorimetric determination of thymoxamine in plasma

Thymoxamine hydrochloride, a specific competitive α-adrenoreceptor blocking drug, and its deacetylated derivative have fluorescent properties which may be exploited for the purposes of recognition and measurement in body fluids and in pharmaceutical formulations. Thymoxamine hydrochloride fluoresces in de-ionized aqueous solution, the pH for maximum fluorescence lying between 7–8. Fluorescence falls more steeply towards alkaline than acid pH and there is another peak at pH 1–1·5 (Fig. 1). Desacetylthymoxamine hydrochloride has the same excitation and emission wavelengths as thymoxamine hydrochloride.

The pK_a values of thymoxamine were found to be about 8·6 and 2·0. Concentrations between 100 ng to 50 μg extracted from water gave a straight line relationship with fluorescence, beyond which quenching occurred. No change in fluorescence of the extract was observed for up to 24 h at room temperature (20°), while the aqueous solution continues to increase in fluorescence for at least this length of time. Benzene with 1·5% isoamylalcohol gave the most consistent recovery of the drug from standard aqueous and plasma solutions, when compared with heptane, chloroform and ethyl acetate. Materials used were:

Thymoxamine hydrochloride 50 mg capsules, solution for intravenous injection 5 mg/ml and powder (Opilon), (William Warner & Co.); desacetylthymoxamine hydrochloride powder, (William Warner & Co.); benzene and isoamylalcohol, Grade A, Analar, (May & Baker Ltd.); sodium hydroxide solution, 1 N in deionized water; and hydrochloric acid, 0·1 N in de-ionized water.

Standard thymoxamine solution was prepared from 10 mg of powder in 10 ml of de-ionized water.

Plasma (2·5 to 4 ml) separated from heparinized blood was extracted by shaking with 1 ml of 1N sodium hydroxide and 12 ml of benzene containing 1·5% isoamylalcohol for 10 min on an automatic shaker. After centrifugation, 10 ml of the supernatant organic layer were added to 1·5 ml of 0·1N hydrochloric acid in another set of stoppered centrifuge tubes for extraction of the drug into the acid phase by shaking for 10 min. After centrifugation, 1·2 ml of the acid phase from the bottom layer was transferred to test tubes and immersed in a boiling water bath for 30 min. The tubes were then cooled to room temperature and the fluorescence of the acid phase measured with an Aminco-Bowman spectrophotofluorimeter at maximum excitation 295 nm and maximum emission 335 nm (uncorrected).

For each subject, duplicate plasma samples were taken at different times before and after administration of the drug. Internal standards were obtained by adding known amounts of thymoxamine to plasma samples together with control blanks which did not contain the drug and these were treated in the same way.

A straight line relationship was obtained with concentrations between 0·2 and 1 μg of drug in plasma and its fluorescence (Fig. 2).

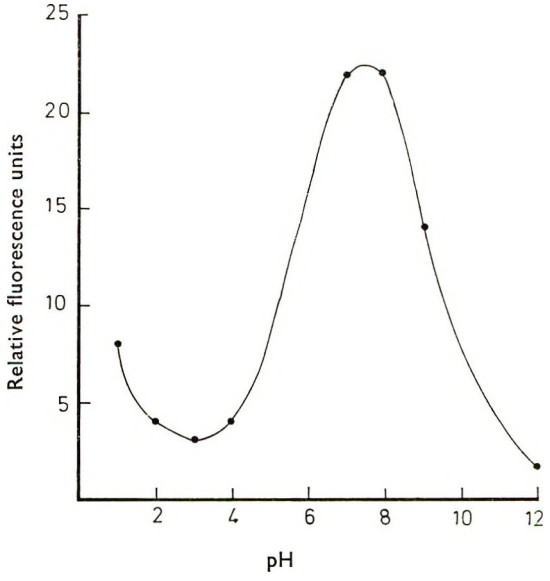

FIG. 1. Relation between fluorescence of a solution of thymoxamine hydrochloride (25 μg/ml in water) and its pH.

