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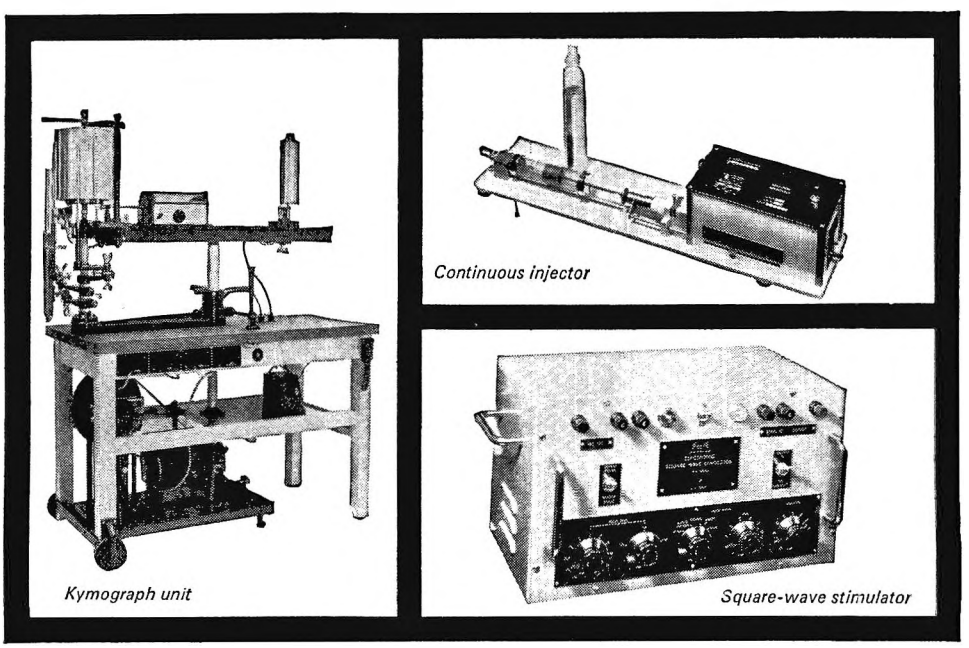

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The chromatographic identification of psychotropic drugs

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The thin-layer chromatography of three classes of psychotropic drugs—phenethylamines, tryptamines and erganes—has been investigated. Published methods are reviewed and Rf data, normalized by a graphical technique, are reported for extensions and modifications of some of these systems. Optimum forensic sorting procedures are recommended.

Recent papers from workers concerned with forensic identification of drugs seized in suspicious circumstances have reported the chromatographic mobility of a variety of psychotropic materials. For forensic purposes it is important to distinguish between potent psychotropic drugs for which restrictions are primarily concerned with supply, and other substances of closely related structure for which their actual or potential abuse has required their unauthorized possession to be made an offence. Chromatography on thin layers on glass plates or commercially coated polyester sheets provides rapid sorting methods for laboratories with limited time, sample and equipment.

It is convenient to summarize the published evidence, and to discuss our own contributions, in relation to three broad classes of bases—phenethylamines, tryptamines and erganes.

EXPERIMENTAL

Support

I.	Silica gel.
II.	Alkaline silica: I (30 g) with 0·1 N sodium hydroxide (60 ml).
III.	Alkaline silica: I (30 g) with N sodium hydroxide (60 ml).
IV.	Acidic silica: I (30 g) with 0·1 N potassium bisulphate (60 ml).
V.	Silica coating on polyester sheet (Eastman "Chromagram" 6061).
VI.	V, incorporating fluorescent indicator (Eastman "Chromagram" 6060).
VII.	V, to which 2 μl 0·1 N sodium hydroxide was added at each origin point.
I–IV.	Coating thickness 0·25 mm; plates 20 × 20 cm; coat applied with "Quickfit" and Shandon spreaders. Plates were dried at least ½ h at room temperature, activated for 1 h at 110° and cooled in a desiccator cabinet.
V–VII.	Commercially prepared layers 0·10 mm thickness. Sheets from opened packets were stored in desiccator cabinet without activation.

Solvents (all of "Analytical Reagent" grade)

A. Methanol. B. Methanol–ammonia (sp.gr. 0·88) (100:1·5). C. Chloroform–methanol (9:1). D. Acetone. E. Cyclohexane–benzene–diethylamine (15:3:2).

About 100 ml of solvent was exposed in small (4 litre) paper-lined tanks and allowed to reach equilibrium before use. For solvent B, a tightly lidded chamber was necessary

to maintain an approximately constant ammonia concentration; but, providing internal normalization of Rf values (see below) was employed, the solvent could be used without renewal for several runs within a period of a few days.

Visualization

The following systems were employed: "a" 254 nm illumination; "b" 350 nm illumination; "c" sprayed with 1% iodine–methanol; "d" sprayed with mixture 10 ml platinic chloride (5%), 5 ml HCl (sp.gr. 1·18) and 240 ml potassium iodide (2%); "e" sprayed with 0·5 g dimethylaminobenzaldehyde dissolved in 5 ml HCl (sp.gr. 1·18) and 95 ml ethanol (99%); the plates heated for 5 to 10 min at 105°; "f" sprayed with sulphuric acid (sp.gr. 1·84).

The colour code employed in Table 2 refers consecutively to the initial colour, to the colour formed within 1 min, and to the time in minutes during which the second colour fades: g = grey, m = mauve or purple, n = brown, o = orange, p = pink or pinkish brown, y = yellow, yy = dark yellow.

Normalization

Although distortion of solvent fronts was prevented by lining the tanks with paper and allowing the solvent to come to equilibrium in the vapour phase (as recommended by French & Wehrli, 1965) there was still sufficient variation between runs to warrant including a reference substance on each plate. Sunshine (1963) used a single marker substance for each class of drug he examined (barbiturates, carbamates, phenothiazines) but he computed relative (Rx) values, equating the marker Rf to unity (i.e., some Rx values were greater than one). Many other workers include a standard substance of previously established Rf value when they wish to cite relative mobility in specified conditions. In a recent symposium on the standardization of TLC procedures, Stahl (1968) again referred to the use of a standard blend of defined substances in fixed concentrations in the same solvent. At the same symposium Gasparič (1968) recommended an internal standard comprising compounds selected evenly to cover the whole Rf range. We had independently decided to select a blend of related substances showing a range of Rf values; the apparent novelty of our technique lies in the graphical treatment, whereby a best-fit line takes account of all the marker points. By this technique, the spread of Rf values for a given substance and system is markedly reduced. The three mixtures routinely employed for phenethylamines, tryptamines and erganes are listed under Method. An example of the graphical normalization for one particular plate is shown in Fig. 1.

Method

Mean values for three or more reference spots were determined using optimum conditions previously established in at least 10 runs. For subsequent runs, 2 to 5 μg reference substances were applied accumulatively at each of two points trisecting the start-line, and the unknown substances symmetrically distributed either side of them. The plate was then developed and the uncorrected Rf values measured. Using the established mean values for the reference substances as abscissa (x-axis), the two observed Rf values for each of the standard spots were plotted from the ordinate (y-axis) and a smooth curve drawn through the family of intersections. Finally, entering all uncorrected Rf values from the y-axis, the "normalized" value (Rf*) was read from the x-axis.

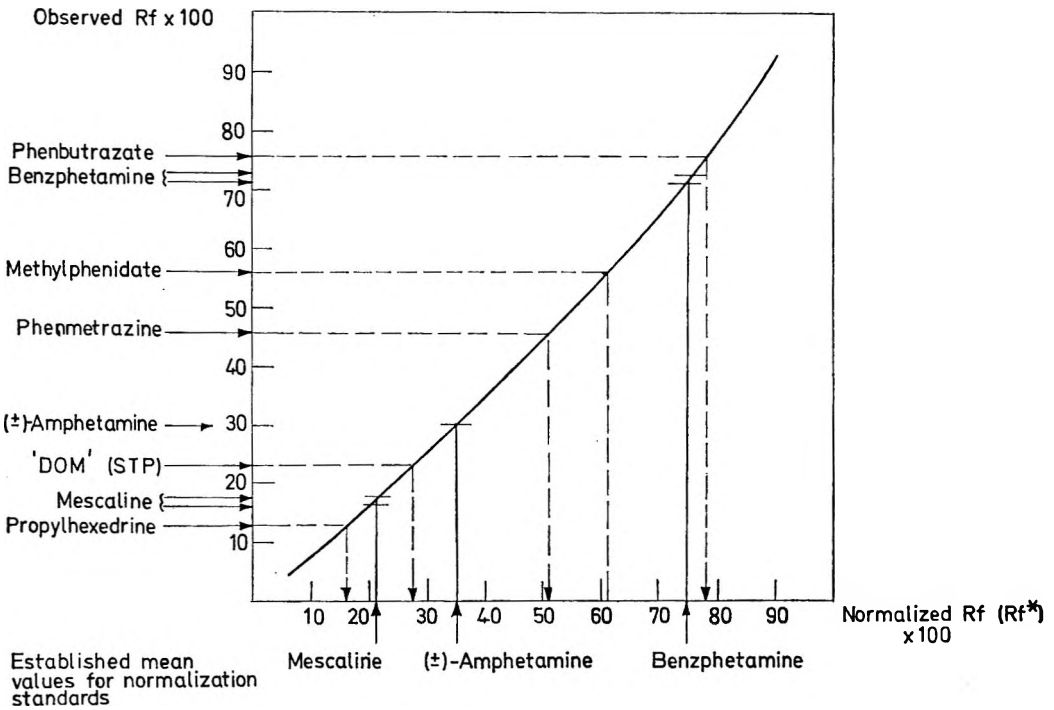

FIG. 1. Illustration of a normalization procedure for a typical plate in the phenethylamine series.

Standard substances selected for this normalization procedure were: (i) for phenethylamines—a blend of mescaline, amphetamine and benzphetamine; (ii) for tryptamines—*N*-methyl-, *NN*-dimethyl- and *NN*-dibutyltryptamine; (iii) for erganes—ergometrine (3 spots) and ergotamine (8β- and 8α-isomers).

PHENETHYLAMINES

Several workers have described the chromatographic resolution of psychotropic drugs in physiological fluids. Although Sunshine (1963) had analysed with a variety of thin-layer systems extracts from blood, urine and human stomach contents containing an extensive series of barbiturates, carbamates, phenothiazines and some narcotics and medicinal alkaloids, he did not examine psychotomimetics and psychotonic amines. Acetone–methanol chromatography on silica plates has been used for the separation of amphetamine, methylamphetamine and ephedrine from the urine of athletes (Beckett, Tucker & Moffat, 1967) and from horse urine (Karawya, El Keiy & others, 1968); for quantitative work the latter group removed zones and estimated the amines colorimetrically. Haywood & Moss (1968)—who also are especially concerned with racehorse "doping"—resolved alkaloids, stimulants and other psychotrophes in extracts from equine urine. They applied the Curry & Powell (1954) citrate buffer technique to thin layers of cellulose powder as well as using their published paper partition method.

Working in the main with the pure drug form, Clarke (1967a) applied convenient paper partition (Curry & Powell, 1954) and thin layer (Sunshine, 1963) chromatographic screening procedures to a number of amphetamines substituted in the aromatic ring. He later extended (Clarke, 1967b) these two techniques to twenty substances

recognized by the Home Office as being subsumed by the generic definition in the 1964 Drugs (Prevention of Misuse) Act. Between these two paper and TLC systems the twenty amines exhibited only moderately different mobilities: about half of each were concentrated within 10% of the respective paper and plate, although only five substances were common to this overlap. In his comprehensive scheme (Clarke & Berle, 1969) for the identification of basic drugs, Clarke recommends a variety of visualization procedures which in practice help to distinguish most amines that show comparable mobility and further assistance may be obtained from classical crystal tests or modern instrumental techniques. Nevertheless, for forensic purposes two distinct thin-layer separations are desirable.

Grant (1968) has recently reported a new solvent mixture, acetonitrile–benzene–ethyl acetate–ammonia for the TLC of extracts of stimulants: he achieved an adequate separation of methylamphetamine and ephedrine (Rf 0·34 and 0·26), but on the other hand phenylpropanolamine—which is well separated with methanol—fell between these two bases. In another recent paper, Genest & Hughes (1968) describe the TLC separation of 2,5-dimethoxy-4-methylamphetamine† from amphetamine, methylamphetamine and the pyschotomimetics mescaline, bufotenine and *NN*-dimethyltryptamine. They employed silica and alumina plates and three different solvent systems.

