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Structure-activity relations for anticholinergic dioxolans

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4-Dimethylaminomethyl 1,3-dioxolan derivatives have been examined for anticholinergic potency. Quaternary derivatives in which C-2 was substituted with two bulky substituents were found to have anticholinergic potencies similar to those of atropine in the peripheral nervous system. A comparison of the enantiomeric *cis* and *trans* 2-cyclohexyl-2-phenyl-4-dimethylaminomethyl-1,3-dioxolan methiodides showed that the observed configurational selectivity depended mainly on the configuration at C-2 and not on the geometrical relation between C-2 and C-4.

Various classes of compounds with high muscarinic activity may be converted into compounds with high anticholinergic activity by the replacement of small groups such as methyl with suitably placed bulky substituents. Thus the anticholinergic benzilic acid derivative (I) and related compounds (Abramson, Barlow & others, 1969) may be considered to be derived from acetylcholine (II) and the 2,2-diphenyl-1,3-dioxolan derivative (III) (Brown & Werner, 1949) may be derived from the potent muscarinic agent (IV) (Belleau & Puranen, 1963). Similarly anticholinergic compounds such as (V) have been prepared which are based on the oxotremorine (VI) structure (Dahlbom, Karlen & others, 1964). Although the stereochemical requirements for high muscarinic activity are well established (Barlow, 1964; Bebbington & Brimblecombe, 1965; Chothia, 1970) the optimal stereochemical requirements for high anticholinergic activity are less certain, but obviously play a very important role as indicated by the differences in pharmacological activity displayed by anticholinergic enantiomers (Ariëns, 1966). It is also not known which of the structural features in the muscarinic compounds also contribute to the activity of their anticholinergic derivatives. To obtain a better understanding of the stereochemical requirements for high anticholinergic activity, series of anticholinergic compounds derived from different classes of muscarinic compounds have been examined. This paper reports on the anticholinergic activity of compounds based on the 1,3-dioxolan structure, special emphasis being placed on pharmacological comparisons of enantiomers and diastereoisomers of known absolute configuration and optical purity.

Although it was first shown by Brown & Werner (1949) that anticholinergic compounds based on the 1,3-dioxolan structure could be formed and although subsequently other studies of this type of anticholinergic compound have been reported (van Rossum & Ariëns, 1959; Kimura, Hirai & Takai, 1968; May, Ridley & Triggle, 1969) to our knowledge no detailed stereochemical study of structure-activity relation in this class of compounds has been reported previously.

Nomenclature

The geometry of the racemic compounds in this paper may be described according to the I.U.P.A.C. Tentative rules for the Nomenclature of Organic Chemistry,

I

II

III

IV

V

VI

Section E. Thus compound VIIm is 2-benzyl-*r*-2-phenyl-t-4-dimethylaminomethyl-1,3-dioxolan methiodide and VIIIm is 2-benzyl-*r*-2-phenyl-*C*-4-dimethylaminomethyl-1,3-dioxolan methiodide. For convenience all the compounds will be designated *cis* when the 2-phenyl and 4-dimethylaminomethyl substituents are on the same side of the plane of the 1,3-dioxolan ring and *trans* when the same substituents are on opposite sides of the ring.

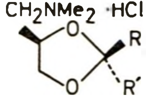
The optically active compounds may be described unequivocally by use of the *R* and *S* nomenclature (Cahn, Ingold & Prelog, 1956). However, without due care this nomenclature can lead to some confusion. For example, the 4-toluene-*p*-sulphonyl-oxymethyl-1,3-dioxolans derived from 1-*O*-toluene-*p*-sulphonyl-D-glycerol have the *R* configuration at C-4, whereas the corresponding 4-dimethylaminomethyl derivatives have the *S* configuration at C-4. This does not imply any change in absolute configuration on introduction of the dimethylamino-group but is simply a consequence of the nomenclature system. To avoid confusion and to facilitate comparison with papers on the stereochemistry of cholinergic dioxolans which generally use D and L nomenclature, a combination of the two forms of nomenclature will be used. Thus compound XImL, the *cis*-isomer from 1-*O*-toluene-*p*-sulphonyl-L-glycerol is L-*cis* (2*S*, 4*R*)-2-cyclohexyl-4-dimethylaminomethyl-2-phenyl-1,3-dioxolan methiodide and compound XmD the *trans*-isomer from 1-*O*-toluene-*p*-sulphonyl-D-glycerol is D-*trans* (2*S*,4*S*)-2-cyclohexyl-4-dimethylaminomethyl-2-phenyl-1,3-dioxolan methiodide.

EXPERIMENTAL

Preparation of compounds

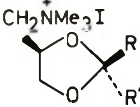
The racemic *cis* and *trans*-2-phenyl-4-dimethylamino-1,3-dioxolans from which the hydrochlorides and methiodides, listed in Tables 1 and 2, were formed in the usual manner, were prepared by reaction of the corresponding 4-toluene-p-sulphonyloxy-methyl-1,3-dioxolans and dimethylamine in ethanol at 100° for 8 h in a sealed tube. The preparation, separation and structural assignments of the racemic *cis* and *trans*-2-

Table 1. *Analytical data for* 1,3-*dioxolans of general structure*



Compound	R	R′	Analysis	m.p. (crystal-lization solvent)
VII	–CH_2Ph	Ph	C, 69·1; H, 7·0	126°
			$C_{19}H_{24}O_2NCl$ requires C, 68·4; H, 7·2	(acetone)
VIII	Ph	–CH_2Ph	C, 58·1; H, 7·2; N, 3·9	180–184°
			$C_{19}H_{24}O_2NCl$ requires C, 68·4; H, 7·2; N, 4·2	(acetone)
IX	Ph	Ph	N, 4·02	198–199°
			$C_{18}H_{22}O_2NCl$ requires N, 4·38%	(acetone)
X	Cyclohexyl	Ph	C, 66·6; H, 8·7; N, 4·4	195–197°
			$C_{18}H_{28}O_2NCl$ requires C, 66·3; H, 8·7; N, 4·3	(acetone)
XI	Ph	Cyclohexyl	C, 66·6; H, 8·9; N, 4·4	026–210°
			$C_{18}H_{28}O_2NCl$ requires C, 66·3; H, 8·7; N, 4·3	(acetone)
XII	Ph	Me	C, 59·9; H, 8·4; N, 5·4	142–144°
			$C_{13}H_{20}O_2NCl$ requires C, 60·5; H, 7·9; N, 5·4	(ethanol/ ether)
XIII	Me	Ph	Hygroscopic—no definite melting point	
XIV	Me	–CH_2Ph	C, 61·9; H, 7·9; N, 4·9	141–142°
			$C_{14}H_{22}O_2NCl$ requires C, 61·9; H, 8·2; N, 5·2	(acetone)
XV	–CH_2Ph	Me	C, 61·1; H, 8·3; N, 5·3	135–136°
			$C_{14}H_{22}O_2NCl$ requires C, 61·9; H, 8·2; N, 5·2	(acetone)

Table 2. *Analytical data for* 1,3-*dioxolans of general structure*



Compound	R	R′	Analysis	m.p. (crystal-lization solvent)
VIIm	–CH_2Ph	Ph	C, 54·4; H, 6·3; N, 3·2	204°
			$C_{20}H_{26}O_2NI$ requires C, 54·7; H, 6·0; N, 3·2	(ethanol)
VIIIm	Ph	–CH_2Ph	C, 54·3; H, 6·2; N, 3·1	203°
			$C_{20}H_{26}O_2NI$ requires C, 54·7; H, 6·0; N, 3·2	(ethanol)
IXm	Ph	Ph	C, 53·6; H, 5·7; N, 3·1	202°
			$C_{19}H_{24}O_2NI$ requires C, 53·7; H, 5·7; N, 3·3	(ethanol)
Xm	Cyclohexyl	Ph	C, 53·0; H, 7·0; N, 2·9	248–251°
			$C_{19}H_{30}O_2NI$ requires C, 52·9; H, 7·0; N, 3·2	(ethanol)
XIm	Ph	Cyclohexyl	C, 53·2; H, 7·0; N, 3·0	235°
			$C_{19}H_{30}O_2NI$ requires C, 52·9; H, 7·0; N, 3·2	(acetone)
XIIIm	Me	Ph	C, 46·0; H, 6·3; N, 3·9	
			$C_{14}H_{22}O_2NI$ requires C, 46·3; H, 6·1; N, 3·9	
XIVm	Me	CH_2Ph	C, 47·9; H, 6·5; N, 3·5	170° (ethanol)
XVm	CH_2Ph	Me	C, 47·6; H, 6·7; N, 3·6	185–186° (ethanol)
XVIm	H	Ph	No analysis obtained	168° (ethanol)
XVIIm	Ph	H	C, 44·7; H, 5·7; N, 4·1	158–162°
			$C_{12}H_{20}NIO_2$ requires C, 44·7; H, 5·8; N, 4·0	(ethanol)

phenyl-4-toluene-*p*-sulphonyloxymethyl 1,3-dioxolans have been described previously (Inch & Williams, 1970).

The optically active *cis* and *trans*-2-cyclohexyl-2-phenyl-4-dimethylaminomethyl-1,3-dioxolan methiodides listed in Table 3 were prepared from 1-*O*-toluene-*p*-sulphonyl-D-glycerol (Baer, 1952) and 1-*O*-toluene-*p*-sulphonyl-L-glycerol (Belleau & Puranen, 1963). The optically active glycerol derivatives were condensed with cyclohexylphenylketone and the *cis* and *trans* isomers separated as described for the corresponding racemates before reactions in sequence with dimethylamine and methyl iodide.

All the compounds examined have satisfactory nmr and infra-red spectra.

Table 3. *Analytical data for optically active 1,3-dioxolan derivatives*

	Compound	$[\alpha]_D$*	Analysis	m.p.
(XImL)	$CH_2\overset{+}{N}Me_3I$, Ph, C_6H_{11}	Not measured (+3·33°)	No analysis obtained	235° (ethanol)
(XImD)	C_6H_{11}, Ph, CH_2NMe_3I	+2·96° (—3·12°)	C, 53·0; H, 6·8; N, 3·0 $C_{19}H_{30}O_2NI$ requires C, 52·9; H, 7·0; N, 3·2	238° (ethanol)
(XmL)	CH_2NMe_3I, C_6H_{11}, Ph	—3·62° (—25·7°)	C, 53·4; H, 7·0; N, 3·1 $C_{19}H_{30}O_2NI$ requires C, 52·9; H, 7·0; N, 3·2	248° (ethanol)
(XmD)	Ph, CH_2NMe_3I, C_6H_{11}	+3·43° (+26·1°)	C, 53·0; H, 7·0; N, 2·8 $C_{19}H_{30}O_2NI$ requires C, 52·9; H, 7·0; N, 3·2	244° (ethanol)

* All rotations were measured in chloroform. The figures in parentheses are the specific rotations of the 4-toluene-*p*-sulphonyloxymethyl-1,3-dioxolans from which the products listed were derived.

Tests for anticholinergic activity

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

Antagonism of acetylcholine-induced contractions of the isolated guinea-pig ileum. Affinity constants for the anticholinergic drugs were measured using essentially the method described by Barlow, Scott & Stephenson (1963). A 2 cm segment of ileum was taken from a freshly-killed guinea-pig at a point about 5 cm from the ileo-caecal junction. This was 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 responses were obtained to two different concentrations of acetylcholine, then the anticholinergic drug was dissolved in the Ringer-Tyrode solution and the concentrations of acetylcholine increased to obtain comparable responses. It was then possible to determine the dose ratio corresponding to a particular dose 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 of 100 μg/kg of oxotremorine. Animals were examined at 5, 10 or 15 min after the oxotremorine injection for the presence of salivation or tremors, or both. No attempt was made to grade the severity of either response it was noted as being either present or absent. Four groups, each containing 5 mice were used, and ED50s for block of salivation and of tremors were calculated by probit analysis.

Production of mydriasis in mice. Male mice (18–25 g) were used. Drugs were injected into a tail vein. Preliminary experiments were made on single animals to obtain an indication of suitable dose levels. Then, using groups of 10 mice at each of 3 dose levels, the pupil diameter was measured at different times after injection to cover, as far as possible, the total period of action of the drug. The eyes were held 20 cm from a Watson microscope lamp and the measurement was made using an eyepiece graticule in a + 20 microscope. The mean pupil diameter from the two eyes was used and the mice were kept in the dark before and between readings. The duration of effect varied with dose so in calculations of potency relative to atropine the maximum mean pupil diameter reached at each dose was used, irrespective of time, and the results calculated on the basis of a six-point assay.

RESULTS

DL-Pairs of *cis* and *trans*-2-substituted-4-dimethylaminomethyl-1,3-dioxolan hydrochlorides and methiodides of previously established geometry were first examined for anticholinergic activity by the methods described. Affinity constants were not determined for the hydrochlorides because preliminary screening on the guinea-pig isolated ileum revealed only very weak antagonist activity and in no case would log K have exceeded 6. From the results obtained (Tables 4 and 5), *cis* and *trans*-2-cyclohexyl-4-dimethylaminomethyl-2-phenyl-1,3-dioxolan methiodides were selected for further study and were prepared in optically pure forms. The pharmacological results for the optically pure isomers and the racemates are listed in Table 6.

DISCUSSION

Methiodides

The data in Table 5 indicate that 4-dimethylaminomethyl-1,3-dioxolan methiodide derivatives which carry two bulky substituents at C-2, resemble atropine in anticholinergic potency in the peripheral nervous system. (For comparison, the corresponding values for atropine are: log K = 9·0, ED50 for antagonism of oxotremorine

Table 4. *Pharmacological results for* 1,3-*dioxolans of general structure*

CH_2NMe_2HCl, R, R′

Compound	R	R′	Antagonism of oxotremorine effects in mice ED50 (μmol/kg with 95% confidence limits)		Mydriatic effect in mice (Potency relative to atropine with 95% confidence limits)
			Salivation	Tremors	
VII	CH_2Ph	Ph	-ve	-ve	0·005 (0·004–0·006)
VIII	Ph	CH_2Ph	-ve	-ve	0·007 (0·006–0·009)
IX	Ph	Ph	61·7 (3·9–98·7)	-ve	0·006 (0·005–0·007)
X	C_6H_{11}	Ph	46·6 (33·2–65·5)	93·3 (66·4–131)	0·018 (0·015–0·021)
XI	Ph	C_6H_{11}	40·2 (23·4—70·1)	107 (76·2–150)	0·015 (0·013–0·018)
XII	Ph	Me	-ve	-ve	-ve
XIII	Me	Ph	-ve	-ve	-ve
XIV	Me	CH_2Ph	-ve	-ve	-ve
XV	CH_2Ph	Me	-ve	-ve	-ve
			(-ve indicates >100)		(-ve indicates <0·001

salivation = 0·44 μmol/kg, mydriatic potency = 1·0). However, where C-2 only carries one bulky substituent as with the *cis* and *trans*-4-dimethylaminomethyl-2-phenyl-1,3-dioxolan methiodides (compounds XVIIm and XVIm respectively) and compounds XIIIm, XIVm and XVm only weak anticholinergic activity was observed. No evidence was obtained from enzyme studies with acetylcholinesterase from bovine erythrocytes to indicate that any of the compounds in Table 5 were acetylcholinesterase inhibitors, although stereochemically many of the compounds in Table 5 closely resemble cholinesterase inhibitors such as IV, at least on one side of the molecule.

The results with the pure *cis* (XVIIm) and *trans* (XVIm) racemates of 4-dimethyl-aminomethyl-2-phenyl-1,3-dioxolan methiodide showing equipotent but very weak

Table 5. *Pharmacological results for* 1,3-*dioxolans of general structure*

$CH_2\overset{+}{N}Me_3I$, R, R′

Compound	R	R¹	Guinea-pig ileum Affinity constant (log K) Mean values—number of determinations in brackets	Antagonism of oxo-tremorine-induced salivation in mice ED50 (μmol/kg with 95% confidence limits)	Mydriatic effect in mice (Potency relative to atropine with 95% confidence limits)
VIIm	$-CH_2Ph$	Ph	8·05 (2)	13·8 (7·5–42·3)	0·21 (0·17–0·27)
VIIIm	Ph	CH_2Ph	8·35 (2)	6·1 (1·0–12·0)	0·62 (0·47–0·86)
IXm	Ph	Ph	8·02 (4)	15·5 (6·9–28·2)	0·50 (0·39–0·64)
Xm	C_6H_{11}	Ph	8·41 (4)	15·0 (8·6–27·1)	0·61 (0·44–0·94)
XIm	Ph	C_6H_{11}	8·34 (6)	6·2 (4·3– 9·0)	0·98 (0·79–1·28)
XIIIm	Me	Ph	5·30 (2)	-ve	-ve
XIVm	Me	CH_2Ph	<4	-ve	-ve
XVm	CHPh	Me_2	5·11 (2)	-ve	-ve
XVIm	H	Ph	4·52 (2)	-ve	-ve
XVIIm	Ph	H	4·31 (2)	-ve	-ve

Table 6. *Pharmacological results for racemic and optically active* cis *and* trans-2-*cyclohexyl-4-dimethylaminomethyl-2-phenyl-*1,3-*dioxolan methiodides*

Compound	Configuration	Guinea-pig ileum Affinity constant (log K ± s.d.) Number of determinations in brackets	Antagonism of oxo-tremorine-induced salivation in mice ED50 (μmol/kg with 95% confidence limits)	Mydriatic effect in mice (Potency relative to atropine with 95% confidence limits)
XIm	*trans*-racemate	8·41 ± 0·17 (4)	15·0 (8·6–27·1)	0·61 (0·44–0·94)
XmD	D-*trans* (2S,4S)	8·56 ± 0·14 (4)	4·2 (2·4– 7·3)	1·12 (0·85–1·64)
XmL	L-*trans* (2R,4R)	8·24 ± 0·17 (4)	20·2 (15·3–26·7)	0·29 (0·22–0·38)
XIm	*cis*-racemate	8·34 ± 0·05 (6)	6·2 (4·3– 9·0)	0·98 (0·79–1·28)
XImD	D-*cis* (2R,4S)	8·34 ± 0·13 (4)	16·6 (11·5–23·9)	0·74 (0·56–1·07)
XImL	L-cis (2S,4R)	8·73 ± 0·15 (4)	2·9 (2·1–4·1)	2·29 (1·79–3·02)

antagonistic activity are not inconsistent with those reported previously (May & others, 1969) for partially purified *cis* and *trans* racemates. As has been demonstrated with many other classes of compounds, however, the more active the compound the more pronounced is the relation between biological activity and stereochemistry (Pfeiffer, 1956) and thus it was not surprising that small differences in anticholinergic potency were observed with the more active *cis* and *trans* pairs VIIm and VIIIm, and Xm and XIm. To ascertain the significance of these differences, if any, a careful pharmacological comparison of the optical isomers of Xm and XIm was made.

Comparison of isomers

It will be seen from Table 6 that although the differences in the values obtained using three assay procedures are small and in some cases statistically insignificant, it is reasonable to arrange the isomers in descending order of anticholinergic potency thus:

$$\text{XImL} > \text{XmD} > \text{XImD} > \text{XmL}$$

Since the racemates Xm and XIm had anticholinergic potencies intermediate between their corresponding optical isomers, some confidence can be placed in the accuracy of the assay procedures. The significance of the results is immediately apparent when the steric factors which can contribute to anticholinergic potency are considered separately. The three main factors are (a) the absolute configuration at C-2, (b) the absolute configuration at C-4 and (c) the geometrical relation between the C-2 and the C-4 substituents. Since compounds XImL and XmD differ in configuration at C-4 and have a different geometrical relation between C-2 and C-4 (XImL is a *cis*-isomer whereas XmD is a *trans* isomer) it is apparent that it is the absolute configuration at the acetal carbon which contributes most to anticholinergic potency. In the cholinergic dioxolans such as IV it is the configuration at C-4 and not the configuration at the acetal carbon which most affects anticholinergic activity (Belleau & Lavoie, 1968). In both XImL and XmD the acetal carbon C-2 has the *S* configuration. It must be emphasised that since it is the absolute configuration at the acetal carbon atom and not the geometrical relation of the substituents at C-2 and C-4 that is of prime importance for high anticholinergic potency, pharmacological comparisons of racemates in this series of compounds can provide little information about structure activity relations.

The result that in anticholinergic dioxolans it is the configuration at the acetal carbon atom which is of prime importance for anti-cholinergic activity may be

compared with the findings of Ellenbroek, Nivard & others (1965) who showed that in esters of 2-cyclohexyl-2-hydroxy-2-phenylacetic acid such as XVIII it was the configuration of the asymmetric benzylic centre which contributed most to the anti-cholinergic activity of the whole molecule and the configuration of the aminoalkyl portion was of far less importance. The fact that in the active isomer of XVIII the benzylic centre has the *R* configuration suggests that in the dioxolans O-1 is equivalent to the -OH group in XVIII. The implications of this result will be discussed in a subsequent publication.

XVIII XImL

Hydrochlorides

The results given in Table 4 indicate that in comparison with their corresponding methiodides, the hydrochlorides of these dioxolans showed only weak anticholinergic activity with the most active compounds having less than one-fiftieth the activity of atropine. Consequently, despite their non-quaternary nature, the compounds showed little or no central activity in blocking oxotremorine-induced tremors in mice.

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The synthesis and anti-acetylcholinesterase activities of (+)-physostigmine and (+)-physovenine*

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(+)-Physostigmine and (+)-physovenine have been synthesized. The anti-acetylcholinesterase activities of these two bases, which have been investigated *in vitro* using erythrocyte acetylcholinesterase, have been found to be much lower than the corresponding activities of the alkaloids (—)-physostigmine and (—)-physovenine. Possible reasons for these activity differences are discussed.

(—)-Physostigmine (eserine), the major alkaloid of *Physostigma venenosum* seeds (Calabar beans), which has recently (Hill & Newkome, 1969; Longmore & Robinson, 1969a, b; Newkome & Bhacca, 1969) been found to have the absolute configuration shown in I (R=Me·NH·CO, R′=Me, X=NMe), and a large number of synthetic analogues have been evaluated for anti-acetylcholinesterase activity (Long, 1963; Long & Evans, 1967; Stempel & Aeschlimann, 1956) and the chemical features essential for such activity have been established (Long, 1963; Long & Evans, 1967; Stempel & Aeschlimann, 1956). The structures and absolute configurations of the minor alkaloids of *Physostigma venenosum* seeds, (—)-physovenine, (—)-N_a-norphysostigmine, (—)-eseramine and (—)-geneserine have also recently been established to be as shown in I (R=Me·NH·CO, R′=Me, X=O; R=Me·NH·CO, R′=H, X=NMe; R=Me·NH·CO, R′=Me, X=N·CO·NH·Me) (Longmore & Robinson, 1969a, b; Robinson, 1968) and II (Longmore & Robinson, 1969a, b; Hootelé, 1969; Robinson & Moorcroft, 1970), respectively. Furthermore, *in vitro* studies using erythrocyte acetylcholinesterase have shown (Robinson & Robinson, 1968) that whereas the anti-acetylcholinesterase activities of (—)-physostigmine, (—)-N_a-norphysostigmine and

I II

(—)-physovenine are approximately the same, (—)-eseramine and (—)-geneserine are devoid of anti-acetylcholinesterase activity under the same experimental conditions. These enzymological results, together with comparative data on the chemical behaviour of this series of alkaloids under conditions of varying pH, led to the suggestion (Robinson & Robinson, 1968) that the reactive species responsible for the anti-acetylcholinesterase activity of (—)-physostigmine is not the N_b-protonated species

* Alkaloids of *Physostigma venenosum*, Part X; for Part IX see Robinson & Moorcroft (1970).

(I; R=Me·NH·CO, R′=Me, X=$\overset{\oplus}{\text{N}}$H·Me) (modified to show the absolute configuration) as had previously (Wilson & Bergmann, 1950) been supposed, but the ring C-opened 3*H*-indolium cation III (R=Me, X=$\overset{\oplus}{\text{N}}H_2$Me) (showing the absolute configuration), the analogous cations (III; R=H, X=$\overset{\oplus}{\text{N}}H_2$Me and R=Me, X=OH) being the active forms of (—)-N_a-norphysostigmine and (—)-physovenine, respectively. It was suggested (Robinson & Robinson, 1968) that the opening of the C-ring to give the 3*H*-indolium cations may occur at the acetylcholinesterase surface. The observation (see Table 1) that the anti-acetylcholinesterase activity of 1,1,3,3-tetramethyl-5-methylcarbamoyloxyindolinium iodide (IV) (Ahmed & Robinson, 1965) is far greater than that of (—)-physostigmine adds further support to this suggestion (see also discussion section).

III IV

A number of enantiomeric pairs of compounds have been assessed as cholinesterase inhibitors (Long, 1963): it has been found that the (—)-isomer of isomethadone (V) is thirty times more active than the (+)-isomer (Greig & Howell, 1948; Long, 1963), the (+)-isomers of active (—)-amino acids are far weaker inhibitors than the (—)-isomers (Bergmann, Wilson & Nachmansohn, 1950; Long, 1963) and it is the (—)-isomer of miotine (VI) which is the active isomer (Easson & Stedman, 1933; Long, 1963). As the absolute configurations of (—)-physostigmine and (—)-physovenine are established (see above), we have now synthesized (+)-physostigmine (VII, R=Me·NH·CO, X=NMe) and (+)-physovenine (VII; R=Me·NH·CO, X=O) and compared their anti-acetylcholinesterase activities with those of (—)-physostigmine and (—)-physovenine. The results of these studies will help in the further elucidation of the stereochemical requirements of the acetylcholinesterase active centres, which in some cases are similar to those of muscarinic cholinergic receptor sites (Beckett, Harper & Clitherow, 1963; Belleau, 1964; Belleau & Puranen, 1963; Pauling, 1968; Robinson, Belleau & Cox, 1969).

$Me_2N\cdot CH_2\cdot CHMe\cdot C(Ph)_2\cdot CO\cdot Et$

V VI VII

CHEMISTRY

Of the several successful syntheses of (—)-physostigmine (Julian, Pikl & Boggess, 1934; Julian & Pikl, 1935a, b; Kobayashi, 1938; Harley-Mason & Jackson, 1954), only one (Kobayashi, 1938) has been extended to use in a synthesis of (+)-physostigmine. However, in the report of that work, the salicylate of (—)-physostigmine is

quoted as been dextrorotatory, whereas it is now well established (see *e.g.* British Pharmacopoeia, 1968) that it is laevorotatory, and the salicylate of (+)-physostigmine is quoted as being laevorotatory. Furthermore, (+)-,(−)- and (±)-physostigmine were not liberated from their salicylate salts in this work and so physical data for the free bases were not obtained. Therefore Kobayashi's work leaves much to be desired.

The route chosen for the synthesis of (+)-physostigmine in the present studies is based upon the method, originally used (Julian & others, 1934; Julian & Pikl, 1935a, b) in the first synthesis of (−)-physostigmine, which appears to be more facile and affords better yields of products than the later (Kobayashi, 1938; Harley-Mason & Jackson, 1954) syntheses. (±)-5-Hydroxy-1,3-dimethyloxindole (Robinson, 1965; Longmore & Robinson, 1967) was successively *O*-ethylated, 3-cyanomethylated, catalytically hydrogenated and *N*-monomethylated, via formation of the benzylidene derivative, to afford (±)-5-ethoxy-1,3-dimethyl-3-(2-methylaminoethyl)oxindole, as already described elsewhere (Julian & others, 1934; Julian & Pikl, 1935a, b). Attempts to resolve this latter compound with (+)-camphor-10-sulphonic acid, as described earlier (Julian & Pikl, 1935b), were unsuccessful. It was therefore reduced with sodium in ethanol to afford (±)-eserethole (racemic I; R=Et, R′=Me, X=NMe), which was resolved (Kobayashi, 1938) with (+)-tartaric acid to give (+)-eserethole (VII; R=Et, X=NMe). This was de-ethylated by boiling a solution of it in light petroleum in which powdered anhydrous aluminium chloride was suspended (Julian & Pikl, 1935a, b) [the yield from this reaction was greatly increased when the reaction mixture was stirred continuously throughout the boiling period (*cf.* Hill & Newkome, 1969)] and the resulting phenol, (+)-eseroline (VII; R=H, X=NMe), was converted into (+)-physostigmine (VII; R=Me·NH·CO, X=NMe) by reaction with methyl isocyanate in the presence of a "speck" of sodium (Robinson, 1968, and refs. therein).

(−)-Physovenine (I; R=Me·NH·CO, R′=Me, X=O) has recently (Longmore & Robinson, 1966) been synthesized from (−)-eserethole (I; R=Et, R′=Me, X=NMe). By an analogous route, the (+)-eserethole (VII; R=Et, X=NMe)

Et·O Me N OH H Me NMe2

VIII

prepared above was converted into its methiodide (VII; R=Et, X=$\overset{\oplus}{N}Me_2I^-$), which upon treatment with aqueous sodium hydroxide gave (−)-eserethole methine(VIII). The methiodide of this, upon treatment with boiling aqueous sodium hydroxide, yielded (+)-5-ethoxy-2,3,3a,8a-tetrahydro-3a,8-dimethylfuro[2,3-*b*]indole (VII; R=Et, X=O), which was de-ethylated by the action of anhydrous aluminium chloride to the phenol, conversion of this into (+)-physovenine (VII; R=Me·NH·CO, X=O) being effected by treatment with methyl isocyanate in the presence of a "speck" of sodium.

EXPERIMENTAL

Melting-points were measured with a Kofler hot-stage apparatus and are uncorrected. Ultraviolet spectra were measured in ethanolic solution with a Perkin-Elmer model 137 spectrophotometer, mass spectra were recorded with an A.E.I. MS. 9

spectrometer and optical rotatory dispersion spectra were obtained in 95% ethanol with a Bendix-N.P.L. "Polarmatic" spectropolarimeter. Optical rotations were measured with a Bellingham and Stanley polarimeter. Where mentioned, solutions were dried with anhydrous magnesium sulphate and solvents were removed on a steam-bath under reduced pressure (water pump). Solid analytical samples were dried (6 h) at room temperature /0·1 mm over phosphorus pentoxide.

(±)-5-*Ethoxy*-1,3-*dimethyl*-3-(2-*methylaminoethyl*)*oxindole*. This was prepared from (±)-5-hydroxy-1,3-dimethyloxindole (Longmore & Robinson, 1967; Robinson, 1965) by the reaction sequence already described (Julian & others, 1934; Julian & Pikl, 1935a).

(±)-*Eserethole*. This was prepared by reduction of (±)-5-ethoxy-1,3-dimethyl-3-(2-methylaminoethyl)oxindole with sodium in ethanol by the method previously described (Julian & Pikl, 1935a). However, the product was purified and obtained in 74% yield by column chromatography on alumina (Grade H) with ether as eluant.

Resolution of (±)-*eserethole*. This was effected by the method of Kobayashi (1938) with (+)-tartaric acid. After initial crystallization of the salt [from (±)-eserethole (20g) and (+)-tartaric acid (12g)] from dehydrated ethanol containing ether (7·5%), eight recrystallizations from ethanol containing (+)-tartaric acid (1·5%) gave fine white needles (5·77g; 36%), which exhibited constant rotation, $[\alpha]_D^{20} = +115° \pm 0·6°$ (water) and had m.p. 172–174° [lit. $[\alpha]_D^{16} = 115·5° \pm 0·7°$ (water); m.p. 173–174° (Kobayashi, 1938)].