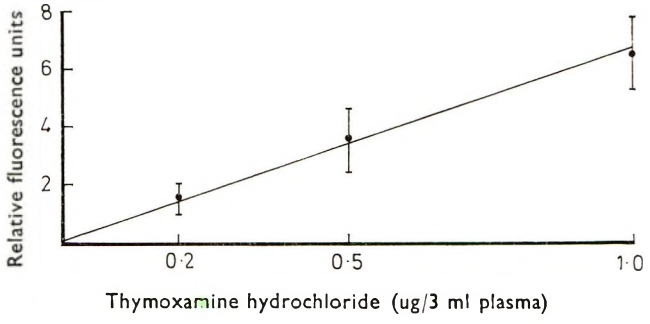

FIG. 2. Relation between concentration of thymoxamine hydrochloride added to plasma, and the fluorescence obtained after extraction. Each point represents a mean ± standard deviation of 16 observations.

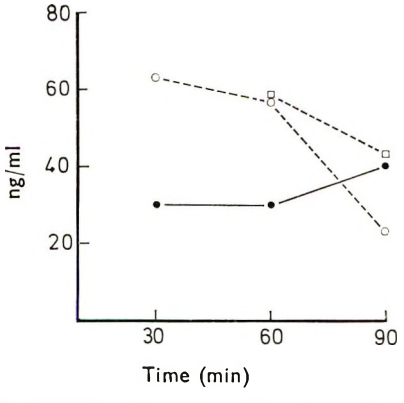

FIG. 3. Plasma levels (ng/ml) obtained at 30, 60 and 90 minutes after administration of thymoxamine hydrochloride 150 and 300 mg to three subjects. □, ○ 150 mg; ● 300 mg.

Four female volunteers (20–30 years) were given 150 mg of thymoxamine in capsule form as a single dose, half an hour after a standard lunch. Blood samples of 15 ml were taken in heparinized tubes before and at 30, 60 and 90 min after ingesting the drug; the procedure was repeated one week later with 300 mg of thymoxamine. In only two of the four subjects were blood concentrations detected after the 150 mg dose, and in these none was found after 300 mg. In one subject, in whom none was found after 150 mg, the drug was detected after 300 mg (Fig. 3).

Two male volunteers (33 years), lying down, were given 0·2 mg/kg of the drug intravenously over 2 min. In one volunteer it was given as a solution of strength 5 mg/ml, and in the other it was diluted in 20 ml of normal saline. Blood samples of 15 ml each were taken in heparinized tubes before and at 2, 7 and 15 min after the end of injection. Plasma concentrations at 2, 7 and 15 min were 140, 83 and 50 ng/ml respectively in the first subject, and 100, 40 and 30 ng/ml in the other.

The fluorescence of thymoxamine is much less than that of deacetylated thymoxamine, although their emission and excitation characteristics are similar. The demonstration that physical and chemical procedures such as boiling and acid hydrolysis increased the fluorescence of thymoxamine in water (Arbab, 1970, unpublished observations) suggests that it is the deacetylated form of thymoxamine which is being extracted and measured in the procedure described in this communication. Deacetylation may well be an important metabolic pathway for thymoxamine, and this extraction method does not distinguish the unchanged drug from its metabolite.

Unfortunately, the calibration curve relating fluorescence to plasma concentration with the extraction procedure described is shallow (Fig. 2), and does not permit accurate determinations of plasma concentrations at the nanogram amounts per ml obtained with therapeutic doses of the drug, but duplicate determinations provide at least a semi-quantitative measurement. The greater intensity of fluorescence at a pH of 7–8 than at the pH of about 1·5 used in this method suggests that the sensitivity of the procedure might be increased if it was modified to permit a final extraction at the alkaline pH.

We thank William Warner & Co. Ltd. for financial support and supplying thymoxamine hydrochloride and its deacetylated derivative. A.G.A. is in receipt of a Colombo Plan Scholarship.