With chloroform–methanol (1 : 1) on alumina "DOM" is well resolved from the other five substances but in butanone–dimethylformamide–ammonia on silica the discrimination from amphetamine is only 0·04 Rf units, and in chloroform–methanol–acetic acid on silica methylamphetamine and "DOM" coincide.

Genest & Farmilo (1964) devised a system (IIC; see Discussion) to separate erganes from psychotonic amines and narcotics. Attempts in this laboratory to apply this system to the psychotomimetic ingredient isolated from a single tablet described as "STP" were not convincing: Maunder (1967) reported the TLC behaviour of the extracted base to be essentially similar to methoxamine, mescaline and (±)-amphetamine. Provision by the (then) U.S. Bureau of Drug Abuse Control of a reference sample of "DOM"† permitted a detailed examination of the behaviour of 2,5-dimethoxy-4-methylamphetamine in a variety of thin-layer systems. Phillips & Mesley (1969) have already reported the observed overlapping of this substance in relation to a number of α-methylphenethylamine (amphetamine) derivatives in three systems. We now report the investigation of nine systems and describe the application of two of these to thirty-four psychotropic amines of related structure; some are psychotonic (stimulant), others are sympathomimetics and a few psychotomimetic (hallucinogenic).

Results

Nine systems have been investigated; Table 1 shows the mean Rf values obtained in preliminary studies with eight of these systems, using eleven phenethylamine bases or their salts. In each system, the optimum loading was about 2 to 5 μg.

Table 2, most of which has appeared in the Government Chemist's Report for 1968, enumerates the Rf* values established with systems IIA and IB for thirty-four phenethylamine bases prepared as 0·1–0·2% solutions of (usually) a convenient salt in undiluted, or 50% aqueous, methanol or ethanol. Each Rf* value cited represents the mean of at least three runs and has been normalized by our graphical method.

† The Dow Co. experimental product "DOM" and the active ingredient of the illicit pyschotomimetic drug "STP" (Maunder, 1967; Phillips & Mesley, 1969).

Table 1. *Preliminary trials with phenethylamine derivatives*

Chromatographic system:	IIA	IIC	IIE	IIIA	IIIC	IVA	IVD	VIB
Phenylephrine hydrochloride	23	—	08 (43)	—	—	36	10, 13	—
Adrenaline sulphate	14, 70	—	09	—	—	07, 22	21	30, 61
Mescaline sulphate	00, 17, 46	15	—	—	17, 63, 79	—	—	00, 27
Methoxyphenamine hydrochloride	17	11	—	70	66	—	—	30
Ephedrine	22	—	—	—	—	09, 20	04, 31, 35	—
Ephedrine hydrochloride	07	06	—	—	45	—	—	31
Pseudoephedrine	—	—	—	—	—	08	—	—
N-Methylephedrine	—	10	—	—	—	—	—	—
(+)-Methylamphetamine	24	—	—	—	—	08, 35	06	—
(±)-Methylamphetamine	28	13	36	—	—	10, 35	08	—
(±)-Methylamphetamine hydrochloride	—	—	—	—	31	—	—	33
Phentermine	—	15	—	—	—	—	—	—
"STP" extract	21	16, 27[b]	—	65	48, 73	12, 65	—	—
"DOM" hydrochloride	21	17	—	—	—	—	—	39
(+)-Amphetamine	36	17	—	55	—	11	22	—
(±)-Amphetamine	35	19	38	—	—	08	19	—
(±)-Amphetamine hydrochloride	31	—	—	—	—	—	—	41
(±)-Amphetamine sulphate	31	—	—	50	69	—	—	—
Methoxamine hydrochloride	—	07, 40[b]	—	69	50, 74, 82	—	—	41
Benzphetamine hydrochloride	73	80	75	—	—	64	(11), 27	81

Observed mean Rf values (×100) normalized to (+)-amphetamine marker only.
Visualization in all systems by method "c" except additional spots (b) revealed by method "b" (see p. 794).
For key to support, solvent and visualization code, refer to Experimental section.

Table 2. *Rf* values* × 100 *for phenethylamine derivatives*

		Control status	System IB	System IIA	Visualization c
1	Oxethazaine	S4B	05; (15)	02; (14)	g–p30
2	Propylhexedrine hydrochloride	PX	16	09; (11)	g–n5
3	Mephentermine sulphate	DPM	18	17	g–n5
4	Adrenaline sulphate	PX	21	21	y–yy30
5	Mescaline sulphate	DPM	21	13	g–n5
6	Methoxyphenamine hydrochloride	PX	21	16	g–p30
7	Ephedrine hydrochloride	PX	25	19	g–o30
8	Pseudoephedrine hydrochloride	PX	27	19	g–o30
9	Methylamphetamine hydrochloride	DPM	27	19	g–p5
10	2,5-Dimethoxy-4-methylamphetamine hydrochloride	S4B	28	19	g–p5
11	3-Methoxy-4,5-methylenedioxyamphetamine hydrochloride	S4B	29	23	g–p5
12	*N*-Methylephedrine hydrochloride	PX	30	28	g–y30
13	Phentermine hydrochloride	DPM	32	29	g–y5
14	3,4-Methylenedioxyamphetamine	S4B	33	24	g–p5
15	Methoxamine hydrochloride	(none)	34	28	g–y30
16	Dexamphetamine sulphate	DPM	33	28	g–p5
17	Levamphetamine sulphate	DPM	34	28	g–p5
18	(±)-Amphetamine hydrochloride	DPM	35	29	g–p5
19	Isoprenaline sulphate	PX	35	34; (48)	y–yy30
20	Chlorphentermine hydrochloride	DPM	38	(13); 34	g–y5
21	Phenylpropanolamine hydrochloride	PX	40	33	g–y5
22	Prolintane hydrochloride	DPM	43	42	g–o30
23	Fenfluramine hydrochloride	S4B	46	38	g–n5
24	Pipradrol hydrochloride	DPM	47	45	g–p30
25	Phenmetrazine theoclate	DPM	49	47	g–p5
26	Fencamfamin hydrochloride	DPM	(38); 51	(35); 50	g–p5
27	Fenethylline hydrochloride	DPM	59	53	g–o30
28	Methylphenidate hydrochloride	DPM	61	57	g–p5
29	Tranylcypromine hydrochloride	DPM	62	64	y–yy30
30	Doxapram hydrochloride	DPM	70	69	g–p30
31	Diethylpropion hydrochloride	DPM	73	73	g–y30
32	Benzphetamine hydrochloride	DPM	75	73	g–o30
33	Phenbutrazate hydrochloride	DPM	77	(66); 77	g–o30
34	Famprofazone	DPM	80	80	g–o30

For details of thin-layer chromatographic system see Experimental section.
Values for minor spots in parentheses.
DPM = Drugs (Prevention of Misuse) Act 1964.
S4B = Schedule 4B of Poisons (No. 2) Rules 1968.
PX = Part I, Poisons List (No. 2) 1968 but full or qualified exemption from S4B.

The visualization procedure "c"† easily reveals 1 μg of the amines cited in Table 2; the lower detection limit has not been fully investigated but is less than 0·5 μg for mescaline, (±)-amphetamine and benzphetamine.

Discussion

Preliminary investigations

With both methanol and acetone on the acid plate, IV, (bisulphate impregnated: Fike, 1966), amine bases not unexpectedly showed limited mobility. Moreover, a considerable proportion of salts or amines remained at the start when run in neutral conditions. More surprisingly, limited mobility was also found to apply to the basic plate, using chloroform–methanol (9:1) (IIC) (Genest & Farmilo, 1964). Better mobility was found with the solvent mixture E (Fike, 1966) but this did not appear to separate amphetamine from methylamphetamine. Experiments with methanol and chloroform–methanol (9:1) on a more strongly basic plate, III, showed a greatly enhanced mobility but poor separation of the bases examined. Methanol as solvent on the less basic plate, II, appeared to us to be the most useful system, IIA, to compare with the Sunshine (1963) system, IB, for which Clarke's results (1967b) with some similar bases were encouraging.

The frequent use in this and other laboratories (e.g., Schweda, 1967) of silica precoated polyester sheets, which have the advantage of permitting reduced sample loading (Maunder, 1969) as well as minimal preparation time, flexibility, durability and permanent record, prompted an investigation of an application to the chromatography of psychotropic drugs. However, with a number of amines, the advantage of the appreciably higher Rf values with ammoniacal methanol on Eastman "Chromagram" 6061 sheets was offset by poorer visualization: even on sheets incorporating fluorescent indicator ("Chromagram" 6060) there was little or no response to ultraviolet illumination and the iodine spray gave imperfectly resolved spots on a murky background.

Optimum systems

The differential resolution that is possible by use of the systems IB and IIA is apparent from Table 2. Especial interest attaches to the separation in system IIA of mescaline (compound 5) and methoxyphenamine (6) from adrenaline (4); of the hallucinogenic pair MMDA (11) from DOM‡ (10); of MDA (14) from dexamphetamine (16); of isoprenaline (19) from (±)-amphetamine (18); of the stimulant pair methylphenidate (28) from tranylcypromine (29); and the anoretic fenfluramine (23) from the stimulant pipradrol (24)—all of which show groups of overlapping mobilities in system IB. The converse is true for separation on IB of certain conjunctions not resolved on IIA, e.g., mephentermine (3) from methoxyphenamine (6); the sympathomimetic drugs *N*-methylephedrine (12) from methoxamine (15) and isoprenaline (19) from phenylpropanolamine (21); and MDA (14) from MMDA (11)—this last pair being hallucinogens bearing mescaline-like aromatic substitution in an amphetamine structure. Preliminary identification based upon these relatively slight differences in mobility is assisted by distinct nuances in the colour reaction with methanolic iodine spray on system IB: the observed sequences have been simplified in the code itemized in Table 2. It is of interest that the colour development can be arrested

† For visualization code, see Experimental section (p. 794).
‡ See footnote, p. 796.

by superimposing a second plate on the top of the silica gel layer. From the colour sequence it may be possible to suspect one component of a pair not resolved on either system. In particular it should be noted that methylamphetamine (9) may thereby be distinguished from ephedrine (7) and pseudo-ephedrine (8), dexamphetamine (16) and levamphetamine (17) from methoxamine (15), and pipradrol (24) from phenmetrazine (25).

The discrepancies between Rf* values we have observed in system IB for nine amines and those reported by Clarke (1967a) have been investigated. The principal cause appears to lie with the dissolution solvent chosen: while all our substances have been dissolved in aqueous or anhydrous alcohols, Clarke preferred to use 2 N acetic acid. Table 3 shows the spots resolved from both solvents and compared with Clarke's results; we have used both methanolic iodine visualization (c) (which we prefer), and the iodoplatinate reagent (d) used by Clarke. In our experience spots obtained from the acetic solutions exhibited considerable tailing, complicating the assignment of meaningful Rf values, as well as generally exhibiting higher mobility. Moreover, the iodoplatinate seemed a less sensitive reagent for these particular substances and did not always visualize the same spots as did the iodine spray. The van Urk reagent (visualization "e") is also less sensitive than "c".

Table 3. *Effect of dissolution solvent and visualization on mobility on system IB. Rf values × 100 for nine phenethylamines*

Solution in:	ethanol (0·1%)		2 N acetic acid (1%)		Clarke†
Visualization:	c	d	c	d	d
Mephentermine	18	(15)	(40); 50^t	40^t; 62	24
Amphetamine	35	—	44^t; 60	45^t; 63	48
Prolintane	43	31	50^t; (68^t)	48^t; (57)	48
"DOM"	28	—	63^t	53^t; 65^t	51
Chlorphentermine ..	38	—	(58); 66	50^t; (60)	54
Phentermine	32	—	58	(45^t); 59; (64)	56
Fencamfamin	(38); 51	(35)	70^t	59	60
Pipradrol	47	(32)	79	59	61
Benzphetamine	75	76	—	—	73

† Reference: Clarke (1967a), except "DOM" in Clarke & Berle (1969), p. 537.
t = tailing; relatively weak spots are enclosed in parentheses.

TRYPTAMINES

Martin & Alexander (1968) reported a variety of spectrometric and chromatographic procedures for suspected hallucinogens and related drugs. They described the separation of bicarbonate–chloroform extracts of *NN*-dimethyl- and diethyltryptamine (DMT, DET) on silica plates with aqueous ammoniacal ethanol (3 : 1 : 4), visualizing with formalin in aqueous ethanol and hydrochloric acid. Clarke (1967a) used a paper and a thin-layer system to examine eleven tryptamines, including both naturally occurring and specially synthesized hydroxylated derivatives of DMT which are controlled by the Drugs (Prevention of Misuse) Act, and—for comparison—the natural brain substance serotonin (5-hydroxytryptamine) which is presumed to be antagonized in psychotomimetic reactions. He reported a moderately good separation of these substances by the Curry & Powell (1954) paper partition method, but found bunching of equivalent mobilities using ammoniacal methanol on silica thin layer (Sunshine, 1963).