The free base was liberated from the above salt by the addition of sodium hydroxide and was subsequently extracted into ether. After drying and removal of the solvent from the combined ethereal extracts, (+)-*eserethole* was obtained as a pale-yellow oil, which completely crystallized at 0° but which melted on warming to room temperature (20°), $[\alpha]_D^{20} = +101·5° \pm 0·7°$ (dehydrated ethanol) [lit. for (−)-eserethole, $[\alpha]_D = -81°$ (ethanol) (Polonovski, 1915), $[\alpha]_D^{28} = -81·6° \pm 0·5°$ (Julian & Pikl, 1935b)].

(+)-*Physostigmine*. This was prepared by the following modification of the method used to convert (−)-eserethole into (−)-physostigmine (Julian & Pikl, 1935b; Polonovski & Nitzberg, 1916). (+)-Eserethole (208 mg) was dissolved in sodium-dried light petroleum (b.p. 60–80°) (10ml), finely powdered anhydrous aluminium chloride (250 mg) added and the mixture boiled under reflux with continuous stirring for 10 h. After cooling, the solvent was evaporated and the residue decomposed by the addition of ice. Addition of sodium bicarbonate caused the formation of a thick gel, which, after dilution with a little water, was extracted with peroxide-free ether (4 × 20 ml). The combined ethereal extracts were washed with water (3 × 10 ml), dried and evaporated to give (+)-*eseroline* as a pale-brown oil, which partially crystallized (151 mg; 81%).

This product was immediately dissolved in sodium-dried ether (10 ml), and a "speck" of sodium added, followed by methyl isocyanate (2 ml). The reaction mixture was kept under nitrogen at room temperature (20°) with occasional shaking for three days, after which it was filtered, the filtrate evaporated and the residue subjected to column chromatography on aluminia (Grade H) with ether as eluant. A small quantity of (+)-*eserethole* was eluted with the solvent front, followed by (+)-*physostigmine* (48·0 mg; 26%), which, after evaporating the ether, was obtained as a clear glassy solid which readily crystallized as pale-yellow prisms upon trituration with a mixture of ether-light petroleum (b.p. 30–40°). Recrystallization from the solvent mixture gave white prisms (35 mg), m.p. 104–106° [lit. m.p. for the enantiomer,

106° (Polonovski & Nitzberg, 1916)], m.p. on admixture with (—)-physostigmine, 72–104°. The product had ultra-violet and mass spectra and Rf value (0·83 on a thin layer of alumina, ethyl acetate being used as solvent and iodine vapour as developer) identical with those of (—)-physostigmine, but its optical rotatory dispersion spectrum was the mirror image of that reported (Longmore & Robinson, 1969b) for (—)-physostigmine. Found: C, 65·2; H, 7·5; N, 15·1. $C_{15}H_{21}N_3O_2$ requires C, 65·5; H, 7·6; N, 15·2%.

(—)-*Eserethole methine.* This was prepared by a method analogous to that used to convert (—)-eserethole into (+)-eserethole methine (Polonovski & Polonovski, 1918, 1923a; Hoshino & Kobayashi, 1934; Longmore & Robinson, 1966). Methyl iodide (3·0 g) was added to a solution of (+)-eserethole (615 mg) in ether (6 ml). The solution immediately became turbid, and the (+)-*methiodide* gradually formed as a deposit of white plates. Recrystallization of these from acetone–ether gave white plates (940 mg; 97%), m.p. 172–174° [lit. m.p. for the enantiomer, 168–169° (Hoshino & Kobayashi, 1934); 171° (Polonovski & Polonovski, 1918); 170–171° (Polonovski & Polonovski, 1923a)]. Found: C, 49·2; H, 6·3; N, 6·9. $C_{16}H_{25}IN_2O$ requires C, 49·5; H, 6·5; N, 7·2%.

To a solution of (+)-eserethole methiodide (900 mg) in water (25 ml) was added 3N sodium hydroxide (3 ml). The solution, which immediately became turbid, was then heated (steam-bath) for ½ h. The reaction mixture, after cooling, was extracted into ether (3 × 15 ml) and the ether evaporated from the combined ethereal extracts, after drying, to afford a pale-yellow oil, which crystallized almost immediately to afford (—)-*eserethole methine* as large pale-yellow fern-like crystals (643 mg, quantitative), m.p. 83–86° [lit. m.p. for the enantiomer, 81–82° (Hoshino & Kobayashi, 1934), 80° (Polonovski & Polonovski, 1918), 89° (Polonovski & Polonovski, 1923a)], $[\alpha]_D^{20°}$ = — 7·6° (dehydrated ethanol) [lit. for enantiometer, $[\alpha]_D$ = + 10° (alcohol) (Polonovski & Polonovski, 1918)].

(+)-5-*Ethoxy*-2,3,3*a*,8*a-tetrahydro*-3*a*,8-*dimethylfuro*[2,3-b]*indole.* This was prepared by a method similar to that used in the synthesis of the enantiomer (Polonovski & Polonovski, 1918, 1923a, b; Longmore & Robinson, 1966). Methyl iodide (3 ml) was added to a solution of (—)-eserethole methine (600 mg) in ether (8 ml). After 16 h the methiodide had separated from the mixture as a pale-yellow oil, which upon further standing and trituration partially crystallized. After collection, the product, a very hygroscopic solid (900 mg; quantitative), was dissolved in water (30 ml), 7N sodium hydroxide (3 ml) added, and the solution boiled under reflux for 4 h, during which time trimethylamine was evolved and an oil separated out from the aqueous phase. After cooling, the aqueous reaction mixture and condenser washings (the product is steam-volatile) were extracted into ether (4 × 25 ml), the combined ethereal extracts washed with water (2 × 5 ml), dried and evaporated to afford a pale-yellow oil (296 mg; 60%), $[\alpha]_D^{20}$= + 100 ± 1° (95% ethanol) [lit. for enantiometer, $[\alpha]_D^{25}$= — 98° (95% ethanol) (Polonovski & Polonovski, 1923a, b)].

(+)-*Physovenine.* Finely powdered anhydrous aluminium chloride (100 mg) was added to a solution of (+)-5-ethoxy-2,3,3a,8a-tetrahydro-3a,8-dimethylfuro[2,3-*b*] indole (77 mg) in sodium-dried light petroleum (b.p. 60–80°) (10 ml) and the mixture boiled under reflux with continuous stirring for 10 h. After cooling, the reaction mixture was "worked-up" and the product reacted with methyl isocyanate in the presence of a trace of sodium as already described in the synthesis of (±)-physovenine (Longmore & Robinson, 1967). (+)-*Physovenine* was obtained as pale-yellow prisms

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(20 mg; 23%) which, after two recrystallizations from ether-light petroleum (b.p. 30°–40°), gave white prisms (12 mg), m.p. 120–122° [lit. m.p. for the enantiomer, 120–121·5° (Longmore & Robinson, 1966), 123° (Salway, 1911), 124–125° (Robinson, 1964)], m.p., on admixture with an equal weight of (—)-physovenine, 135–141° [*cf.* lit. for the racemate, m.p. 142–143° (Longmore & Roinson, 1967)]. The product had ultraviolet and mass spectra and Rf value (0·84 on a thin layer of alumina with ethyl acetate as solvent and iodine vapour as developer) identical with those of (—)-physovenine, but its optical rotatory dispersion spectrum was the mirror image of that reported (Longmore & Robinson, 1969b) for (—)-physovenine.

ENZYME STUDIES

In vitro anti-acetylcholinesterase activities were determined using erythrocyte acetylcholinesterase and the pH-stat method for measuring acetylcholine hydrolysis rates as already described (Robinson & Robinson, 1968). The results obtained are given in Table 1, in which the enzyme-inhibitor dissociation constants (K_i) of the three synthetic compounds investigated are compared with those of (—)-physostigmine and (—)-physovenine. All measurements were made after pre-incubation of the inhibitor with the enzyme for 1 min and in every case purely competitive inhibition was observed; after pre-incubation for 3 min all compounds showed mixed inhibition and after pre-incubation for 10 min the inhibition kinetics were purely non-competitive. This change in kinetics has already (Robinson & Robinson, 1968) been explained by the formation of a carbamoylated enzyme by transfer of the carbamoyl group from the inhibitor to the enzyme.

Table 1. *Enzyme-inhibitor dissociation constants of* (—)-*physostigmine*, (—)-*physovenine, their enantiomers and* 1,1,3,3-*tetramethyl*-5-*methylcarbamoyloxyindolinium iodide.*

Inhibitor	Molor concn ($\times 10^7$)	Dissociation constants ($\times 10^7$) after pre-incubation of the inhibitor with acetylcholinesterase for 1 min
(—)-Physostigmine	1·416	5·1
(+)-Physostigmine	14·87	97
(—)-Physovenine	1·527	4·6
(+)-Physovenine	15·33	95
1,1,3,3-Tetramethyl-5-methylcarbamoyloxyindolinium iodide	0·0106	0·052

DISCUSSION

From Table 1 it can be seen that the value of K_i obtained for (—)-physotigmine and (—)-physovenine agree closely with those obtained earlier (Robinson & Robinson, 1968). Also, since a decrease in the value of K_i can be taken as an increase in inhibitory strength, (+)-physostigmine and (+)-physovenine are far less active anti-acetylcholinesterases than their natural enantiomers, whereas 1,1,3,3-tetramethyl-5-methylcarbamoyloxyindolium iodide is much more active than these alkaloids as an anti-acetylcholinesterase.

When the above alkaloids and their enantiomers are acting as anti-acetylcholinesterases, irrespective of whether the active form has ring C intact or open (see earlier), the marked differences in inhibitory activities between the members of the two enantiomeric pairs of bases is obviously caused by the optical asymmetry of the enzyme surface or receptor site or both. It could be that the difference in activity between the (+)- and (–)-isomers reflect the preferential enzymic opening of ring C in the (−)-isomers, which affords the 3*H*-indolium cations (III), the biologically active species, at or near the acetylcholinesterase receptor site.

The high anti-acetylcholinesterase activity of 1,1,3,3-tetramethyl-5-methylcarbamoyloxyindolinium iodide (IV) compared with that of (−)-physostigmine and of (−)-physovenine can be ascribed to the additional steric and/or ionic factors present in the alkaloids, or their ring C-opened 3*H*-indolium cations, at the $C_{(3)}$ atom of the indoline or 3*H*-indole nucleus, respectively, which adversely affect their binding to the receptor site. The asymmetric modification of these factors in (+)-physostigmine and (+)-physovenine, caused by optical inversion at $C_{(3)}$, further decreases the anti-acetylcholinesterase activities, probably by further adversely affecting binding of the inhibitor at the receptor.

Further studies investigating the relation between the asymmetry at $C_{(3)}$ and anti-acetylcholinesterase activity of other methylcarbamoyloxylated indoline derivatives are in progress.

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Combined gas-liquid chromatography—mass spectrometry in the study of barbiturate metabolism

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Combined gas-liquid chromatography-mass spectrometry is shown to be a useful technique for the identification of barbiturates and their metabolites, when applied to *NN'*-dimethyl derivatives.

Mass spectrometry offers a means of investigating the structures of organic compounds which are available in only small amounts (Budzikiewkz, Djerassi & Williams, 1964; McLafferty, 1967), hence its utility in the study of drug metabolism (Kabasakalian, Taggart & Townley, 1968, Neal, 1968; Schwartz, Vane & Postma, 1968). Combination with a gas-liquid chromatograph enables useful mass spectra to be obtained from components of a mixture which are present in sub-microgram quantities, since manipulative losses are reduced to a minimum (Holmes & Morrell, 1957). It was decided to evaluate the usefulness of this technique in determining the structure of metabolites of barbiturate drugs by ascertaining the information which could be derived from barbituric acid derivatives of known structure.

Although barbiturates themselves are amenable to gas liquid chromatography, it was found that greatly improved results were obtained when the *NN'*-dimethyl derivatives were used (Neville, 1970). This did not affect interpretation of the mass spectra. The molecular separator working on the opposed jet principle (Ryhage, 1964) gave superior results with polar compounds to the fritted glass type (Watson & Biemann, 1964) and hence was used in the investigation.

EXPERIMENTAL

The *NN'*-dimethyl derivatives of the barbiturates were prepared by addition of an ethereal solution of diazomethane to a methanolic solution of the barbiturate. After 15 min the solution was evaporated and a 10% solution of the derivative in methanol was prepared for GLC injection in 0·5 μl quantities. GLC analysis showed 85–90% conversion to the *NN'*-dimethyl derivative. A 5 foot stainless steel column packed with 2% SE 52 on Embacel 60/100 was used, and the temperature was maintained at either 175° or 200°. The molecular separator was contained in the GLC oven. The inlet line to the mass spectrometer source was kept at 200°. The mass spectrometer was an A.E.I. M.S. 902, running at a source temperature of 220° and a beam energy of 70 eV. Resolving power was 1000 (10% valley definition). The inlet pressure of the helium carrier gas was 2·5 p.s.i. and flow rate 50 ml/min. The column, separator and mass spectrometer inlet system were pretreated with 50 μl of hexamethyldisilazane.

The formula of ions encountered in the fragmentation schemes outlined in the discussion was confirmed by accurate mass measurements at a resolving power of 20 000 (10% valley definition). Although for a given GLC peak, a single accurate mass measurement could be made on a compound passing through the system, for multiple determinations it was more convenient to make such measurements on samples admitted via the direct inlet system of the mass spectrometer.

RESULTS AND DISCUSSION

Table 1 lists the compounds and their relative retentions, Table 2 their mass spectra. Those compounds with saturated alkyl side chains (I–IV), and in addition *NN'*-dimethylcyclobarbitone (VI) do not give observable molecular ions in their mass spectra. This is because of the ease of elimination of a molecule of ethylene from the molecular ion via a McLafferty rearrangement (McLafferty, 1959) in those compounds (I–IV) where an ethyl side-chain is present. Although (VI) has such an ethyl side-chain, this is in the β-position to the double bond of the cyclohexenyl substituent, hence fission of the ethyl group without transfer of hydrogen (Reed, 1966) is as

Table 1. *Compounds examined and their relative retention times*

Compound	R^1	R^2	Relative retention 175°	Relative retention 200°
NN'-Dimethylbarbitone (I)	Et	Et	0·48	0·6
NN'-Dimethylbutobarbitone (II)	Et	Bu^n	0·78	0·82
NN'-Dimethylpentobarbitone (III)	Et	$Me{\cdot}CH_2{\cdot}CH_2{\cdot}CH(Me)$-	1·0	1·0
NN'-Dimethylamylobarbitone (IV)	Et	$Me{\cdot}CH(Me){\cdot}CH_2{\cdot}CH_2$-	0·87	0·89
N-Methylhexobarbitone (V)	Me	Cyclohexenyl-	2·09	1·77
NN'-Dimethylcyclobarbitone (VI)	Et	Cyclohexenyl-	2·55	2·04
NN'-Dimethylphenobarbitone (VII)	Et	Ph	2·43	1·9
NN'-Dimethylquinalbarbitone (VIII)	$CH_2{:}CHCH_2$-	$Me{\cdot}CH_2{\cdot}CH_2CH(Me)$-	1·18	1·1
NN'-Dimethyl-5-(1-methylbutyl)-5-methoxycarbonylmethybarbituric acid (IX)	$-CH_2{\cdot}CO_2{\cdot}Me$	$Me{\cdot}CH_2{\cdot}CH_2{\cdot}CH(Me)$-		1·81
NN'-Dimethylnealbarbitone (X)	$CH_2{:}CHCH_2$-	$Me_3C{\cdot}CH_2$-	0·9	0·9
NN'-Dimethyl-3'-hydroxybutobarbitone (XI)	Et	$Me{\cdot}CH(OH){\cdot}CH_2CH_2$-		1·45
NN'-Dimethyl-3'-hydroxypentobarbitone (XII)	Et	$Me{\cdot}CH(OH){\cdot}CH_2{\cdot}CH(Me)$-		1·57

expected the dominant process in this case. The other alkyl substituent present in compounds I–IV, and the hydroxylated alkyl substituents present in compounds XI and XII are able to take part in a McLafferty rearrangement, whereby they are eliminated, but with transfer of a hydrogen atom to the residual charge-carrying portion of the molecule. This gives in all cases an ion of m/e 184 which decomposes further as in Scheme 1: the fragmentation of *NN'*-dimethylpentobarbitone. These two McLafferty rearrangement processes are therefore of great diagnostic value in determining the nature of the substituents attached to the 5-position of the barbiturate ring.

N-Methylhexobarbitone (V) and *NN'*-dimethylcyclobarbitone (VI) behave similarly in the mass spectrometer. Just as VI loses the ethyl group by β-fission, so does V lose its methyl substituent. The cyclohexyl substituent in both cases is involved in a McLafferty rearrangement, being eliminated as a C_6H_8 moiety to give ions of m/e 170 and 184 respectively. *NN'*-dimethylphenobarbitone (VII) is able to lose a molecule of ethylene from the ethyl substituent in the same manner as the previous compounds. An $(M-C_6H_5)$ ion is not observed, but the presence of a phenyl substituent can be inferred by the presence of a $C_6H_5^+$ ion at m/e 77, and ions at m/e 51 and 39, typical of phenyl-substituted compounds.

NN'-Dimethylquinalbarbitone (VIII), loses the 1-methylbutyl side-chain from the molecular ion as the neutral olefin, to give the base peak of the spectrum, at m/e 196. The molecular ion is also able to lose the allyl group to give an ion of m/e 225. *NN'*-Dimethylnealbarbitone (X), behaves similarly.

Table 2. *Mass spectra of the compounds examined.* m/e values are followed by relative intensities in parentheses. Only those peaks with relative intensities greater than 2 for m/e above 150 and relative intensities greater than 5 for m/e below 150 are included.

NN′-*Dimethylbarbitone* (I)
212 (1), 185 (9), 184 (97), 183 (30), 170 (9), 169 (100), 156 (2), 155 (3), 126 (25), 112 (15), 98 (5), 69 (7), 58 (7), 55 (10), 41 (12).

NN′-*Dimethylbutobarbitone* (II)
212 (1), 211 (10), 210 (8), 185 (8), 184 (63), 183 (12), 170 (16), 169 (100), 156 (2), 155 (3), 154 (2), 126 (11), 112 (21), 97 (7), 83 (7), 69 (7), 58 (13), 55 (21), 42 (7), 41 (24), 39 (10).

NN′-*Dimethylpentobarbitone* (III)
226 (8), 185 (11), 184 (88), 183 (12), 170 (9), 169 (100), 156 (3), 155 (3), 126 (9), 112 (13), 97 (9), 69 (12), 58 (10), 55 (12), 53 (5), 43 (25), 41 (34).

NN′-*Dimethylamylobarbitone* (IV)
227 (7), 226 (8), 211 (5), 185 (18), 184 (81), 183 (12), 170 (22), 169 (100), 156 (2), 155 (3), 126 (12), 112 (19), 97 (8), 83 (7), 69 (12), 58 (15), 56 (22), 43 (15), 41 (33), 39 (12).

N-*Methylhexobarbitone* (V)
250 (9), 236 (16), 235 (100), 178 (7), 171 (23), 170 (18), 169 (16), 137 (9), 136 (11), 135 (5), 112 (6), 108 (11), 93 (14), 91 (18), 81 (62), 80 (20), 79 (29), 77 (22), 67 (10), 66 (7), 65 (12), 58 (23), 56 (14), 55 (8), 53 (19), 52 (10), 51 (11), 41 (30), 39 (27).

NN′-*Dimethylcyclobarbitone* (VI)
236 (15), 235 (100), 185 (2), 184 (2), 183 (2), 179 (2), 178 (8), 170 (3), 169 (29), 150 (3), 149 (2), 121 (5), 112 (6), 91 (6), 81 (9), 79 (10), 77 (7), 58 (5), 53 (5), 41 (8), 39 (6).

NN′-*Dimethylphenobarbitone* (VII)
260 (7), 245 (4), 233 (15), 232 (100), 203 (4), 188 (9), 175 (20), 147 (4), 146 (24), 118 (22), 117 (22), 115 (7), 103 (10), 91 (8), 89 (6), 77 (8), 58 (6), 51 (4).

NN′-*Dimethylquinalbarbitone* (VIII)
266 (4), 248 (4), 237 (5), 225 (5), 224 (7), 223 (6), 209 (5), 197 (12), 196 (100), 195 (72), 183 (8), 181 (25), 169 (10), 138 (22), 126 (4), 112 (7), 111 (26), 110 (14), 109 (5), 97 (10), 82 (10), 81 (9), 80 (9), 58 (17), 55 (16), 53 (20), 43 (36), 41 (54), 39 (19).

NN′*Dimethyl*-5-(1-*methylbutyl*)-5-*methoxycarbonylmethylbarbituric acid* (IX)
298 (0·3), 267 (2), 229 (2), 228 (14), 227 (1), 225 (2), 197 (5), 196 (100), 195 (2), 183 (2), 169(10), 112 (5), 43 (8), 41 (7).

NN′-*Dimethylnealbarbitone* (X)
266 (4), 251 (21), 233 (3), 225 (5), 210 (15), 209 (64), 196 (15), 195 (80), 181 (5), 170 (13), 169 (100), 168 (8), 152 (6), 138 (14), 112 (20), 83 (10), 67 (13), 58 (12), 57 (65), 56 (8), 55 (18), 43 (13), 41 (60).

NN′-*Dimethyl*-3′-*hydroxybutobarbitone* (XI)
241 (2), 239 (2), 228 (2), 227 (6), 211 (3), 210 (3), 209 (12), 195 (2), 186 (4), 185 (28), 184 (40), 183 (6), 171 (5), 170 (14), 169 (82), 157 (5), 156 (4), 155 (10), 154 (4), 152 (4), 141 (5), 126 (22), 112 (32), 97 (24), 83 (19), 81 (11), 70 (16), 69 (34), 68 (15), 67 (18), 58 (72), 57 (21), 56 (29), 55 (64), 54 (23), 53 (34), 45 (100), 44 (17), 43 (84), 42 (36), 41 (68), 40 (18), 39 (52), 31 (19), 30 (16), 29 (60).

NN′-*Dimethyl*-3′-*hydroxypentobarbitone* (XII)
255 (2), 241 (2), 239 (2), 226 (26), 225 (6), 224 (30), 209 (3), 197 (2), 186 (7), 185 (44), 184 (89), 183 (18), 171 (5), 170 (11), 169 (100), 168 (2), 166 (3), 157 (4), 156 (6), 155 (4), 154 (2), 128 (7), 126 (11), 112 (21), 111 (7), 97 (18), 83 (9), 71 (10), 70 (19), 69 (85),67 (10), 59 (7), 58 (27), 57 (7), 56 (9), 55 (29), 53 (9), 45 (46), 44 (6), 43 (23), 42 (21), 41 (48), 40 (7), 39 (20), 30 (11), 29 (19).

The ester (IX), derived from quinalbarbitone by oxidation and subsequent methylation with diazomethane, undergoes fragmentations typical of the barbiturates and of esters (Scheme 2). Again the McLafferty rearrangement is in evidence in the typical elimination of the 1-methylbutyl side chain as an olefin from the molecular ion. The ester side chain in the resulting ion then loses a molecule of methanol.

The compounds (XI) and (XII), with hydroxylated side-chains, behave in a similar manner to each other. Thus the side-chains fragment by loss of the terminal methyl; by loss of CH_3CHOH; by loss of water and by complete elimination via the McLafferty rearrangement. This latter gives an intense peak in both spectra, and elimination of a methyl radical from this ion gives rise to the base peak. The breakdown of *NN′*-dimethyl-3′-hydroxybutobarbitone (XI) is shown in Scheme 3.

(III), m/e 254 (not observed)

$-C_2H_4$ → m/e 226

$-C_5H_{10}$ → m/e 184

m/e 184 → $-CH_3NCHO^{\bullet}$ → $Me\text{-}\overset{+}{N}\text{-}C(=O)\text{-}C(CH_2CH_3)=C=O$ m/e 126

m/e 184 → m* $-CH_3^{\bullet}$ → m/e 169

m/e 169 → $-CH_3NCO$ → m/e 112

m/e 112 → $-CH_3^{\bullet}$ → m/e 97

Scheme 1

(IX), m/e 298

$-CH_3O^{\bullet}$ → m/e 267

m* $-C_5H_{10}$ → m/e 228

m/e 267 → m* $-C_5H_{11}$ → m/e 196

m/e 228 → $-CH_3OH$ → m/e 196

m/e 228 → m* $-CH_3CO_2^{\bullet}$ → m/e 169 → m/e 112 → m/e 97

as in Scheme 1

Scheme 2

Scheme 3

In conclusion, it is evident that mass spectrometry, combined with gas-liquid chromatography, offers a very powerful method of determining the structures of barbiturate metabolites present in quite complex mixtures, since the side-chains fragment by processes which enable their structures to be deduced.

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The stabilization-lysis action of anti-inflammatory steroids on lysosomes

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The effect of anti-inflammatory steroids on lysosomal enzyme release has been investigated. Most of the steroids stabilized lysosomes at pharmacological concentrations (10^{-4}–10^{-6}M) but lysed them at higher concentrations. Etiocholanolone, a steroid pyrogenic in man, had no stabilizing effect. The concentration of steroid would therefore seem critical in determining its subcellular action. Experiments with albumin suggest that anti-inflammatory steroids (at 5×10^{-4}M) have little effect in aiding its thermal denaturation whereas other steroids greatly increase denaturation. Increasing concentrations of cortisol and prednisolone however caused greater denaturation of albumin. Although the correlation between albumin solutions and lysosomal membrane proteins is tenuous it is suggested that the lytic effect of anti-inflammatory steroids could be due to protein denaturation. Their stabilizing effect, however, probably involves steroid-lipid interactions.

Corticosteroids stabilize lysosomes and this property has been proposed as the basis of their anti-inflammatory activity (Weissmann & Dingle, 1961). Many of the *in vitro* experiments made on isolated lysosomes have been at a fixed steroid concentration. However, there is some evidence that at high concentrations the stabilizing action of these steroids on lysosomes is lost (Seeman, 1968). This investigation was designed to determine the action of steroids on lysosomes over a wide range of concentrations. In addition, the effect of steroids on the stability of acid phosphatase (EC 3.1.3.2) and of albumin has been examined because steroid-protein interactions may be involved in the action of steroids on lysosomes.

EXPERIMENTAL

The action of steroids on lysosomes

The action of dexamethasone, 9α-fluoroprednisolone, β-methasone alcohol, methyl prednisolone acetate, prednisolone, prednisolone stearoylglycolate and triamcinolone acetonide on lysosomes was examined. These steroids have a stabilizing action on lysosomes when at a final concentration of 5×10^{-4}M in our lysosomal preparation (Symons, Lewis & Ancill, 1969). Etiocholanolone, a steroid with a lytic action on lysosomes, was also examined.

Lysosome enriched suspensions in 0·05M tris-acetate buffered 0·25M sucrose (pH 7·4) were prepared from rabbit liver by methods previously described (Symons & others, 1969). The protein concentration of each suspension was determined by the method of Lowry, Rosebrough & others (1951). Steroids were deposited as thin films in 50 ml conical flasks by evaporating to dryness portions of a 1,4-dioxan solution. Portions (5 ml) of the lysosome suspensions were added to the flasks, which were then incubated for 90 min in a shaking reaction incubator at 37° (100 oscillations/min). In other experiments the flasks were incubated at 20° and 45°. Intact

lysosomes and debris were then removed by centrifuging at 20 000 *g* for 20 min at 4° in a Beckman Model L2 Ultracentrifuge. The supernatants were examined for acid phosphatase activity and β-glucuronidase activity (EC 3.2.1.31) by methods previously described (Symons & others, 1969). In one experiment *N*-acetyl-β-glucosaminidase (EC 3.2.1.30) activity was assayed. A 0·1 ml portion of the supernatant was added to 0·5 ml of 1·5 mM p-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (Koch–Light) and 0·5 ml of 0·2M acetate buffer pH 4·5. After incubation for 30 min at 37° in a shaking reaction incubator 5 ml of 0·1N NaOH was added and the amount of *p*-nitrophenol released was determined at 410 nm.

The effect of steroids on acid phosphatase

A lysosome suspension was prepared from rat liver by methods previously described (Symons & others, 1969). The suspension was frozen and thawed six times and the debris removed by centrifuging at 20 000 *g* for 20 min at 4°. The acid phosphatase activity of the supernatant was determined and 2 ml portions were transferred to stoppered test-tubes. The steroids were added to the tubes dissolved in 0·1 ml of ethanol, except for prednisolone stearoylglycolate which was dissolved in 0·1 ml of 1,4-dioxan. The solvent alone was added to the control tubes. The tubes were heated for 90 min in a water bath at 37°. Portions of the solutions were then examined for acid phosphatase activity. The experiment was repeated at 45°.

The effect of steroids on the stability of albumin solutions

The steroid was dissolved in 0·2 ml of either ethanol, 1,4-dioxan or propane-1,2-diol and added to stoppered test-tubes containing 5 ml of 1% w/v egg albumin dissolved in 0·9% w/v sodium chloride solution buffered at pH 5·2 with 0·1M phosphate. The solvent alone was added to the control tubes. The tubes were gently shaken to disperse the steroid and the extinction of the solution determined at 420 nm in a spectrophotometer (Unicam S.P. 500). The tubes were placed in a water bath, at a temperature, and for a period determined by preliminary experiments. After heating, the absorbances of the solutions were again determined. To avoid delay in the determination of the absorbances of the solutions only two steroids were compared with one control in each experiment.

RESULTS

The action of steroids on lysosomes

The effect of etiocholanolone, dexamethasone, methyl prednisolone acetate on the release of acid phosphatase and β-glucuronidase from lysosomes is shown in Fig. 1. The effect of prenisolone stearoylglycolate and β-methasone alcohol on the release of these two enzymes plus that of triamcinolone acetonide on acid phosphatase is shown in Fig. 2. Fig. 3 shows the effect of prednisolone on the two enzymes and the effect of 9α-fluoroprednisolone on the release of β-glucuronidase. The control values have been fixed at 100% and values in excess of 100% represent a lytic action and values below 100% represent a stabilizing action by the steroids on the lysosomes. The anti-inflammatory steroids, with the exception of prednisolone stearoylglycolate, gave stabilization-lysis curves where the steroids stabilized the lysosomes at the lower steroid concentrations but lysed them at higher concentrations. The optimum stabilization concentration range for all the anti-inflammatory steroids examined was 10^{-4}–10^{-6}M.

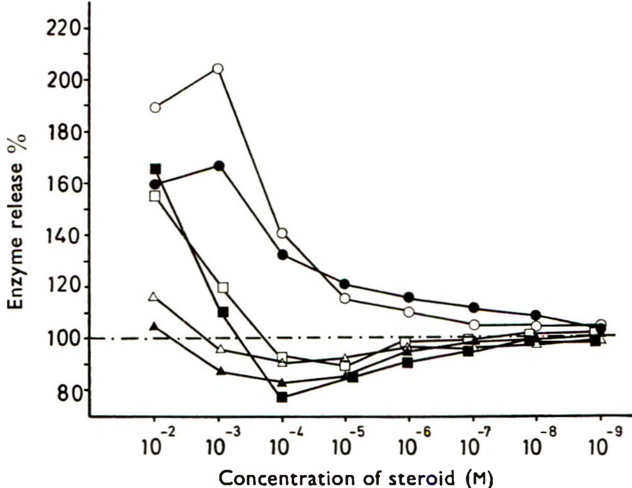

FIG. 1. The effect of etiocholanolone (circles), dexamethasone (triangles), and methyl prednisolone acetate (squares), on the release of lysosomal enzymes. Closed symbols represent acid phosphatase and open symbols β-glucuronidase. The protein concentration of the three lysosomal preparations were 4·1, 4·0 and 6·6 mg/ml respectively.