Division of Clinical Pharmacology,
Medical Professorial Unit,
St. Bartholomew's Hospital,
London, E.C.1, U.K.

A. G. ARBAB
P. TURNER

May 21, 1971

Experimental gastric ulcers and uropepsinogen excretion in the rat

Excretion of uropepsinogen has been examined in animals and man (Müller & Braun, 1964; Calandra, Fonnesu & Cozzolino, 1965; Pastor Franco, 1966; Abbott, Harrisson & Brogle, 1967) and though it is generally accepted that its excretion rises in patients with gastric or duodenal ulcers, the value of the parameter as a diagnostic test has not been assessed.

Table 1. *Relation between gastric ulcers and uropepsinogen excretion in the rat.* The urines were collected for 18 h after the administration of reserpine or hydrochlorothiazide; after the second administration of phenylbutazone and the tenth injection of prednisolone; after 4 fasting days; after the plaster bandage and 24 h after the injection of CCl_4 or renal monolateral lesion respectively from 20 h fasted rats. Atropine and oxyphencyclimine were given simultaneously to the reserpine or to the second phenylbutazone administration. Morphological examination was made at the end of the urine collection.

Treatment	Uropepsinogen (mg tyrosine/rat/18 h) mean ± s.e.	Morphological examination Ulceration Index mean ± s.e.	% Ulcerated animals
Controls	1·65 ± 0·20 (20)	0·15 ± 0·08 (20)	15
Hydrochlorothiazide (5 mg/kg orally	1·51 ± 0·19 (20)	0·10 ± 0·07 (20)	10
Controls	1·53 ± 0·21 (20)	0 (20)	0
Renal monolateral	1·67 ± 0·17 (20)	0 (20)	0
Controls	1·70 ± 0·20 (20)	0 (20)	0
CCl_4 (4 mg/kg, i.p. as 1 : 1 v/v) mixture with olive oil)	1·73 ± 0·17 (20)	0 (20)	0
Controls	1·55 ± 0·20 (20)	0 (20)	0
Plaster bandages	2·53 ± 0·21 (20)*	3·25 ± 0·28 (16)	100
Controls	1·38 ± 0·12 (20)	0·10 ± 0·07 (18)	10
Fasting (4 days)	2·49 ± 0·16 (20)*	1·74 ± 0·40 (19)	63·1
Controls	0·78 ± 0·09 (10)	0 (10)	0
Prednisolone (10 mg/kg daily s.c. for 10 days)	1·28 ± 0·11 (10)*	2·00 ± 0·44 (10)	80
Controls	1·91 ± 0·18 (20)	0 (20)	0
Reserpine (5 mg/kg, i.p.)	4·02 ± 0·27 (20)*	2·35 ± 0·23 (20)	100
Reserpine (5 mg/kg, i.p.) + atropine (10 mg/kg orally)	2·78 ± 0·42 (20)*†	0·90 ± 0·23 (20)*	95
Reserpine (5 mg/kg, i.p.) + oxyphencyclimine (10 mg/kg orally)	2·22 ± 0·30 (20)†	0·35 ± 0·13 (20)*	45
Controls	1·03 ± 0·14 (20)	0 (20)	0
Phenylbutazone (100 mg/kg orally twice in 8 h)	2·55 ± 0·25 (20)*	3·40 ± 0·21 (20)	100
Phenylbutazone (100 mg/kg orally twice in 8 h) + atropine (10 mg/kg orally)	1·28 ± 0·07 (20)†	2·05 ± 0·33 (20)*	85
Phenylbutazone (100 mg/kg orally twice in 8 h) + Oxyphencyclimine (10 mg/kg orally)	1·24 ± 0·05 (20)†	1·90 ± 0·38 (20)*	65

* Significance relative corresponding control group, † Significance relative to reserpine or phenylbutazone treated group ($P \leqslant 0·01$). Numbers of animals are in parentheses.