We have investigated five TLC systems with twenty tryptamine derivatives, most of which have been shown to exhibit psychotomimetic activity.

Results

The Rf* values enumerated in Table 4 were obtained on five systems with tryptamine and a variety of derivatives of the corresponding primary, secondary and tertiary amines. Data for systems IB and VIB are the mean of at least three runs; they have all been normalized to standard values for DMT and its monomethyl and di-n-butyl analogues. The data for IIC and IIA refer to less than three runs for some compounds. The optimum loading was 2 to 5 μg of salts from 0·2% ethanol solutions; 1 μg was easily detectable using visualization "c", and also with "a" on "Chromagram" sheets, but higher loadings were necessary for "a" on plates.

Discussion

The system IIC, which proved so successful in the ergane series (see the third part of this paper), provided very limited mobility for seventeen of the twenty tryptamines examined. When visualized in 254 nm ultraviolet illumination the basic plate seemed to minimize the already limited fluorescence of the tryptamines but spray "c" gave satisfactory visualization. Following our experience with phenethylamines (first part of this paper), we tried the more polar solvent methanol on the basic plate (i.e., system IIA) and found that generally mobility was increased but bufotenine (5-hydroxy-DMT) was no longer separated from 7-methyltryptamine. Employment of the Sunshine (1963) system, IB, further increased the mobilities (maximum Rf* 0·66 for *N*-dibutyltryptamine) but was without significant alteration of the sequence of bases. Clarke (1967a) used this system for his tryptamine series; for those five substances common to our investigation the Rf values are in substantial agreement. Fig. 2 illustrates the relative mobility in our three thin layer plate systems and with the polyester sheet equivalent (VIB) of IB. For the plates it is evident that IB is the system of first choice but that in some instances discrimination is possible from a second chromatogram using the system IIC.

Distinction between tertiary, and primary and secondary, bases is also possible by visualization "c"; the tertiary bases gave an orange colour which faded through yellow to a permanent but very faint yellow, whereas the primary and secondary bases gave an initial pale yellow which darkened and then slowly faded to a permanent fawn colour. This behaviour should be contrasted with the grey changing to orange or yellow and fading within 30 min, that we have reported for phenethylamine derivatives. With the ultraviolet 254 nm visualization the hydroxylated tryptamines (5-HT and bufotenine) gave a pinkish fluorescence contrasting with the dark purple of the other tryptamines.

On polyester sheets the tryptamines exhibited enhanced mobility (see Table 4) and all the main spots could be resolved by visualization "c" on "Chromagram" 6061 sheets. On sheets "6060" these tryptamines fluoresced (254 nm) strongly, appearing as blue or purple spots clearly distinguished from the orange background. The Rf values on the two types of sheet were essentially the same. Considering the speed and simplicity of the operation, this medium with ammoniacal methanol solvent and 254 nm visualization (i.e., system VIB a) is recommended for the forensic identification of tryptamine drugs. Mobility is compared with the three plate systems in Fig. 2.

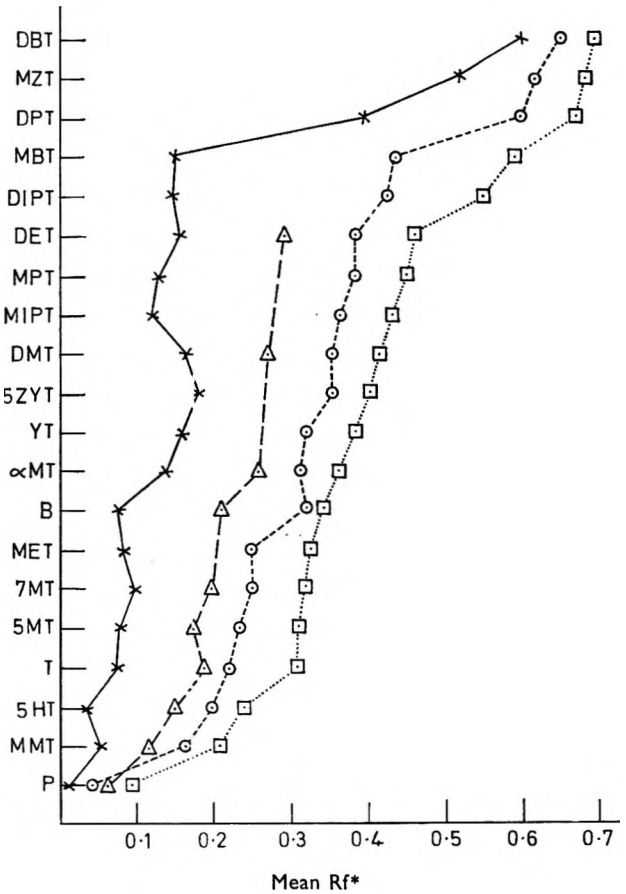

FIG. 2. Relative mobility of tryptamine derivatives in four TLC systems: ×, II C; △, II A; ○, IB and □, VIB. For system code refer to experimental section. Full names of the compounds are given in Table 4.

The series of tryptamine derivatives is sufficiently extensive to permit deduction of a limited correlation between structure and mobility. Two homologous series may be distinguished. For the tertiary amines mobility increases smoothly through dimethyl, diethyl and di-isopropyl tryptamines and then somewhat faster through the di-n-propyl and di-n-butyl derivatives. The pyrrolidino analogue is slower than its uncyclized equivalent, diethyltryptamine. With the secondary (i.e., monoalkyl) tryptamines there is a regular and (except for the propyl isomers) well resolved, series: methyl, ethyl, isopropyl, n-propyl, n-butyl, benzyl.

Ring substitution has less effect. Introduction of a 5-hydroxyl group marginally reduces the mobility of tryptamine and DMT; Clarke's (1967a) data also show this slight difference. It may be attributable to a reduction in the basicity of the indole nitrogen and/or participation of a semiquinone mesomer. On the other hand, substitution of a methyl group at positions 5 or 7 slightly increases the mobility. In the single instance of side chain substitution, an α-methyl appears to have somewhat more effect: thus, α-methyltryptamine (the indolyl analogue of amphetamine) runs ahead of its 5- and 7-isomers. The variation of mobility within the two homologous series and with ring or chain substitution is illustrated in Fig. 3.

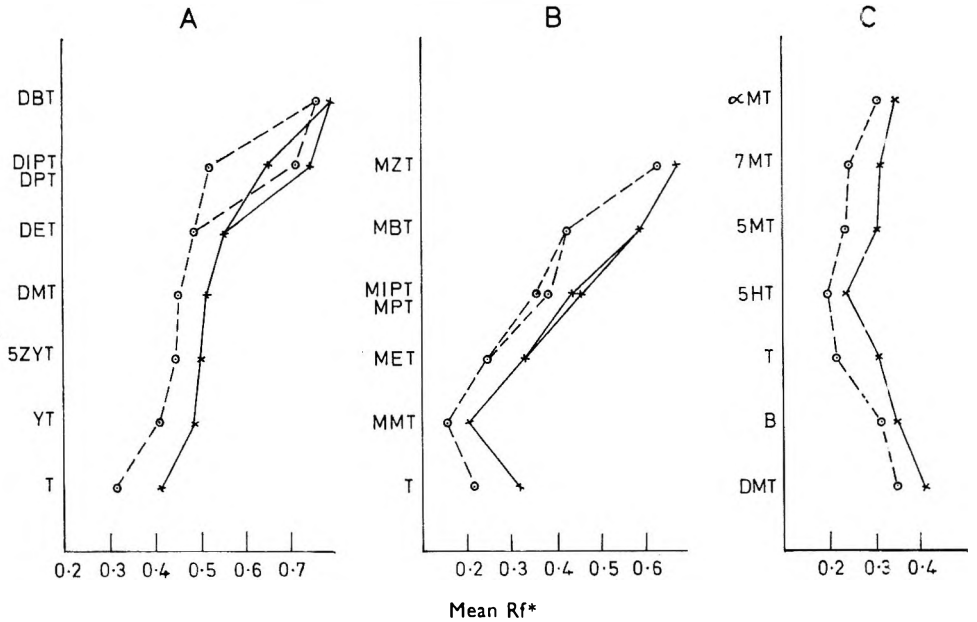

FIG. 3. Correlation of structure with mobility for tryptamine derivatives. A, tertiary amine series; B, secondary amine series; C, ring and side-chain substituents; ○ refers to system IB and × to system VIB. Full names of the bases are given in Table 4.

Table 4. *Rf* values* × 100 *for tryptamine derivatives in four TLC systems*

	Code	Psychotomimicry	IB	VB/VIB	IIA	IIC
Primary amines						
Tryptamine	T	− (i)	22	31	19	07
Serotonin (5-hydroxytryptamine)	5HT	− (i)	20	24	15	03
5-Methyltryptamine	5MT	− ? (iv)	23	31	18	08
7-Methyltryptamine	7MT	− ? (iv)	25	32	20	10
α-Methyltryptamine	αMT	+ (i)	31	36	26	14
Secondary amines						
N-Methyltryptamine	MMT	− ? (iv)	16	21	12	05
N-Ethyltryptamine	MET	+ (ii)	25	33	—	08
N-Isopropyltryptamine	MIPT	+ (ii)	36	43	—	12
N-Propyltryptamine	MPT	+ (ii)	38	45	—	13
N-Butyltryptamine	MBT	+ (ii)	43	59	—	16
N-Benzyltryptamine	MZT	+ (ii)	62	67	—	52
Tertiary amines						
Psilocybin	P	+ (i)	04	09	—	01
Bufotenine (5-hydroxy-DMT)	B	+ (i)	32	35	21	07
3-(2-Pyrrolidinoethyl)indole	YT	+ (iii)	32	38	—	16
5-Benzyloxy-3-(2-pyrrolidinoethyl)-indole	5ZYT	+ (iii)	35	40	—	18
NN-Dimethyltryptamine	DMT	+ (i)	35	41	27	16
NN-Diethyltryptamine	DET	+ (i)	38	46	29	16
NN-Diisopropyltryptamine	DIPT	+ (ii)	42	56	—	15
NN-Dipropyltryptamine	DPT	+ (i)	60	65	—	39
NN-Dibutyltryptamine	DBT	+ (i)	66	69	—	60

References: (i) Downing, 1964 (Review); (ii) Brimblecome & others, 1964; (iii) Hunt & Brimblecombe, 1967; (iv) R. W. Brimblecombe (unpublished work).
For system codes refer to Experimental section.

The results cited in Table 4 refer to authentic samples obtained either direct from the manufacturer or by the courtesy of other official laboratories. Examination by these systems of seizure samples of DMT (not at the moment a prohibited drug in the U.K.) and psilocybin have normally indicated a homogeneous product, implying commercial origin; but at least one DMT sample of U.S. origin contained secondary spots (Rf 0·02, 0·07) suggesting a probable illicit synthesis. Solutions of DMT and MMT have not produced additional spots on storage, but after 9 months in the dark at laboratory temperature psilocybin and bufotenine solutions each developed a relatively immobile (Rf 0·03 and 0·01 respectively) secondary spot.

ERGANES

According to Lerner (1967), Sandoz Pharmaceuticals recommended the solvent mixture dichloromethane–methanol (93 : 7) for the thin-layer chromatography of lysergide (LSD) (Rf value on silica 0·6), but the U.S. Food & Drug Administration (FDA) preferred chloroform-acetone (1 : 4) (Rf 0·4–0·5). Martin & Alexander (1967) subsequently reported that the FDA use acetone mobile phase for the separation of LSD, lysergamide (LAA) and lysergic acid seized from clandestine laboratories. Genest & Farmilo (1964) devized a different system to separate LSD and LAA from a number of ergot alkaloids and their various 8α-isomers, as well as from amphetamine, methylamphetamine and six narcotics. They used a basic plate (silica impregnated with 0·1 N sodium hydroxide) developed with chloroform–methanol (9 : 1); spots—not always the same ones—were visualized with dimethylaminobenzaldehyde spray and in ultraviolet light. Satisfactory resolution of a larger number of erganes was described by Clarke (1967a), who employed both the Genest & Farmilo (1964) thin-layer system and a modification of the Curry & Powell (1954) ascending buffered paper partition method. Clarke included methysergide, a potential hallucinogen arguably subsumed (Phillips, 1967) by the Drugs (Prevention of Misuse) Act, and methylergometrine—a synthetic homologue of the natural oxytocic ergometrine.