Prednisolone stearoylglycolate stabilized the lysosomes at 37° over the whole concentration range examined although this action declined at higher concentrations, but the curve was similar to that for the other steroids. The stabilizing properties of the steroid were much less at 20° than at 37°. At 20° the steroid had a lytic action on the lysosomes at the higher concentrations. At 45° the steroid had a lytic action over the entire concentration range (Fig. 4).

Etiocholanolone had a lytic-action on the lysosomes over the range of steroid concentrations examined which increased with concentration except for the highest concentration where there was a slight decline.

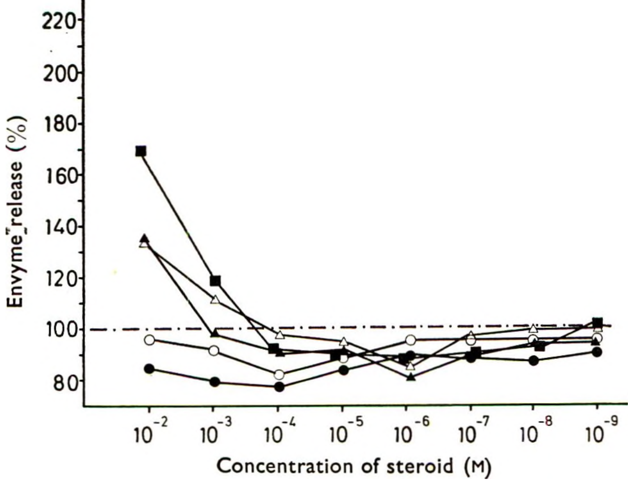

FIG. 2. The effect of prednisolone stearoylglycolate (circles), β-methasone alcohol (triangles), and triamcinolone acetonide (squares), on the release of lysosomal enzymes. Closed symbols represent acid phosphatase and open symbols β-glucuronidase. The protein concentration of the three lysosomal preparations were 6·4, 4·7 and 5·2 mg/ml respectively.

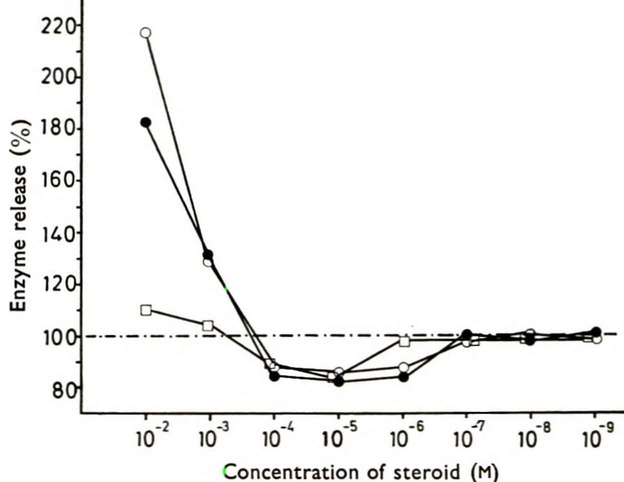

FIG. 3. The effect of prednisolone (circles), and 9α-fluoroprednisolone (squares), on the release of lysosomal enzymes. Closed symbols represent acid phosphatase and open symbols β-glucuronidase. The protein concentration of the two lysosomal preparations were 3·7 and 6·7 mg/ml respectively.

The effect of steroids on acid phosphatase

The results in Table 1 are in general agreement with those of Weissmann (1965) who has reported that steroids do not affect acid phosphatase activity at 37°. From Table 1 it would appear that low acid phosphatase levels in the lysosome experiments were due to membrane stability rather than inactivation of the enzyme. In other experiments where higher concentrations of steroids were used the results were similar. Presumably steroid solubility was a limiting factor in these experiments.

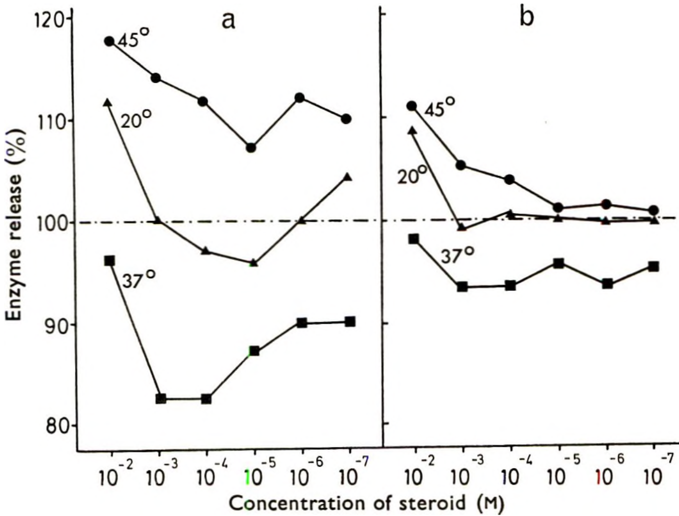

FIG. 4. a. The effect of prednisolone stearoylglycolate on the release of acid phosphatase from lysosomes at 20°, 37° and 45°.

b. The effect of prednisolone stearoylglycolate on the release of *N*-acetyl-β-glucosaminidase from lysosomes at 20°, 37° and 45°. Protein concentration of lysosomal preparation was 6·9 mg/ml.

Table 1. *Effect of steroids on acid phosphatase.* Each value is the mean of four experiments ± standard deviation.

Steroid added to final concentration of 5×10^{-4}M	Activity (%) of acid phosphatase after 90 min incubation at 37°	45°
None (control)	100	100
Cortisol	104 ± 5	112 ± 3
Cortiscosterone	100 ± 1	109 ± 5
Dexamethasone	113 ± 3	103 ± 1
Etiocholanolone	106 ± 2	114 ± 3
Epiandrosterone	100 ± 4	99 ± 3
9α-Fluorocortisol	103 ± 3	106 ± 2
β-Methasone alcohol	107 ± 2	106 ± 2
Methyl testosterone	96 ± 7	99 ± 1
Methyl prednisolone acetate	110 ± 2	115 ± 5
Prednisolone stearoylglycolate	108 ± 2	121 ± 5
Triamcinolone acetonide	110 ± 2	117 ± 3

The effect of steroids on the thermal stability of albumin solutions

The effect of steroids on the thermal denaturation of albumin solutions is shown in Table 2. The difference between the final and initial absorbance values of the solutions to which steroids were added have been calculated as a percentage of the increase of the absorbance values of the controls. Values in excess of 100% indicate that the steroid increased the amount of denaturation and values below 100% indicate that the steroid decreased the amount of denaturation. Steroids with a lytic action on lysosomes, namely etiocholanolone, dehydroepiandrosterone, testosterone (Weissmann, 1965) and methyl testosterone (personal observation) greatly increased the rate of thermal denaturation of the albumin solution. Some of the anti-inflammatory steroids also increased the rate of denaturation of the protein but less so than the androgens. Prednisolone stearoylglycolate effected a decrease indicating a protective action on the protein at the concentration examined. The denaturing action of cortisol, prednisolone and etiocholanolone increased with concentration (Table 3).

DISCUSSION

The results show clearly that steroid concentration is a critical factor in the action of steroids on lysosomes. In addition they show that many anti-inflammatory steroids have a biphasic action on lysosomes, in that they stabilize them at relatively low concentrations but lyse them at higher concentrations. The investigations of Bangham, Standish & Weissmann (1965) on the interaction of steroids with artificial lipid membranes suggest that the action of steroids on biological membranes may result directly from their interaction with lipid, independent of other membrane components. The chemical composition of rat liver lysosome membranes has been reported to be similar to that of plasma membranes. Typical analysis figures reported are phospholipid 0·43 mg/mg protein and cholesterol 0·13 mg/mg protein (Thinès-Sempoux, 1967). The lysosomal membrane is therefore rich in both lipid and protein. It is probably an over simplification to regard the lysosomal membrane as consisting of inert structural protein and lipid. At low concentrations the actions of steroids on lysosomes parallel closely the action of steroids on the permeability of artificial lipid membranes. Therefore it is unlikely that the mild protein denaturing actions of anti-inflammatory steroids we observed with albumin are of significance in their actions on lysosomes at low concentrations. However, it is possible that

Table 2. *Effect of steroids on the thermal denaturation of albumin.* Each value is the mean of three experiments ± standard deviation.

Steroid added to give a final concentration of 5×10^{-4}M	Action on lysosomes at pharmaceutical concentrations (10^{-4}–10^{-6}M)	Heating time min	Temp. °C	Solvent	Increase in denaturation of albumin (%) compared to control value of 100
Cholic acid	Lytic	15	60	Ethanol	266 ± 12
Dehydroepiandrosterone ..	"	10	60	Propane-1,2-diol	197 ± 12
Etiocholanolone	"	10	59	Ethanol	243 ± 40
Etiocholanolone	"	10	60	Propane-1,2-diol	225 ± 2
Methyl testosterone ..	"	10	60	Propane-1,2-diol	237 ± 10
Methyl testosterone ..	"	15	55	Ethanol	267 ± 21
Testosterone	"	10	60	Propane-1,2-diol	219 ± 3
Corticosterone	Stabilization	10	59	Ethanol	111 ± 2
Corticosterone	"	5	62	Propane-1,2-diol	127 ± 2
Corticosterone	"	10	60	Propane-1,2-diol	121 ± 4
Cortisol	"	5	62	Propane-1,2-diol	142 ± 2
Cortisol	"	10	59	Ethanol	116 ± 5
Cortisone	"	10	59	Ethanol	129 ± 7
9α-Fluorocortisol	"	5	62	Propane-1,2-diol	98 ± 4
9α-Fluorocortisol	"	10	59	Ethanol	96 ± 8
9α-Fluoroprednisolone ..	"	10	59	Ethanol	108 ± 2
Fluoxymesterone	"	10	59	Ethanol	98 ± 4
Fluoxymesterone ..	"	5	62	Propane-1,2-diol	98 ± 4
Methyl prednisolone acetate	"	15	55	Ethanol	128 ± 5
Prednisolone	"	10	60	Propane-1,2-diol	112 ± 1
Prednisolone	"	10	59	Ethanol	122 ± 3
Prednisone	"	5	62	Propane-1,2-diol	121 ± 4
Prednisone	"	15	55	Ethanol	124 ± 8
Prednisolone stearoyl-glycolate	"	15	55	1,4-Dioxan	76 ± 9

Table 3. *The effect of increasing steroid concentrations on the thermal denaturation of albumin.* Each value is the mean of three experiments ± standard deviation. Albumin solutions were heated at 50° for 15 min.

Steroid added	Concentration of steroid M	Increase in denaturation of albumin (%) compared to control value of 100
Cortisol	0	100
	10^{-4}	106 ± 1
	2×10^{-4}	112 ± 2
	3×10^{-4}	118 ± 5
	4×10^{-4}	120 ± 3
	5×10^{-4}	123 ± 0
Etiocholanolone	0	100
	10^{-4}	105 ± 1
	2×10^{-4}	135 ± 3
	3×10^{-4}	146 ± 3
	4×10^{-4}	152 ± 3
	5×10^{-4}	157 ± 1
Prednisolone	0	100
	10^{-4}	103 ± 1
	5×10^{-4}	108 ± 3

the stronger denaturing actions of androgens may have aided their lytic actions on lysosomes. In the presence of excess steroid (e.g. at high concentrations) the possibility that steroid-protein interactions occur increases. It has been shown in this investigation that the denaturing action of three steroids on albumin increased with concentration. Therefore it is possible that many steroid-protein interactions may affect the stability of the lysosomal membrane. Denaturation leads to a disorganization of protein structure and this will lead to a loss in stability of the membrane.

Although most of the steroids examined denatured albumin, prednisolone stearoylglycolate stabilized the protein and also stabilized the lysosomes over the entire concentration range examined (10^{-2}–10^{-9}M) at 37° while at 45° it had a lytic action on the lysosomes. The reason for this is not clear. It is possible that high acid phosphatase levels result from the stabilizing action of the steroid on the enzyme at 45° (Table 1). A recent report (Brown & Schwartz, 1969) stated that dexamethasone has a lytic action on lysosomes at 10^{-4}–10^{-5}M after incubation for 90 min at 45°. Although prednisolone stearoylglycolate stabilized the lysosomes at 10^{-4}–10^{-5}M at 20° the stabilization was much less than that at physiological temperature.

Whether the lytic action of steroids at high concentrations on lysosomes is of pharmacological significance is not clear. The concentrations of steroids needed to produce a lytic action on lysosomes in the *in vitro* experiments were well in excess of concentrations likely to occur *in vivo* as a result of short term steroid therapy. However, it is possible that long term steroid therapy may result in an accumulation of steroids in cells or membranes. It is of interest that the oral administration of steroids occasionally gives rise to gastrointestinal ulceration. Ulceration would be accelerated by lyosomal damage since acid hydrolases (e.g. cathepsins) would be released intracellularly. Many other drugs show a biphasic pattern of stabilization-lysis on various membranes. This subject has recently been reviewed by Seeman (1966).

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The actions of some non-steroidal drugs on lysosomes

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Several non-steroidal acidic anti-inflammatory drugs had a lytic action on lysosomes at high concentrations but no apparent action at lower concentrations. The drugs also inhibited acid phosphatase activity but only at high concentrations. High concentrations of drugs accelerated the thermal denaturation of albumin solutions but partially protected the albumin against denaturation at lower concentrations. The possibility that the denaturing action of drugs on proteins at high concentrations is related to lysosomal damage and that this damage may be associated with ulceration *in vivo* is discussed. Salicylate was found to inhibit the stabilizing action of cortisol on lysosomes.

The basis of the pharmacological action of non-steroidal anti-inflammatory drugs has been related to the property of many of them to uncouple oxidative phosphorylation (Adams & Cobb, 1958). Mizushima & Suzuki (1965) proposed that the ability of many of the drugs to stabilize proteins was a basis for their action. Other mechanisms have been reviewed recently (Whitehouse, 1968). The anti-inflammatory action of steroids has been related to their ability to stabilize lysosomes at certain concentrations (Weissmann & Dingle, 1961). Some non-steroidal drugs, for example phenylbutazone (Tanaka & Iizuka, 1968), stabilize lysosomes, but the evidence for the stabilizing action of salicylates on lysosomes is contradictory (Miller & Smith, 1966; Weissmann, 1968; Tanaka & Iizuka, 1968). Harford & Smith (1970) suggest that differences in experimental conditions may be responsible for the contradictory results reported by various authors.

It is apparent from investigations with steroids (Lewis, Symons & Ancill, 1970), that concentration and structure are important factors in the actions of these drugs on lysosomes. I have incubated non-steroidal drugs over a wide concentration range with lysosomes, and examined the effect of aromatic acids on the thermal stability of albumin and acid phosphatase (EC 3.1.3.2 orthophosphoric monoester phosphohydrolase). Aromatic acids interact strongly with proteins and it is possible that protein-drug interactions may effect the actions of drugs on lysosomes, particularly at high concentrations.

EXPERIMENTAL

The action of anti-inflammatory drugs on lysosomes

A lysosome suspension in tris-acetate (0·05M) buffered sucrose (pH 7·4) (0·25M) was prepared from rat liver (Lewis & others, 1970). An aqueous solution (adjusted to pH 7·4) of the sodium salt of the drug was prepared and portions added to 50 ml stoppered flasks and evaporated to dryness under reduced pressure. To this 5 ml of the lysosome suspension was added and the flasks were incubated for 90 min at 37° in a shaking reaction incubator. The suspensions were centrifuged at 20 000 *g*

for 20 min at 4° in a Beckman Model L2 Ultracentrifuge and the supernatants examined for acid phosphatase activity (Symons, Lewis & Ancill, 1969) and protein (Lowry, Rosebrough & others, 1951). The amount of protein present in the initial lysosome suspension was also determined and the preparation was diluted, if necessary, to give a protein concentration of 5 mg/ml.

In one experiment the action of salicylate on the stabilizing action of cortisol on lysosomes was examined. The initial lysosome suspension was divided into two portions and sodium salicylate was added to one portion to a final concentration of 5×10^{-4}M. Portions (5 ml) of both the salicylate-treated and untreated lysosomes were separately added to flasks containing various amounts of cortisol. After incubation at 37° for 90 min the supernatants were examined for acid phosphatase activity and β-glucuronidase (EC 3.2.1.31 β-D-glucuronide glucuronohydrolase) activity (Symons & others, 1969).

The effect of anti-inflammatory drugs on the thermal stability of acid phosphatase and albumin

Albumin solutions. A 1% w/v solution of egg albumin (which preliminary experiments showed to behave like bovine albumin) in 0·9% sodium chloride solution was prepared and buffered at pH 5·2 with 0·1M phosphate. Aqueous solutions of the sodium salts of the drugs were prepared and 0·1 ml portions added to 5 ml of the buffered protein solution. Water alone was added to the controls. The absorbances of the solutions were measured at 420 nm. The solutions were then heated and the absorbance values at 420 nm determined at various times.

Acid phosphatase activity. A lysosome suspension in tris acetate buffered sucrose was prepared and allowed to freeze and thaw six times. The suspension was then centrifuged at 20000 *g* for 20 min at 4° to remove lysosomes and lysosomal debris. Portions (2 ml) of the solutions were added to test tubes containing 0·1 ml of an aqueous solution of the sodium salt of the drug. The control tubes contained 0·1 ml of water. After their contents had been mixed the tubes were placed in a water bath at 37° and samples examined at various times for acid phosphatase activity. The initial acid phosphatase activity of the solutions was also determined.

RESULTS AND DISCUSSION

The drugs inhibited acid phosphatase activity at 10^{-1}M but had little effect at 10^{-5}M (Table 1). Salicylate, up to 10^{-3}M, has been reported to have no effect on acid

Table 1. *Effect of non-steroidal drugs on acid phosphatase.* Only at the higher drug concentration is the enzyme inhibited.

Incubation period (min)	% Activity of acid phosphatase preparation remaining after incubation in the presence of added drugs										
	None	* Ibufenac		† Ibuprofen		‡ Fluprofen		2-*p*-(2-Methyl biphenylyl)-propionic acid		Salicylic acid	
	(control)	10^{-1}M	10^{-8}M	10^{-1}M	10^{-8}M	10^{-1}M	10^{-5}M	10^{-1}M	10^{-5}M	10^{-1}M	10^{-5}M
0	100	—	—	—	—	—	—	—	—	—	—
90	66 ± 4	15 ± 3	61 ± 3	9 ± 4	64 ± 3	9 ± 2	63 ± 2	9 ± 1	64 ± 2	31 ± 3	57 ± 5
210	47 ± 3	5 ± 2	46 ± 1	2 ± 1	42 ± 2	1 ± 1	42 ± 4	2 ± 2	46 ± 4	15 ± 2	43 ± 2
330	30 ± 3	2 ± 2	31 ± 5	1 ± 1	30 ± 4	1 ± 1	31 ± 2	2 ± 2	32 ± 1	7 ± 1	27 ± 4

Each result is the mean value ± standard deviation of four experiments.
Abbreviations: *4-isobutylphenylacetic acid; †2-(4-isobutylphenyl)propionic acid; ‡2-*p*-(2-fluorobiphenyl)propionic acid.

phosphatase activity (Robinson & Willcox, 1969). Since the drugs inhibited the action of acid phosphatase, this enzyme could not be used to measure lysosome stability at high drug concentrations. However, the amount of enzyme released from lysosomes incubated with drugs over a concentration range of 10^{-4}–10^{-7}M was similar to the control values. The drugs do not appear to have stabilized the lysosomes at these concentrations. The amounts of protein released from the lysosomes after incubation with the drugs also showed no significant difference from control values at drug concentrations below 10^{-3}M but above this the results show the drugs to have a lytic action on lysosomes (Table 2). Although these high concentrations are not usually of physiological significance, it is possible that local high concentrations of the drugs occur after oral administration. A high concentration would certainly be present in the liquid film coating particles of the drugs in the stomach or elsewhere. It is therefore possible that gastrointestinal ulceration, a side-effect of these drugs, may be associated, at least in part, with lysosomal damage

Table 2. *Release of protein from lysosomes by anti-inflammatory drugs.*

	Amount of protein (as % with control value adjusted to 100) in supernatant after treating the lysomes with the drugs below					
Drug concn. (M)	Aspirin	Ibufenac	Ibuprofen	Fluprofen	2-p-(2-Methyl biphenylyl) propionic acid	Salicylic acid
None (control)	100	100	100	100	100	100
5×10^{-1}	—	—	—	—	—	208 ± 10
10^{-1}	385 ± 2	—	—	—	—	—
5×10^{-2}	—	183 ± 16	170 ± 2	175 ± 6	156 ± 5	104 ± 2
10^{-2}	210 ± 6	—	—	—	—	—
5×10^{-3}	—	99 ± 7	105 ± 4	130 ± 6	125 ± 2	94 ± 5
10^{-3}*	118 ± 4	—	—	—	—	—

* Concentrations of drug below this (to 10^{-8}) have no effect on protein release.

Table 3. *Effect of non-steroidal drugs on the thermal denaturation of albumin*

Concentration (M)	Aspirin (at 54°)	Ibuprofen (at 57°)	Ibufenac (at 58°)	Salicylic acid (at 55°)
None (control)	147 ± 3	185 ± 1	210 ± 2	162 ± 4
10^{-1}	1000 ± 50	—	—	1200 ± 200
5×10^{-2}	—	—	—	244 ± 15
$2 \cdot 5 \times 10^{-2}$	—	1209 ± 80	428 ± 10	—
10^{-2}	158 ± 3	—	—	—
5×10^{-3}	—	—	—	172 ± 2
$2 \cdot 5 \times 10^{-3}$	—	216 ± 4	220 ± 12	—
10^{-3}	144 ± 5	—	—	—
5×10^{-4}	—	—	—	158 ± 2
$2 \cdot 5 \times 10^{-4}$	—	179 ± 2	196 ± 4	—
10^{-4}	138 ± 2	—	—	—
5×10^{-5}	—	—	—	155 ± 3
$2 \cdot 5 \times 10^{-5}$	—	170 ± 3	178 ± 6	—
10^{-5}	140 ± 3	—	—	—
5×10^{-6}	—	—	—	164 ± 2
$2 \cdot 5 \times 10^{-6}$	—	178 ± 2	172 ± 9	—
10^{-6}	141 ± 2	—	—	—

The values represent the percentage increase in the absorbances of the solutions after heating for 15 min at various temperatures.

induced by high concentrations. Further experiments showed that salicylate and ibufenac caused a rapid release of protein from gut and stomach tissue suspended in oxygenated Ringer–Tyrode solution. However, it was not possible to determine the cellular source of this protein.

The drugs stabilized albumin against thermal denaturation at the lower concentrations but rapidly increased the rate of denaturation at high concentrations (see Table 3). It is possible that a denaturing action by the drugs on lysosomal membrane proteins may be responsible for the lytic actions of these drugs at high concentrations. Cortisol stabilized lysosomes over a concentration range of 10^{-3}–10^{-8}M but induced lysis at 10^{-2}M. The stabilizing action of cortisol was abolished in the presence of salicylate. At a concentration of 10^{-2}M some cortisol was taken up by the lysosome, despite the presence of salicylate, since some stability was conferred on the organelle. It would appear that salicylate blocked the uptake of cortisol at the lower concentrations. At a cortisol concentration of 10^{-2}M sufficient cortisol was taken up by the lysosome to stabilize it but insufficient to induce lysis.

This result may have some clinical interest since salicylates and steroids are occasionally used together in the treatment of rheumatoid arthritis.

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The effects of salicylate on the concentrations of amino-acids in mouse tissues

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The concentrations of amino-acids in chopped preparations of mouse liver incubated with 0 to 20 mM salicylate were measured. The changes, observed with salicylate concentrations of 10 mM and above, were increased concentrations of aspartate, glutamine, tyrosine and ornithine and decreased concentrations of glutamate and γ-aminobutyrate. The effects of the intraperitoneal injection of salicylate, in doses ranging from 75 to 600 mg/kg body weight, on the amino-acid concentrations in mouse blood, kidney, liver and brain were studied. With a dose of 600 mg/kg, the amino-acid concentrations were decreased in the blood (except glutamate and aspartate which increased) and in the kidney, were increased in the liver (except glutamine, glutamate, glycine and alanine which decreased) and were unchanged in the brain (except alanine, valine and leucine which decreased and γ-aminobutyrate which increased). These changes may result from a combination of an inhibitory effect of salicylate on the renal tubular transport of the amino-acids and intracellular actions of the drug on aminotransferase and other enzyme activities.

The administration of salicylate has been shown to produce amino-aciduria in man (Andrews, Bruton & Knoblock, 1961) and in the rat (Berry & Guest, 1963). It has been suggested that this is due to the drug blocking the reabsorption of amino-acids in the kidney tubules (Segal & Blair, 1963). However, Andrews, Bruton & de Baare (1962) reported that the plasma concentrations of amino-acids were elevated in salicylate-poisoned patients suggesting that there was an intracellular accumulation of amino-acids leading to an increased entry of these metabolites into the circulation and hence an increased rate of urinary excretion. There is considerable evidence that salicylate inhibits several pathways of amino-acid metabolism *in vitro*. These include transamination reactions (Gould & Smith, 1965; Gould, Dawkins & others, 1966) and incorporation into proteins (Dawkins, Gould & Smith, 1966; Burleigh & Smith, 1970). In addition, there are some limited studies indicating that the administration of salicylate *in vivo* alters the concentrations of some amino-acids in rat tissues (Yoshida, Metcoff & Kaiser, 1961; Huggins & Smith, 1963). The present investigation is concerned with the effects of salicylate on the patterns and concentrations of amino-acids in mouse tissues, *in vitro* and *in vivo*, using an automated method for the separation and estimation of the amino-acids.

EXPERIMENTAL

Materials. Amino-acids were obtained from BDH Limited, Poole, Dorset; ninhydrin, A.R. grade, from Koch-Light Limited, Colnbrook, Bucks; hydrindantin was prepared by the method of Connell, Dixon & Haines (1955), reduced and oxidized gluatathione were obtained from the Sigma Chemical Co., St. Louis, and methylcellosolve from Union Carbide Chemicals Division, Rickmansworth, Herts. All

other chemicals were of analytical grade and glass-distilled water was used throughout.

Animals and design of experiments. Male albino mice, 25 to 30 g, of the King's College Hospital strain, maintained on MRC cube diet no. 41B, were killed by cervical fracture. In the *in vitro* experiments the livers were removed as rapidly as possible, washed with Krebs-Ringer phosphate (Cohen, 1957), pH 7·4, blotted and either homogenized immediately in 10 vol of saturated picric acid or cut into blocks 0·3 mm × 0·3 mm × 0·7 mm with a tissue chopper (McIlwaine & Buddle, 1953). The wet weight of tissue initially used for each experiment was 2·0 g and the chopped tissue was suspended in either 5 ml of the phosphate medium or 5 ml of the medium containing sufficient sodium salicylate to give final concentrations of between 2·5 and 20 mM. Each mixture was incubated in a 50 ml glass-stoppered conical flask for 1 h at 37° with shaking and the tissue killed by the addition of 20 ml of saturated picric acid solution. Corresponding control mixtures were obtained by adding the picric acid solution either at zero time or after incubation for 1 h. After the addition of the picric acid the mixture was homogenized in an all-glass Potter homogenizer, centrifuged at 3000 *g* for 20 min and the supernatant removed for subsequent analysis.

In the *in vivo* experiments each animal was given an intraperitoneal injection (0·5 ml) of either saline or sodium salicylate in doses ranging from 75 to 600 mg/kg body weight. Each injection was adjusted by the addition of appropriate amounts of sodium chloride to give a final sodium content of 0·52 g-ions per 100 ml. The animals were killed at time intervals varying from 0·5 to 4 h after the injection and the livers removed, homogenized in 10 vol of saturated picric acid solution and centrifuged as described above. In some experiments the kidneys and brains and samples of blood were also removed and treated as described for liver except that the brains were homogenized in 20 vol of picric acid.

Preparation of tissue extracts for analysis. Aliquots (5 ml) of each picric acid supernatant were allowed to pass through 12 cm × 0·5 cm diameter columns of Dowex 1-acetate and the amino-acids eluted with 160 ml of 0·03 M acetic acid. Under these conditions, only the picric acid and glutathione remained on the column. It was necessary to remove glutathione because the tripeptide, either in the reduced or oxidized form, interfered with the subsequent analysis. Reduced glutathione obscured aspartate and oxidized glutathione could not be separated from glycine and alanine. The total effluent was evaporated to dryness in a rotary evaporator under reduced pressure at 40° and the residue stored at − 20° until required for analysis. The stability of the amino-acids under these conditions of storage was investigated by analysing aliquots of a bulk, one in ten homogenate of mouse liver, freed from picric acid and glutathione, at intervals of one and three months.

Amino-acid analysis. The apparatus was constructed from standard Technicon Autoanalyser units arranged according to the conventional plan. The column was filled to a height of 132 cm with Chromobeads A. The Haake heating bath was modified to allow the insertion of two contact thermometers controlled by a relay and time switch so that the apparatus could be operated for various time intervals at different temperatures. The Milton Roy pump was set to deliver 0·5 ml/min. The column was regenerated by filling the line from the Autograd to the tylock connection at the top of the column (volume 15 ml) with 0·2N NaOH, connecting up to a reservoir of pH 3·0 buffer and pumping for 2 h at a flow rate of 0·5 ml/min. The manifold consisted of a sample line (Orange Clear Tygon, 0·42 ml/min), a nitrogen line (Red Clear Tygon 0·8 ml/min) ninhydrin reagent line (Yellow Solvaflex, 1·06 ml/min) and a

return line (Yellow Solvaflex, 1·06 ml/min) and was cleaned as recommended by Technicon. The pump tubes were stretched one hole after each run. Nitrogen gas (white spot, British Oxygen Company) was continuously passed through the standard Technicon ninhydrin reagent. The heating bath was operated at 95°, only a single coil being used. Buffers were prepared according to the directions given by Technicon except that varying quantities of HCl or NaCl were added to give a range of pH values and salt concentrations.

The residue from the tissue extracts was dissolved in 5 ml of 0·1 M HCl containing 0·1 mM norleucine as an internal standard and 1 ml aliquots loaded on the top of the Chromobead column by forcing in under pressure. The amino-acids were eluted with the buffer gradient produced from the Autograd*.

The temperature of the column was maintained at 35° for the first 2 h and then allowed to cool to room temperature (20°) by the time control device switching off the Haake heating bath. After a further 2 h, the temperature of the column jacket was raised to 60° and kept at this temperature for the remaining 16 h of the run. Under these conditions the breakdown of glutamine was reduced to a minimum, and a good resolution of aspartate, threonine, serine and asparagine plus glutamine was obtained with the recorder pen returning to the base line between each peak. In addition, proline was eluted before and completely separated from glutamate, citrulline appeared as a separate peak between glutamate and glycine, β-alanine was separated after phenylalanine, γ-aminobutyrate, ethanolamine and ammonia occurred as separate peaks in that order and the elution of tryptophan was accelerated so that it formed a separate peak between ammonia and ornithine.