We now report the results of experiments made in rats in which different kinds of experimental gastric ulcers have been induced and in some instances treated with anti-acetylcholine drugs. We also examined the effect of the diuretic hydrochlorothiazide on the rate of uropepsinogen excretion in normal rats, and also the influence of lesions other than gastric ulcers on the assay of acid proteolytic enzyme activity in the urine of rats with hepatic or renal damage.

Male or female Sprague-Dawley rats, 160–170 g, had gastric ulcers produced by reserpine (Radouco-Thomas, Lataste-Dorolle & others, 1960), by phenylbutazone, by prednisolone, by fasting or by immobilization in plaster bandages (Müller & Braun, 1964). Groups of rats with reserpine- or phenylbutazone-induced ulcers were treated orally with atropine sulphate or oxyphencyclimine (1-methyl-1,4,5,6-tetrahydro-2-pyrimidylmethyl-α-cyclohexyl-α-phenylglycolate hydrochloride) both solubilized in 5% acacia gum. Further groups of normal rats were treated with hydrochlorothiazide; the hepatic damage was produced by CCl_4 and a monolateral renal lesion according to Coppi, Bonardi & Fresia, (1966). Uropepsinogen activity was measured with the haemoglobin method of Anson (1963) and the amount of uropepsinogen excreted was expressed as mg of tyrosine/rat per 18 h. On additional groups of rats in the same experimental conditions the number of animals with gastric lesions and the severity of these graded by an arbitrary scale from 0 to 4+ was recorded. All the results were statistically evaluated by the Student's *t*-test.

Hydrochlorothiazide, CCl_4 or renal lesion did not affect the uropepsinogen excretion or the integrity of the gastric mucosa (Table 1).

The excretion of uropepsinogen always increases in animals with any gastric ulcers, however induced. Oxyphencyclimine and, to a lesser extent, atropine decreased the ulceration index as well as the excretion of uropepsinogen in the animals given reserpine or phenylbutazone. In these groups there was a good relation between the enzyme excretion and the severity of gastric lesions, although the two parameters were not always in proportion.

Thus the evaluation of the uropepsinogen excretion, together with a morphological examination, could provide a useful tool in the screening of anti-ulcer drugs.

Research Laboratories of Istituto De Angeli,
Via Serio 15, Milan,
Italy.

G. COPPI
G. BONARDI
M. GAETANI

June 14, 1971

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The effect of amantadine on spontaneous locomotor activity in the rat

Direct observation of mice (Vernier, Harmon & others, 1969) and rats (Abuzzahab, 1971) after injection of the new anti-parkinsonian agent, amantadine, has suggested that this drug increases spontaneous locomotor activity, but more objective measures have failed to confirm this increase in mice treated with 100 mg/kg (Svensson & Stromberg, 1970) or in rats treated with 10 or 100 mg/kg (Abuzzahab, 1971). We have investigated the effect of various doses of this compound on spontaneous locomotor activity and have found a significant increase in activity which is dose and time dependent.

Male Wistar rats (Woodlyn Farms, Ontario, Canada), 275–325 g, were injected subcutaneously with amantadine hydrochloride (25, 50 or 100 mg/kg of the salt in a volume of 1 mg/kg of distilled water), or with distilled water. One half hour after the injection, each animal was placed in a circular cage (14″ in diameter) equipped with two sets of photocells which recorded one count each time a photobeam was broken. Counts were automatically recorded every half hour for 3 h.

Amantadine produced a significant dose-related increase in activity ($F = 7{\cdot}96$, d.f. $= 3, 44$, $P < 0{\cdot}01$). All doses used produced a significant increase in the cumulative 3 h activity (25 mg/kg: $t = 2{\cdot}19$, d.f. $= 22$, $P < 0{\cdot}05$; 50 mg/kg: $t = 4{\cdot}23$, d.f. $= 22$, $P < 0{\cdot}01$; 100 mg/kg: $t = 2{\cdot}94$, d.f. $= 22$, $P < 0{\cdot}01$).