Our preliminary results with the Genest & Farmilo (1964) system have been reported (Government Chemist, 1968) and revealed satisfactory agreement with other published Rf values (Genest & Farmilo, 1964; French & Wehrli, 1965; Clarke, 1967a). We now publish fuller details of studies undertaken with authentic and seizure samples, comment on visualization techniques and secondary spots, and describe a convenient adaptation of the Genest & Farmilo (1964) conditions to commercially available silica impregnated polyester ("Chromagram") sheets as a rapid screening procedure for suspected erganes.

Results

Table 5 contrasts mean Rf* values for ten erganes and some of their 8α-isomers in three systems: methanol and ammoniacal methanol on neutral plates (systems IA and IB) and chloroform–methanol (9 : 1) on an alkaline plate (system IIC). For IB and IIC each value is the mean (±0·02) of at least nine runs (except 8β- and 8α-lysergamide, 8α-ergometrine and dihydroergotamine) normalized to values standardized for the three spots observed with commercial 8β-ergometrine (averaged from seventeen runs) and the two spots for 8β- and 8α-ergotamine (mean of fourteen runs). The erganes were applied as 2–10 μg of the salt from 0·1% solutions in aqueous methanol (1 : 1) or aqueous ethanol (7 : 3). In 254 nm illumination each main spot

Table 5. *Rf* values* × 100 *for erganes; subsidiary (impurity) spots in parentheses*

Chromatographic system:	Prepared plates			Precoated polyester sheets				
Visualization:	IA a,c	IB a,b,c	IIC a,b,c,e,f	VIA a	VIC a	VIB a	VB a	VIIC a,c
Lysergic acid	—	78 (15, 62)	02 (33†, 66†)	—	—	65	64	02 (22†, 42†, 72†)
8β-Lysergamide	60 (75)	—	24 (32, 55‡)	—	—	—	—	24 (30, 37, 51‡)
8β-Ergometrine maleate	63 (74)	74 (58)	25 (16, 31, 48‡)	76	36	69	70	25 (33, 40‡, 49)
Methylergometrine bimaleate	—	—	30 (37†, 42†, 55†)	—	—	—	—	31 (20†, 40†, 46†)
8α-Lysergamide	—	—	55 (08, 19, 24‡, 30†, 40)	—	—	—	—	53 (25‡, 41, 66)
8α-Lysergide‡§	27	62	42	—	44	—	—	34
8α-Ergometrine	—	—	48 (09, 20, 25‡, 35, 55)	—	—	—	—	39 (11, 20, 31, 67)
Methysergide bimaleate	—	75 (58)	47 (13†, 24, 31†, 35†, 54†, 62†, 67†)	—	—	74	73	51 (19†, 29†, 40†, 63†, 70†)
8β-Ergotamine tartrate	65 (73‡)	—	58 (30†, 42†, 48†, 61†, 66†, 76‡)	84	68	—	—	58 (37, 67, 76‡)
Dihydroergotamine	57 (65)	72	57 (17†, 25†, 35‡)	—	—	—	—	50 (19†, 27†, 34‡)
8β-Lysergide tartrate	61 (74)	71	63 (33†, 42‡, 71, 76†)	79	75	70	71	60 (34‡, 73)
1-Acetyl-lysergide	58	—	72 (45†, 49, 61, 77†, 85)	—	—	—	—	74 (58, 76, 82)
"Ergotoxine" ethanosulphate (8β-ergocristine)	73	—	76 (24†, 80†)	—	81	—	—	70
8α-Ergotamine tartrate‡§	73	—	76	—	—	—	—	76

† Unidentified spots observed after solution had been allowed to stand in the dark for several weeks.
‡ Spot weak or absent in freshly prepared solution and attributed to 8-epimer.
§ Values for these compounds are inferred from results for the 8-epimer.

was visualized by its bright blue fluorescence except dihydroergotamine, which had almost no fluorescence, and 1-acetyl-lysergide (ALD) which had bright green fluorescence; secondary spots from freshly prepared solutions, or those appearing in solutions that had been allowed to stand in the dark for several weeks, usually exhibited different fluorescent colours, but spots corresponding to 8-epimers gave the characteristic lysergic blue colour. On systems IA and IB loadings of 2–5 μg of the specified erganes gave, with spray "c", slowly fading yellow spots and significant tailing. In system IIC each main spot appeared purple to visualizations "e" and "f" and a slowly darkening yellow with "c", but few subsidiary spots could be visualized with these sprays.

Rf* values obtained with precoated polyester sheets are also summarized in Table 5. To simulate the basic plate of system II, 2 μl 0·1 N sodium hydroxide was pipetted on to each origin spot and the sheets dried before applying 5–10 μg of seven erganes.

Discussion

Ammoniacal methanol had been shown by Sunshine (1963) to be eminently suitable for the resolution of phenothiazines and alkaloids. However, our preliminary investigation indicated that neither this solvent nor methanol alone adequately separated a number of erganes, including lysergic acid itself, although all ran well ahead of tryptamines and most phenethylamines. Moreover, assignment of Rf values was obscured by considerable tailing. The principal application would therefore be an initial separation where these latter classes of psychotropic drugs were suspected of being mixed with, say, lysergide. It would appear that the system IIC is the one of choice for resolution of erganes on thin-layer plates.

Results with precoated polyester sheets confirmed our experience with silica gel on glass plates, namely that methanol (solvent A) and ammoniacal methanol (solvent B) gave poor resolution of substances that appeared as blue primary, but

suppressed secondary, spots with 254 nm illumination, and as blotchy badly resolved yellow areas with visualization "c". The sequence obtained on alkali-treated sheets (VII) with solvent C was similar to that with the plate system IIC but the observed Rf values were somewhat lower, with an appreciable proportion of the sample immobile. Although VII is not strictly comparable with the uniformly impregnated silica plates II, when adjusted to the previously well established mean values for ergometrine–ergotamine in system IIC the other principal spots showed good correlation but there was less satisfactory agreement for the subsidiary ones (see Table 5). The "Chromagram" 6061 sheets (without fluorescence indicator) are preferred because very few of the subsidiary spots could be detected by 254 nm visualization on the "6060" (with F.I.) sheets. The van Urk spray (visualization "e") gave, on warming, purple brown principal spots—except methysergide and 1-acetyl-lysergide, for which the faint grey brown colour may tentatively be attributed to substitution at the indole nitrogen modifying aromatic conjugation with the dimethylaminobenzaldehyde adduct.

Taking advantage of the sensitivity of system IIC for subsidiary spots, a special investigation of the homogeneity of lysergide samples was undertaken. From the results (see Table 6) it is clear that it may be possible, for a new seizure, to distinguish between recently diverted licitly manufactured material, and lysergide that has either been crudely synthesized or has been stored under adverse conditions. Subdivision of the latter category would depend upon detailed investigation of the likely by-products of various synthetic routes and breakdown mechanisms; this study has not yet been undertaken.

Table 6. *Multiplicity of spots observed in lysergide from various sources. Rf* values* $\times$ 100 *in system IIC; colour given by visualization "a"* (see p. 794)

Origin of sample		Main spot	8α-isomer	Subsidiary spots estimated less than 1% in fresh solutions		
Sandoz manufacture		63; blue	40†; faint blue (5%)	70†; faint green	76; orange	—
1966	Official synthesis	63; blue	40†; faint (1%)	71; green	76†; orange	30†; v. faint
1966	Illicit synthesis	64; blue	42; faint blue (5%)	72; green	76†; orange	28†; v. faint
1968	Stained blotting paper	63; blue	45; blue (20%)	71; faint	75; v. faint	—
1967	Seized capsules	62; blue	42; blue (30%)	69; v. faint	73; v. faint	—
1969	Seized powder	64; blue	41; blue (50%)	71; v. faint	75; v. faint	—

† Spots observed only after solution had been standing for several weeks in the dark. Percentages refer to estimated proportion of 8α-lysergide.

In addition it may be possible tentatively to identify observed secondary spots in some other erganes. Thus, the impurities in ALD with Rf* 0·61 and 0·45 are probably 8β- and 8α-lysergide; and for pharmacopoeial purposes, conformers of lysergamide and ergometrine may be detected in ergometrine and methylergometrine.

The possibility that use of the basic plate (II) for chromatography of erganes might facilitate unintentional isomerization to 8α-conformers was considered.

Table 7. *Effect of basic plate on 8β-lysergide conformation. Uncorrected Rf values* × 100 *in methanol*

		Neutral plate (I)					Basic plate (II)				
Fluorescence colour:		orange	blue†	orange	blue	orange	orange	blue†	orange	blue	orange
Official synthesis	fresh solution	—	57	—	—	—	—	67	—	—	—
	old solution	70	57	40	—	27	77	66	59	—	47
Illicit synthesis	fresh solution	—	57	42	29	—	—	68	—	54	—
	old solution	71	58-(tail)-		29	—	77	68	—	52	—

† Major spot corresponding to 8β-lysergide.

Experiments with two samples of lysergide, one substantially free of the 8α form and one containing about 5%, are summarized in Table 7. Applications of 5 μl of methanolic solutions to neutral (I) and basic (II) plates were developed with methanol and the spots visualized in 254 nm illumination. Under conditions in which secondary spots arising in old solutions can be readily detected, there was no significant difference in the distribution of spots between the neutral and basic plates.

CONCLUSION

For reliable identification it is essential to run simultaneously a reference substance of well established mobility. Graphical normalization from multiple spot markers enables consistent correlation with previous data. When only a small amount of salt is available, a technique avoiding extraction of the free base is an advantage. For the resolution of *phenethylamine* drugs we recommend two separate chromatographic examinations of alcoholic solutions of the free bases or their salts, using ammoniacal methanol on silica plates and methanol on 0·1 N sodium hydroxide impregnated silica, visualizing spots in both systems with methanolic iodine spray. A blend of mescaline, amphetamine and benzphetamine is a convenient reference mixture; if mescaline is not available methoxyphenamine is an appropriate substitute.

For the preliminary sorting of tryptamines and erganes, silica coated polyester sheets (such as Eastman "Chromagram") provide a convenient, rapid and durable medium. The preferred solvent for *tryptamines* is ammoniacal methanol and for *erganes* we recommend chloroform–methanol (9 : 1) development after dosing the applied spots with 2 μl of 0·1 N sodium hydroxide solution.

It is desirable to repeat the chromatography (for the erganes preferably on a glass plate) concurrently with a known sample of the provisionally identified drug. Ultimate confirmation by chemical and spectrometric procedures will, of course, depend upon the particular substance suspected.

Acknowledgements

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Identification of a urinary metabolite of perazine as a piperazine-2,5-dione derivative

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A non-basic perazine metabolite has been isolated from the urine of schizophrenic patients ingesting perazine. Identification of this compound as 10-[3′-(2″,5″-dioxo-4″-methyl-piperazinyl)-propyl]-phenothiazine sulphoxide was achieved using ultraviolet, infrared, nuclear magnetic resonance and mass spectroscopy.

In a previous paper, Breyer (1969) has described the isolation and identification of those perazine (Taxilan) metabolites that result from demethylation, sulphoxidation, *N*-oxidation and aromatic hydroxylation followed by glucuronide conjugation of the molecule. A further metabolic product, referred to as substance VIII, which seemed to possess an altered piperazine ring, was also regularly detected and isolated (Breyer, 1969; Kanig & Breyer, 1969). This paper gives details of the isolation of substance VIII on a milligram scale and of the elucidation of its structure by chemical and physico-chemical methods.

EXPERIMENTAL

Urine was collected from patients receiving orally 300–600 mg of perazine daily.

Chemical reactions were as described by Breyer (1969). Alkaline hydrolysis was achieved by heating with 0·1 N NaOH/tetrahydrofuran (1 : 1, v/v) to 100° for 30 min.