Ninhydrin-positive peaks were initially identified by comparing their elution times with those of authentic compounds. Samples containing five times the normal amount of amino-acids were loaded on the Analyser and eluted in the normal way for peak identification. The column effluent was split so that one tenth passed into the analytical system and the remainder was collected in a fraction collector holding 200 tube positions and set to collect a fresh sample every six min. The manifold for the analytical system consisted of a sample line (Orange Blue Clear Tygon, 0·05 ml/min); a nitrogen line (Black Clear Tygon 0·32 ml/min); a ninhydrin reagent line (White Solvaflex, 0·56 ml/min) and a return line (Orange Solvaflex, 0·42 ml/min). The recorder was fitted with an event marker to denote the changing of tubes in the fraction collector. The contents of tubes corresponding to each peak on the recorder chart were bulked and the combined fraction was desalted by passing it through a 15 cm × 0·5 cm diameter column of Zeokarb 225 H^+ and washing the resin with 30 ml distilled water. The amino-acids were then eluted with 30 ml 2N ammonium hydroxide. The residue, obtained after evaporating the ammoniacal solution to dryness in a rotary evaporator, was dissolved in 1 ml of 10% (v/v) isopropanol in 0·1 N HCl and transferred to a bijou bottle. The resulting solution was evaporated to dryness in a vacuum desiccator, containing concentrated H_2SO_4 and a beaker of NaOH pellets, and the residue dissolved in 50 μl of the acidified isopropanol. The amino-acids in 5 μl aliquots were identified by paper chromatography and high voltage electrophoresis. Identification of some of the minor peaks necessitated bulking the samples from several separate analyses.

* Chambers Nos. 1–4 contained 0·2M sodium citrate† buffer pH 3·0; No. 5, 0·2M citrate† buffer pH 4·0; Nos. 6 and 7, 0·18M citrate buffer pH 5·0; Nos. 8 and 9, 0·2 M citrate buffer pH 5·0 containing M sodium chloride (Volume 75 ml). All buffers contained 0·1% (w/v) phenol and 1% (v/v) Brij 35 detergent. Those marked † also contained 0·5% (v/v) thiodiglycol.

Recovery of the amino-acids was studied by analysing portions of a mouse liver extract before and after adding known amounts of standard amino-acids and comparing their differences with separate determinations of the standard amino-acid solution. The results ranged from 93% for aspartate and glutamate to 105% for β-alanine, the overall mean recovery being 99%.

RESULTS

Amino-acid concentrations in mouse liver. The concentrations of the amino-acids in mouse liver preparations either homogenized in picric acid immediately after death, chopped into blocks before the addition of the picric acid or after incubating the chopped samples for 1h at 37° in Krebs-Ringer phosphate before adding the picric acid, are given in Table 1. The concentrations of the amino-acids in the picric acid homogenate were greater than those in the chopped preparation but subsequent incubation of the latter preparation for 1 h at 37° in the phosphate medium caused an appearance of γ-aminobutyrate, decreased concentrations of aspartate and glutamine,

Table 1. *Amino-acid concentrations in mouse liver preparations.* The whole liver was either homogenized in 10 vol of saturated picric acid or 2 g portions cut into blocks with a tissue chopper followed by homogenization in 20 ml picric acid either before or after incubation at 37° for 1 h in 5 ml of Krebs-Ringer phosphate buffer, pH 7·4. After centrifugation at 3000 *g* for 20 min each supernatant was passed through a Dowex 1-acetate column to remove the picric acid, the effluent evaporated to dryness and dissolved in 5 ml of 0·1 M HCl containing 0·1 mM norleucine. Aliquots (1 ml) were analysed for amino-acids using a Technicon AutoAnalyser system modified as described in the text. The results are given as the means ± s.d.

	Amino-acid concentration (μmol/g wet weight liver)		
Amino-acid	Whole liver (15 animals)	Chopped liver (7 samples)	Chopped liver after 1 h incubation (11 samples)
Tau*	11·07 ± 2·40	—	—
Asp	0·59 ± 0·18	0·46 ± 0·17	0·32 ± 0·03
Thr	0·18 ± 0·05	0·15 ± 0·08	0·44 ± 0·03
Ser	0·42 ± 0·07	0·42 ± 0·15	0·68 ± 0·12
Gln	3·43 ± 0·52	1·09 ± 0·54	0·87 ± 0·19
Pro	0·13 ± 0·06	0·16 ± 0·06	0·34 ± 0·11
Glu	1·93 ± 0·38	1·78 ± 0·48	2·98 ± 0·65
Cit†	0·03 ± 0·01	0·04 ± 0·02	0·06 ± 0·04
Gly	2·07 ± 0·29	1·38 ± 0·65	2·67 ± 0·33
Ala	2·08 ± 0·57	1·78 ± 0·53	6·04 ± 0·74
Val	0·18 ± 0·04	0·11 ± 0·04	0·56 ± 0·10
Met	0·03 ± 0·01	0·02 ± 0·01	0·10 ± 0·02
Ile	0·09 ± 0·03	0·04 ± 0·02	0·25 ± 0·03
Leu	0·18 ± 0·05	0·10 ± 0·04	0·45 ± 0·06
Tyr	0·07 ± 0·02	0·03 ± 0·01	0·02 ± 0·01
Phe	0·07 ± 0·02	0·04 ± 0·02	0·15 ± 0·02
β-Ala‡	0·14 ± 0·03	0·15 ± 0·03	0·57 ± 0·32
Amb§	0·00	0·00	0·82 ± 0·31
Eth‖	0·10 ± 0·04	0·14 ± 0·04	0·60 ± 0·38
Orn	0·34 ± 0·06	0·24 ± 0·11	0·39 ± 0·10
Lys	0·44 ± 0·12	0·25 ± 0·11	0·76 ± 0·11
His	0·43 ± 0·07	0·25 ± 0·12	0·45 ± 0·10
Arg	0·03 ± 0·01	0·01 ± 0·01	0·01 ± 0·01

* Taurine. † Citrulline. ‡ β-Alanine. § γ-Aminobutyrate. ‖ Ethanolamine.

and increased concentrations of the remainder except tyrosine and arginine. No significant changes in the concentrations of the amino-acids in the picric acid extract of mouse liver homogenate ocurred after storage at −20° for up to three months.

Effects of salicylate on the concentrations of amino-acids in mouse liver in vitro

The concentrations of the amino-acids in chopped preparations of mouse liver incubated for 1 h at 37° were measured in the presence and in the absence of salicylate concentrations ranging from 2·5 to 20 mM. No significant changes were observed with salicylate concentrations of 2·5 and 5 mM; at salicylate concentrations of 10 mM and above the most prominent effects were increased concentrations of aspartate, glutamine, tyrosine and ornithine and diminished contents of glutamate and γ-amino-butyrate. Alanine was reduced in the presence of only 20 mM salicylate (Table 2.)

Table 2. *Effect of salicylate on the amino-acid concentrations of chopped mouse liver preparations* in vitro. Experimental conditions as in Table 1. The results are expressed as percentages of the corresponding control values. Only those with values for $P < 0.05$ (Student's t-test) are included.

Amino-acid	Salicylate concn (mM)	
	10	20
Asp	162	159
Gln	151	155
Glu	76	83
Ala	—	69
Tyr	314	496
Amb	60	26
Orn	145	123

Effects of salicylate injections on the concentrations of amino-acids in mouse tissues

A high dose of salicylate, 600 mg/kg weight, was chosen for the first set of experiments and the amino-acid concentrations in the livers of animals killed between ½ and 4 h after the injection are given in Table 3. Taurine, aspartate, proline, ethanolamine and histidine were not affected and have been excluded from the table but all the remainder showed changes. Glutamine and glutamate were significantly decreased over the 4 h period, glycine and alanine were reduced initially and the others were increased at one or more time intervals. The most widespread changes were observed at 1 h after injection. This time interval was used in the subsequent experiments in which the amino-acids were measured in blood, kidney, liver and brain of mice injected with either saline or salicylate. The results, after the administration of 600 mg/kg body weight salicylate are given in Table 4 and any changes observed with lower doses of the drug are described below. In blood the 600 mg/kg dose caused either significant decreases or no change in the concentrations of the amino-acids with the exception of aspartate and glutamate which increased significantly. This effect was less marked with lower doses of salicylate, with a 300 mg/kg dose, threonine, serine, glutamine, citrulline, alanine, valine, isoleucine, leucine and lysine were significantly decreased and with 150 mg/kg salicylate, serine, glutamine, alanine, valine, isoleucine and leucine were reduced. Similar changes were observed in the kidney except that glutamate and aspartate decreased but the brain showed a different pattern

Table 3. *Effects of 600 mg per kg body weight of salicylate on the amino-acids of the liver in mice killed at varying time intervals after the injection.* Each animal was given an intraperitoneal injection (0·5 ml) of either saline or sodium salicylate and killed at either ½, 1, 2 or 4 h. The liver was removed and homogenized in 10 vol of saturated picric acid and subsequently treated as described in Table 1. At each time interval six mice injected with saline and six with salicylate were used. The results are expressed as mean percentage differences from the corresponding control group. They have been analysed by Students' *t*-test and those marked * show a significant difference ($P < 0{\cdot}05$) between the control and salicylate-treated animals.

Amino-acid	Time after injection (h) ½	1	2	4
Thr	+27	+64*	+79*	+141*
Ser	+5	+61*	+36*	+59*
Gln	−60*	−64*	−54*	−41*
Glu	−64*	−47*	−44*	−18*
Cit	+84*	+104*	+125	+131
Gly	−20*	−27*	−21	+8
Ala	−26*	−56*	−5	−11
Val	+19	+72*	+65*	+113*
Ile	+8	+61*	+72*	+51*
Leu	+9	+33*	+24	+21
Tyr	−15	+35*	+21	+8
Phe	+2	+52*	+21*	+24*
β-Ala	+51	+73*	+89*	+97*
Orn	+52	+50*	+56*	+63*
Lys	+92*	+62*	+52*	+56*
Arg	−19	+79*	+27	+47

in that the only significant changes were decreased concentrations of alanine, valine and leucine and an increased concentration of γ-aminobutyrate at the 600 mg dose. An additional experiment was performed in which mice received a lethal dose of salicylate, 800 mg/kg, and were killed when they started to convulse, between 75 min and 2 h after the injection. The only changes observed in the brain amino-acids were decreased concentrations of alanine and valine. The results in the liver were similar to those given in Table 3 except that with lower doses, 300 and 150 mg/kg, only glutamine was significantly reduced and methionine and β-alanine increased.

DISCUSSION

Suspensions of animal tissues prepared with a mechanical chopper are superior to tissue homogenates for studying some aspects of metabolism because cellular structure is largely retained (McIlwaine & Buddle, 1953) and are convenient systems for the investigation of possible effects of substances added *in vitro*. The present results (Table 1) show that the concentrations of many of the amino-acids are lower in the chopped liver than in the liver homogenized in picric acid directly after removal from the animal. This decrease is probably due to transfer of amino-acids from the cut tissues into the wet filter paper on which the liver is placed during the chopping procedure. The values found in the chopped liver before incubation have been used as zero time values for comparison with those found after incubation and are not strictly comparable with values in the picric acid homogenate, which have been taken to be

Table 4. *Effects of salicylate on the concentrations of amino acids in mouse blood, liver, kidney and brain.*

Each animal was given an intraperitoneal injection (0·5 ml) of either saline or sodium salicylate and killed after 1 h. The livers, kidneys, brains and weighed samples of whole blood from groups of five animals were homogenized in either 10 or 20 vol of saturated picric acid and subsequently treated as described in Table 1. Only the results for the 600 mg per kg body weight are given and they are expressed as the means ± standard deviations, the numbers in parentheses being the numbers of groups, each of five animals. They have been analysed by Student's *t*-test and * indicates a significant difference ($P < 0·05$) between the control and salicylate-treated animals.

Amino-acid	Amino-acid concentration (μmol/g wet weight)							
	Blood		Kidney		Liver		Brain	
	Control	Salicylate	Control	Salicylate	Control	Salicylate	Control	Salicylate
Tau	0·52 ± 0·09 (7)	0·56 ± 0·13 (5)	6·26 ± 1·71 (9)	3·07 ± 0·48 (10)*	10·69 ± 3·18 (9)	11·24 ± 2·53 (10)	7·02 ± 1·19 (7)	6·53 ± 0·39 (5)
Asp	0·11 ± 0·03 (7)	0·16 ± 0·03 (4)*	1·41 ± 0·20 (9)	1·07 ± 0·25 (8)*	0·46 ± 0·11 (7)	0·52 ± 0·08 (5)	3·88 ± 0·28 (7)	4·09 ± 0·13 (5)
Thr	0·17 ± 0·01 (5)	0·09 ± 0·02 (5)*	0·27 ± 0·04 (9)	0·16 ± 0·02 (8)*	0·24 ± 0·07 (7)	0·40 ± 0·03 (5)*	0·49 ± 0·05 (7)	0·48 ± 0·04 (5)
Ser	0·19 ± 0·02 (5)	0·13 ± 0·03 (5)*	0·44 ± 0·07 (9)	0·20 ± 0·05 (10)*	0·43 ± 0·09 (7)	0·63 ± 0·06 (5)*	1·10 ± 0·14 (7)	1·09 ± 0·06 (5)
Gln	0·54 ± 0·05 (7)	0·33 ± 0·05 (5)*	1·49 ± 0·26 (9)	0·28 ± 0·07 (10)*	4·63 ± 0·71 (5)	1·81 ± 0·16 (5)*	5·79 ± 0·58 (7)	6·20 ± 0·79 (5)
Pro	0·14 ± 0·08 (6)	0·06 ± 0·02 (4)	0·08 ± 0·05 (5)	0·03 ± 0·02 (4)	0·21 ± 0·07 (7)	0·26 ± 0·04 (4)	0·13 ± 0·02 (6)	0·12 ± 0·03 (6)
Glu	0·21 ± 0·04 (7)	0·29 ± 0·04 (4)*	5·63 ± 0·75 (9)	3·00 ± 0·66 (12)*	1·87 ± 0·43 (7)	1·28 ± 0·15 (5)*	13·42 ± 2·41 (7)	13·60 ± 1·74 (5)
Cit	0·07 ± 0·01 (7)	0·05 ± 0·01 (5)*	0·04 ± 0·01 (4)	0·04 ± 0·03 (3)	0·02 ± 0·01 (7)	0·04 ± 0·01 (5)*	0·05 ± 0·01 (4)	0·05 ± 0·02 (4)
Gly	0·46 ± 0·04 (5)	0·35 ± 0·09 (5)*	3·86 ± 0·45 (9)	0·98 ± 0·18 (12)*	1·93 ± 0·27 (7)	1·51 ± 0·19 (5)*	2·11 ± 0·54 (7)	2·08 ± 0·15 (5)
Ala	0·35 ± 0·04 (5)	0·14 ± 0·04 (5)*	0·70 ± 0·11 (9)	0·31 ± 0·06 (12)*	2·55 ± 0·61 (7)	1·75 ± 0·27 (5)*	0·89 ± 0·11 (7)	0·66 ± 0·08 (5)*
Val	0·17 ± 0·02 (6)	0·09 ± 0·01 (5)*	0·16 ± 0·02 (9)	0·12 ± 0·03 (12)*	0·24 ± 0·08 (7)	0·43 ± 0·05 (5)*	0·11 ± 0·01 (7)	0·07 ± 0·02 (5)*
Met	0·03 ± 0·01 (5)	0·02 ± 0·01 (3)	0·07 ± 0·02 (9)	0·04 ± 0·01 (9)*	0·04 ± 0·01 (5)	0·09 ± 0·02 (4)*	0·04 ± 0·03 (7)	0·02 ± 0·02 (5)
Ile	0·06 ± 0·01 (7)	0·03 ± 0·01 (5)*	0·08 ± 0·01 (9)	0·05 ± 0·01 (12)*	0·13 ± 0·05 (7)	0·24 ± 0·03 (5)*	0·04 ± 0·02 (7)	0·03 ± 0·01 (4)
Leu	0·13 ± 0·01 (6)	0·07 ± 0·01 (5)	0·25 ± 0·05 (9)	0·12 ± 0·02 (12)*	0·29 ± 0·04 (7)	0·42 ± 0·03 (5)*	0·12 ± 0·03 (7)	0·08 ± 0·01 (5)*
Tyr	0·08 ± 0·02 (7)	0·07 ± 0·01 (5)	0·08 ± 0·02 (9)	0·08 ± 0·02 (12)	0·10 ± 0·04 (7)	0·17 ± 0·03 (5)*	0·10 ± 0·01 (7)	0·11 ± 0·02 (5)
Phe	0·06 ± 0·01 (7)	0·05 ± 0·02 (5)	0·07 ± 0·03 (9)	0·07 ± 0·02 (11)	0·10 ± 0·04 (7)	0·18 ± 0·03 (5)*	0·08 ± 0·02 (7)	0·08 ± 0·02 (5)
β-Ala	0·02 ± 0·01 (3)	0·03 ± 0·01 (2)	0·44 ± 0·12 (9)	0·15 ± 0·09 (9)*	0·14 ± 0·03 (7)	0·20 ± 0·03 (5)*	0·09 ± 0·03 (2)	0·11 ± 0·02 (2)
Amb	0·00	0·00	0·00	0·00	0·00	0·00	3·04 ± 0·45 (7)	3·59 ± 0·25 (5)*
Eth	0·03 ± 0·01 (3)	0·03 ± 0·01 (3)	0·50 ± 0·24 (9)	0·37 ± 0·16 (12)	0·11 ± 0·05 (7)	0·13 ± 0·05 (5)	0·73 ± 0·01 (3)	0·74 ± 0·03 (3)
Orn	0·08 ± 0·01 (6)	0·05 ± 0·01 (5)*	0·05 ± 0·01 (9)	0·04 ± 0·01 (12)*	0·31 ± 0·08 (7)	0·43 ± 0·08 (5)*	0·05 ± 0·03 (5)	0·04 ± 0·01 (4)
Lys	0·34 ± 0·04 (6)	0·26 ± 0·05 (5)*	0·22 ± 0·02 (9)	0·17 ± 0·03 (12)*	0·48 ± 0·18 (7)	0·85 ± 0·12 (5)*	0·34 ± 0·02 (7)	0·30 ± 0·04 (5)
His	0·05 ± 0·01 (7)	0·06 ± 0·03 (5)	0·14 ± 0·03 (9)	0·07 ± 0·01 (12)*	0·43 ± 0·13 (7)	0·41 ± 0·04 (5)	0·10 ± 0·03 (7)	0·11 ± 0·02 (5)
Arg	0·20 ± 0·02 (5)	0·14 ± 0·01 (5)*	0·16 ± 0·03 (9)	0·09 ± 0·02 (11)*	0·03 ± 0·01 (7)	0·05 ± 0·01 (5)*	0·21 ± 0·03 (7)	0·19 ± 0·03 (4)

more representative of concentrations of amino-acids existing *in vivo*. After incubation of the chopped mouse liver preparations in Krebs-Ringer phosphate medium, the concentrations of almost all the amino-acids increased markedly. The appearance of γ-aminobutyrate in the chopped liver preparations after incubation is consistent with the results of other workers who have shown that the presence of the amino-acid is not restricted to the brain and may occur in the liver of man (Zachmann, Tocci & Nyhan, 1966) and of the cat (Tallan, Moore & Stein, 1954) and that it appears in homogenates and sterile autolysates of mouse liver and tumours after incubation for varying periods of time (Roberts & Frankel, 1950). The mechanisms controlling the amino-acid concentrations in chopped preparations of mouse liver incubated in phosphate medium are not very sensitive to salicylate. Drug concentrations below 10 mM produced no effects and the changes observed with 10 and 20 mM salicylate were restricted to a few amino-acids (Table 2).

In contrast to the relative insensitivity of chopped preparations of mouse liver to salicylate *in vitro* the injection of salicylate into whole animals produced more marked and widespread effects on the amino-acid concentrations in the liver (Table 3). Previous work (Sturman, Dawkins & others, 1968) indicated that the expected concentrations of the drug in the livers of mice given an intraperitoneal injection of 600 mg/kg body weight of salicylate would range from 3 mM at 30 min to 0·5 mM at 4 h after the injection. Significant differences in the concentrations of a variety of the amino-acids occurred at each time interval, the effects being most marked at 1 h, when only aspartate, proline, ethanolamine and histidine were not affected. One h between injection and killing was therefore used for the later experiments which were extended to include other tissues (Table 4) and a range of salicylate doses.

The concentration of most of the free amino-acids in kidney and blood were significantly reduced after injection of high doses of salicylate, aspartate and glutamate were increased in blood. The essential amino-acids, threonine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine and arginine may be considered as a group with respect to their response to salicylate in that most of these compounds were decreased in the blood and kidney, unaffected in the brain and increased in the liver. Non-essential amino-acids which behaved in a similar manner included taurine, serine, citrulline, β-alanine and ornithine. In contrast to the group of amino-acids whose concentrations increased in the liver, there was a small group comprising glutamine, glutamate, glycine and alanine whose concentrations decreased in the liver. The decreased plasma concentrations of the amino-acids in the salicylate-treated animals may be caused by a primary action of the drug in blocking the renal tubular reabsorption of the amino-acids. Salicylate causes amino-aciduria in man (Elliot & Murdaugh, 1962; Ben-Ishay, 1964) but it has been reported (Andrews & others, 1962) that the drug also increases the plasma α-amino nitrogen concentrations and that the pattern of amino-aciduria resembled that found in hepatic injury rather than in renal tubular damage (Andrews & others, 1961). However, in the rat a dose of 450 mg/kg weight of salicylate induces a gross generalized amino-aciduria (Berry & Guest, 1963) which suggests a renal origin, although the authors considered that the pattern of amino-acid excretion was similar to those observed in several types of human liver disease. The intravenous administration of salicylate also causes amino-aciduria in the dog (Simoes & de Barros, 1955) and in this species there is some evidence that there may be competition between salicylate and some amino-acids for active sites in the renal tubular transport system (Weiner, Washington & Mudge, 1959).

Segal & Blair (1963) have also shown that salicylate *in vitro* inhibits the transport of phenylalanine, lysine and histidine into slices of rat and human kidney cortex and also accelerates the efflux of the amino-acids from these tissues. This effect may explain the decreased concentrations of amino-acids in the mouse kidney observed in the present work (Table 4). If the amino-aciduria observed in animals given high dose levels of salicylate is due to an overflow mechanism, the plasma concentrations of the amino-acids would be expected to either exceed or equal those in the controls. However, the present results do not support such a mechanism, except for aspartate and glutamate, where an overflow mechanism cannot be excluded. Webber (1963) has shown that in the dog there is a separate mechanism of renal tubular reabsorption for the acidic compared to the basic and neutral amino-acids, and the failure to see a decrease in these may be due to this transport system not being affected by salicylate. The changes in the blood amino-acid concentrations caused by the acute dose of salicylate described here, are qualitatively similar to changes in the plasma amino-acid levels produced by chronic doses of salicylates which did not produce amino-aciduria. Rats fed on a diet containing 0·5% aspirin showed elevated levels of plasma glutamate while the peak concentrations of the other amino-acids were depressed (Vaughan, Korty & Steele, 1969).

If salicylate depletes the intracellular amino-acid pools in the mouse kidney cells, other than the tubules, by decreasing influx from and increasing efflux to the circulation, this effect of the drug is far less evident in the brain, and in the liver must be antagonized and overcome by other actions of salicylate. Such actions must lead to increased formation of amino-acids in the hepatic cells and may comprise an increased rate of protein breakdown, inhibition of protein synthesis and interference with transamination. There is experimental evidence that salicylate may promote all these. The drug is known to cause a marked depletion of liver glycogen in whole animals (Lutwak-Mann, 1942) and it may also increase other catabolic processes in the liver, including protein breakdown, as part of a general stimulating action on substrate breakdown due to its uncoupling action on oxidative phosphorylation reactions (Smith, 1966). Salicylate has been shown to inhibit the incorporation of radioactive amino-acids into the protein of cell-free preparations from rat liver (Dawkins & others, 1966; Reunanen, Hanninen & Hartiala, 1967) by a mechanism involving an interference with the formation of aminoacyl-t-RNAs (Burleigh & Smith, 1970). The drug also inhibits the activities of rat tissue aminotransferases involving the interaction of 2-oxoglutarate with leucine, isoleucine, valine, serine, threonine, methionine, phenylalanine, ornithine and arginine (Gould & Smith, 1965). If salicylate differentially interfered with aminotransferase activities in the mouse liver, then this could explain the increased concentrations of the above amino-acids plus tyrosine, β-alanine and lysine, and the decreased concentrations of glutamate and alanine, observed in the present work. Any effects of salicylate on amino-acid patterns due to interference with aminotransferase activities would be expected to be most pronounced in the liver since this organ is concerned with the metabolism of dietary amino-acids entering it from the portal circulation.

One prominent difference between the effects of salicylate on the amino-acid levels in brain and the other tissues is the lack of sensitivity of the brain towards the drug. It is known (Sturman & others, 1968) that salicylate penetrates the mouse brain after injection and attains concentrations approximately equivalent to those in the other tissues. The levels of aspartate and glutamate are very much higher in the

brain than in other tissues and there are different mechanisms controlling the levels of the amino-acids in brain (Haslam & Krebs, 1963; Balazs, 1965) compared with other tissues (Williamson, Lopes-Vieira & Walker, 1967). In the brain the non-essential amino-acids are formed mainly from glucose (Gaitonde, Dahl & Elliott, 1965). The levels of essential amino-acids in this tissue are maintained to a large extent by protein breakdown within the brain (Vrba & Cannon, 1970) and the rate of entry of these amino-acids from the blood across the blood brain barrier is a much slower process than in other tissues (Neuberger & Richards, 1964). In addition, there is evidence for compartmentation of some of the amino-acid pools within the brain (Berl & Purpura, 1966) and not all the compartments may be accessible to salicylate.

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Formulation and pharmacological studies of a controlled release pentagastrin injection

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Four formulations were designed to release pentagastrin at different rates after subcutaneous injection. These formulations were tested *in vitro* for rate of diffusion across a dialysis membrane into pH 7·3 phosphate buffer, and *in vivo* in rats for rate of excretion in the bile and on dogs for pharmacological responses. Good agreement was observed between the *in vitro* data, elimination rate and dose response data for the rate of release and duration of action of the four formulations. The results agreed with the theory that by controlling the rate of release of a short acting drug, one could sustain the pharmacological response to the drug and increase the total response by better utilization of an administered dose.

The synthetic pentapeptide, pentagastrin (Peptavlon ICI 50 123), is active in stimulating gastric secretion (Morley, Tracy & Gregory, 1965). It produces maximal stimulation of gastric secretion after subcutaneous injection in man at a dose of 6 μg/kg dose (Multicentre Pilot Study, 1967). The rate of secretion reaches a maximum between 20 to 40 min after injection and returns to basal levels in 90 to 120 min (Makhlouf, McManus & Card, 1966). Intravenous infusion of a pentagastrin solution at a dose of 0·01 μg/kg min^{-1} in 0·15M sodium chloride solution at a rate of 150 ml h^{-1} produces stimulation which approaches the maximal gastric output in most human subjects (Wormsley, Mahoney & Ng, 1966; Aagaard & Schmidt, 1967). This evidence indicates that the subcutaneous dose of pentagastrin is not fully utilized in the body for the desired function. Most of the subcutaneous dose may pass from the site of injection into the blood stream at a rate faster than that required to produce the maximal pharmacological response and is rapidly eliminated or metabolized. The possibility of controlling the rate of release of pentagastrin by formulation techniques and thus achieving more efficient use of the dose has been investigated.

Theory

Many drugs in solution when injected into subcutaneous or intramuscular sites behave as if their absorption were taking place passively by diffusion in one direction (Sund & Schou, 1964a, b; Ballard & Menczel, 1967; Ballard, 1968). The disappearance rate of the drug from the injection site can be described by Fick's Law. In practice, it may be assumed that the rate of absorption, R_t, of a subcutaneous dose of drug from the site of injection into the blood is proportional to the amount of drug at the site, A_s, and the relation may be expressed as

$$R_t = \frac{dN}{dt} = p(A_s)$$

where dN/dt is the penetration rate and N is the amount of drug penetrating the tissue at time, t. The penetration coefficient, p, depends upon the diffusion coefficient

of the drug in the particular environment, the area of absorption membrane exposed to the solution and the concentration of drug at the site.

Fig. 1 shows graphically an hypothetical exponential relation between the release rate, R, and time, t, after subcutaneous or intramuscular injection of a drug. R_1 represents the rate of release at which a particular drug produces its maximum pharmacological action. A drug diffusing into the blood at a higher rate will not cause a further rise in pharmacological response. R_2 is the release rate required to

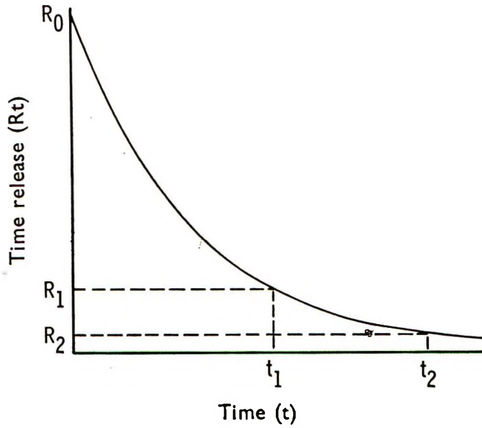

FIG. 1. Exponential relation between rate of release and time after subcutaneous injections.

maintain the threshold blood level for producing a pharmacocological response. In the period t_0 to t_1, the drug is released into the blood at a rate faster than it can be utilized. The total dose is represented by the area under the curve. The total dose, utilized dose and dose efficiency can be calculated as follows:

$$\text{Total dose} = \int_0^\infty R(t)dt$$

$$\text{Utilized dose} = R_1t_1 + \int_1^2 R(t)dt$$

$$\text{Dose efficiency} = \frac{\text{Utilised dose}}{\text{Total dose}} = \frac{R_1t_1 + \tau(R_1 - R_2)}{R_0\tau}$$

The dose efficiency represents the fraction of the dose being used for its pharmacological action. The difference between total dose and utilized dose represents overdosing. τ = time constant.

Overdosing frequently happens in the administration of short-acting drugs. In order to produce a desirable duration of action, a drug is often given at excessively high dose. By maintaining the rate of diffusion of the drug from the site of injection at a constant level or by prolonging the diffusion half-life, the dose efficiency can be improved and this is demonstrated by a prolongation of pharmacological action without increase in dose.

EXPERIMENTAL

Material. Pentagastrin (n-t-butyloxycarbony-β-Ala.Try.Met.Asp.Phe.NH_2). The equilibrium solubilities of pentagastrin are 7·5 ppm in 0·1 N hydrochloric acid and

$4 \cdot 0 \times 10^3$ ppm in isotonic phosphate buffer of pH 7·3 at 25° as determined by the method of Martin (1960). Radioactive pentagastrin (0·6 μCi/mg) [^{14}C] labelled at the methylenyl carbon of the tryptophan moiety was synthesized for these experiments and its purity was confirmed by electrophoresis and thin-layer chromatography. Propylene glycol B.P. was distilled at 0·5 mm mercury pressure. All other reagents were analytical quality.