The effect of the various doses of amantadine across time varied (Fig. 1). Amantadine at 25 mg/kg produced a significant increase in activity only in the early part of the 3-h period. The 50 mg/kg dose produced a significant effect throughout the period, while the 100 mg/kg group was consistently more active only during the last hour. The long latency required for the 100 mg/kg dose to elicit locomotor stimulation may have been due to toxic effects and may account for the failure by Abuzzahab (1971) to find a significant increase in activity.

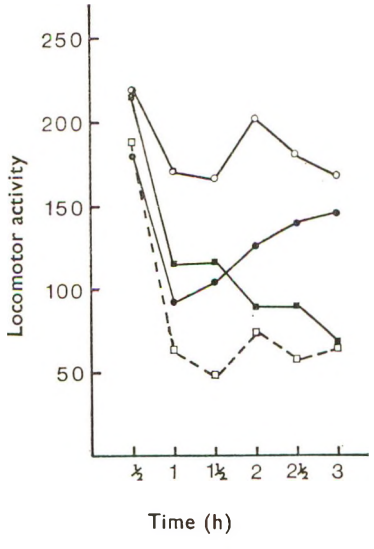

FIG. 1. The effect of amantadine HCl on spontaneous locomotor activity (30 min periods) over 3 h. Each point represents the mean of 12 animals. Control □ - - - □; 25 mg/kg ■——■; 50 mg/kg ○——○; 100 mg/kg ●——●.

These results indicate that amantadine HCl has a significant stimulant effect on locomotor activity in rats. In mice, amantadine appears to increase spontaneous locomotor activity only after reserpine pretreatment (Svensson & Stromberg, 1970; Stromberg, Svensson & Waldeck, 1970). The mechanism by which amantadine exerts this effect is not clearly understood. It is interesting, however, that this drug has recently been shown to resemble (+)-amphetamine in some of its actions on dopamine and noradrenaline metabolism in brain (Scatton, Cheramy & others, 1970; Stromberg & others, 1970). α-Methyl-*p*-tyrosine, a drug which is known to block amphetamine-mediated locomotor stimulation (Weissman, Koe & Tenen, 1966) also appears to antagonize amantadine-mediated excitation in rats (Fibiger, Fox, McGeer and McGeer, unpublished observations) and in reserpine pretreated mice (Stromberg & others, 1970).

We were impressed by the variability of the response to amantadine, a point also discussed by Abuzzahab (1971) both in locomotor activity and in brain dopamine concentrations.

We wish to thank E. I. Du Pont de Nemours & Co. (Inc.) Wilmington, Delaware, U.S.A., for amantadine hydrochloride (Symmetrel).

This work was supported by Medical Research Council of Canada Grants MA-3633 and MA-4013 and an MRC Fellowship to one of us (HCF).

The Kinsmen Laboratory of Neurological Research,
Department of Psychiatry,
The University of British Columbia,
Vancouver 8, B.C., Canada.

H. C. FIBIGER
M. FOX
E. G. MCGEER
P. L. MCGEER

May 13, 1971

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An automated method for the determination of dissolution rate and urinary concentration of sulphonamides

Several methods of determining dissolution rate have been automated (Schroeter & Wagner, 1962; Niebergall & Goyan, 1963; Michaels, Greely & others, 1965). We have developed a method similar to that described by Barzilay & Hersey (1968) except that our method uses an AutoAnalyzer dialyser.

Determination of sulphonamides in blood by means of automated methods has been described by Falk & Kelly (1965) and Probst, Rehm & others (1965), the sulphonamide being diazotized and coupled according to the procedure of Bratton & Marshall (1939) on which our automated procedure is also based. We have compared manual and automated procedures in the assessment of dissolution rates and urine concentrations of sulphonamides.

Sulphathiazole was dissolved, with heat if necessary, in fresh, protein-free urine to give concentrations of 5, 10, 25, 50, 75 and 100 mg%. Also, the urine collected under normal urine conditions (Goossens & van Oudtshoorn, 1970) from subjects who had ingested 500 mg sulphathiazole (tablet) was analysed both for free and total sulphathiazole by both methods.