Isolation of substance VIII. Urine (1 litre) was adjusted to pH 2 by addition of 5 N HCl and extracted with successive portions of 200 and 100 ml of 1,2-dichloroethane. The combined organic layers were washed with 30 ml of N ammonia and evaporated under reduced pressure at 40°. The dark brown residue was subjected to thin-layer chromatography (TLC) on a 400 × 200 mm silica gel H (Merck) plate with dichloroethane–ethyl acetate–ethanol–acetic acid–water (30 : 28 : 8·5 : 8·5 : 5 by volume) as developing solvent. After the solvent had been allowed to rise twice to a height of 23 cm above the application line, the position of substance VIII was determined using a reference strip which was sprayed with concentrated HCl. The band containing compound VIII was removed and treated as described for the other metabolites (Breyer, 1969). The isolated organic material was further purified by TLC in isopropanol–chloroform–water–25% ammonia (35 : 30 : 4 : 1 by volume) on 200 × 200 mm silica gel G (Merck) plates. Extraction residues from several chromatograms were combined, dissolved in ethanol and centrifuged at 3000 rev/min for 15 min, in order to remove traces of silica gel. The solution was evaporated under a stream of nitrogen and the oily residue obtained was warmed with benzene, whereupon it crystallized. Recrystallization from methanol resulted in nearly colourless crystals

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which melted at 255°. The yield was about 1 mg from 1 litre of urine. The material proved to be homogeneous upon TLC in 5 different solvent systems (Breyer, 1969). Elementary analysis*: C, 62·5; H, 5·5; N, 11·0%

Ultraviolet spectroscopy. Small amounts of substance VIII were dissolved in 0·1 N HCl, the solution extracted with dichloroethane–methanol (1 : 1 v/v) and the material obtained by evaporation of the solvent. The spectrum was obtained using a Zeiss PMQ II spectrophotometer from 220 to 380 nm.

Infrared spectroscopy. Spectra were recorded with a Perkin-Elmer model 621 grating spectrophotometer. The sample of substance VIII was prepared with KI (1 mg in 200 mg KI). The spectrum was compared with the spectra of perazine and perazine sulphoxide which were recorded similarly.

Nuclear magnetic resonance spectroscopy. A Varian HA-100 spectrometer was used. Substances were dissolved in $CDCl_3$–CD_3OD (1 : 1 v/v), and tetramethylsilane served as internal reference compound.

Mass spectroscopy. The spectrum was obtained with a Varian MAT SM 1 mass spectrometer at 70 eV, using the direct oven inlet system H heated to 220°; the source temperature was 250°. Exact mass measurements were made using a resolution of 15000.

RESULTS AND CONCLUSIONS

Chemical properties. Substance VIII is extracted into dichloroethane from acidic (pH 2) as well as from alkaline (pH 11) aqueous solutions. In pure form it is slightly soluble in dichloroethane, chloroform and alcohols and easily soluble in a chloroform–methanol mixture (1 : 1 v/v). It fails to react with sulphur dioxide (no *N*-oxide), methyl iodide, acetic anhydride–pyridine (no N–H or O–H), diazotized sulphanilic acid (no aromatic hydroxyl) or hydrogen peroxide (no sulphide or tertiary amine), but zinc–HCl produces a substance with higher Rf values in TLC with basic solvent systems. With NaOH in tetrahydrofuran compound VIII is decomposed to a more polar product, judging from the behaviour in TLC.

Ultraviolet spectrum. This closely resembles that of perazine sulphoxide and the other sulphoxides described by Breyer (1969), but absorption maxima and minima are slightly shifted. (For compound VIII, λ_{max} 228, 272, 301, 344, λ_{min} 256, 285, 317; for perazine sulphoxide λ_{max} 233, 271, 298, 341, λ_{min} 256, 283, 314 nm, for 5×10^{-5} M solutions in 0·1 N HCl). The fluorescence properties of compound VIII and the colour reaction given upon spraying TLC plates with concentrated HCl are also consistent with the sulphoxidic character, while the reaction product formed with zinc–HCl behaves like a sulphide in fluorescence and HCL staining.

Infrared spectrum. That the aromatic system of the parent compound perazine was preserved is shown in the spectrum of VIII by the presence of ν(CH) bands at 3115, 3070 and 3015 cm^{-1}, strong γ(CH) bands at 772 and 756 cm^{-1}, as well as bands at 1610, 1586 and 1572 cm^{-1}. The latter are typical of aromatic rings. In contrast to perazine, the spectra of perazine sulphoxide and VIII show strong bands at 1015 and 1030 $cm,^{-1}$ respectively. These bands and the absence of a ν(OH) vibration at short wave lengths indicate the presence in VIII of a S=O group. A conspicuous difference between the spectrum of VIII and those of perazine and perazine sulphoxide is the presence of two strong bands at 1681 and 1668 cm^{-1} in VIII. These bands stem from ν(C=O) vibrations. The long wavelength is characteristic of the amide group. The

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absence of short wave ν(NH) and amide II absorptions near 1550 cm^{-1} indicate that C_2NCO groups are probably present. The intensity of the aliphatic ν(CH) bands is much less with VIII than with perazine or perazine sulphoxide. Further, the strong long wave ν(CH) bands which are present in the comparison spectra are missing in the spectrum of VIII. These bands are characteristic of CH_2 groups in amines. Their absence can be seen as an indication of C=O groups in the piperazine ring.

Nuclear magnetic resonance spectrum. This is indicative of the presence of two chemically equivalent *ortho*-disubstituted phenyl rings: there is a quartet at 2·06 τ (2 H) coupled to a multiplet at 2·72 τ (2 H) with about 7·5 Hz and to a multiplet at 2·4 τ (4 H) with about 1·5 Hz. The multiplet at 2·72 τ is coupled with about 6·4 Hz and with ~1·9 Hz to the multiplet at ~2·4 τ. The trimethylene fragment gives rise to two triplets at 5·56 τ (2 H) and 6·46 τ (2 H) coupled with 7·0 and 6·8 Hz, respectively, to a multiplet at 7·75 τ (2 H). Further there are a singlet at 6·56 τ indicative of isolated CH_2 groups and another singlet at 6·98 τ which probably belongs to a N-CH_3 group. The chemical shift of this latter signal is more consistent with an amide N-CH_3 than with one belonging to a trialkylamine. The N-CH_3 of sarcosine anhydride (2,5-dioxo-1,4-dimethylpiperazine) which was measured for a comparison forms a singlet at 7·04 τ.

Mass spectrum. This confirmed that the metabolite must be an oxidation product. The molecular ion was found at m/e 383 with an accurate mass of 383·1281 corresponding to an elemental composition of $C_{20}H_{21}N_3O_3S$ (calculated: 383·1300). Loss of oxygen, OH and H_2O from the molecular ion is in agreement with the assumption of a sulphoxide (Bowie & others, 1966). The base peak at m/e 238 with an elemental composition of $C_{15}H_{12}NS$ may be best rationalized as the stable △1-propenylen-(3)-phenothiazinium ion depicted in Fig. 1 showing that further oxidation took place in the piperazine ring. Accurate mass measurements of the most prominent ions (Table 1) allow discussion of the fragmentation scheme presented in Fig. 1 where the oxidized piperazine ring is symbolized by R.

Table 1. *Accurate mass data of the molecular ion and the main fragments of substance VIII obtained in mass spectroscopy*

m/e	Composition	Calculated	Found
383	$C_{20}H_{21}N_3O_3S$	383·130356	383·128056
367	$C_{20}H_{21}N_3O_2S$	367·135441	367·134787
366	$C_{20}H_{20}N_3O_2S$	366·127616	366·125301
365	$C_{20}H_{19}N_3O_2S$	365·119791	365·119658
238	$C_{15}H_{12}NS$	238·069044	238·069509
226	$C_{14}H_{12}NS$	226·069044	226·068430
212	$C_{13}H_{10}NS$	212·053394	212·053632
198	$C_{12}H_8NS$	198·037744	198·037188
180	$C_{13}H_{10}N$	180·081320	180·081404
169	$C_8H_{13}N_2O_2$	169·097696	169·097157
167	$C_8H_{11}N_2O_2$	167·082047	167·081928

Conclusions. The formula $C_{20}H_{21}N_3O_3S$ derived from the molecular peak in the mass spectrum is in accordance with the elementary analysis (calculated C, 62·7; H, 5·5; N, 11·0; found C, 62·5; H, 5·5; N, 11·0%). The excellent agreement indicates that the relatively strong peak at m/e 367 is due rather to thermal release of the sulphoxide oxygen during mass spectroscopy than to admixture of the sulphide with the purified substance VIII. This possibility can further be excluded by consideration of the ultraviolet spectrum and the homogeneity on TLC.

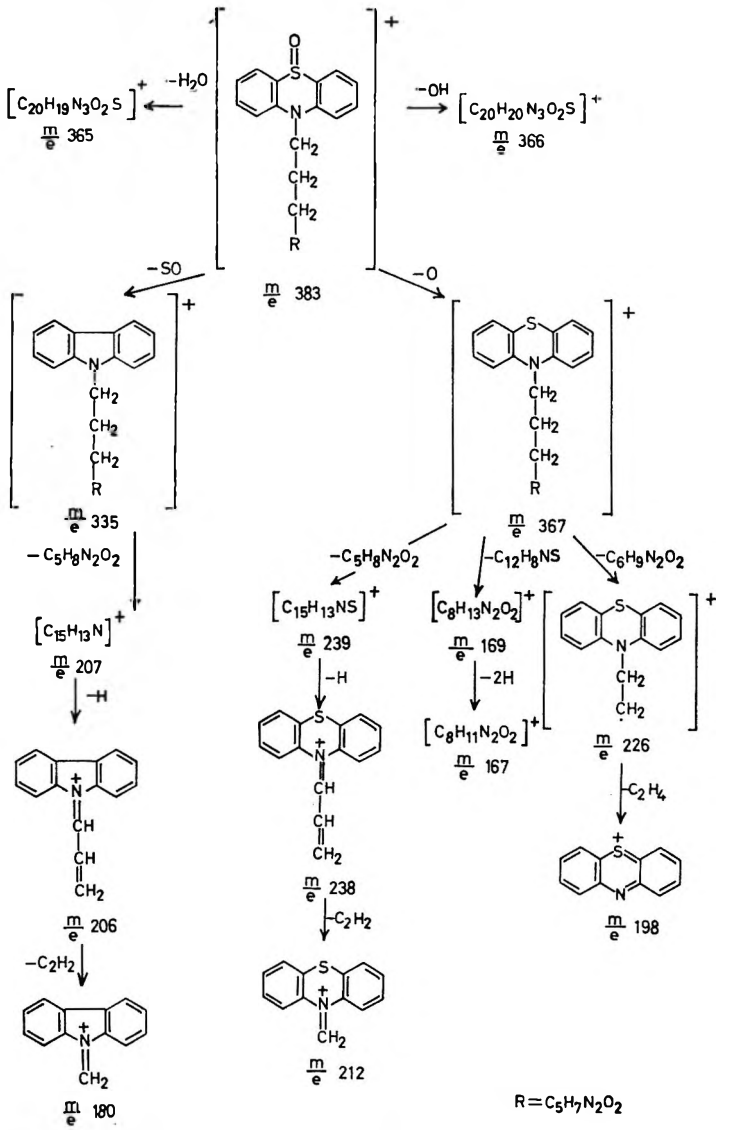
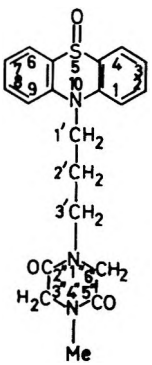


FIG. 1. Tentative fragmentation scheme of substances VIII in mass spectrometry.

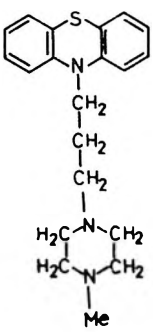
From the formula, $C_{20}H_{21}N_3O_3S$, it follows that three oxygen atoms have been added to the parent molecule, $C_{20}H_{25}N_3S$, and four hydrogen atoms have been removed. Since one oxygen is bound to the sulphur, the two others have each replaced two hydrogens. The occurrence of ether functions is ruled out by the infrared spectrum, so that the formation of CO from CH_2 groups is the most probable oxidative reaction. This is confirmed by the amide bands at 1681 and 1668 cm^{-1} in the infrared spectrum and by the loss of basic character. The elemental composition of the ions m/e 169 $C_8H_{13}N_2O_2$ and m/e 167 $C_8H_{11}N_2O_2$ is further support for the assumption that two oxygen functions occur in the side-chain. That both CO groups are situated in the piperazine ring is proved by the nmr spectrum showing a CH_2–CH_2–CH_2 sequence which must be due to the propyl chain on N–10 and a peak at 6·98 τ indicative of a methyl group.

There are four possibilities left for the position of the two keto-groups in the piperazine ring: 2″, 3″-, 3″,5″-, 2″,6″-, and 2″,5″-. Vicinal CO groups (2″,3″-) would mean vicinal CH_2 groups, too, but this is not compatible with the nmr spectrum suggesting isolated CH_2 groups. The possibility of two CO groups adjacent to one nitrogen (3″,5″- or 2″,6″-) is ruled out by the absence of basic character, since the other N atom would be surrounded by methylene (and possibly methyl) groups and should be basic. The absence of ν(CH) bands at high wavelengths in the infrared spectrum which would indicate CH_2 groups in amines also points to the same conclusion.