Preparation of injections. To conserve the labelled pentagastrin, it was diluted to a specific activity of 0·24 μCi/mg with unlabelled material. Four types of injectable formulation were prepared using this material for the *in vitro* testing and for the determination of absorption and excretion kinetics. The same formulations using unlabelled pentagastrin were used in the studies of gastric response in dogs. These formulations were as follows:

Formulation I. Pentagastrin (10 mg) was dissolved in 0·1N ammonia (0·04 ml) and adjusted to 10 ml with normal saline. The injection was filtered through a sterile 0·22 μm Millipore filter.

Formulation II. Pentagastrin (10 mg) was dissolved in 0·1N ammonia (0·04 ml), diluted with propylene glycol (4 ml) and a sufficient quantity of normal saline was added to make 10 ml of solution which was filtered through a sterile 0·22 μm Millipore filter.

Formulation III. Propylene glycol (4 ml) and normal saline (4 ml) were added to pentagastrin (10 mg) dissolved in 0·1N ammonia (0·04 ml) and then filtered. An equivalent quantity of 0·1N acetic acid was added to neutralize the ammonia. The solution was stored for 24 h at 4° to allow crystallization of pentagastrin in its acid form to be completed. Sterile normal saline was then added to adjust the volume to 10 ml.

Formulation IV. Crystals were produced as in Formulation III. To the suspension, 0·1M aluminium potassium sulphate (0·32 ml) was added and the preparation adjusted to pH 5·0 with 0·1N sodium hydroxide the volume being made up to 10 ml with normal saline.

In vitro *release rate.* An apparatus (Fig. 2) was designed to study passive diffusion in one direction. Compartment A, constructed from Perspex, was clamped to compartment B but separated from it by a Millipore filter (H.A. 02560, 0·45 μm) exposing an efficient diffusion area of 3·14 cm². Before use, the diffusion membrane was soaked in the formulation for 24 h and rinsed twice with 50 ml portions of distilled water. One ml of Formulations I to IV, prepared with labelled pentagastrin, was separately placed in compartment A and was allowed to diffuse through the diffusion

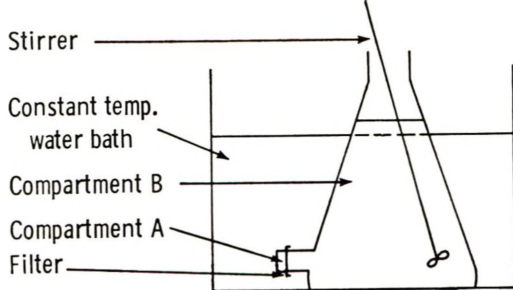

FIG. 2. Schematic diagram of apparatus for the study of drug release by diffusion.

membrane into 1000 ml isotonic phosphate buffer of pH 7·3 in compartment B which was stirred at 750 rev/min with a glass paddle. The apparatus was at 37° in a water bath. One ml samples were removed from compartment B over 8 h. The amount of pentagastrin diffusing through the membrane was calculated from the radioactivity detected in the samples. Two tests were run on each formulation and the average results of the two runs were reported.

Biliary excretion in rats after subcutaneous injection. Polyethylene cannulae were inserted into the common bile duct of four anaesthetized rats (Alderley Park strain), each to receive a different formulation prepared with labelled pentagastrin. Two h after recovery from the anaesthetic, the rats, housed in restraining cages, were injected with one ml of the formulation. Bile samples were collected hourly for 24 h, diluted to 5·0 ml with normal saline and the radioactivity of 0·10 ml of each aliquot measured.

Measurement of radioactivity. Radioactivity was assayed in a Packard Tri-Carb Automatic Liquid Scintillation Spectrometer. The samples were counted in a mixture of 6·6 ml of 0·6% Butyl-PBD [2-(4′-t-butylphenyl)-5-(4″-biphenyl)-1,3,4-oxdiazole] in toluene and 3·3 ml Triton X-100.

Stimulation of gastric secretion in dogs. The two dogs used had been provided with an innervated pouch of the oxyntic gland area of the stomach several months previously. The dogs were deprived of food, but not water for 18 h before the test. Basal secretion, if any, was collected for 15 min before the injection of the test formulation. Each formulation was then given by subcutaneous injection and the gastric juice produced in each 15 min interval was collected until the secretion had returned to the basal level or to the end of 4½ h period. The volume of secretion was measured, and aliquot titrated against 0·1N sodium hydroxide to the phenolphthalein end point and total acid output was computed for each time period. In each test, Formulation I was compared with one of the other formulations on the same dog in duplicate runs.

RESULTS AND DISCUSSION

Formulations I and II were solutions of pentagastrin whereas, Formulations III and IV contained crystalline pentagastrin. Comparative studies were conducted on these four formulations to illustrate the effects of 40% propylene glycol (Formulations II, III and IV), crystallinity (Formulations III and IV) and a protective coating of aluminium hydroxide (Formulation IV) on the rate of release of the drug from the site of injection. The crystals of Formulation III and IV had a similar appearance under the microscope. The needle-shaped crystals have a particle size distribution of 3–20 μm equivalent spherical diameter and a mean diameter of 8·7 μm as analysed by a Coulter Counter using an 140 μm orifice.

The results of the *in vitro* diffusion study are summarized in Fig. 3. Formulations I and II followed Fick's Law in contrast to Formulations III and IV which showed an initial period of constant release rate. The observed release pattern for Formulation III is believed to be due to the combined effects of dissolution and diffusion, with dissolution dominating the initial stage. Dissolution factors also govern the initial release rate of Formulation IV. On coming into contact with the buffer solution at pH 7·3 a gel of aluminium hydroxide is formed around the crystals. This gel provides a barrier controlling the rate of release of pentagastrin at a relatively steady level.

Using the same sets of data, the *in vitro* rates of release of the four formulations at various times were determined. Since the *in vitro* release of Formulations I and II followed first order kinetics, their rates were determined with the aid of a digital

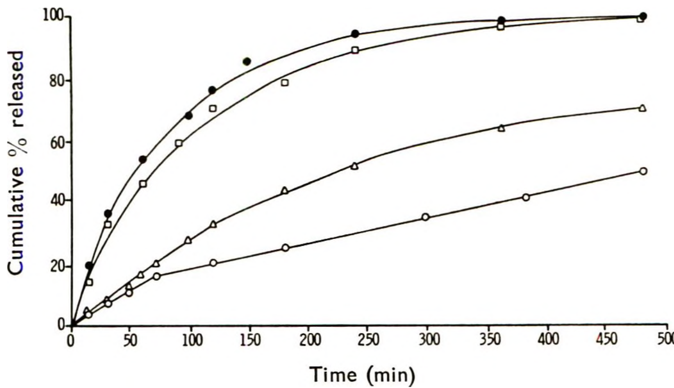

FIG. 3. *In vitro* release of pentagastrin from Formulation I (●), Formulation II (□), Formulation III (△), Formulation IV (○).

computer. The release rates of Formulations III and IV at various times were determined by graphical approximation. Fig. 4 compares the *in vitro* release rates, R_t, of the four formulations over the test period. Formulations I and II show a theoretical initial release rate of 11·7 and 9·8 μg/min respectively. Formulation III and IV gave gradual release of the drug at rates not exceeding 2·4 μg/min over a long period of time.

Fig. 5 shows the total ^{14}C excreted in the bile of rats after subcutaneous dosing of the four radio-labelled formulations. The absorption and elimination may be represented by the following model:

$$\underset{\text{Site of injection}}{(A)} - K_1 \rightarrow \underset{\text{Blood}}{(B)} - K_2 \rightarrow \underset{\text{Liver}}{(C)} - K_3 \rightarrow \underset{\text{Bile}}{(D)}$$

The blood level half-life of pentagastrin is less than 1 min as determined by tracer technique in this laboratory. Therefore it may be taken that K_2 is very high in comparison to K_1 and K_3. In practice, the absorption and elimination kinetics can

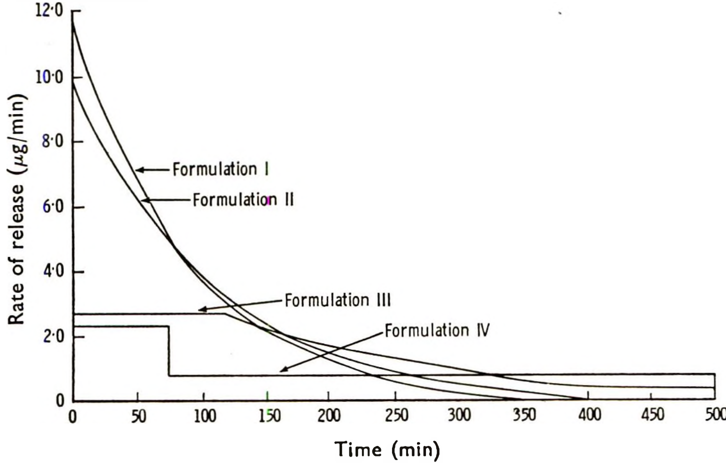

FIG. 4. *In vitro* rate of release of pentagastrin formulations.

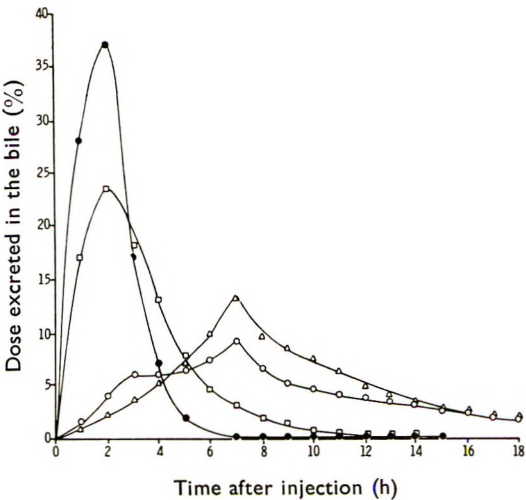

FIG. 5. Biliary excretion in rats of labelled pentagastrin after subcutaneous dosing. Formulation I (●), formulation II (□), formulation III (△), formulation IV (○).

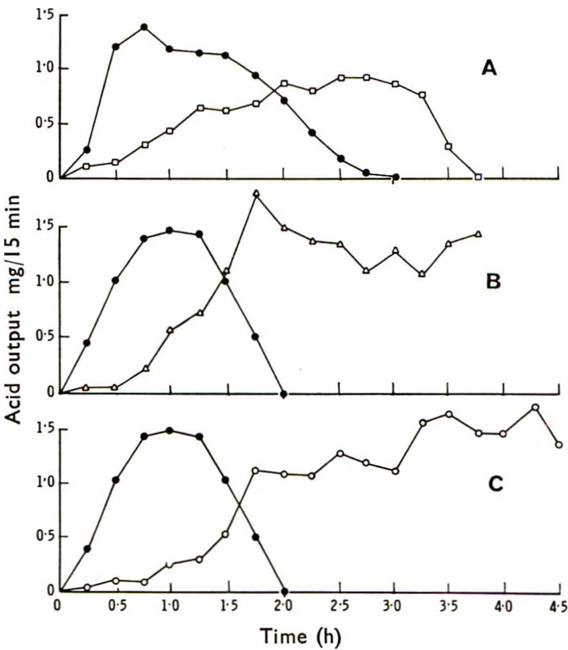

FIG. 6. Secretion of gastric acid in dogs after subcutaneous injection of various pentagastrin formulations. (A) Comparison of formulations I (●) and II (□). (B) Comparison of formulations I and III (△). (C) Comparisons of formulations I and IV (○).

be simulated using the following simplified model:

$$(A) \xrightarrow{K_1} (C) \xrightarrow{K_3} (D)$$

Site of injection Liver Bile

An analogue computer was used to evaluate K_1 and K_3 based on the biliary excretion data. Very close approximations to the biliary excretion results were achieved with Formulations I and II when a time lag of 0·3 h was chosen for the onset of action and 93·5% was taken as the maximum excretion of ^{14}C. The K_1 and K_3 values for Formulation I were 1·0 h^{-1} and 5·0 h^{-1} respectively and the corresponding figures for the experiment on Formulation II were 0·45 h^{-1} and 5·0 h^{-1} respectively. The calculations demonstrated that the K_1 values for Formulation III and IV do not follow first order kinetics.

Fig. 6 compares the gastric secretory response in the dog of Formulations II, III and IV against Formulation I after subcutaneous injection at 10 μg/kg. Each point is the average mean acid output from two experiments on the same dog. There is good agreement between the *in vitro* data, elimination data and dose-response data for the duration of action of the formulations. The maximum secretion can be sustained by controlling the rate of release. This agrees with the theoretical implication that by controlling the rate of release of a short-acting drug, the maximum response to the drug can be sustained and the total response increased by better utilization of an administered dose.

Acknowledgement

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Modification of morphine analgesia by drugs affecting adrenergic and tryptaminergic mechanisms

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The effects of drugs that modify adrenergic or tryptaminergic mechanisms were tested on the analgesic action of morphine in mice. Analgesia was assessed by the hot plate method and phenylquinone-writhing method. Reserpine antagonized the analgesic action of morphine in both tests, the maximal effects occurring 6–8 h after the administration of reserpine. *p*-Chlorophenylalanine antagonized the analgesic action of morphine as assessed by the writhing method but not by the hot plate method. The analgesic action of morphine was not modified in either test by pretreatment with α-methyl-*p*-tyrosine, propranolol, phentolamine or methysergide. These results suggest that the analgesic action of morphine, as measured in the writhing test, may be mediated by 5-hydroxytryptamine but that other mechanisms may be involved in the hot plate test.

Conflicting reports have appeared concerning the effect of reserpine on morphine analgesia. Most authors have reported antagonism (Schneider, 1954; Takagi, Tashima & Kimura, 1964; Verri, Graeff & Corrado, 1968), whereas potentiation of the analgesic action of morphine by reserpine was observed when heat was employed as the nociceptive stimulus (Tripod & Gross, 1957; Garcia Lema & Rocha e Silva, 1961). This effect of reserpine on morphine analgesia, be it antagonism or potentiation, has led most workers to conclude that the analgesic action of morphine may be mediated by the release of catecholamines within the central nervous system. This view has been reinforced by a number of other findings: (a) morphine releases noradrenaline in the central nervous system (Vogt, 1954; Maynert & Klingman, 1962); (b) morphine analgesia is antagonized by pretreatment with α-methyl-*p*-tyrosine, a specific depletor of catecholamines (Verri & others, 1968); (c) intracerebral injection of catecholamines produces analgesia in mice (Handley & Spencer, 1969); (d) morphine analgesia is antagonized by phenoxybenzamine (Heller, Saavedra & Fischer, 1968).

On the other hand, some authors have suggested that the antagonism of morphine analgesia by reserpine may involve changes in tryptaminergic mechanisms. There is evidence in support of this contention: (a) morphine reduces the levels of 5-hydroxytryptamine (5-HT) in the central nervous system (Türker & Akçasu, 1962); (b) *p*-chlorophenylalanine, a specific depletor of 5-HT, antagonizes morphine analgesia (Tennen, 1968); (c) intraventricular administration of 5-HT not only prolongs morphine analgesia but abolishes the effect of reserpine in antagonizing morphine analgesia (Sparkes & Spencer, 1969).

The levels of 5-HT in mouse brain were depressed by morphine in a dose of 0·85 mg/kg, that corresponded to the ED50 for analgesia in the phenylquinone-writhing test, whereas a higher dose (8·5 mg/kg), which was the ED50 for analgesia in the hot plate test, depressed levels of both 5-HT and noradrenaline (Lee & Fennessy, 1970).

It was suggested that a tryptaminergic mechanism may be involved in the analgesic action of morphine when phenylquinone is the stimulus, whereas a combination of adrenergic and tryptaminergic mechanisms may be involved when heat is the stimulus. In view of these findings we decided to study the effect of reserpine on morphine analgesia in both the hot plate and phenylquinone-writhing tests. In addition we have investigated the effects of a number of drugs that interfere with adrenergic and tryptaminergic functions on the analgesic activity of morphine.

EXPERIMENTAL

Swiss albino mice (Commonwealth Serum Laboratories strain) of either sex and weighing 18–25 g were randomly assigned to groups of 10. Two analgesic tests were used: the hot plate method (Eddy & Leimbach, 1953) and the phenylquinone-writhing method (Hendershot & Forsaith, 1959). The criteria used for assessing analgesia in these tests were as described by Lee & Fennessy (1970). The ED50 values for morphine analgesia in all tests were determined 30 min after subcutaneous administration of morphine and were calculated by the method of Litchfield & Wilcoxon (1949).

p-Chlorophenylalanine (BDH) was dissolved in half the required volume of 0·9% NaCl solution adjusted to pH 10 with 5M NaOH titrated to pH 4·5 with 5M HCl and two drops of polysorbate 80 were added; α-methyl-*p*-tyrosine (Aldrich) was suspended in 0·5M phosphate buffer at pH 7·4 in a concentration of 50 mg/ml and was dissolved by the addition of 3M NaOH, then the pH was adjusted to 7·4 with M HCl; phenylquinone (phenyl-*p*-benzoquinone, Sigma) was dissolved in 5% ethanol in water; these solutions were injected intraperitoneally. All other drugs were dissolved in 0·9% NaCl solution and were injected subcutaneously in a volume of 0·1 ml/10 g of mouse. Doses of morphine are expressed in terms of morphine sulphate (DHA); doses of propranolol hydrochloride (ICI), phentolamine mesylate (Ciba), reserpine (Ciba), 5-hydroxytryptamine creatine phosphate complex (Sigma), noradrenaline bitartrate monohydrate (Winthrop) and methysergide maleate (Sandoz) are expressed in terms of the base.

RESULTS

Phenylquinone-writhing test

The time course of the antagonistic effect of reserpine (1 mg/kg) on morphine analgesia is shown in Fig. 1. The increase in the analgesic ED50 of morphine was significantly ($P \leqslant 0·05$) above the control ED50 of morphine between 2 and 48 h after reserpine administration. Maximal antagonism of morphine analgesia was observed about 8 h after reserpine when the analgesic ED50 value for morphine was 2·55 mg/kg, compared with the control value of 0·85 mg/kg. Reserpine produced marked sedation but did not affect the writhing response to phenylquinone.

Pretreatment of mice with three doses each of 316 mg/kg of *p*-chlorophenylalanine, injected 72, 48 and 24 h before morphine, produced a significant ($P < 0·05$) increase in the analgesic ED50 of morphine (Fig. 2). This dose regime of *p*-chlorophenylalanine has been shown by Koe & Weissman (1966) to inhibit tryptophane hydroxylase selectively. These mice did not differ noticeably in behaviour from control animals, nor was there any effect on the writhing response to phenylquinone.

Treatment with α-methyl-*p*-tyrosine (100 mg/kg) has been reported to inhibit noradrenaline synthesis specifically by inhibition of dopa decarboxylase (Spector, Sjoerdsma & Udenfriend, 1965). This dose, given 2 h before morphine did not

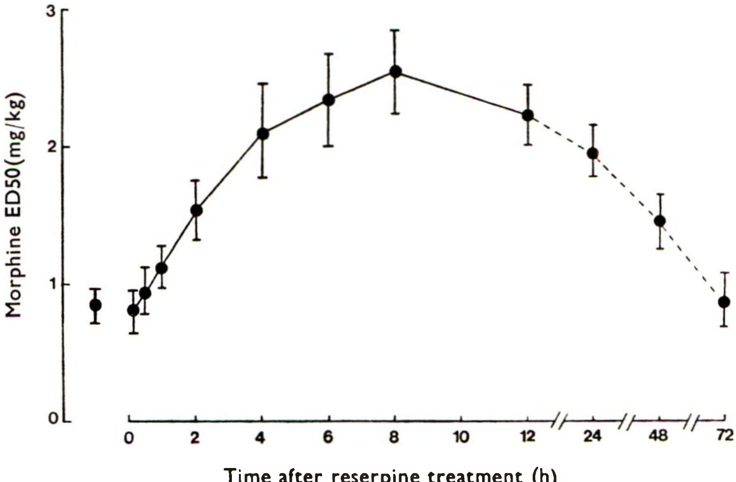

FIG. 1. The analgesic ED50 of morphine in the phenylquinone-writhing test at various times after subcutaneous injections of reserpine (1 mg/kg). Vertical lines are the 95% confidence limits.

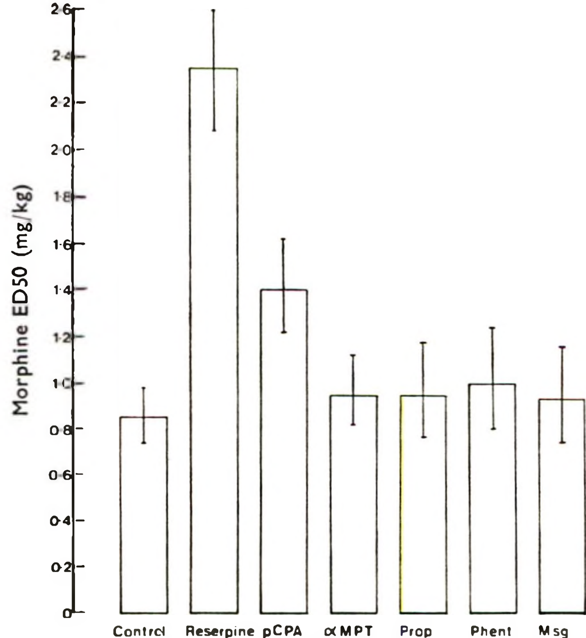

FIG. 2. The effects of pretreatment with various drugs on the ED50 value of morphine in the phenylquinone-writhing test. The doses of drugs and the intervals between pretreating for morphine and analgesia are as follows: reserpine, 1 mg/kg, 6 h pretreatment; *p*-chlorophenylalanine (pCPA), 316 mg/kg, 72, 48 and 24 h pretreatment; α-methyl-*p*-tyrosine (αMPT), 400 mg/kg, 2 h pretreatment; propranolol (Prop), 5 mg/kg, 30 min pretreatment; phentolamine (Phent), 10 mg/kg, 30 min pretreatment; methysergide (Msg), 5 mg/kg, 30 min pretreatment. Vertical lines are the 95% confidence limits.

significantly affect the analgesic action of morphine (Fig. 2). α-Methyl-*p*-tyrosine did not possess any analgesic activity by itself; however, the mice were sedated and they had an increased frequency of defaecation.

The blocking drugs, phentolamine, propranolol and methysergide, when used in doses reported to produce receptor blockade, did not significantly ($P \geqslant 0{\cdot}7$) affect morphine analgesia (Fig. 2).

Hot plate test

Pretreatment with reserpine (1 mg/kg) caused a slight and statistically insignificant enhancement of the analgesic action of morphine after 0·5 and 1 h and then antagonized significantly ($P < 0{\cdot}05$) the analgesic action of morphine from 4 to 18 h after administration of reserpine (Fig. 3). Maximal antagonism was observed between 6 to 8 h after reserpine. The analgesic ED50 for morphine 6 h after reserpine pretreatment was 19·3 mg/kg compared to the control ED50 of 8·5 mg/kg.

Neither *p*-chlorophenylalanine, α-methyl-*p*-tyrosine, propranolol, phentolamine nor methysergide affected morphine analgesia (Fig. 4).

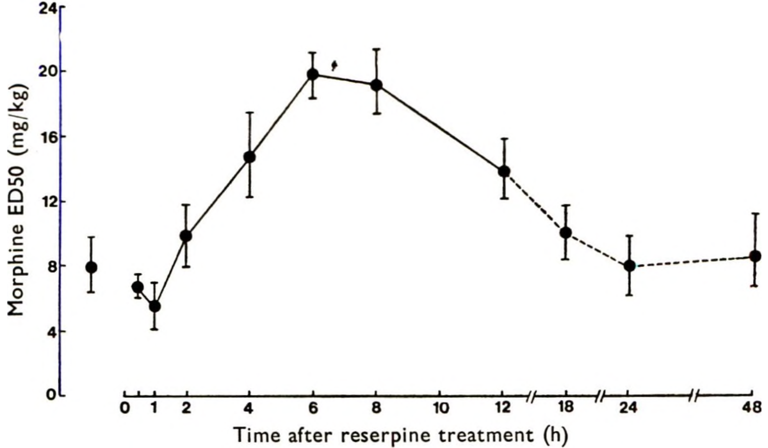

FIG. 3. The analgesic ED50 of morphine in the hot-plate test at various times after reserpine (1 mg/kg), subcutaneously. Vertical lines are the 95% confidence limits.

DISCUSSION

Reserpine antagonized the analgesic action of morphine in mice when either a heat method or a writhing method was used in the assessment of analgesia. The antagonism of morphine analgesia in the hot plate test with mice after pretreatment with reserpine confirms the results of Verri & others (1968) and Medaković & Banic (1964) but disagrees with the reports of Garcia Lema & Rocha e Silva (1961), Tardos & Jobbágyi (1958) and Ross & Ashford (1967) who demonstrated potentiation of the analgesic action. The conflicting reports of the effect of reserpine on morphine analgesia using the hot plate method do not appear to depend on the laboratory of origin since Verri & others (1968) obtained opposite results to Garcia Lema & Rocha e Silva (1961) even though both groups worked in the same laboratory and used the same strain of mice. Antagonism of morphine analgesia after reserpinization of mice using the writhing method has previously been reported by Rudzik & Mennear (1965).

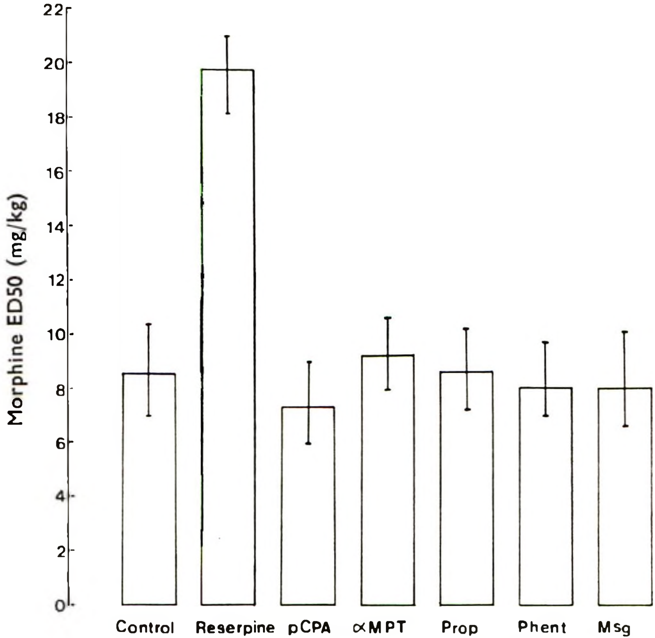

FIG. 4. The effects of pretreatment with various drugs on the ED50 value of morphine in the hot plate test. The doses of drugs and intervals between pretreatment and testing of morphine analgesia are as follows: reserpine, 1 mg/kg, 6 h pretreatment; *p*-chlorophenylalanine (pCPA), 316 mg/kg, 72, 48 and 24 h pretreatment; α-methyl-*p*-tyrosine (αMPT), 400 mg/kg, 2 h pretreatment; propranolol (Prop), 5 mg/kg, 30 min pretreatment; phentolamine (Phent), 10 mg/kg, 30 min pretreatment; methysergide, (Msg) 5 mg/kg, 30 min pretreatment. Vertical lines are the 95% confidence limits.

It is possible that the antagonism of morphine analgesia produced by reserpine in the writhing test may be due to decreased levels of 5-HT in the central nervous system since *p*-chlorophenylalanine, but not α-methyl-*p*-tyrosine, antagonized morphine analgesia. *p*-Chlorophenylalanine has been shown to inhibit tryptophane decarboxylase specifically, resulting in depletion of 5-HT (Koe & Weissman, 1966), whereas α-methyl-*p*-tyrosine specifically inhibits dopa decarboxylase and leads to a decrease in levels of noradrenaline (Spector & others, 1965). We have suggested previously (Lee & Fennessy, 1970) that morphine analgesia as determined by the writhing method may involve 5-HT since the ED50 of morphine caused a reduction in brain levels of this amine but not of noradrenaline. The present findings support this suggestion since the analgesic action of morphine was depressed by drugs which depleted the brain levels of 5-HT.

The observation that reserpine, but not *p*-chlorophenylalanine or α-methyl-*p*-tyrosine, reduced the analgesic action of morphine when the hot plate method was used is more difficult to explain. We were unable to confirm the findings of Medaković & Banic (1964) and Verri & others (1968) who reported an antagonism to morphine analgesia after pretreatment of mice with α-methyl-*p*-tyrosine using the hot plate method. Doses of morphine required to produce analgesia by this method significantly reduced brain levels of both 5-HT and noradrenaline (Lee & Fennessy, 1970). If both of these amines were concerned conjointly in morphine analgesia as determined in the hot plate method, then neither *p*-chlorophenylalanine nor α-methyl-*p*-tyrosine would be expected to cause antagonism. There is other evidence for difference

between the two tests. The ED50 of morphine was ten times greater and the persistance of effect for the reserpine-induced antagonism was shorter when the nociceptive stimulus was thermal than when it was chemical. In the hot plate test, there was slight early enhancement of analgesia that was not observed in the writhing test. The time course of antagonism of analgesia in the hot plate test by reserpine was closely parallel to the effect of reserpine in depleting catecholamines (Iversen, Glowinski & Axelrod, 1965).

Phentolamine, an α-adrenoreceptor antagonist, propranolol, a β-adrenoreceptor antagonist, and methysergide, a 5-HT antagonist, did not affect the analgesic action of morphine in either of the two tests. A possible explanation for the lack of effect is that the adrenergic and tryptaminergic receptors in the central nervous system, if they are involved in the production of analgesia, may be different to those in peripheral systems. However, other workers have observed effects of blocking drugs on morphine analgesia, although the reports are conflicting. Tolazoline was reported to antagonize morphine analgesia whereas phenoxybenzamine was without effect (Contreras & Tamayo, 1966). On the other hand, Gupta & Deshpande (1965) reported potentiation of morphine analgesia by phenoxybenzamine and tolazoline and antagonism by pronethalol.

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We gratefully acknowledge the Sigma Company Limited for a scholarship for one of us (J.L.) and Professor M. J. Rand for valuable criticism of the manuscript. We also thank Miss Sandra Stewart for her technical assistance. Part of the expenses were defrayed by a grant from the National Heath and Medical Research Council to Professor M. J. Rand.

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LETTERS TO THE EDITOR

Effects of diethyldithiocarbamate and carbon disulphide on brain tyrosine

Diethyldithiocarbamate (DDC), an inhibitor of dopamine-β-oxidase when injected into rats, brings about a significant decrease in noradrenaline, a slight increase in dopamine (Carlsson, Lindquist & others, 1966), and a significant increase in tyrosine (Goodchild, 1969a) in the brain. As DDC *in vitro* inhibits tyrosine hydroxylase (Taylor, Stubbs & Ellenbogen, 1969), it was suggested by Goodchild (1969a), that the inhibition of tyrosine hydroxylase by DDC was responsible for the *in vivo* accumulation of tyrosine.

However, an earlier observation by Goodchild (1969b), that after the simultaneous administration of 3-iodotyrosine, a competitive inhibitor of tyrosine hydroxylase, the depletion of noradrenaline was significantly slower than that obtained with DDC alone could not be explained by an inhibitory effect of DDC on tyrosine hydroxylase.

In the experiments reported here the concentrations of tyrosine, noradrenaline and dopamine were estimated at intervals after the administration of a single dose of DDC and after repeated exposures to CS_2—a compound which has the same effects on brain catecholamines as DDC (Magos & Jarvis, 1970).