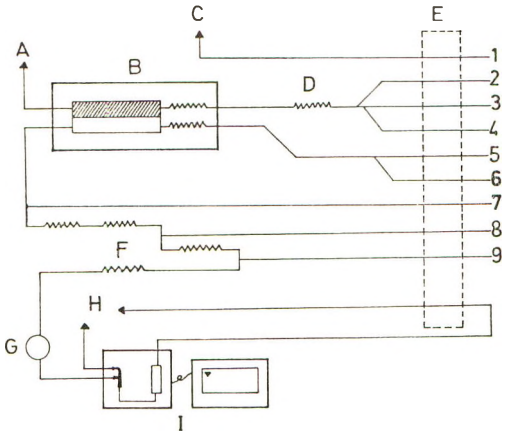

FIG. 1. Flow diagram for automated determination of sulphonamides in urine. A, Waste. B, dialyser. C, Fluid receptacle. D, Mixing coil. E, Proportioning pump. F, Mixing coils. G, 5 min time delay coil. H, Waste. I, Spectrophotometer-recorder. 1, 1% Tween 2·0 ml/min. 2, Sample 0·1 ml/min. 3,5, 0·2N HCl 1·6 and 2·9 ml/min. 4,6, Air 0·8 ml/min. 7, 0·1% $NaNO_3$ 0·6 ml/min. 8, 0·5% ammonium sulphamate 0·6 ml/min. 9, 0·1% *N*-(1-naphthyl)ethylenediamine dihydrochloride 0·6 ml/min.

Standard solutions were prepared by dissolving the sulphonamide (at about 60°) in 0·1N hydrochloric acid. For the manual procedure the Bratton and Marshall method was used, except that the coupling reagent was dissolved in 0·1N hydrochloric acid. In all cases a urine blank was read. All solutions were read in a 10 mm cell.

For the automated method a Technicon AutoAnalyzer (flow diagram Fig. 1) was connected to a Beckman DBG spectrophotometer and 10 inch recorder. The sampler was set to withdraw 20 samples/h. In every run, samples of both standard and urine solutions were pumped through, the standard samples being separated by one and the urine samples by 2 cups of distilled water. The absorption of the coloured solutions were recorded at 545 nm in a 10 mm flow cell using a dialyser temperature of 20° to obviate any hydrolysis of conjugated sulphonamides. A standard curve was plotted for every run of samples and the sulphonamide concentration of each urine sample read from this curve.

For the evaluation of the two methods, a dilution factor of 250 was used throughout with the manual method. For the excreted sulphathiazole, dilution factors were chosen to obtain absorptions between 0·2 and 0·5. Total sulphathiazole was determined after diluting the urine sample and hydrolysing with an equal volume of N hydrochloric acid.

The urinary excretion data correlate well, the biggest difference between the methods being 2·89 mg for the amount of sulphathiazole excreted in a specific period of time.

With the automated method, it is important to keep the fluid receptacle of the sampler filled with a surface-active reagent such as a 1% solution of Tween 80. This prevents high recovery of sulphathiazole from the urine, presumably because of the difference between the surface activity of the urine and that of the hydrochloric acid used in the standard solutions. Sulphamethizole and sulphamethoxazole in urine, but not sulphafurazole, could also be determined by the method.

The dissolution apparatus used resembled that of Ganderton, Hadgraft & others (1967). One 500 mg tablet in a 2 litre beaker containing 1000 ml of 0·1N hydrochloric acid, at 37°, was stirred at 100 rev/min.

For the manual method, 1 or 2 ml samples were withdrawn at 5 min intervals through a sintered glass filter (porosity 3) and the beaker volume was immediately

made up with 0·1N hydrochloric acid at 37°. After a suitable dilution the samples were analysed.