Therefore the only constitution which agrees with all findings is that of 10-[3′-(2″,5″-dioxo-4″-methylpiperazinyl)-propyl]phenothiazine sulphoxide (VIII).



VIII



Perazine

DISCUSSION

Piperazine-2,5-diones have been described as urinary metabolites resulting from amino-acids (Kibrick, Hashiro & others, 1965; Perry, Richardson & others, 1965), as metabolites from microbial cultures (Birkinshaw & Mohammed, 1962) and as products of protein hydrolysis simulating fermentation metabolites (Mitscher, Kunstmann & others, 1967). Apparently they have not yet been detected as oxidative metabolites of piperazine or piperazine derivatives despite the widespread use of these compounds as anthelmintics (see, for instance, Oelkers, 1959) and for other purposes.

The easy hydrolysis of diketopiperazines to dipeptides (Sykes, Robertson & others, 1966) would suggest the possibility that hydrolysis products of substance VIII might occur in the urine of patients ingesting perazine. Since as amino-acids these would not be extracted into organic solvents they are not easily detected. Radioactive labelling would indicate the presence of further metabolites in addition to those isolated. Until now, however, labelled perazine has not been available so that no conclusions about the occurrence of such polar degradation products can be made.

Acknowledgements

The authors wish to express their gratitude to Miss G. Taigel and Dr. J. C. Jochims, Max-Planck-Institut für medizinische Forschung, Heidelberg, for measurement and interpretation of the nmr spectrum. They are indebted to Professor Dr. K. Kanig, Abteilung für Neurochemie, Universitäts-Nervenklinik, Homburg/Saar, for valuable discussions and to Mr. P. Johann for technical assistance in the isolation procedure.

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The effects of etorphine and of morphine on respiration, blood carbon dioxide tension, and carbon dioxide sensitivity in the conscious rabbit

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The effects of intravenous doses of morphine and etorphine on respiration, blood pCO_2 and sensitivity to carbon dioxide were investigated in the conscious rabbit. Both morphine and etorphine depressed respiratory rate and elevated pCO_2. Etorphine was about 5000 times more potent than morphine as a depressant of respiratory rate. With etorphine, depression of respiratory rate was always accompanied by a large increase in tidal volume. In the normal rabbit, inhalation of mixtures of carbon dioxide up to 15% (in oxygen) produced a concentration-dependent increase in tidal volume accompanied by no increase in respiratory rate. After injection of morphine followed by inhalation of carbon dioxide there was a dose-dependent depression of carbon dioxide sensitivity which was concurrent with the depression of respiratory rate; 8 mg/kg completely abolished the respiratory stimulant effects of concentrations of carbon dioxide up to 12%. Whilst high doses of etorphine depressed carbon dioxide sensitivity, a dose of 0·5 μg/kg (which depressed respiratory rate by about 30%) did not depress carbon dioxide sensitivity. Doses of 1 μg/kg of etorphine administered to pregnant animals produced an increase in respiratory rate and a depression of tidal volume. The significance of the differences between the respiratory pharmacology of etorphine and that of morphine is discussed.

The effects of several narcotic analgesics on pH homoeostasis in the rabbit have been described (Rees, 1967; 1968). The present report describes the respiratory pharmacology of the highly potent narcotic analgesic etorphine in the conscious rabbit in which blood carbon dioxide tension (pCO_2), pH, standard bicarbonate, respiratory minute volume and respiratory rate were measured concurrently. The effects of inhalation of carbon dioxide (CO_2) on the respiratory depression produced by etorphine (M99) is also described and compared to those observed after morphine.

EXPERIMENTAL

Apparatus

pH studies. The apparatus was identical to that used by Rees (1967), based upon the method of Astrup, Jørgensen & others (1960).

Respiratory measurements. The method of Gaddum (1941) was used to determine respiratory rate and minute volume. This method measures pressure changes across a capillary which is acting as a resistance to inspiratory flow. Measurements were

made while applying an air-tight face mask over the snout of the animal intermittently for periods of between 1 and 2 min, a procedure which the animals tolerated better than continuous application. Application of the face mask did not alter respiratory rate. Pressure changes were recorded via a tambour on a smoked drum. The apparatus was calibrated against a water manometer using a suction pump which was periodically calibrated against a spirometer.

Method

Groups of not less than four and not more than twenty Flemish rabbits, weighing 2·5–3·5 kg were used. Each group consisted of an equal number of male and female animals. In nearly every instance in which the effects of etorphine were investigated on pH homoeostasis each rabbit was used on only one occasion. No rabbit was used more than twice.

Controls. Before the determination of control values each rabbit was allowed 20 min for its respiration to settle. Although the rabbits were unrestrained they were encouraged to remain stationary, and those for whom such encouragement was inadequate were not used. During the following 20 min two or three control values for blood pCO_2, pH and standard bicarbonate were determined by the method of Astrup & others (1960) in the blood obtained from the marginal vein of the warmed ear (Rees, 1967). Immediately after each blood sampling the control values for respiratory rate, minute volume and tidal volume were obtained.

Effect of changing the diameter of the capillary resistance in the Gaddum apparatus. A capillary bore of 2·00 mm was used routinely, but in some experiments capillary bores of 1·75 and 2·25 were also used. Each capillary was calibrated and then used in succession to determine respiratory minute volume.

Effects of inhaled gases. The exact concentration of CO_2 in mixtures containing approximately 5, 8 and 15% CO_2 (in O_2) was measured either by Haldane's method or by the use of an infrared CO_2 analyser. Mixtures of these CO_2/O_2 concentrations were fed into the Gaddum respirometer circuit avoiding positive pressure. Respiratory rate and minute volume were recorded until no further increase in minute volume was seen over a 30 s period (between 2 and 3 min exposure) both in control animals and in animals treated with morphine or etorphine. The effects of inhalation of 100% O_2 on the respiratory effects of morphine and etorphine were also investigated.

Time course of investigations. After stable control values for all parameters had been obtained drugs were administered intravenously. Samples of blood were obtained 7 and 15 min after injection and then at 15 min intervals until control values were regained. Respiratory rate, minute volume and the effects of inhaled gases were measured immediately after blood sampling. In some experiments changes in respiratory rate and minute volume were recorded continuously for the first 15 min after injection. The effect of repeated doses of etorphine was also investigated in a group of animals. Etorphine, 0·5 μg/kg, was injected and minute volume and rate followed until a peak change in rate was recorded at which time a second dose of 0·5 μg/kg was administered and the same procedure followed until a maximum change was observed. A dose of 1·0 μg/kg was then injected and when peak change in rate was recorded blood was sampled and pCO_2 measured. In all instances the interval between injections was between 5 and 10 min.

Expression of results. Changes in respiratory rate and minute volume are expressed as percentage change from control values. Changes in tidal volume were calculated

from these two parameters and expressed in the same way. Changes in pH, pCO_2 and standard bicarbonate are expressed as the difference between each respective reading and its control.

Drugs

Drugs used were morphine sulphate injection B.P. (BDH) and etorphine hydrochloride [7α(1-(*R*)-hydroxy-1-methylbutyl)-6,14-*endo*ethenotetrahydro-oripavine hydrochloride] (Reckitt & Sons Ltd.). A stock solution of 10 μg/ml was buffered at pH 4·0. Dilutions of both drugs were made in sterile saline. All doses are expressed in terms of the salts.

RESULTS

Effect of changing the diameter of the capillary resistance. Fig. 1 shows the calibration slopes of mm excursion of the pointer against air flow in ml/min for each of the three capillary resistances. The relation was linear up to about 1200 ml/min. Respiratory minute volumes in excess of this figure were rarely encountered in these experiments. Table 1 shows the changes in respiratory minute volumes measured with each of the capillaries in a variety of experimental types (some of which are not described in this report). There was no significant difference between changes in respiratory minute volumes calculated from each capillary nor was there a consistent trend in the values related to the capillary bore.

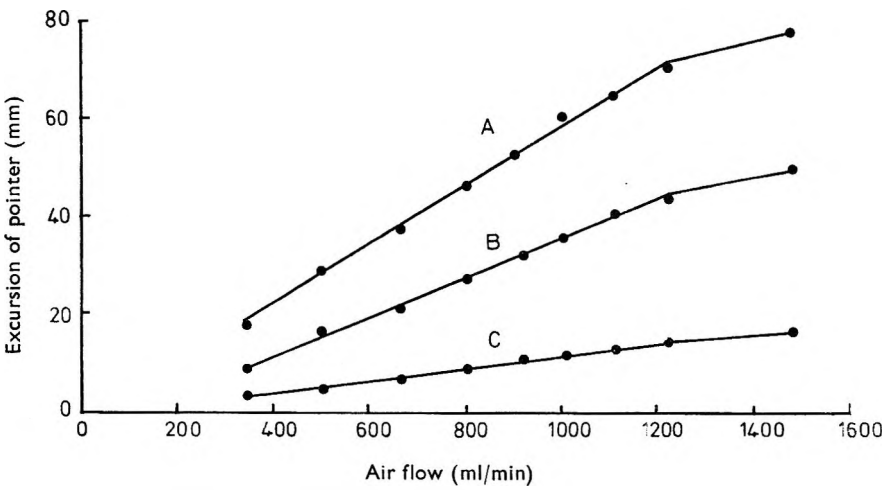

FIG. 1. The relation between airflow through the Gaddum apparatus and the excursion of the pointer, measured using three capillary tubes of different bore. A = a capillary of internal diameter 1·75 mm; B = internal diameter 2·00 mm; C = internal diameter 2·25 mm.

Effects of inhaled CO_2 in control animals. The percentage changes in respiratory minute volume, rate and tidal volume on exposure of rabbits to various concentrations of CO_2 up to 15% are shown in Fig. 2. The inhalation of 5 or 8% CO_2 had little effect on respiratory rate, but 15% produced a fall. On the other hand, increasing the ambient CO_2 percentage produced an increase in tidal volume roughly proportional to the concentration of CO_2. At 15% this represented a 2·5-fold increase. The net effect on minute volume was therefore a concentration-dependent increase.

Table 1. *The values of percentage change in minute volume recorded under a variety of experimental conditions during which three different capillary resistances were incorporated into the Gaddum apparatus and used consecutively. Means of 4 animals ± s.e.*

Experiment	Resistance (letters refer to key in Fig. 1; internal capillary diameter is given in mm) A (1·75 mm)	B (2·00 mm)	C (2·25 mm)
% depression of respiratory minute volume produced by 4 mg/kg morphine	31·0 ± 1·1	30·9 ± 1·8	29·7 ± 2·3
% depression of minute volume produced by 30 mg/kg pentobarbitone (*)	37·9 ± 2·2	39·1 ± 1·7	39·5 ± 2·7
% increase in respiratory minute volume response to 12% CO_2 after 4 mg/kg morphine	34·4 ± 1·8	38·0 ± 2·6	36·8 ± 2·2
% increase in respiratory minute volume response to 12% CO_2	51·1 ± 1·4	53·9 ± 1·0	54·9 ± 1·7
% increase in respiratory minute volume response to 7·2% CO_2 in animals pretreated with progesterone (*)	42·7 ± 1·0	41·4 ± 1·4	41·4 ± 1·1
% increase in respiratory minute volume response to 4% CO_2 in animals pretreated with progesterone (*)	30·5 ± 1·2	29·7 ± 1·8	29·7 ± 1·2

(*) Readings taken from studies not described in this paper.

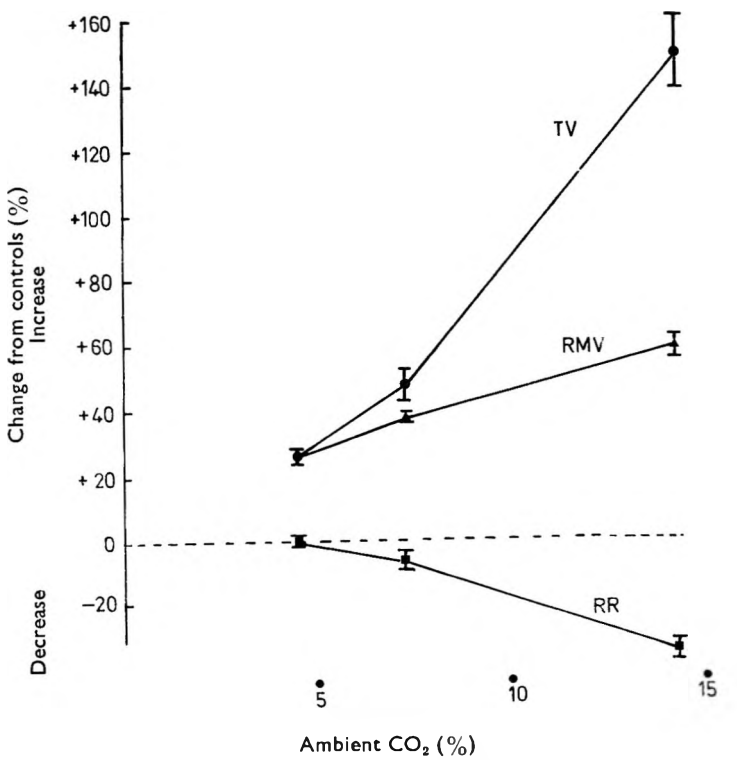

FIG. 2. The effect of elevating the ambient carbon dioxide concentration on tidal volume (circles, TV), respiratory minute volume (triangles, RMV) and respiratory rate (squares, RR) in the conscious rabbit. Means of at least 20 determinations ± standard error.