Male albino rats of Porton-Wistar strain, 45–55 days old were used. Sodium diethyldithiocarbamate (Hopkin & Williams Limited, Chadwell Heath, Essex) was given intraperitoneally in 3·5% aqueous solution. Controls were given saline. Exposures to 2·0 mg/litre of CS_2 were made for 4 h each day in a vertical type constant flow inhalation chamber described with the operation procedure for CS_2 elsewhere (Magos, Emery & others, 1970). Control rats were kept in an inhalation chamber without CS_2 for the same period as animals exposed to CS_2. Animals were killed by decapitation either immediately after the last exposure to CS_2, or 1 to 4 h after the administration of DDC. Dopamine and noradrenaline were estimated by a modified version of Chang's (1964) method. This modification with some of the results obtained from CS_2-exposed animals was published by Magos & Jarvis (1970). Tyrosine was estimated by the method of Waalkes & Udenfriend (1957) but time allocated to every step in the procedure was standardized and internal standards were used for every sample. For the three compounds estimated in the brain, normal values $\pm$ s.e. were: noradrenaline, 0·380 ($\pm$0·0090) μg/g; dopamine, 0·709 ($\pm$0·0138) μg/g; tyrosine, 21·52 ($\pm$0·6555) μg/g. Concentrations in the experimental animals were expressed as the percentage of the paired controls.

Fig. 1 shows that after the administration of 500 mg/kg sodium diethyldithiocarbamate, the dopamine concentration in the brain increased, and the noradrenaline concentration decreased. The dopamine concentration reached the maximum 1 h and the noradrenaline the minimum $1\frac{1}{2}$–2 h after injection, and dopamine but not adrenaline returned to the control level 4 h after injection. The decrease in the noradrenaline concentration was significant at any time from $\frac{1}{2}$ to 4 h after injection, but the increase in the dopamine concentration was significant only at 1 to $1\frac{1}{2}$ h. The latter result explains why Carlsson, Lindquist & others (1966), who made the earliest analysis 2 h after DDC, found no significant difference in the brain dopamine concentration.

Tyrosine concentration like that of dopamine reached the maximum 1 h after the injection of DDC, and the increase was followed by a rapid decrease. It reached the control level between 2 and 3 h after injection and at 4 h decreased to 80% of the controls. A similar two-phased change in the brain concentration of tyrosine was observed after repeated daily exposure to 2·0 mg/litre CS_2 but the changes were smaller (Fig. 2). After two days exposure, tyrosine concentration increased to

106·0% and after 10 days exposure it was only 88·8% of the controls. Had the increase in tyrosine arisen from a direct effect on tyrosine hydroxylase by CS_2 or its metabolites, ten days exposure should not have had the opposite effect to a two days exposure. Consequently it seems that, both after a single dose of DDC or repeated exposures to CS_2, at the first phase of the reaction the increase in the

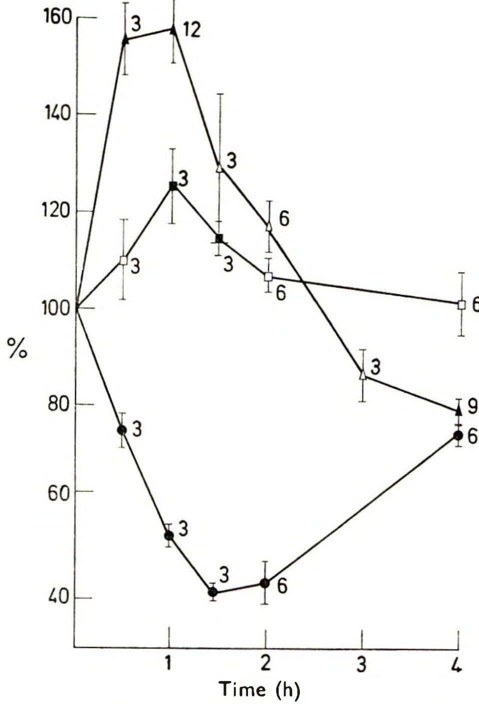

FIG. 1. Effects of 500 mg/kg of DDC on the brain levels of tyrosine (triangles), noradrenaline (circles) and dopamine (squares). Concentrations in the experimental animals are expressed as the percentage of the paired controls. Vertical lines indicate the standard error of means. Solid symbols designates statistically significant differences from the controls at $P < 0.05$ level using the Student *t*-test. Numbers besides the symbols show the number of pairs tested.

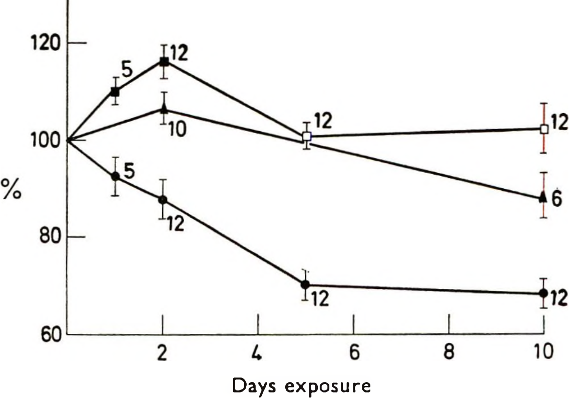

FIG. 2. Effects of repeated daily 4 h exposures to 2·0 mg/litre CS_2 on the brain levels of tyrosine (triangles), noradrenaline (circles) and dopamine (squares). Concentrations in the experimental animals are expressed as the percentage of the paired controls. Vertical lines show the standard error of means; solid symbols designate significant differences from controls at $P < 0.5$ level using the Student *t*-test. Numbers besides the symbols show the number of pairs tested.

dopamine concentration slowed down the conversion of tyrosine to dopa by a feedback mechanism resulting in a subsequent decrease in the concentrations of both noradrenaline and dopamine. When dopamine approached or reached the control level, the feedback effect of noradrenaline became dominant which by increasing the conversion of tyrosine to dopa restored a new balance on the one hand between catecholamines and tyrosine, and on the other hand between noradrenaline and dopamine. Since the deflection in tyrosine concentration after CS_2 was not so extensive as after DDC, and at least after the first or second exposures the increase in dopamine corresponded to the decrease in noradrenaline, CS_2 seems to be more suitable than DDC as an agent for the study of dopamine receptors.

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Blockade of adrenergic transmission by dehydroemetine

The synthetic amoebicidal drug, dehydroemetine, is effective as the natural alkaloid, emetine, in the treatment of amoebiasis and is better tolerated by the patient (Powell, Wilmot & others, 1967), but, as with emetine, gastrointestinal complications like diarrhoea and abdominal colic occur after injections of dehydroemetine in therapeutic doses (Herrero, Brossi & others, 1960). Ng (1966a,b) demonstrated an adrenergic neuron-blocking action for emetine and suggested that diarrhoea produced by this drug might reflect a reduction in intestinal sympathetic activity. I now report that dehydroemetine also has this adrenergic neuron-blocking action.

Segments of rabbit jejunum (Finkleman, 1930) were suspended in 70 ml aerated Tyrode solution (NaCl, 8·0; KCl, 0·2; $CaCl_2$, 0·2; $NaHCO_3$, 1·0; $MgCl_2$, 0·1; NaH_2Po_4, 0·5; Glucose, 1·0 g/litre) at 37°. The periarterial nerves were stimulated with square pulses (20 V; 0·5 ms) for 15 s every 3 min. Cats were anaesthetized with chloralose (80 mg/kg) and pentobarbitone sodium (5 mg/kg, i.v.). The cervical sympathetic nerve was stimulated with square pulses of 20 V and 0·5 ms for 10 s every 2 min, and isotonic contractions of the nictitating membrane were recorded on smoked paper.

Dehydroemetine (0·5–10 μg/ml) had no effect on the tone and pendular movements of the rabbit jejunum but antagonized the relaxation of tone and cessation of pendular movements produced by sympathetic stimulation. The inhibition of pendular movements produced by added noradrenaline (0·2–2 μg/ml) was either not affected or increased. Concentrations of dehydroemetine higher than 10 μg/ml reduced the tone of the intestine and the amplitude of the pendular movements in addition to antagonizing the effects of sympathetic stimulation. The sympathetic block was

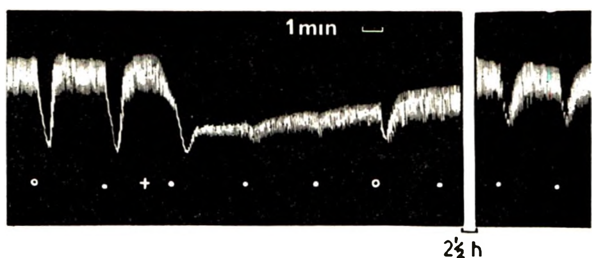

FIG. 1. Pendular movements of isolated rabbit jejunum. Dehydroemetine (17 μg/ml) added at (+) reduced the tone of the intestine, the magnitude of the pendular movements, and the effect of sympathetic stimulation (20 V, 0·5 ms, 50/s, for 15 s every 3 min, at the white dots). After the effect of sympathetic stimulation had been completely abolished, the effect of adding noradrenaline 1 μg/ml (open circles) persisted. Partial recovery from the effect of dehydroemetine occurred after repeated washing and stimulation over 2½ h.

slow to develop with the lower concentrations of the drug but developed more rapidly as the concentration was increased. The effect of dehydroemetine was not reversed by cocaine (0·2–20 μg/ml) but could be reversed by repeated washing and stimulation over 1–3 h. The longer recovery times were associated with the higher drug concentrations (Fig. 1).

Dehydroemetine (3–5 mg/kg) reduced the contractions of the cat nictitating membrane produced by preganglionic or post-ganglionic stimulation of the cervical sympathetic nerve. The blockade, once established, was persistent, no reversal being obtained during observations lasting 3–4 h, and the block was also not antagonized by 0·5 mg/kg cocaine given intravenously. At the height of the blockade produced by dehydroemetine, responses to noradrenaline (50 μg, i.v.) were either unchanged or slightly increased when compared with control responses.

The results show that dehydroemetine has an adrenergic neuron-blocking action which, however, differs from that of bretylium and guanethidine in that it is not antagonized by cocaine (Day, 1962).

A similar action has previously been observed with emetine (Ng, 1966b), and the diarrhoea which has been found to complicate parenteral administration of dehydroemetine in therapeutic doses might, at least in part, be due to this action.

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Vagotomy and atropine antagonism of (±)-chloroacetyl carnitine chloride mediated blood pressure reduction in the rat

Carnitine and carnitine derivatives have been reported to possess cholinergic activity (Dallemagne, Philippot & others, 1955; Yoshimi, Takaori & Shimamoto, 1965). Experiments now reported suggest that (±)-chloroacetylcarnitine chloride [$Me_3N^+ \cdot CH_2 \cdot CH(CH_2 \cdot COOH)O\text{-}COCH_2Cl \cdot Cl$; CAC] produces cholinergic-like effects on rat blood pressure *in vivo*. Furthermore, blood pressure reduction is antagonized by vagotomy and by atropine pretreatment.

CAC (28 mg/kg, i.v.) significantly reduces blood pressure of 200–250 g female albino Sprague Dawley rats (Table 1). After vagotomy, about five times the dose is required to reduce blood pressure by 13%; after atropine pretreatment, administration of approximately half the dose which produces 50% lethality elicits a non-significant 4% reduction in blood pressure.

The data show that CAC possesses acetylcholine-like activity as reported for other acetyl carnitine derivatives (Dallemagne & others, 1955; Fritz, 1963; Yoshimi & others, 1965). Blood pressure reduction does not appear to be mediated by ganglionic blockade since CAC did not inhibit the response of the cat nictitating membrane to pre- and post-ganglionic sympathetic electrical stimulation. Antagonism of CAC-produced blood pressure reduction in atropine treated and vagotomized rats suggests central mediation of the effect through cholinergic mechanisms. Since passage of these quaternary ammonium compounds into the brain may be difficult, a post-ganglionic site of action of CAC may be an alternative hypothesis.

Table 1. *Antagonism of (±)-chloroacetylcarnitine chloride fall in blood pressure by vagotomy and atropine.* Each value represents the mean of at least two determinations. Animals were anaesthetized with sodium pentobarbitone (35 mg/kg, i.p.). Carotid artery blood pressure was monitored using the E & M Physiograph. The test compound was administered into the jugular vein. Responses of all preparations were standardized with methacholine, acetylcholine and adrenaline.

Intact		Vagotomized		Atropine pretreated	
Dose (mg/kg)	Decrease %	Dose (mg/kg)	Decrease %	Dose (mg/kg)	Decrease %
7·1	0	28·4	4	14·0	0
14·2	13	35·7	11	29·0	15
28·4	53	71·4	13	36·0	0
42·6	33			72·0	0
				171·0	4

(±)-Carnitine and (±)-acetylcarnitine are converted to β-methylcholine by carnitine decarboxylase in liver, muscle and kidney rat mitochondrial preparations (Khairallah & Wolf, 1967). Thus biotransformation of CAC may be important in mediation of the reported cholinergic effect, since perfusion of rat hand limb vasculature with CAC results in reduced flow (Louis-Ferdinand, Cutroneo & others, 1970).

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Uptake of [7, 8-^{3}H]dihydromorphine by rat cerebral cortical slices and eye tissue

Morphine and its congeners have recently been reported by Scrafani & Hug, (1968) to be accumulated in rat cerebral cortical slices by an active transport process. We now report an inability to saturate the uptake system at concentrations ranging from 2·5 to 20·0 μg/ml likely to be encountered *in vivo* and the inability of glucose-substrate or metabolic inhibitors to depress the uptake of [7,8-^{3}H]dihydromorphine.

Male Holtzman rats, 140–200 g, were decapitated, the brains placed in a cold chamber and tissues sliced by an apparatus (O'Neill, Simon & Cummins, 1963) adjusted to give two outer slices, one dorsal and one lateral (weight, 15–25 mg, thickness, 0·2 mm) from each hemisphere. The slices were transferred to incubating beakers (20 ml) containing 2 ml oxygenated calcium-free Ringer (Elliot, Kokka & Way, 1963), previously kept in ice, and dihydromorphine, and incubated in Dubnoff metabolic shaker at 37° under oxygen with a shaking rate of 140 strokes/min. Control samples prepared as above were kept at 0° without shaking. At the end of the incubation, beakers were quickly placed on ice, tissue slices rinsed several times with saline, transferred to tared aluminium foil and dried to a constant weight at 105° and their radioactivity assayed.

The accumulation of dihydromorphine (2·2 μCi/mg) by slices in oxygen at 37° reached a steady-state distribution between tissue and medium in about 30 min (Fig. 1a). The uptake of dihydromorphine by slices was linear and unsaturable at concentrations of 2·5 to 20 μg/ml (Fig. 1b). The tissue/medium ratio (T/M) showed slight changes as dihydromorphine concentration was increased in the incubating solution. These findings were not altered with the omission of glucose from the media. At low concentrations of dihydromorphine (1·25 μg/ml) the T/M ratio was greater than at other concentrations studied. Neither the addition of calcium ($1{\cdot}3 \times 10^{-3}$M) to incubating solution nor 3-day fasting of animals changed the effect of glucose on the uptake of dihydromorphine. Glucose had no effect on accumulation of dihydromorphine in tissue slices when the concentration of dihydromorphine was 1·25 μg/ml. Increasing glucose concentrations from 0 to 12 mM at 37° produced a corresponding decrease in dihydromorphine uptake, which could not be accounted for on the basis of slight pH changes alone during incubation for 30 min.

Dinitrophenol, nitrogen atmosphere and high potassium content in the medium (an additional 1×10^{-1}M) significantly inhibited the accumulation of dihydromorphine in the slices (Table 1). Sodium cyanide, sodium malonate, iodoacetate, fluoroacetate had no effect on accumulation of dihydromorphine in slices with or without glucose in incubating medium.

Although nalorphine at a concentration of 2·5 μg/ml and with a 15 min preincubation period before the addition of dihydromorphine to the medium, did not alter the accumulation of dihydromorphine in the tissue, pretreatment of rats with a subcutaneous injection of nalorphine, 20 mg/kg, 30 min before decapitation, produced a significant decrease in tissue accumulation of dihydromorphine when the incubating solution contained no glucose but there was no effect when glucose was in the medium.

Our failure to inhibit [^{3}H]dihydromorphine transport by omission of glucose from incubation media, or by known metabolic inhibitors and also the failure to saturate this transport system in the concentration range 2·5–20·0 μg/ml of [^{3}H]dihydromorphine furnish evidence that an active transport mechanism is not involved. These results are at variance with those of Scrafani & Hug (1968), but agree with those of Bell (1958) and Miller & Elliott (1954).

For the measurement of uptake of [^{3}H]dihydromorphine in rat eye tissue, the rats were injected subcutaneously with a dose of 10 mg/kg of the drug and after the required time, decapitated, the eyes removed, freed from adhering tissue, washed with physio-

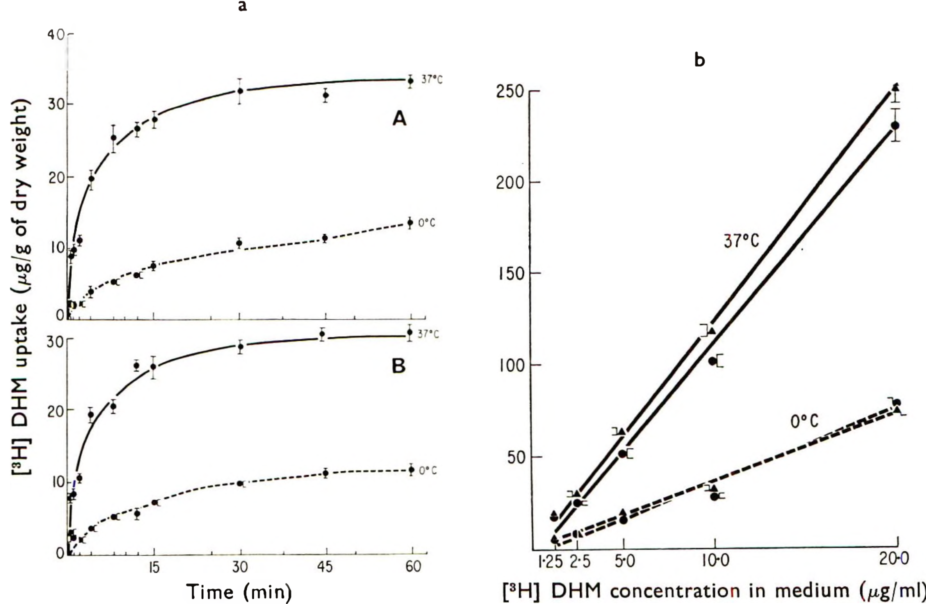

FIG. 1a. Rate of uptake of [^{3}H]dihydromorphine by rat brain cortical slices. Dihydromorphine concentration in medium 2·5 μg/ml, pH 7·4 in (A) absence and (B) presence of 12 mM glucose. Each point represents the mean of 7 or 8 samples. b. Relation of concentration of [^{3}H]dihydromorphine to its uptake by rat brain cortical slices. Incubation time is 30 min, pH 7·4. Each point represents the mean of 7 or 8 samples.

Table 1. *Effect of various agents upon the uptake of* [^{3}*H*]*dihydromorphine* (2·5 μ*g*/*ml*) *by rat brain cortical slices after* 30 *min incubation at* 37° *at pH* 7·4

Agents	Concentration	% of controls* (± s.e.)	$P \leq$
Dinitrophenol	1×10^{-3}	74·3 ± 2·3	0·001
Nitrogen atmosphere	—	88·9 ± 2·3	0·01
Nitrogen atmosphere (without glucose)	—	79·8 ± 1·2	0·001
Nalorphine (*in vivo*)	20 mg/kg s.c.	85·9 ± 4·4	0·02
Additional potassium chloride ..	1×10^{-1}	90·6 ± 4·0	NS
Additional potassium chloride (without glucose) ..	1×10^{-1}	78·5 ± 3·8	0·001
Sodium cyanide	1×10^{-3}	95·5 ± 4·4	NS

* Mean of 8 samples. NS = not significant.
Iodoacetic acid (1×10^{-3}M), malonic acid (1×10^{-3}M), sodium fluoride (1×10^{-3}M), sodium fluoroacetate ($3·3 \times 10^{-3}$M), calcium chloride ($1·3 \times 10^{-3}$M) and nalorphine (2·5 μg/ml) did not alter the uptake.

logical saline, gently wiped with tissue paper and, after weighing, transferred to counting vials. Eyes and slices moistened with 2–3 drops of water were dissolved in 0·5–1·0 ml NCS solution (a quaternary ammonium base supplied as a 0·6N solution in toluene by Amersham/Searle Corp., Des Plaines, Illinois) by gentle warming and shaking at 45°. After dissolution of the tissue, 10 ml of phosphor-toluene was added, the contents were well mixed, and the radioactivity assayed in a liquid scintillation counter. The values were corrected for quenching by the addition of 1 ml of internal standard [^{3}H]toluene. Aliquots of incubating fluid were similarly assayed. The uptake of [^{3}H]dihydromorphine (10 mg/kg s.c.) in two eyes for each animal each at different time intervals after injection was (ng/g of tissue): 980, 1451, 1059, 437, 245, 168 at times after injection of 30, 60, 120, 240, 360 and 720 min respectively. The values obtained give no indication of the amount of drug actually present in the lens. The peak value of drug was reached approximately 60 min after injection.

Thin-layer chromatography of organic extracts of homogenized eyes and cortical tissue slices with solvent system ethyl acetate–methanol–conc. ammonia (17 : 2 : 1 v/v) which can resolve oxidation and dealkylation products of dihydromorphine, and paper chromatography with n-butanol–conc. ammonia and water (4 : 1 : 3 v/v), did not give evidence of metabolic conversion of dihydromorphine. A single peak of radioactivity due to dihydromorphine was observed in both cases.

The general pattern of uptake of dihydromorphine *in vivo* in eye tissue is similar to that for brain and plasma levels (Sanner & Woods, 1965; Hug & Mellett, 1962). The presence of a high concentration of reduced glutathione and its active synthesis has been demonstrated in mammalian lens by several workers and it has been shown (Kinsey & Merriam, 1950; Salmony, 1960; Sippel, 1966) that the content of this tripeptide in experimental or senile cataract is drastically diminished. Evidence for interaction of morphine and reduced glutathione to form a peptide-conjugate has recently been obtained (Misra & Woods, 1970). In view of the absence of biotransformation products of dihydromorphine in eye tissue, the formation of reversible lenticular opacity by morphine-like analgesics may conceivably be due to some interference with lens metabolism or inhibition of glutathione synthesis.

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The uptake of anti-inflammatory steroids by lysosomes

Recently, Lewis, Symons & Ancill (1970) showed that the concentration of steroids was a critical factor in their action on lysosomes, and at high concentrations (10^{-3}M) the stabilizing action of the steroids was lost. It was assumed that at the higher concentrations a structural change was produced in the membrane. We now report the uptake of cortisol and cortisone by our sub-cellular preparation.

Lysosome suspensions in 0·05M tris-acetate buffer (pH 7·4) sucrose (0·25M) were prepared from rabbit liver (Symons, Lewis & Ancill, 1969) (1 ml ≡ 1 g of liver) and 1·5 ml portions transferred to dialysis bags, which were placed in stoppered test tubes containing 10 ml of [^{3}H]labelled steroid solutions prepared in the same sucrose buffer. The steroid was omitted in some tubes and in others the lysosome suspension was replaced by the sucrose-buffer. The tubes were rotated at 1 rev/min for 4 h at 37°. After 4 h, the observed time for maximum values, portions (0·1 ml) were removed from the dialysis bags and added to 15 ml of scintillation fluid [0·1 g 1,4-di-2-(4-methyl-5-phenyloxazolyl)-benzene; 5 g 2,5-diphenyloxazole in 1 litre of toluene and 500 ml methanol]. After being corrected for quenching, the amount of steroid taken up by the organelles was calculated after subtracting control values, and related to the protein concentration (Lowry, Rosebrough & others, 1951). Free

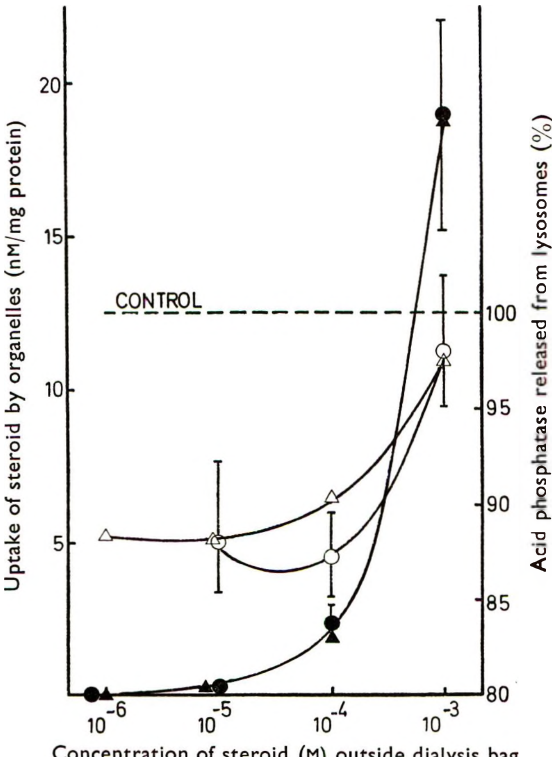

FIG. 1. The effect of cortisone and cortisol concentration on the uptake of the steroids by the organelles in our homogenate and the release of acid phosphatase from lysosomes.

Cortisone results are indicated with triangles and cortisol with circles. Open symbols represent enzyme release, and closed symbols uptake. Acid phosphatase released in the presence of the steroid has been expressed as % of that released in control experiments without steroid. In six experiments the enzyme released in controls was 49·3% ± 4·2% (s.d.) of the "total" released by freezing and thawing the homogenate four times. Each point is the mean of three experiments. Ranges are shown for cortisol results.

acid phosphatase was measured in the supernatant obtained from centrifuging the contents of the dialysis bag at 20 000 *g* for 20 min at 4° (Symons & others, 1969).

The subcellular fraction concentrated the steroids from the surrounding medium and the amount of steroid taken up was proportional to the steroid concentration of the medium (Fig. 1). Also, it seems that our previous assumption was correct in that a high concentration of steroid within the lysosome led to a loss of stability of its membrane and higher levels of free acid phosphatase. At maximum values the amount of steroid taken up by the fraction was equivalent to 0·1 mg/g fresh liver.

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A comparison of the β-adrenoceptor stimulant properties of salbutamol, orciprenaline and soterenol with those of isoprenaline

The discovery of different structure-activity relations for the actions of catecholethanolamines at β-adrenoceptors in different tissues led Lands and co-workers (Lands, Arnold & others, 1967; Lands, Luduena & Buzzo, 1967) to suggest the existence of two types of β-receptors, namely β_1 and β_2. In the present work we have examined the activities of four β-adrenoceptor agonists on tissues thought to contain β_1 or β_2 adrenoceptors. The drugs used were isoprenaline, orciprenaline, salbutamol and soterenol.

Brief details of experimental methods are given in Table 1. Full dose-response curves were obtained for isoprenaline and for one of the other drugs on each preparation. The activities of the drugs were compared at 50% of the maximum effect or, if this was not possible, at suitable equi-effective dose-levels.

The dose-ratios for the β-adrenoceptor agonists compared with isoprenaline are given in Table 1. As shown in the Table the activities of the four drugs on guinea-pig ileum and colon have not been included because it was found that a major proportion of the response to isoprenaline is mediated through stimulation of α-receptors (Farmer & Levy, 1970b).

Salbutamol was selective in its actions, being much more active at β–2 than at β–1 receptors. The difference in the mean dose-ratios for activity in the β–1 and β–2 groups of adrenoceptors was highly significant ($P = 0{\cdot}002$). Some separation of effects in the β–1 group is indicated in the relatively greater action of salbutamol on rate than on force in isolated rat atria. The mean of the activities for orciprenaline at β–2 receptors was seven times greater than at β–1 receptors and this difference was just significant ($P = 0{\cdot}015$). Soterenol had high activity at β–2 adrenoceptors

Table 1. *Dose ratios for salbutamol: isoprenaline, orciprenaline: isoprenaline and soterenol: isoprenaline at β–1 and β–2 adrenoceptor sites.* (Each value is the mean of at least five determinations).

Preparation	Ref.	Receptor class	Salbutamol	Orci-prenaline	Soterenol
Guinea-pig					
Right atria—rate	(a)	β–1	500	125	130
Left atria—force	(b)	β–1	2500	63	>10 000
Rat					
Right atria—rate	(a)	β–1	54	24	>10 000
Left atria—force	(b)	β–1	8000	38	>10 000
Guinea-pig					
Ileum	(c)	Excluded due to α-receptor involvement in response to β-stimulant drugs (Farmer & Levy, 1970)			
Colon	(d)				
Rabbit					
Intestine (+ 1 μg/ml phentolamine)	(e)	β–1	800	500	30
Guinea-pig					
Trachea	(f)	β–2	6	14	6
Vas deferens	(g)	β–2	1	20	2
Rat					
Diaphragm	(h)	β–2	5	40	3
Uterus	(i)	β–2	3	4	1
Dog					
Hind limb blood flow	(j)	β–2	5	33	3
Chick					
Colon	(d)	β–2	9	10	5

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and except for high positive chronotropic activity on guinea-pig isolated right atria, low activity at β–1 adrenoceptors. The significance of this exception is not clear.

Lands and others have already shown that structural modifications of the ethanolamine side chain of catecholamines can produce selectivity of action. The present work shows that replacement of the 3-hydroxy group by a hydroxymethyl or methanesulphonamide-group produces an even greater selectivity of action. On the other hand, replacement of a catechol by a resorcinol function, as in orciprenaline, produces much less separation of effects.

The results, in general, support the concept of two main types of β-adrenoceptor. The question whether the receptors within the groups are identical or differ slightly from one another remains to be finally answered. Evidence obtained by the use of β-adrenoceptor blocking agents indicates that a simple two-receptor hypothesis would be inadequate (Farmer & Levy, 1970a). However, the concept that different tissues have different β-adrenoceptor populations is substantiated by these results.

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Amphetamine toxicity and endogenous noradrenaline concentrations in isolated and aggregated mice

There is indirect evidence for the participation of noradrenaline in the mechanism by which aggregation augments amphetamine toxicity in mice. Experiments with amphetamine toxicity in mice pretreated with a variety of pharmacological agents known to modify the metabolism, storage, or action of noradrenaline indicate that noradrenaline released from endogenous stores plays a role in the aggregation effect (Sethy & Sheth, 1968). Although reduced tissue concentrations of noradrenaline in mice treated with amphetamine are consistent with this view (Moore, 1963, 1964; Beauvallet & Solier, 1964; Lal & Chessick, 1964; Menon & Dandiya, 1967), the relation between the enhanced toxicity of amphetamine in aggregated mice and noradrenaline depletion is not clear. We aimed to examine this relation by determining whether amphetamine-induced lethality, in either isolated or aggregated mice, is correlated with the degree of noradrenaline depletion in tissues. This approach differs from that used earlier (Moore, 1963, 1964; Lal & Chessick, 1964) in one important aspect. A distinction has been made between the degree of noradrenaline depletion in mice that died as a consequence of amphetamine treatment and in mice that survived the treatment.