The automated procedure described was used for all but one of the sulphonamides examined, sulphafurazole, for which the volume of 0·2N hydrochloric acid fed into the lower half of the dialyser was reduced to 1·2 ml/min. After the standard solutions were run, the sampling probe was dipped into the dissolution medium, and the dissolution process started. Dissolution medium withdrawn was replaced at the same rate by 0·1N hydrochloric acid. The recording continued until the concentration of sulphonamide dissolved remained constant.

The results of 7 sets of determinations by the manual method and 6 by the automated procedure revealed that for samples from 5–100 mg% of sulphathiazole the mean ± standard deviation was at 5 mg% 5·19 ± 0·57 for the manual method and 4·80 ± 0·13 for the automated procedure, and at 100 mg% 95·44 ± 1·09 and 95·40 ± 0·78 respectively.

In dissolution rate studies good correlation of the manual and automated methods was found with the total amount of sulphonamide dissolved and with the time required for half of the total amount of sulphonamide to be released from the tablet. The automated method is superior to the manual method in that the results must be regarded as more accurate since a continuous recording of the dissolution process takes place, providing a true dissolution profile.

The automated procedure was successfully used in dissolution rate studies of tablets of sulphafurazole, sulphathiazole, sulphamethoxazole, sulphadimidine, sulphadiazine, and sulphamethizole, including a sustained release sulphamethizole tablet in a wax matrix.

We wish to thank the Council for Scientific and Industrial Research and Propan Pharmaceuticals for financial aid in this study. We are indebted to Messrs. F. J. Potgieter, R. H. Lombard and A. P. Lötter for making results on dissolution rate studies available, and to Mmes. A. Coetzee and R. R. van Jaarsveld for assistance with the experimental work.

Department of Pharmaceutics,
Potchefstroom University for C.H.E.,
Potchefstroom, South Africa.

A. P. GOOSSENS.
M. C. B. VAN OUDTSHOORN

May 11, 1971

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Urinary histamine and urine volume in man

In 1944, Anrep, Ayadi & others reported the results of experiments in which diuresis had been induced in dogs and concluded that the amount of histamine excreted in the urine was independent of urine volume. Much work since then has confirmed this finding in other species, including man, and the 24-h excretion of free histamine has been widely used as an index of histamine metabolism. In view of this, the report by Horlington, Kolthammer & Lazare (1970) that there is a correlation between urinary histamine and urine volume in rats will require repeated confirmation by other workers before it can be held to invalidate previous work on urinary histamine. Moreover, it has often been pointed out (e.g. Mitchell, 1965) that the great differences between the rat and man in their metabolism of histamine are an indication for extreme caution in making inferences about human metabolism from observations made on rats. In many hundreds of estimations of free histamine in human urine during the past 20 years, I have repeatedly substantiated Anrep's finding that there is no relation between urine volume and the excretion of free histamine (e.g. Mitchell, 1956). To confirm this observation, a comparison was made between the amounts of free histamine excreted on each of two successive days, the fluid intake being severely limited on the first day and large quantities of fluid being administered on the second day. Free histamine was estimated in duplicate samples by the Decalso method of Roberts & Adams (1950) and expressed in terms of histamine base. The results recorded for five healthy men (Table 1) support the statement that in man the excretion of free histamine bears no relation to urine volume.

Table 1. *The effect of variations in urine volume on the urinary excretion of free histamine by healthy men.*

	Low fluid intake		High fluid intake	
Subject	Urine volume in 24 h (ml)	Urinary free histamine in 24 h (μg)	Urine volume in 24 h (ml)	Urinary free histamine in 24 h (μg)
1	520	9·0	1780	9·6
2	540	22·7	2440	22·2
3	1080	17·3	3770	16·0
4	610	13·2	3500	15·7
5	625	8·9	4100	8·5
Mean	675	14·2	3120	14·4

Department of Child Health,
University of Aberdeen,
Aberdeen AB9 2ZD, U.K.

R. G. MITCHELL

June 8, 1971

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Printed by W Heffer & Sons Ltd Cambridge England

Journal of Pharmacy and Pharmacology

Volume 23 Number 9 September 1971

Original Papers

Letters to the Editor