Effects of etorphine—gross observations. The most obvious effect observed with the doses used in this series (0·25–8·0 μg/kg) was sedation, but no doses produced unconsciousness. With doses of 2 μg/kg and above, a characteristic catatonic state developed a few minutes after injection, and persisted for about a quarter of an hour. With doses of 4 μg/kg some rabbits convulsed two or three times during the first 5 min after injection. These convulsions were less severe than those seen after pethidine in high doses in the rabbit. At a dose of 8 μg/kg three of the four animals convulsed. The fourth showed a catatonic condition and this was the only animal which died.

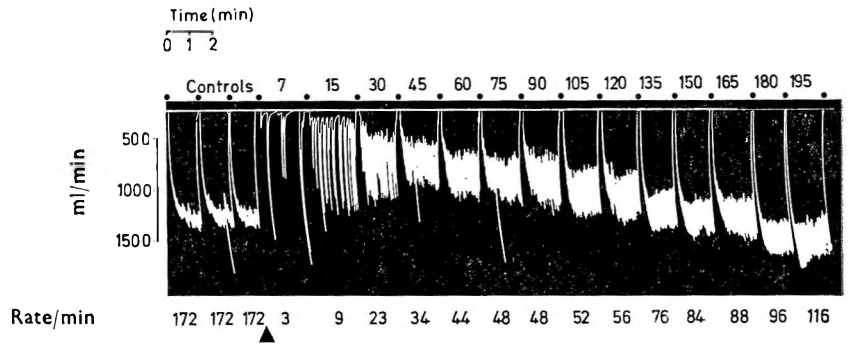

FIG. 3. The effects of 2 μg/kg etorphine on respiratory rate and minute volume measured by the Gaddum respirometer. Minute volume calibration is shown on the left, and respiratory rate is recorded below the trace. Recordings were made during the application of a face mask for periods of between 1 and 2 min (see time scale). At the black circles the face mask was removed and the drum stopped. The time after injection at which recordings were made is stated above the trace. At arrow, etorphine, 2 μg/kg.

Effects of etorphine on respiration. Fig. 3 shows a characteristic recording of the effects of 2 μg/kg on respiratory minute volume and rate. Three control readings are shown. Seven min after injection, the irregular and infrequent gasping respiration

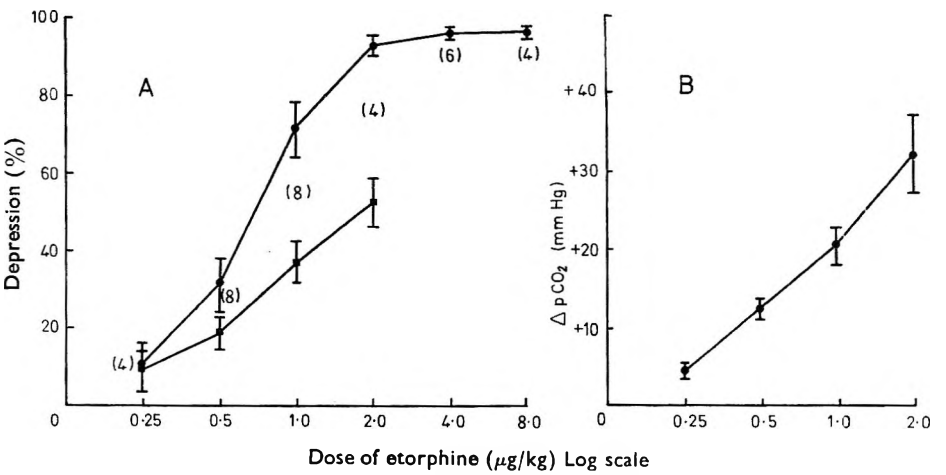

FIG. 4 A. The relation between the dose of etorphine and the percentage depression of respiratory rate (circles) and minute volume (squares) in groups of rabbits. The numbers in brackets refer to the number of rabbits in each group. Means are ± standard error.

B. The relation between the dose of etorphine and the maximal elevation of blood pCO_2. Means are ± standard error.

can be seen during which no accurate determination of minute volume could be made. A marked increase in tidal volume is much in evidence, for although minute volume had returned to the control value 150 min after injection, respiratory rate at this time was still only 49% of the original control.

Cheyne Stokes respiration was frequently observed at all doses. At the highest dose rabbits respired 5 to 10 times during a period of about 10 s, this being followed by an apnoeic period of between 40 and 50 s.

Log dose response relations for both respiratory rate and minute volume are shown in Fig. 4A. Respiratory rate only could be determined at doses in excess of 2 μg/kg because the convulsions produced at these doses precluded application of the face mask. The percentage depression of respiratory rate was always greater than that of minute volume. The maximum change in tidal volume for each dose of etorphine is shown in Table 2. There was a dose-dependent increase in tidal volume.

Table 2. *The maximum change in tidal volume after injection of etorphine in the rabbit*

Dose of etorphine (μg/kg)	Increase in tidal volume (% ± s.e.)	Time after injection (min)	Number of experiments
0·25 (mixed group)	6 ± 4	15	4
0·5 (male group)	14 ± 6	15	4
0·5 (female group)	38 ± 10	7	4
1·0 (male group)	190 ± 73	7	4
1·0 (female group)	170 ± 56	7	4
2·0 (mixed group)	344 ± 44	7	3

Changes in minute volume, rate and tidal volume during the first 15 min after injection of 1 μg/kg are shown in Table 3. The increase in tidal volume appeared immediately after the injection.

Table 3. *Changes in respiratory minute volume, respiratory rate and tidal volume during the first* 15 *min after intravenous injection of* 1 μg/kg *of etorphine into a group of four rabbits*

Time after injection (min)	Decrease in respiratory rate (% ± s.e.)	Decrease in respiratory minute volume (% ± s.e.)	Increase in tidal volume (%)
1	60·1	51·3 ± 5·8	83
2	68·7 ± 4·8	50·8 ± 0·9	201
3	73·1 ± 9·0	52·4 ± 1·0	163
4	74·6 ± 10·8	55·8 ± 2·6	256
5	71·6 ± 9·2	47·5 ± 2·8	189
6	67·2 ± 12·5	45·7 ± 5·3	164
7	68·1 ± 13·0	44·4 ± 6·0	178
8	64·5 ± 16·1	40·5 ± 4·3	190
9	66·9 ± 12·7	37·2 ± 4·9	195
10	65·7 ± 13·8	33·9 ± 5·5	200
15	59·4 ± 16·2	27·7 ± 7·7	140

When 2 μg/kg was injected by the cumulative method (0·5 + 0·5 + 1·0 μg/kg) changes in minute volume and rate were not significantly different from those produced by the same dose given as a single injection. The mean maximum depression of minute volume (± s.e.) when the drug was injected as a single dose was 52·1 ± 6·0%

whilst that produced by cumulative injections was 50·5 ± 1·7%. The respective values for depression of respiratory rate were 93·3 ±2·0 (single injection) and 85·3 ± 6·6 (cumulative injections).

Effects of etorphine on pH homoeostasis. Etorphine caused an increase in blood pCO_2 which was linearly related to log dose (Fig. 4B). When change in respiratory minute volume or tidal volume was plotted against the concurrent change in pCO_2, only the change in tidal volume against change in pCO_2 was found to be linear and passing through the origin.

Table 4. *The maximum fall in blood pH and the concurrent* changes in standard bicarbonate and pCO_2 after intravenous injection of etorphine into groups of four rabbits*

Dose of etorphine (μg/kg)	Change in standard bicarbonate (m-equiv/litre ± s.e.)	Change in pH (pH ± s.e.)	Change in pCO_2 (mm Hg ± s.e.)
0·25 (mixed group)	−0·2 ± 0·3	−0·027 ± 0·005	+4·3 ± 0·8
0·5 (male group)	−0·9 ± 0·3	−0·070 ± 0·012	+11·6 ± 1·4
0·5 (female group)	+0·2 ± 0·8	−0·071 ± 0·015	+13·9 ± 1·4
1·0 (male group)	−5·6 ± 0·9	−0·201 ± 0·023	+18·6 ± 3·1
1·0 (female group)	−5·1 ± 1·6	−0·209 ± 0·043	+17·7 ± 6·8
2·0 (mixed group)	−9·1 ± 0·9	−0·310 ± 0·027	+31·3 ± 6·1

* Note: the changes in standard bicarbonate and pCO_2 concurrent with the maximum change in pH are not necessarily the maximum changes themselves.

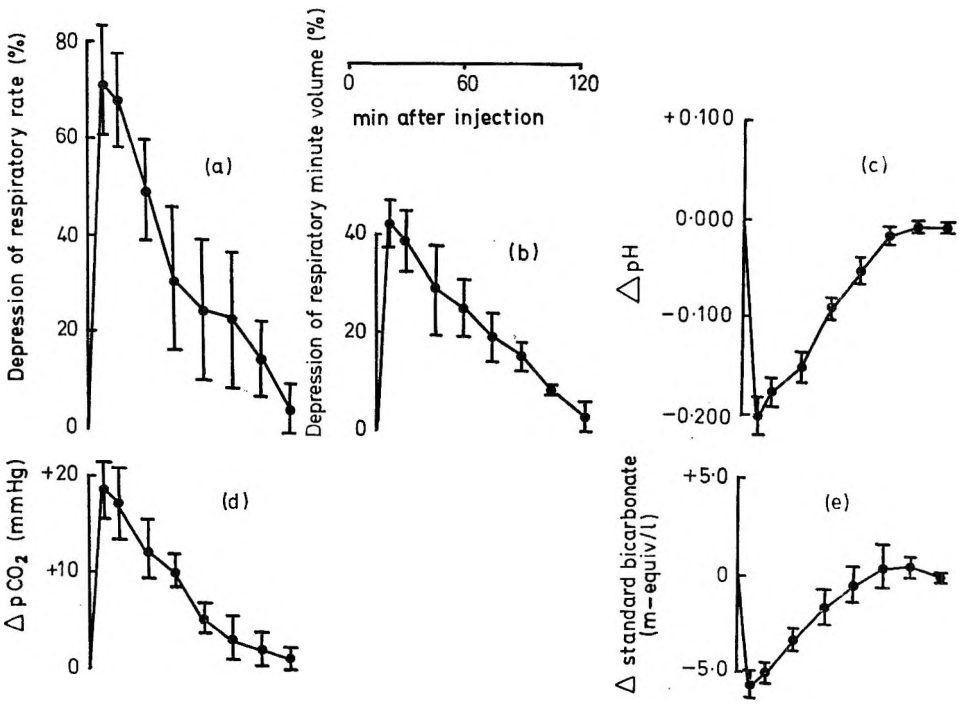

FIG. 5. The time course of the effect of 1 μg/kg etorphine in a group of four male rabbits on respiratory rate (a), minute volume (b), pH (c), blood pCO_2 (d) and standard bicarbonate (e). Means are ± standard error.

The maximum change in pH with the concurrent values for standard bicarbonate are shown in Table 4. At doses of 1 and 2 μg/kg there was a marked fall in bicarbonate associated with a severe acidosis.

Effects of etorphine—time course. A full record of changes in minute volume, rate, pH, pCO_2 and standard bicarbonate is shown for one of the groups (male group given 1 μg/kg) in Fig. 5. The important features are the rapid onset and short duration of effect, the relatively small change in minute volume compared with that of rate and the severe fall in pH.

Effects of etorphine—sex differences. Eight rabbits were investigated after doses of 0·5 and 1·0 μg/kg in order to identify any sex differences. There was no significant difference between the changes in standard bicarbonate observed in the two sexes.