Novice, male, albino mice of a random-bred Swiss strain (Maxfield; Cincinnati, Ohio), 9 to 12 weeks, 25 to 35 g, were housed 15 per cage (45 × 24 × 12 cm) for not less than 30 days; Purina laboratory chow and water were freely available. After an intraperitoneal injection of saline or (+)-amphetamine sulphate (30 or 100 mg/kg) in aqueous solution (1 ml/100 g), mice were either isolated or aggregated (3 per cage) in metal cages (7 × 7 × 7·5 cm) with a wire mesh side for observation. The rationale for using these doses of (+)-amphetamine has been previously discussed (George & Wolf, 1966, 1967). Ambient temperature was 24 ± 1°. After 3 h, surviving animals (survivors) were killed by cervical dislocation; their brains and hearts were removed and frozen in liquid nitrogen within 1 min after death. Mice that died within 3 h (non-survivors) had their tissues removed and frozen immediately after death. The degree of aggregation was maintained constant by replacing non-survivors with untreated mice. Noradrenaline in pooled samples of 4 hearts or 3 whole brains was assayed fluorometrically by the trihydroxyindole method of Anton & Sayre (1962).

Aggregation itself did not deplete brain or heart stores of noradrenaline (Table 1). The levels were not significantly different ($P > 0{\cdot}05$) in saline-treated isolated versus aggregated mice. Furthermore, aggregation did not enhance the noradrenaline-depleting effect of (+)-amphetamine. Brain and heart noradrenaline levels in mice treated with 30 or 100 mg/kg of (+)-amphetamine were not significantly different in isolated versus aggregated animals. This finding differs from that of Lal & Chessick (1964) who found lower brain levels of noradrenaline in aggregated than in isolated mice 30 min after (+)-amphetamine (25 mg/kg). It is possible that high doses of (+)-amphetamine or long treatment times, such as we used, obscured any influence aggregation might have had on the noradrenaline-depleting effect of (+)-amphetamine. Moore (1963), too, reported that aggregation in mice enhanced noradrenaline deple-

Table 1. *Effect of* (+)-*amphetamine on noradrenaline concentrations in brains and hearts of isolated and aggregated mice.* Determinations were made on pooled organs (3 brains, 4 hearts) from mice that died within 3 h (non-survivors) or were killed 3 h (survivors) after treatment. Noradrenaline content expressed as mean values in μg/g ± s.e.; number of determinations is in parentheses.

Treatment	Lethality %	Noradrenaline* Brain Non-survivors	Brain Survivors	Heart Non-survivors	Heart Survivors
Saline					
Isolated	0	—	0·18 ± 0·01 (16)	—	0·46 ± 0·03 (11)
Aggregated	0	—	0·20 ± 0·01 (18)	—	0·48 ± 0·02 (13)
(+)-Amphetamine 30 mg/kg					
Isolated	7	†	0·08 ± 0·01 (8)	†	0·32 ± 0·03 (7)
Aggregated	52	0·04 ± 0·00 (10)	0·06 ± 0·01 (8)	0·10 ± 0·01 (7)	0·25 ± 0·03 (6)
(+)-Amphetamine 100 mg/kg					
Isolated	31	0·13 ± 0·02 (7)	0·02 ± 0·00 (12)	0·22 ± 0·03 (5)	0·16 ± 0·02 (9)
Aggregated	67	0·13 ± 0·01 (10)	0·02 ± 0·00 (6)	0·24 ± 0·02 (6)	0·12 ± 0·02 (4)

* Noradrenaline concn in survivors or non-survivors within each treatment group are not significantly different ($P > 0·05$) in isolated versus aggregated mice. The largest difference shown (heart, survivors, 30 mg/kg dose) has $P = 0·22$.
† Low incidence of lethality did not permit determination of this value.

tion by (+)-amphetamine when brain and heart noradrenaline concentrations were measured 4 h after doses up to 40 mg/kg (+)-amphetamine. But while Lal & Chessick reported no deaths during their relatively short treatment period, there were many deaths during Moore's experiments. However, Moore made no distinction between noradrenaline depletion in survivors and non-survivors, and apparently combined the data from the two groups. In view of the differences we observed in noradrenaline levels in survivors and non-survivors, such a combination would yield misleading results.

The results from animals treated with 100 mg/kg of (+)-amphetamine show that death apparently is not due to a reduction of brain or heart noradrenaline below survival level. After this dose of (+)-amphetamine, noradrenaline concentrations in non-survivors were higher than those in survivors. Thus we find no obvious connection to exist between lethality and noradrenaline depletion in either brain or heart; differences in the degree of noradrenaline depletion may simply reflect the influence of dose and duration of action of (+)-amphetamine. Noradrenaline concentrations in survivors of the 100 mg/kg dose were lower than those observed in survivors of the 30 mg/kg dose, indicating that the depleting effect of (+)-amphetamine is dose-dependent. If the non-survivor and survivor data had been pooled, this dose-dependent relation would have been obscured. Almost all deaths (94%), regardless of the environmental situation, resulting from a dose of 100 mg/kg occurred within 60 min, whereas after 30 mg/kg, 97% of deaths occurred between 90 and 180 min after drug administration. The limited duration of action of (+)-amphetamine could explain why less depletion was seen in non-survivors treated with a dose of 100 mg/kg than in non-survivors that received the 30 mg/kg dose. Similarly, the shorter time available for depletion in non-survivors versus survivors at the 100 mg/kg dose (60 versus 180 min) might explain the higher noradrenaline concentrations observed in non-survivors.

These results suggest that the degree of depletion of endogenous stores of noradrenaline is not causally related to (+)-amphetamine lethality in isolated mice or the enhanced lethality in aggregated mice.

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Effects of mescaline and 2,5-dimethoxy-4-methylphenethylamine on sleeping time in mice

Mescaline shortens while 2,5-dimethoxy-4-methylphenethylamine (DMM-PEA) potentiates the sleeping time of pentobarbitone in mice (Ho, McIsaac & others, 1970; Ho, Tansey & others, 1970). We have now enquired whether the effect is explicable by an alteration in the metabolism of pentobarbitone.

Male Yale-Swiss mice, 25–30 g, were injected intraperitoneally with 50 μmol/kg of mescaline or DMM-PEA in saline. Control animals were given only saline. After 5 min a mixture of 40 mg/kg of sodium pentobarbitone and 100 μCi of [^{14}C]labelled pentobarbitone (New England Nuclear, U.S.A.) in saline was administered by the same route. The animals, groups of eight, were killed 30 and 60 min after pentobarbitone. The tissues from two mice of the same interval were combined and homogenized in three parts of water. Blood samples from a pool of two mice were centrifuged to separate the plasma. Tissue homogenates (0·1 ml) or plasma (25 μl) were treated with methanol and liquifluor, and assayed for ^{14}C by liquid scintillation. All values were corrected for 100% efficiency (channel ratio) and recovery. For chromatography, plasma or brain homogenate was extracted with diethyl ether (Cooper & Brodie, 1957). Sequential sections of paper (1 × 2·5 cm^2) from paper chromatograms were placed in counting vials, treated with methanol and liquifluor, and then assayed for ^{14}C. Sleeping time was recorded as the time between loss and return of the righting reflex after intraperitoneal injection of sodium barbitone (250 mg/kg) to mice.

At 60 min after intraperitoneal injection of [^{14}C]pentobarbitone, significant increases of specific activity were observed in the plasma and brain of animals pretreated with mescaline or DMM-PEA (Table 1). The two compounds also caused increases of radioactivity in liver at both 30 and 60 min intervals. The kidney concentration of pentobarbitone plus metabolites was higher than in the controls at 30 min, but decreased to the same level at 60 min.

From the chromatographic studies, the proportions of unchanged pentobarbitone and metabolites in plasma and brain of both experimental and control animals were calculated (Table 2). The recoveries of metabolites by ether extraction from the plasma and brain homogenates were 90 and 96% respectively. Decreases in the amounts of metabolites and increases in the unchanged pentobarbitone were observed in the 30 min plasma and 60 min brain samples of animals treated with DMM-PEA;

however, these changes in ratio of metabolites and the unchanged compound did not appear in the animals receiving mescaline.

The results in Table 3 show that DMM-PEA doubled the barbitone sleeping time in mice, but mescaline was without effect.

The increase in the pentobarbitone sleeping time in mice by DMM-PEA may arise from its ability to decrease the rate of metabolism of pentobarbitone in brain and plasma. But, the same argument cannot explain the effect of DMM-PEA in lengthening the barbitone sleeping time, because barbitone is largely unmetabolized in mice (Roth Leiper & others, 1949). The prolongation of pentobarbitone sleeping time in mice by oestrogen has been reported (Blackham & Spencer, 1969), and decreases in both the rate of metabolism and the renal clearance of pentobarbitone by this compound were offered in explanation of its action. DMM-PEA-induced reduction of renal excretion of barbiturates is likewise possible, in view of the increases of concentrations of pentobarbitone in brain, kidney, liver and plasma of the experimental animals.

Table 1. *Distribution of radioactivity in mouse tissues*

	nCi Specific activity (nCi/g tissue) ± s.e.					
	Control		DMM-PEA		Mescaline	
Tissue	30 min	60 min	30 min	60 min	30 min	60 min
Plasma†	89·64 ± 6·03	69·71 ± 3·60	92·91 ± 4·32	89·37 ± 1·73*	99·88 ± 6·48	97·66 ± 6·97*
Brain	71·32 ± 8·36	38·43 ± 2·93	72·92 ± 0·87	64·34 ± 7·57*	65·42 ± 2·79	60·93 ± 3·88*
Liver	192·50 ± 9·93	146·52 ± 5·56	224·98 ± 7·44*	201·36 ± 5·86*	236·33 ± 3·74*	198·38 ± 3·67
Kidney	140·41 ± 1·93	152·72 ± 11·57	160·98 ± 13·27*	145·64 ± 4·73	159·91 ± 8·47*	152·14 ± 6·66

* $P < 0{\cdot}01$
† Specific activity: nCi/ml

Table 2. *Percent unchanged pentobarbitone and metabolites in mouse plasma and brain*

	Metabolite		Control		DMM-PEA		Mescaline	
Tissue	No.†	Rf‡	30 min	60 min	30 min	60 min	30 min	60 min
	I	0·12	5·1 ± 0·5	3·2 ± 0·2	3·6 ± 0·2	4·4 ± 0·2	4·3 ± 0·3	4·8 ± 0·5
Plasma	II	0·58	48·1 ± 4·0	61·9 ± 2·1	36·9 ± 1·8*	52·3 ± 4·5	40·6 ± 2·2	58·7 ± 1·6
	III	0·90	46·8 ± 3·7	34·9 ± 2·4	59·5 ± 1·9*	43·2 ± 4·5	55·0 ± 2·6	36·5 ± 2·5
	I	0·12	1·1 ± 0·3	0·7 ± 0·2	0·3 ± 0·2	0·3 ± 0·1	0·2 ± 0·0	0·5 ± 0·1
Brain	II	0·58	4·6 ± 1·6	13·3 ± 1·2	3·3 ± 0·1	8·7 ± 1·2*	3·4 ± 0·6	12·6 ± 0·9
	III	0·90	94·3 ± 1·8	86·0 ± 1·2	96·4 ± 0·5	90·8 ± 1·0*	96·3 ± 0·5	86·9 ± 0·8

* $P < 0{\cdot}05$.
† I, pentobarbitone carboxylic acid; II, pentobarbitone alcohol; III, unchanged pentobarbitone (Cooper & Brodie, 1957).
‡ Paper chromatography in n-butanol saturated with 1% aqueous NH_4OH (Cooper & Brodie, 1957).

Table 3. *Effect of DMM-PEA and mescaline on barbitone and pentobarbitone sleeping time in mice.*

	Sleeping time (min) ± s.e.	
Treatment	Barbitone	Pentobarbitone
Control	237 ± 16 (6)	41·0 ± 1·1
DMM-PEA	427 ± 14* (6)	98·0 ± 8·5*‡
Mescaline	257 ± 16 (6)	34·4 ± 2·1†‡

Number in parentheses represent numbers of animals.
* $P < 0{\cdot}001$.
† $P < 0{\cdot}01$.
‡ Data from Ho, Tansey & others (1970).

Mescaline had no effect on the metabolism of pentobarbitone in mice. Although it also caused increases in accumulation of pentobarbitone in brain, plasma, liver and kidney, the pentobarbitone sleeping time in animals treated with mescaline was shortened. Furthermore, the barbitone sleeping time was unaffected. If the increase of concentration in the tissues of experimental animals was the result of an increase in binding of pentobarbitone by mescaline, a reduction of the "free" pentobarbitone for exerting hypnotic action could account for the resulting decrease of pentobarbitone sleeping time in mice.

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A comparison of the stereochemical requirements of cholinergic and anticholinergic drugs

None of the many theories which have been suggested to explain the observed behaviour of cholinergic and anticholinergic drugs at the muscarinic or postganglionic receptor account satisfactorily for all the experimental data (Goldstein, Aronow & Kalman, 1968). For example, it is difficult to explain why, although the dose-response curves for the antagonism of acetylcholine by atropine on the guinea-pig ileum are indicative of a competitive interaction (with both acetylcholine and atropine having at least one common point of attachment as a receptor site), the well known fact that the rate of washout of atropine from ileum is independent of the concentration of acetylcholine in the rinsing solution is not consistent with such a competitive interaction. There have been many attempts to explain this; for example, it has been suggested recently that the observed apparent competitive antagonism could result if the receptors were quite distinct, but that the presence of an antagonist at a site near to the cholinergic receptor could modify the cholinergic receptor in such a way that the affinity of the agonist for its receptor was reduced (Goldstein & others, 1968). In an attempt to assess whether or not cholinergic and anticholinergic drugs interact with a common receptor we have considered the structure-activity relations of a series of agonists and antagonists which are formally derived from acetylcholine.

Acetylcholine (I) may be converted into an anticholinergic drug by replacement of the acetyl group by a more bulky substituent such as 2-cyclohexyl-2-hydroxy-2-phenylacetyl (II) (Ellenbroek, Nivard & others, 1965). In such anticholinergic drugs the potency is critically dependent on the configuration of the benzylic carbon atom, the *R* enantiomer of II being 100 times as active as the *S* enantiomer (Table 1). Comparison of cholinergic esters of acetic acid and anticholinergic esters of *R*(−)-2-cyclohexyl-2-hydroxy-2-phenylacetic acid may be made in the following manner.

1. Replacement of any of the *N*-methyl substituents in I by other alkyl groups reduces cholinergic activity whereas in II the nature of the *N*-substituents may vary over wide limits without appreciably reducing potency, and in some instances increase potency.

I R = R′ = H II R = R′ = H

III R = Me, R′ = H IV R = Me, R′ = H

V R = H, R′ = Me VI R = H, R′ = Me

Also in anticholinergic drugs the nitrogen may be tertiary or quaternary whereas only quaternary compounds are potent agonists (Abood, 1968).

2. Replacement of one of the α-protons in I with methyl to give acetyl α-methylcholine (III) causes a considerable reduction in muscarinic potency (although the nicotinic potency is little affected.) Also the muscarinic potency is dependent on the absolute configuration of the methyl substituted carbon, the *R* enantiomer of III being 8 times more active than the *S* isomer (Beckett, Harper & Clitherow, 1963). On the other hand, replacement of a α-proton in II with methyl to give IV enhances anticholinergic potency and activity no longer depends on the configuration of the methyl-substituted carbon atom (Table 1).

Table 1. *Affinity of stereoisomeric anticholinergic compounds*

Compound (Configuration)	*log K**
II (*R*)	9·66 (10·4)†
II (*S*)	7·38 (8·4)
IV (*R*-acid, *S*-alcohol)	10·08
IV (*R*-acid, *R*-alcohol)	10·04
VI (*R*-acid, *S*-alcohol)	8·9
VI (*R*-acid, *R*-alcohol)	8·9

* log K values were determined by the method of Barlow, Scott & Stephenson (1963).
† Values in parentheses are pA_2 values recorded by Ellenbroek & others (1965).

3. The *S* enantiomer of acetyl β-methylcholine (V) is equiactive with acetylcholine whereas the *R* enantiomer is much less active (Beckett & others, 1963). Substitution of the β-carbon of II with methyl to give VI affords a product which is less active than II and in which the absolute configuration of the β-substituted carbon is of little importance (Table 1).

4. Replacement of the alcoholic oxygen in acetylcholine with sulphur considerably reduces muscarinic potency but replacement of alcoholic oxygen by sulphur in anticholinergic compounds has little effect on anticholinergic potency (Barlow, 1964).

Thus, apart from the observation that the anticholinergic drugs discussed above are formally derived from acetylcholine by the replacement of acetyl by a bulky substituent,

VII VIII

the stereochemical requirements for high cholinergic and high anticholinergic potency bear no other resemblance and make it unlikely that the two types of drugs share a common receptor. A similar conclusion may be reached from the observation that in the cholinergic 2-methyl-4-trimethylammoniummethyl-1,3-dioxolan iodides (VII) it is the configuration of C-4 on which cholinergic potency depends, whereas in the anticholinergic drugs derived from VII such as the 2-phenyl-2-cyclohexyl derivative VIII the configuration at C-4 is of little importance and anticholinergic potency depends only on the configuration at C-2 (Brimblecombe & Inch, 1970). However against these facts must be weighed the results that *R*(−)-quinuclin-3-yl acetate is a more potent agonist than its *S*-enantiomer (Robinson, Belleau & Cox, 1969; Belleau & Pauling, 1970) and *R*(−)-quinicludin-3-yl diphenylacetate is a much more potent antagonist than its *S*-enantiomer (Randall, Benson & Stefko, 1952).

If the idea that antagonists interact with a different receptor site to the agonist and merely alter the affinity of the agonist for the receptor is correct, it appeared to us to be unlikely that the affinity of all agonists would be altered to the same extent and thus using different agonists and the same antagonist, different affinity constants for that antagonist might be obtained. Using acetylcholine, carbachol, (*R*)-acetyl β-methylcholine and (*S*)-acetyl-β-methylcholine and oxotremorine the same value for the atropine affinity constant was obtained in experiments on guinea-pig ileum although with oxotremorine considerable changes in the rate of reactions on the ileum were apparent.

It appears therefore that anticholinergic drugs act at different receptors to the cholinergic drugs and do not allosterically modify the nature of the cholinergic receptor yet in many respects anticholinergic drugs appear to be competitive antagonists of cholinergic drugs (for example by causing a parallel shift in dose response curves). This seems to us to provide evidence for the view that there must be a large receptor reserve and that maximum biological response must be elicited by fractional receptor occupancy.

Moran & Triggle (1970) have reached a similar conclusion that the agonist and antagonist receptor sites are different but their experiments seem to indicate the absence of a receptor reserve.

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Conformations in solution of some cholinergic and anticholinergic compounds

Interest in the structure-activity relations of cholinergic compounds has prompted detailed X-ray analyses of acetylcholine, $S(+)$-acetyl-β-methylcholine, $R(+)$-acetyl-α-methylcholine, L-(+)-muscarine and L-(+)-2(*S*)-methyl-4(*R*)-trimethylammonium-methyl-1,3-dioxolan iodide (for relevant references see Chothia, 1970). As a result of these studies the conformational similarity of these compounds in the crystal phase is now well established. Additionally it has been suggested, from a consideration of the magnitude of stereodependent vicinal proton–proton coupling constants from nmr studies, that acetylcholine has a similar preferred conformation in D_2O solution (Culvenor & Ham, 1966). However, since the generality and accuracy of this type of approach for determining the conformation in solution of cholinergic (and anti-cholinergic) compounds remains to be established and since it is not known whether all the above cholinergic compounds are conformationally similar in crystal form and in solution we have attempted to use two other methods to assess the solution conformation of the O–C–C–N chain of acetyl α- and β-methylcholines. The first method depends on reports that ^{14}N-H vicinal coupling constants are stereodependent (Mooney & Winson, 1969) and the second method utilizes reports that the molecular rotations of chiral molecules may be calculated and are conformation dependent (Lemieux & Martin, 1970).

Method 1. The quadrupole moment associated with the ^{14}N nucleus (spin I = 1) precludes observation of spin–spin coupling to nitrogen in all but a few compounds. However, when there is a highly symmetrical field gradient about the nitrogen nucleus, such as in quaternary salts, appreciable vicinal coupling constants may be observed particularly at high temperatures with D_2O and DMSO as solvent. Such couplings are stereodependent; for example, for vicinal ^{14}N–H dihedral angles of 60°, 80°, 120° and 0° $J_{^{14}N-H}$ is <1, <1, <1 and 1–3 Hz respectively (Terui, Aono & Tori, 1968), and more recently it has been shown that for compounds in which the vicinal dihedral angle is 180° $J_{^{14}N-H}$ was 6·9–9·7 Hz (Bothner-By & Cox, 1969).

The nmr spectrum of acetyl α-methylcholine (I) has been measured in D_2O at 90° and found to be first order.* The β-protons at δ 4·48 ppm were equivalent and appeared as a pair of superimposed triplets as a consequence of coupling with the α-proton (J = 4 Hz) and with ^{14}N (J = 2 Hz). Depending on values assigned to J_{gauche} and J_{trans} the value of 4 Hz for $J_{H\alpha, H\beta}$ is consistent with a rapidly equilibrating mixture of conformers A and B or of a mixture of A, B and C (Fig. 1). However the vicinal $J_{^{14}N-H}$ value of 2 Hz is similar to that shown by acetylcholine where an antiperiplanar N–C–C–O arrangement was thought unlikely (Culvenor & Ham, 1966), whereas in acetylthiocholine and acetylselenocholine, where an antiperiplanar N–C–C–S(Se) arrangement was favoured, the vicinal $J_{^{14}N-H}$ value was < 0·7 Hz (Cushley & Mautner, 1970). Thus it is unlikely that conformer C of acetyl α-methylcholine (I) is present to any appreciable extent in solution. This result indicates a difference between the solution and crystal structures of I since crystallographic data indicated that both conformers B and C were important (Chothia & Pauling, 1969).

$RCOOCH_2CHMe\overset{+}{N}Me_3$	$RCOOCHMeCH_2\overset{+}{N}Me_3$
I R = CH_3	III R = CH_3
II R = $Ph_2C(OH)$-	IV R = $Ph_2C(OH)$-

* It was necessary to measure ^{14}N-H coupling constants at 90° in order to sufficiently reduce the relaxation time of the N nucleus to allow the appearance of well resolved ^{14}N-H couplings.

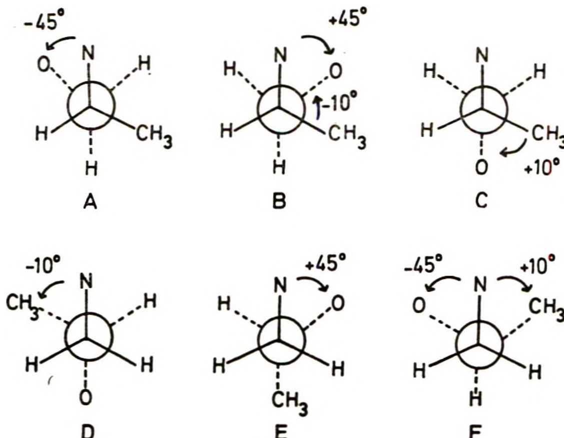

FIG. 1. The conformers of *S*(—)acetyl-α-methylcholine (A, B and C), and *S*(+)acetyl-β-methylcholine (D, E and F) are shown, together with the contributions (e.g. —45°↶) of various gauche contributions to molecular rotation.

It is of interest that an anticholinergic analogue of I, benziloyl α-methylcholine (II), shows a similar spectrum ($J_{H\alpha H\beta}$ = 4Hz, $J_{^{14}N-H}$ = 2Hz) to I and can be confidently assigned a similar conformation in solution.

The spectrum of acetyl β-methylcholine (III) in D_2O at 90° was also first order with non-equivalent α-protons at δ 3·58 and 3·82 ppm with vicinal coupling constants of 2·8 and 8·5 Hz respectively. These coupling constants are consistent with conformers D and E but not with F (Fig. 1). Conformer F was also precluded because no vicinal ^{14}N–H coupling was detected and only F should show such a coupling. Distinction between conformers D and E may be made from a consideration of the magnitude of the J_{gauche} coupling constant. It is well known that gauche coupling constants are smaller when one proton is antiperiplanar to an electronegative substituent, such as in E, than otherwise, such as in D where the gauche coupling would be expected to be nearer 5Hz than the observed 2·8Hz (Booth, 1965). Thus it appears that for acetyl β-methylcholine conformer E is favoured in solution and in crystal form (Chothia & Pauling, 1969a).

The anticholinergic analogue of III, benziloyl β-methylcholine (IV) did not give a first order spectrum and no vicinal ^{14}N–H coupling could be detected. Thus as for III, conformation F is unlikely to be present to any significant extent in solution.

It is encouraging that with the choline-like compounds examined, for which H–H couplings have been measured, there is good agreement between results based on these values and those based on ^{14}N–H couplings. Additionally the conformational assignments to I and III in solution by the nmr method were in agreement with those obtained by molecular rotational considerations. It is recognized that the preferred conformation of acylic molecules in solution can depend on the temperature of the solution but since for the choline like molecules described the vicinal H-H couplings showed no significant variation with temperature increase there is no reason to suppose that the solution conformations at room temperature differed from those at 90°.

Method 2. Molecular rotations ($[M]_D$) of chiral molecules may be calculated using the empirical treatment of Lemieux & Martin (1970). In their approach only relations between *gauche* situated groups are considered to contribute significantly to the overall molecular rotation, and for example a *gauche* C/O relation is assigned a value of 10° and a gauche O/O relation a value of 45°. The signs of these *gauche* contributions to

molecular rotation are illustrated:

Since the molecular rotations of methyl 4,6-*O*-benzylidene-α-D-altropyranoside ($[M]_D$ + 350°) and its 3-deoxytrimethylammonium derivative ($[M]_D$) + 370°) show only a small difference it has been assumed for the purposes of the present discussion that $\overset{+}{N}$/O and $\overset{+}{N}$/C contributions to molecular rotation differ little from O/O and O/C contributions. Thus the measured molecular rotation* of +70° for acetyl S(+)-β-methylcholine suggests that in solution conformer E preponderates (calculated $[M]_D = +45°$, Fig. 1) since the occurrence of conformers D and F or a mixture of conformers would cause a marked reduction in the magnitude or change in the sign of the calculated rotation, or both. Similarly, the measured molecular rotation* of *S*(−)-acetyl-α-methylcholine (−18°) is most nearly consistent with a mixture of conformers A and B in approximately equal proportions (calculated $[M]_D = -5°$, Fig. 1) since no other conformer or mixture of conformer gives a calculated $[M]_D$ of similar sign and magnitude. These results therefore suggest that if more realistic values can be obtained for $\overset{+}{N}$/C and $\overset{+}{N}$/O *gauche* contributions toward molecular rotation, the method of comparing observed and calculated molecular rotations may provide accurate information about the relative proportions of conformers in chiral cholinergic and anticholinergic compounds.

In summary, both the nmr and molecular rotational data indicate that acetyl β-β-methylcholine has a similar conformation in solution to that found in the crystal lattice. For acetyl α-methylcholine the nmr, molecular rotation and X-ray data indicate that two or more conformations are equally favoured and that the preferred conformations in solution and in the crystal lattice may differ.

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* Molecular rotations are calculated from the specific rotations quoted by INCH, T. D. & LEWIS, G. J. (1970), *Carbohydrate Res.* in press.

The effect of amantadine on the uptake of dopamine and noradrenaline by rat brain homogenates

Amantadine hydrochloride (1-adamantanamine hydrochloride), an antiviral agent (Davies, Grunert & others, 1964) has been shown to be effective against parkinsonism (Schwab, England, & others 1969, Parkes, Calver & others, 1970). The mode of action of the drug in improving the condition of the parkinsonian patient has not been elucidated. The results of Vernier, Harmon & others (1969) suggested that, in high doses, amantadine inhibits the uptake of noradrenaline into peripheral nerve endings. It seemed reasonable to suppose that a similar action on the uptake of dopamine into central neurons might, in part, explain the anti-parkinsonian activity of amantadine. To investigate this possibility, preliminary experiments have been performed using a method based on that described by Snyder & Coyle (1969).

Fresh rat brain was homogenized in 10 volumes of oxygenated (95% O_2, 5% CO_2) modified Krebs-Henseleit buffer pH 7·2, using a "Tri-R" tissue homogenizer with a teflon pestle and a clearance of 0·15–0·23 mm at 750 rev/min. After further oxygenation, 1 ml aliquots of the homogenate were placed in small lengths of $\frac{1}{4}$ inch dialysis tubing, tied at both ends to form a sac containing an air bubble to aid mixing. The sacs were placed in stoppered test-tubes containing 5·0 ml Krebs-Henseleit buffer pH 7·2, and were incubated, with constant mixing, at 37°. At the start of each experiment, either dopamine or noradrenaline was added to the external medium, in a concentration of 1·0 pg/ml. In each case, 0·05 Ci/ml of [^{14}C]labelled amine was included in the total concentration. To measure amine uptake, the contents of the dialysis bag were first centrifuged at 48 000 *g* and 0·2 ml of the supernatant fluid was added to 15 ml of scintillation fluid (1 litre toluene: 500 ml methanol: 5 g POPOP: 0·1 g PPO). The pellet was digested in 3 ml M Hyamine solution, and 0·2 ml of the digest was added to 15 ml of scintillation fluid. The number of disintegrations in 10 min at 7° were recorded using a Packard liquid scintillation counter. While the increase in radioactivity in the supernatant, caused by passive diffusion of the amine, rapidly reached a maximum, radioactivity in the pellet reached a maximum after 2 h. Uptake of amines into the pellet was shown to be sodium dependent and temperature dependent, and was inhibited by ouabain (see Fig. 1 A and B); it was therefore assumed to involve an active process. The inclusion in the homogenate and incubating medium of various concentrations of reserpine did not reduce the radioactivity in the pellet by more than 40% and it was further assumed that the uptake which was reserpine-resistant represented active uptake into intact nerve endings.

Fig. 1A shows the effect of amantadine, added to the medium and to the homogenate, on the uptake of noradrenaline into nerve endings. A concentration of 1×10^{-4}g/ml decreased the radioactivity in the pellet by 87%. Concentration between 1×10^{-6} and 5×10^{-5} g/ml produced a 37–44% decrease.

Fig. 1B shows the effect of amantadine on dopamine uptake. All concentrations between 1×10^{-6} and 5×10^{-4} g/ml produced a similar reduction in uptake of 38–52%. A concentration of 1×10^{-3} was required to produce an inhibition of 69%.

If it is assumed that the 40% of the total uptake inhibited by reserpine represents active uptake into synaptic vesicles released by homogenization, then our experiments have not excluded the possibility that amantadine will inhibit the uptake of dopamine and noradrenaline by this process. However, it seems more likely that this reduction in radioactivity represents a non-specific action, probably on passive diffusion, since it was readily produced by widely-varying concentrations of several drugs, including reserpine and amantadine.

What does emerge clearly from our results is that, under the conditions of our experiments, high concentrations of amantadine are required to produce significant

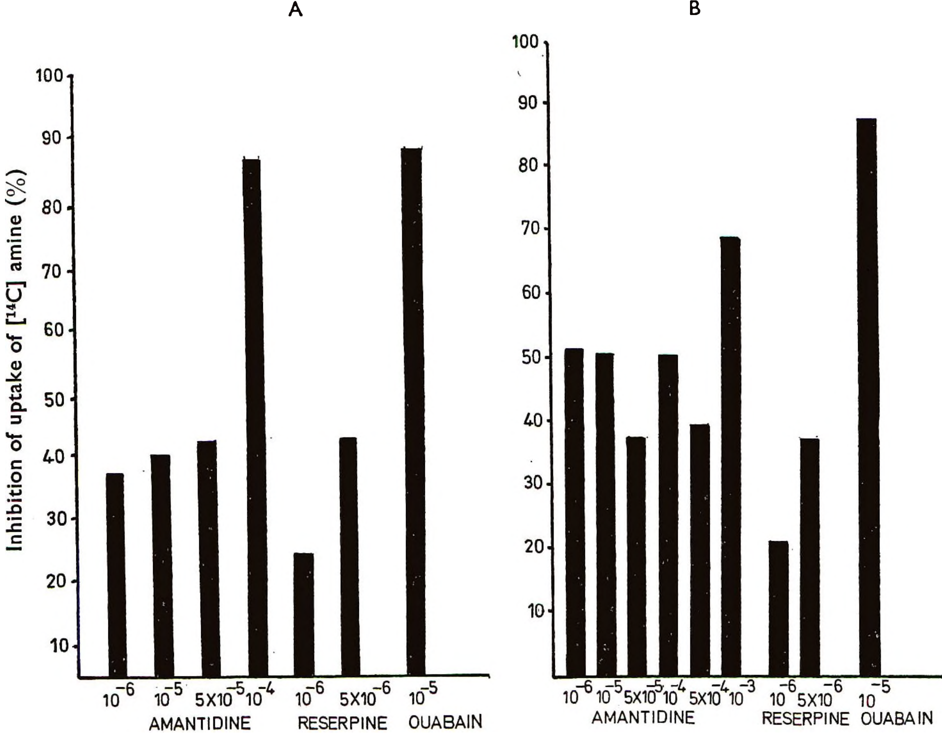

FIG. 1. The effect of amantadine, reserpine and ouabain on the uptake of: A, [^{14}C] labelled noradrenaline; B, [^{14}C] labelled dopamine by rat brain homogenates.

inhibition of noradrenaline and dopamine uptake into brain nerve-endings. In clinical use, amantadine has been found to produce side effects of central hyperactivity (e.g. insomnia, jitteriness) (Schwab & others, 1969) while Simon, Malatray & Boissier (1970) reported similar CNS stimulation in animals. Although Vernier & others (1969) found some evidence of inhibition by amantadine of noradrenaline uptake into peripheral nerve endings, the results of our experiments make it appear unlikely that the central stimulation caused by amantadine could be due to inhibition of uptake into central nerve endings. Equally, the concentration of amantadine required to produce significant inhibition of dopamine uptake suggests that this mechanism of action does not account for the clinical effectiveness of the drug against parkinsonism. This is in agreement with the report of Grelak, Clark & others (1970), who found no evidence for the inhibition by amantadine of dopamine uptake into peripheral nerve endings.

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On the mode of action of amantadine

Amantadine has been shown to have some therapeutic effect in parkinsonism, initially by Schwab, England & others (1969). In animal experiments it has been found to cause moderate central stimulation, reversal of tetrabenazine-induced sedation, a slight block of the noradrenaline uptake in the heart and to be ineffective in decreasing monoamine oxidase activity *in vitro* (Vernier, Harmon & others, 1969) An anti-cataleptic effect is reported (Simon, Malatray & Boissier, 1970; Zetler, 1970) and the drug potentiates L-dopa-induced effects in mice (Svensson & Strömberg, 1970). We have now examined some functional and biochemical aspects of the effect of amantadine on central and peripheral catecholamine neurons, and compared its mode of action with (+)-amphetamine.

We have confirmed the stimulant action of amantadine on the motor activity of mice. To investigate the effect of catecholamine depletion on this effect, amantadine HCl (150 mg/kg) was injected to female mice (strain NMRI, about 20 g) pretreated with reserpine (10 mg/kg) 5 h before. The animals were put into a test cage 90 min later, 10 min after which the motility was measured for the next 30 min by means of an Animex activity meter (Svensson & Thieme, 1969). Some mice received α-methyl-tyrosine methylester (H 44/68), an inhibitor of tyrosine hydroxylase (200 mg/kg) 15 min before amantadine, and some L-dopa (25 mg/kg) 85 min after amantadine. All injections were given intraperitoneally. Controls were given either reserpine and H 44/68 or these drugs together with L-dopa (Fig. 1). Statistical evaluation showed that amantadine caused an increased motor activity in the reserpine-pretreated mice ($P < 0{\cdot}005$), which was inhibited by H44/68 ($P < 0{\cdot}01$). A small dose of L-dopa, causing no motor stimulation by itself, restored the amantadine effect ($P < 0{\cdot}05$). The general picture strongly resembles that of (+)-amphetamine (*c.f.* Hanson, 1966, 1967) and indicates, that amantadine requires small amounts of catecholamines for its motor stimulant effect.

For assay, amantadine HCl or (+)-amphetamine sulphate were injected in mice in various doses 105 and 45 min, respectively, before death. All mice were pretreated with reserpine (10 mg/kg) 22 h and nialamide (100 mg/kg) 4 h before death. L-Dopa (25 mg/kg) was injected subcutaneously 30 min before death. All other drugs were given intraperitoneally. Noradrenaline was determined according to Bertler, Carlsson & Rosengren (1958); dopamine according to Carlsson & Waldeck (1958, as modified by Carlsson & Lindqvist, 1962a); normetanephrine according to Carlsson & Lindqvist (1962b); methoxytyramine according to Carlsson & Waldeck (1964). Amantadine, 50 or 100 mg/kg, or (+)-amphetamine, 0·5 or 1·5 mg/kg, caused a decrease in the noradrenaline accumulation in the brain after L-dopa ($P < 0{\cdot}001$). In the heart, amantadine, 50 mg/kg, caused a decrease in noradrenaline accumulation ($P < 0{\cdot}025$) and so did (+)-amphetamine, 2·5 and 1·5 mg/kg, ($P < 0{\cdot}001$ and $< 0{\cdot}01$, respectively) (Table 1). No decrease of the dopamine accumulation in the brain or in the heart was found after amantadine or (+)-amphetamine but it was enhanced in the brain after 25 mg/kg amantadine ($P < 0{\cdot}025$) and in the heart after 50 mg/kg ($P < 0{\cdot}005$). A decrease in

the dopamine: methoxytyramine ratio with increasing doses of amantadine was also found. This could be interpreted in terms of a net release of dopamine into the extraneuronal space. The normetanephrine analyses showed amounts too small to be measured by the technique used.

The effect of amantadine on brain levels of noradrenaline, dopamine, normetanephrine and methoxytyramine after nialamide pretreatment was also examined.

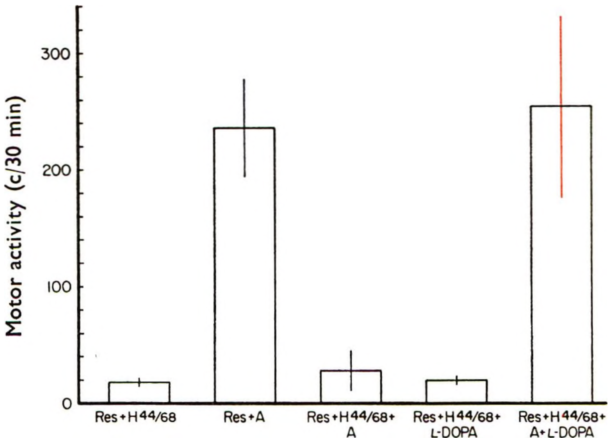

FIG. 1. Effect of amantadine on motor activity in mice after catecholamine depletion. Drugs were given intraperitoneally, using the following doses and time intervals before the start of the measurement: Reserpine (Res) 10 mg/kg 6 h 40 min, H44/68 200 mg/kg 1 h 55 min, amantadine HCl (A) 150 mg/kg 1 h 40 min and L-dopa 25 mg/kg 15 min. Each value represents the mean of activity of 4–6 groups, consisting of three mice, ± s.e.

Table 1. *Effect of amantadine and* (+)-*amphetamine on* L-*dopa induced accumulation of noradrenaline* (NA), *dopamine* (DA), *and methoxytyramine* (MT) *in mice pretreated with reserpine and nialamide.* The drugs were given i.p. in the following doses and time intervals before death: reserpine, 10 mg/kg—22 h, nialamide, 100 mg/kg—4 h, amantadine HCl—105 min, (+)-amphetamine sulphate—45 min. L-Dopa, 25 mg/kg s.c., was given 30 min before death. Shown are the means ±s.e. in μg/g of (n) determinations.

Drug, mg/kg	Heart NA	Heart DA	Brain NA	Brain DA	Brain MT
Amantidine					
100	0·04 (2) ±0·01	0·52 (2) ±0·12	0·05 (2) ±0·01	1·41 (2) ±0·07	
50	0·04 (6) ±0·01	0·82 (6) ±0·10	0·07 (8) ±0·01	1·65 (8) ±0·06	3·03 (4) ±0·38
25	0·05 (2) ±0·01	0·64 (2) ±0·06	0·11 (2) ±0·02	2·20 (2) ±0·00	
0	0·07 (8) ±0·01	0·50 (7) ±0·05	0·10 (10) ±0·01	1·72 (10) ±0·10	2·24 (6) ±0·24
(+)-Amphetamine					
2·5	0·01 (3) ±0·00	0·38 (2) ±0·06	0·05 (3) ±0·01	1·42 (3) ±0·02	
1·5	0·02 (2) ±0·01	0·34 (2) ±0·10	0·06 (4) ±0·01	1·54 (4) ±0·08	2·82 (2) ±0·03
0·34	0·09 (2) ±0·00	0·66 (2) ±0·02	0·10 (2) ±0·01	1·74 (2) ±0·20	

Table 2. *Effect of amantadine on the levels of noradrenaline* (NA), *dopamine* (DA), *normetanephrine* (NM), *and methoxytyramine* (MT) *in nialamide pretreated mice*. Nialamide 100 mg/kg and amantadine HCl (100 mg/kg) were given i.p. 4 and 2 h, respectively, before death. Each value represents the mean of 6 determinations ± s.e. The increase in NM and MT caused by amantadine is statistically significant ($P < 0{\cdot}001$).

Treatment	NA μg/g	DA μg/g	NM μg/g	MT μg/g
Nialamide + amantadine	0·43 ± 0·02	1·15 ± 0·06	0·25 ± 0·02	0·42 ± 0·02
Nialamide	0·47 ± 0·03	1·17 ± 0·05	0·07 ± 0·01	0·26 ± 0·02

Nialamide (100 mg/kg, l.p.) and amantadine HCl (100 mg/kg, l.p.) were given 4 and 2 h respectively, before death. Amantadine caused an increase in both normetanephrine and methoxytyramine ($P < 0{\cdot}001$), but no change in the catecholamine levels (Table 2). The data in Tables 1 and 2 are similar to those found after (+) amphetamine treatment (Carlsson, Fuxe & others, 1966) and indicate an increased release of catecholamines into the extraneuronal space (*c.f.* Carlsson, 1970) after amantadine.

Finally, reserpine (10 mg/kg) and nialamide (10 mg/kg) were injected intraperitoneally in mice 6 and 2 h, respectively, before ^{3}H-NA, 0·5 μg/kg, intravenously. Fifteen min later, amantadine HCl in various doses was given intravenously and the mice were killed after another 15 min. ^{3}H-NA was measured in the heart (Carlsson & Waldeck, 1963). Amantadine released ^{3}H-NA taken up by the heart through a reserpine-resistant mechanism (^{3}H-NA ng/g ± s.e.; 0·32 ± 0·05 n = 5; 0·20 ± 0·06 n = 3; 0·09 ± 0·00 n = 4; 0·13 ± 0·01 n = 5 for amantidine 0, 2·5, 10, 25 mg/kg respectively, $P < 0{\cdot}001$ at the 10 mg dose and $<0{\cdot}005$ at the 25 mg dose compared to control value), an effect shared by (+)-amphetamine (Carlsson & Waldeck, 1966). This specific release is not due to a block of the uptake mechanism at the level of the cell membrane (the so-called membrane pump), since a potent inhibitor of this mechanism, protriptyline, is ineffective in this respect (Carlsson & Waldeck, 1966).

We thus have to consider the possibility that the activity of amantadine against parkinsonism is brought about by an amphetamine-like mechanism, i.e. by release of catecholamines from an extragranular, though intraneuronal pool, apparently dependent on the rate of catecholamine synthesis. As is well known, amphetamine belongs to the traditional armamentarium of drugs used in parkinsonism. An important difference between the two drugs may be a higher selectivity of amantadine for *central* catecholamine neurons for amine release. As indicated above, the actions of amantadine described here cannot be entirely explained by inhibition of the membrane pump. Nevertheless the evidence does not exclude the possibility that amantadine has this action in addition to its amine-releasing properties.

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The effect of prenylamine on adrenaline-induced hypercholesterolemia in mice

The administration of adrenaline causes a rise in serum cholesterol level in many animal species. The hypercholesterolemia is preceded by an elevation of free fatty acid levels in rats (Shafrir, Sussman & Steinberg, 1960; Shafrir & Steinberg, 1960), dogs (Kaplan, Stafford & Gant, 1957; Shafrir, Sussman & Steinberg, 1959), and rabbits (Dury, 1957), and it seems that the rise in serum cholesterol is secondary to the rise in blood free fatty acids (Gidez, Roheim & Eder, 1962; Nestel & Steinberg, 1963; Steinberg, 1963). We now report the effect of adrenaline on cholesterol serum levels in mice, and the modulation of this effect by prenylamine and other adrenergic blocking drugs known to inhibit the free fatty acid response to adrenaline.

Swiss-Webster (ICR) albino male mice (Harlan Industries, Cumberland, Indiana) 25–28 g were housed in groups of five at 25 $\pm$ 3° with freely available food and water. All injections were subcutaneous at varying sites in the dorsal region. The drugs were dissolved or suspended in sesame oil in a volume of 10 ml/kg weight. The doses as base of drug were: (—)-adrenaline, 1 mg/kg; prenylamine, 5, 10, 25 and 50 mg/kg; propranolol, 10 mg/kg; phenoxybenzamine hydrochloride, 1 mg/kg; and phentolamine hydrochloride, 10 mg/kg. The schedule for drug administration was: blocking drug at 5 30 am on days 1, 2 and 3 and again 12 h later on days 1 and 2. Adrenaline was given at 6 am on days 2 and 3 and 12 h later on day 2. Analysis was at 6 pm on day 3. In experiments where either adrenaline or prenylamine (25 or 50 mg/kg) was given alone, in injection of 10 ml/kg of sesame oil replaced the blocking drug; thus eight injections were always made in the three-day period. The controls similarly had only sesame oil. A group of older male mice, 32–36 g was also included.

Blood was obtained from the mice by decapitation, and serum cholesterol measured colorimetrically (Watson, 1960).

No difference was found between control serum levels of cholesterol of either weight groups of mice (Table 1). Whereas adrenaline administration caused no significant change in serum cholesterol level in the older mice (32–36 g), there was a statistically significant ($P < 0{\cdot}001$) elevation in mean cholesterol value above control

Table 1. *Serum cholesterol levels in male mice after various sympathomimetic and sympatholytic drugs*

No. of mice per group	Mice weight (g)	Treatment and dosage	Cholesterol serum levels (mg %) Mean ± s.e.
30	25–28	Sesame oil controls (10 ml/kg)	118 ± 2·7
10	32–36	Sesame oil controls (10 ml/kg)	115 ± 3·0
19	25–28	Adrenaline (1 mg/kg)	150 ± 4·0
20	32–36	Adrenaline (1 mg/kg)	126 ± 7·0
20	25–28	Prenylamine (50 mg/kg)	128 ± 5·4
20	25–28	Prenylamine (25 mg/kg)	121 ± 4·3
20	25–28	Prenylamine (25 mg/kg) + adrenaline (1 mg/kg)	140 ± 4·7
20	25–28	Prenylamine (10 mg/kg) + adrenaline (1 mg/kg)	125 ± 4·0
20	25–28	Prenylamine (5 mg/kg) + adrenaline (1 mg/kg)	140 ± 5·7
20	25–28	Propranolol (10 mg/kg) + adrenaline (1 mg/kg)	125 ± 4·5
18	25–28	Phenoxybenzamine (1 mg/kg) + adrenaline (1 mg/kg)	116 ± 3·8
20	25–28	Phentolamine (10 mg/kg) + adrenaline (1 mg/kg)	110 ± 1·9

level in the younger mice (25–28 g). This response was inhibited by pretreatment with phenoxybenzamine (1 mg/kg) and phentolamine (10 mg/kg), by propranolol (10 mg/kg), and by prenylamine (10 mg/kg). Pretreatment both with higher (25 mg/kg) and lower (5 mg/kg) doses of prenylamine failed to block adrenaline-induced hypercholesterolemia but did reduce the response slightly. In 10 mice pretreated with prenylamine, 50 mg/kg, four deaths occurred shortly after injection of adrenaline. Given alone, prenylamine, 25 and 50 mg/kg, did not raise serum cholesterol levels significantly above the control value ($P < 0{\cdot}1$). The mean weights of drug-treated animals did not differ from that of controls throughout.

Prenylamine differs in its effects from either of the two classes of blocking agents used in that it is reported to produce a reserpine-like depletion of catecholamine storage granules (Schöne & Lindner, 1960; Carlsson, Hillarp & Waldeck, 1963), and was shown to exhibit both α-adrenolytic (Kochsiek, Scheler & Bretschneider, 1960; Lindner, 1963; Kuschke, Eckmann & others, 1964; Obianwu, 1967) and β-adrenolytic (Lindner, 1960, 1964; Haas, 1964; Fleckenstein, Döring & others, 1968) properties. Lindner (1964) and Braunsteiner, Sailer & Sandhofer (1965) showed prenylamine to block the noradrenaline-induced mobilization of free fatty acids in dogs, and to lower free fatty acid blood levels in man. The slight elevation of cholesterol seen in our experiments after prenylamine, 25 and 50 mg/kg, as well as the failure of 25 mg/kg to block the hypercholesterolemic effect of adrenaline may reflect the sympathomimetic properties of prenylamine in discharging endogenous catecholamines. Moreover, the deaths occurring upon administration of adrenaline to the mice pretreated with prenylamine, 50 mg/kg, may arise from a failure of uptake into stores (Carlsson, Hillarp & Waldeck, 1963). Whereas a dose of 5 mg/kg was too low to effect adrenergic blockade, a dose of 10 mg/kg blocked the adrenaline-induced rise of serum cholesterol. It is therefore apparent that at lower doses of prenylamine the sympathetic blocking properties of this drug supervene, whereas at higher doses the sympathomimetic properties become more prominent and overcome the intrinsic blocking effect.

The failure to elicit a rise in serum cholesterol after adrenaline administration to older mice remains a matter of observation only. A similar age dependent effect

of catecholamines on free fatty acid mobilization was observed in the rat by Jelinkova & Hruza (1963, 1964).

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Estimation of drug metabolite elimination kinetics in man by the synthesis-blocking method

It is often necessary for the complete characterization of the pharmacokinetics of a drug (Levy, Amsel & Elliot, 1969) and for drug biotransformation interactions (Amsel & Levy, 1969; Amsel & Levy, 1970) to measure the elimination (or excretion) rate constants of drug metabolites. Many drug metabolites are not readily synthesized or available commercially; they may be unstable in or not well absorbed from the gastrointestinal tract (Levy, Amsel & Elliott, 1969; Levy, Weintraub & others, 1966) and unsuitable for parenteral administration. The elimination rate constants of such metabolites, which include most glucuronides and sulphates, cannot be determined by administering the metabolite as such. Some mathematical techniques have been developed to estimate the rate constants indirectly from the urinary excretion rates of free drug and metabolites (Cummings, Martin & Park, 1967; Martin, 1967) but these estimations are difficult or impossible if little or no drug is excreted in non-metabolized form (Cummings, King & Martin, 1967).

Another approach to this problem is to administer the drug, block the synthesis of metabolite soon thereafter, and follow as a function of time the decline in the plasma concentration or urinary excretion rate of the quantity of metabolite present in the body when further synthesis was blocked (Levy & others, 1969; Amsel & Levy, 1969; 1970). Blocking the synthesis of a drug metabolite may be accomplished by administering a competitive inhibitor such as benzoate which blocks the synthesis of salicylurate from salicylate (Levy, & others, 1969), or salicylamide, which blocks the formation of salicylic glucuronides (Levy & Procknal, 1968). The formation of acetaminophen sulphate (APAPS) from acetaminophen (APAP) can also be blocked by salicylamide, which competes with APAP for sulphate (Levy & Yamada, 1970).

The elimination rate constant of APAPS has been measured in three healthy adult male volunteers who received 1 g APAP orally followed 1·5 h later by 1 g salicylamide in aqueous solution. Urine was collected every 15 min and assayed for APAPS (Levy & Yamada, 1970). Semilogarithmic plots of APAPS excretion rate versus time after salicylamide administration were linear for about 2 h and permitted the calculation of elimination half-life ($t_{\frac{1}{2}}$) and elimination rate constant ($0{\cdot}693/t_{\frac{1}{2}}$). The three subjects yielded elimination rate constants of 1·0, 0·9, and 1·0 h^{-1} respectively, equivalent to half-lives of 0·7, 0·8, and 0·7 h. Repetition of the experiment in the first subject, but with 2 g rather than 1 g salicylamide to make sure that APAPS formation had been blocked completely, yielded a rate constant of 0·9 h^{-1}.

The APAPS elimination rate constants thus obtained are in good agreement with the results of Cummings, King & Martin (1967) who determined these constants indirectly by pharmacokinetic analysis based on the excretion rates of APAP and APAPS after APAP administration. These investigators reported values ranging from 0·8 to 1·5 h^{-1} in four subjects, compared with 0·9 to 1·0 h^{-1} found in our experiments.

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Limitations of liquid penetration in predicting the release of drugs from hard gelatin capsules

In an attempt to explain factors affecting the release of drugs from capsules, Samyn & Jung (1970) used the liquid penetration test of Studebaker & Snow (1955). The former authors concluded that extended dissolution rates are obtained with powder blends that show reduced liquid penetration. We have found that this is not always to be the case. This can be illustrated by the results in Fig. 1a and b where the dissolution of capsules containing a water-insoluble drug plus various additives is compared with the liquid penetration of the same powder blends. Thus, poor liquid penetration does not necessarily ensure poor dissolution of the drug from the capsule. The blend which

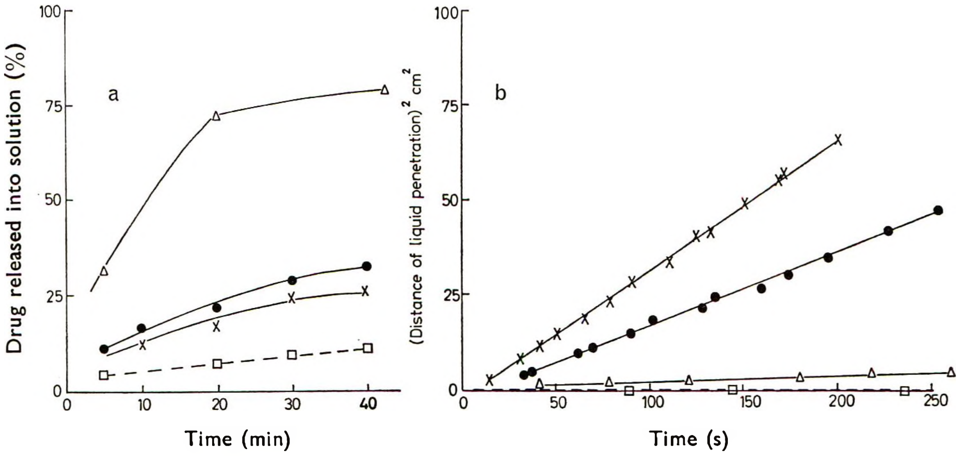

FIG. 1. (a). The percentage of drug released from the capsule into solution at known time intervals. (Dissolution test carried out as described by Newton and Rowley, 1970). (b). The square of the distance of liquid penetration against the time of flow. □------□ Drug + magnesium stearate 0·5 w/w. X——X Drug + magnesium stearate 0·5% w/w, sodium lauryl sulphate 1% w/w, lactose 5% w/w. ●——● Drug + magnesium stearate 0·5 w/w, sodium lauryl sulphate 1% w/w, lactose 20% w/w. △——△ Drug + magnesium stearate 1·0% w/w, sodium lauryl sulphate 1% w/w, lactose 50% w/w.

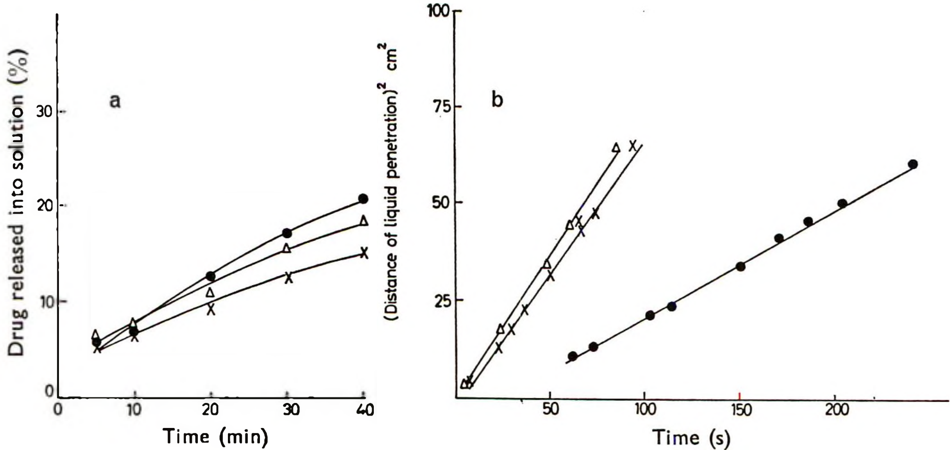

FIG. 2. (a). The percentage of drug released from the capsule into solution, at known time intervals (Dissolution test carried out as described by Newton & Rowley, 1970). (b). The square of the distance of liquid penetration against time of flow. ●——● Drug. X——X Drug + sodium lauryl sulphate 0·5% w/w. △——△ Drug + sodium lauryl sulphate 1·0% w/w.

allows no liquid penetration does, however, have the lowest dissolution. The results also show that rapid liquid penetration does not ensure good dissolution characteristics, further illustrated in Fig. 2a and b, where the presence of wetting agent readily promotes liquid penetration but does not assist dissolution. The liquid penetration test can help in the screening of wetting agents, but we consider that its extension to the prediction of drug release from capsules may not be possible.

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The effect of ethanol and amphetamine mixtures on the activity of rats in a Y-maze

Low doses of sodium amylobarbitone potentiated the stimulant effect of (+)-amphetamine sulphate on the behaviour of rats in the Y-maze (Steinberg, Rushton & Tinson, 1961; Steinberg, 1963; Rushton & Steinberg, 1963). Since the pharmacological properties of ethanol are similar to those of the barbiturates, it was of interest to see whether it shared with barbiturates the ability to potentiate the effect of amphetamine on the performance of rats in a Y-maze.

The experiment was made in a darkened room, the maze being illuminated by diffuse light from a lamp held in close proximity. The Y-maze, painted a uniform grey, was of the dimensions described by Rushton & Steinberg (1963). Male rats of the Wistar strain, initially weighing 150g, were maintained on a reversed 12 h lighting schedule. Food and water were freely available apart from the 3 min period when the rats were in the maze. The rats were kept singly in polythene cages throughout the experiment. In a preliminary experiment the time when the animals showed peak activity in the Y-maze was found to be 13·30 h and therefore all subsequent trials were conducted at this time. Only those rats with an activity score greater than 5 per 3 min trial were used. Each rat was run in the maze once a week for 5 weeks. This frequency was found in a preliminary experiment to be insufficient for the animals to become habituated to the maze. The rats were divided into 4 groups, each containing 5 animals. The effects of alcohol and amphetamine, alone and in combination, were tested over a period of five weeks. For one week in the five, the rats received no drugs, and hence acted as their own controls. During the other 4 weeks, ethanol and amphetamine were given in doses of 50–800 mg/kg and 4 mg/kg respectively, or in combinations of varying amounts of alcohol with 4 mg/kg of amphetamine.

All rats were pretreated with ethanol, (+)-amphetamine sulphate or the mixture for 15 min before being run in the maze. The drugs were given intraperitoneally in a volume of less than 0·5 ml/rat; the control group was injected with 0·5 ml of physiological saline.

Ethanol alone caused a slight increase in the Y-maze activity compared to the controls, in the lower dose used, but higher doses caused a decrease in exploratory activity (Fig. 1). Amphetamine, even in the lower dose used, increased the Y-maze activity. When these drugs were administered in combination, it was apparent that the depressant effect of ethanol was antagonized by amphetamine but at no dose

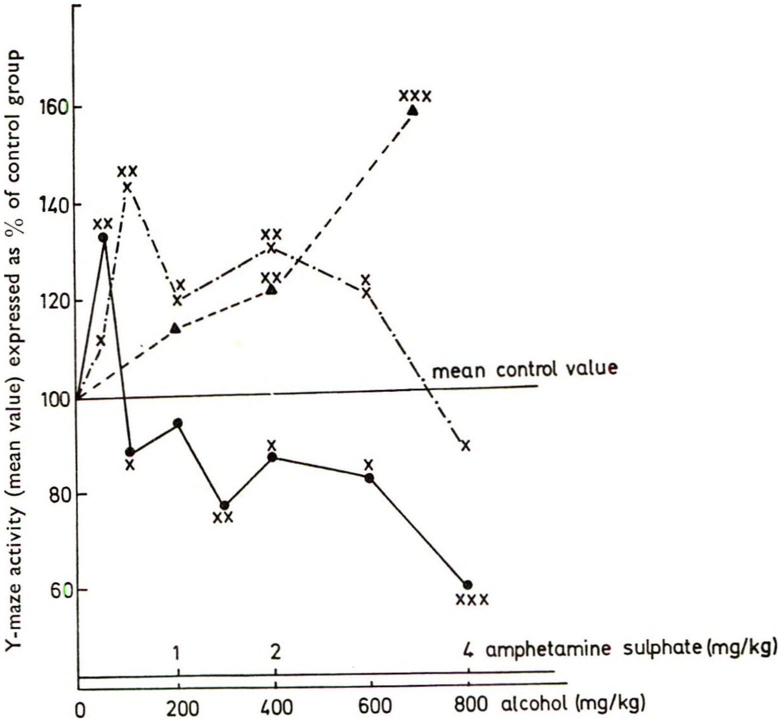

FIG. 1. Effect of (+)-amphetamine sulphate, ethanol and mixtures of the two on the Y-maze activity of rats. Each point is the mean score for 5 animals. ▲–▲(+)-Amphetamine. ●–● Ethanol. X–X Amphetamine + ethanol. * $P < 0.05 > 0.02$. ** $P < 0.02 > 0.01$. *** $P < 0.01 > 0.005$. The significance of the differences were determined by Student's *t*-test.

combination did the activity of the rats approach that produced by amphetamine (4 mg/kg) alone. The dose of amphetamine used was similar to that shown by Rushton & Steinberg (1963) to be potentiated by amylobarbitone. It would therefore appear that ethanol has an effect in amphetamine-pretreated rats different from that caused by sodium amylobarbitone. This suggests that the potentiation of amphetamine by amylobarbitone is probably a specific mechanism which may not be shared by other depressant drugs.

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