Two of the female rabbits given 0·5 μg/kg gave anomalous results with respect to all parameters. Later it transpired that they were in the later stages of pregnancy at the time of the experiment. The effects in these two animals are of interest. The mean maximum depression of rate ($\pm$ s.e.) in the group of normal females was 42·1 $\pm$ 4·9%. The corresponding changes in the two pregnant animals represented a 32 and 66% *increase* in respiratory rate. Whilst there was a 12% increase in tidal volume in the normal group there was a 33 and 44% *decrease* in the pregnant animals. The elevation of pCO_2 and depression of minute volume in the two pregnant animals were both substantially less than those obtained from the normal group.

Effects of morphine on respiration. The effects of 2, 4 and 8 mg/kg of morphine on respiratory rate and minute volume are shown in Fig. 6. These changes should be compared with the parallel changes produced by etorphine (Fig. 4).

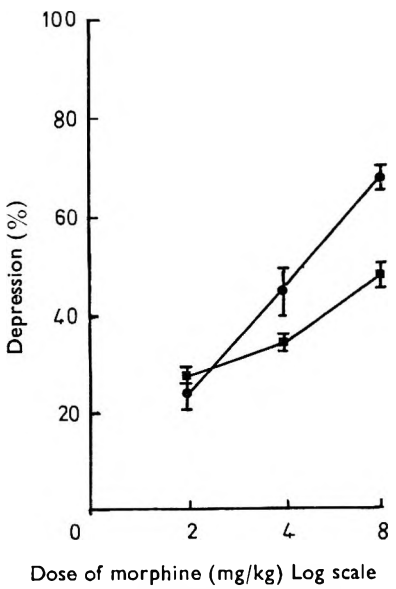

FIG. 6. The relation between the dose of morphine and the percentage depression of respiratory rate (circles) and respiratory minute volume (squares) in groups of rabbits. Means are $\pm$ standard error.

Effects of inhaled gases in animals treated with morphine and etorphine. Inhalation of 100% O_2 did not significantly alter the changes in minute volume produced by 4 mg/kg of morphine or 1 μg/kg of etorphine.

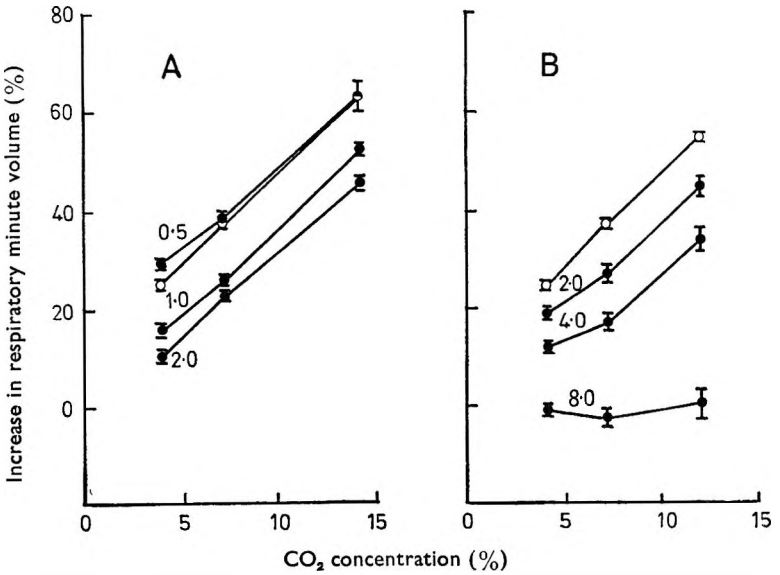

FIG. 7. The percentage increase in respiratory minute volume during inhalation of carbon dioxide in groups of rabbits treated with etorphine or morphine. Open circles are the control readings, closed circles show the values after drug treatment. The figures above each line refer to the dose of A, etorphine (μg/kg) and B, morphine (mg/kg). Means are $\pm$ standard error.

Doses of 2 and 4 mg/kg of morphine produced a parallel shift in the CO_2 concentration/respiratory minute volume slope (Fig. 7). A dose of 8 mg/kg completely abolished the stimulant action of CO_2 on minute volume up to concentrations of 12%.

On the other hand, when animals treated with etorphine were exposed to CO_2 a quantitatively different response was seen (Fig. 7). In animals given 0·5 μg/kg of etorphine there was no depression of CO_2 sensitivity up to concentrations of 14%. Indeed, there was a significant *increase* in sensitivity to 4% CO_2. Doses of 1 and 2 μg/kg produced a parallel shift of the CO_2 concentration/minute volume slope.

DISCUSSION

The Gaddum respirometer measures inspiratory flow rate by recording the pressure drop across a capillary tube which is acting as a resistance to inspiratory flow. It was possible that the inclusion of this resistance would place an inspiratory load upon the animal, causing a change in respiration which might not remain constant after drug treatment. To ensure that this did not influence our own work three different resistances were used in some experiments. The calibration line for each resistance is shown in Fig. 1. All gave a linear relation over the minute volume ranges encountered in this study. If the introduction of a capillary resistance into the inspiratory flow were to produce an abnormal respiratory pattern the extent of the abnormality should be related to the bore of the capillary. Table 1 shows that when each resistance was used during six different experimental types there was no significant difference between the percentage changes in minute volume determined with each resistance, nor was there a trend in the values related to capillary bore. It is concluded that the capillary resistance does not influence drug-induced changes in respiration.

The capillary resistance used routinely had an internal diameter of 2 mm—that originally recommended by Gaddum (1941) for the rabbit.

The remarkable potency of etorphine as a narcotic analgesic under all test conditions is well known (Blane, Boura & others, 1967). In this report 0·72 μg/kg caused a 50% depression of respiratory rate in the rabbit. The dose of morphine needed to produce a 50% depression of rate in the rabbit was found to be 3·7 mg/kg (Hunter, Pleuvry & Rees, 1968) and therefore etorphine is about 5000 times more potent than morphine in the present test conditions.

The marked periodic breathing produced by higher doses of etorphine has frequently been reported for other narcotic analgesics. Breckenridge & Hoff (1952; 1953) have reported periodic breathing after both morphine and levorphanol and concluded that this was indicative of "pharmacologic decerebration" caused by inactivation of cortical and subcortical suppressor mechanisms normally impinging upon the respiratory centre. Orkin, Egge & Rovenstine (1955) observed that this phenomenon was prominent after intravenous injection and in patients with depressed cerebral function.

The mechanism by which narcotic analgesics depress respiration has yet to be fully established. The depressant action on the sensitivity of the respiratory centre to carbon dioxide and/or hydrogen ion seems to be of primary importance (Eckenhoff & Oech, 1960), but even this has been questioned (Krueger, 1955). It is evident that the primary target for etorphine's respiratory depressant activity lies amongst the factors controlling respiratory rate. In all instances there was an immediate and often striking increase in tidal volume and the percentage depression of minute volume was far less than that of rate.

Such a marked increase in tidal volume produced by etorphine is not a characteristic action of narcotic analgesics. Hunter & others (1968) investigated the intravenous effects of nine narcotic analgesics in the rabbit, concluding that three of these produced a slight decrease in tidal volume, two a slight increase and four had no effect. Doses of morphine up to 20 mg given by either the intravenous or intramuscular routes caused a depression of tidal volume (Loeschcke, Sweel & others, 1953; Dripps & Comroe, 1945; Huggins, Spencer & others, 1957; Eckenhoff, Helrich & others, 1955). Alphaprodine and pethidine have been shown to produce a persistent depression of tidal volume (Orkin & others, 1955).

To preclude the possibility that etorphine initially depressed tidal volume but that it had returned to normal or even increased by the time of our first measurements —a phenomena reported after intravenous morphine (Foldes, Swerdlow & Siker, 1964)—tidal volume changes were continuously investigated after injection of etorphine (Table 3). Our results clearly show that the increase in tidal volume was instantaneous.

Hunter & others (1968), who showed that narcotic analgesics had no consistent effect on tidal volume in the rabbit, used a cumulative method of drug administration. However, we could show that when etorphine was injected either as a single dose, or in divided doses the effects on the respiratory parameters were the same.

In the light of the relatively enormous changes in tidal volume after etorphine the rabbit's normal response to an elevated ambient CO_2 mixture is of considerable interest. With CO_2 mixtures up to 15% a dose-dependent increase in tidal volume was observed which was never accompanied by an increase in respiratory rate. It is not unreasonable to assume that this was a direct consequence of the increased

alveolar and therefore arterial pCO_2. The fact that this change in tidal volume still occurred when respiratory rate was depressed by etorphine could imply that depression of the CO_2-sensitive medullary chemoreceptors contributes less to the mechanism of etorphine's respiratory depression than it does to other narcotic analgesics.

This suggestion is supported by the effects of CO_2 in rabbits given morphine or etorphine. The effects of CO_2 inhalation after morphine were predictable (Bellville & Seed, 1960, and references cited therein). There was a dose-dependent depression of CO_2 sensitivity *which was concurrent* with respiratory depression. A dose of 8 mg/kg totally abolished the stimulatory effect of concentrations of CO_2 up to 12%. On the other hand, a dose of 0·5 μg/kg of etorphine which produced considerable respiratory depression did not depress CO_2 sensitivity. Indeed, there was a significant increase in sensitivity to 4% CO_2. Higher doses of etorphine depressed CO_2 sensitivity but this was always quantitatively less than that caused by equi-respiratory depressant doses of morphine. A dose of 2 μg/kg of etorphine, which produced over 90% depression of rate, depressed CO_2 sensitivity less than did 4 mg/kg of morphine—a dose which only depressed rate by 44%.

Rees (1967; 1968) examined the effects of narcotic analgesics on blood pCO_2. It was shown that for drugs with a long duration of action (morphine) the rise in pCO_2 was accompanied by an increase in standard bicarbonate. A short-acting drug (dextromoramide) showed no such change whilst a drug of intermediate duration (phenazocine) exhibited a sex hormone-dependent sex difference in which only the female animals showed an increase in bicarbonate. The absence of any increase in standard bicarbonate after etorphine is compatible with this earlier pattern since etorphine has a similar duration of action as dextromoramide.

The finding that etorphine causes an increase in respiratory rate in pregnant females, but a depression in normal females is of interest. A sex hormone imbalance in favour of progesterone will result in a shift to the left of the slope relating alveolar pCO_2 to minute volume (Doring, Loeschcke & Ochwadt, 1950). This effect is in direct contrast to that produced by morphine. It is possible therefore that in the presence of progesterone the effect of etorphine would be reduced.

This provides another possible involvement of steroids in the mechanism of action of the narcotic analgesics (Rees, 1968, and references cited therein). The narcotic analgesic receptor proposed by Beckett & Casy (1954) would seem to be an over-simplification. With the advent of the highly potent narcotic analgesics (typified by etorphine), the hydroxyl group on the C-19 is of considerable importance to the compound's pharmacological activity and an additional part of the receptor must be considered (Leadbeater & Davis, 1968; Harris & Dewey, 1967).

We consider that the stereochemical similarities between the steroidal hormones and the most potent of the narcotic analgesics are too striking for their interactions to be dismissed as being coincidental. This is of interest in view of the recent finding by Craig (1968) that a dimethyl-aminomethyl substituted steroid possesses analgesic activity, causes a Straub tail in mice, reduces gastrointestinal propulsion and depresses respiration in rabbits—an effect which may be antagonized by nalorphine.

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A perfused tail artery preparation from the rat

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The isolated perfused tail artery of the rat responds by constriction to 1·0 ng (−)-noradrenaline, and would be suitable for the assay of sympathomimetic amines. Electrical stimulation of this preparation is shown to activate solely postganglionic adrenergic nerve terminals. Tachyphyllaxis to angiotensin, vasopressin and bradykinin preclude the use of this preparation for their assay.

Initial studies on the hypotensive actions of diuretics (Lockett & Nicholas, 1968) were made in rats. Consequently, arteries from rats were preferred for *in vitro* studies of the direct arterial actions of these drugs. The isolated arteries most used are perfused preparations made from the central ear artery of the rabbit (de la Lande & Rand, 1965) and the mesenteric vessels of the rat (McGregor, 1965). Unfortunately the mesenteric artery of the rat is insensitive to catecholamines *in vitro*. Hence the purpose of the present work was to find a simple, rapidly prepared, reliable, rat isolated arterial preparation with a noradrenaline threshold sensitivity of about 20 ng. The tail artery has satisfied these requirements.

EXPERIMENTAL

Methods

Preparation of the artery. Male Wistar rats (230–260 g) were killed by a blow on the head. Blunt scissors were used to make a ventral incision extending for 7 cm from the base of the tail. The tail artery and vein were together separated from the fascia. Two ligatures were tied around the caudal artery and vein, 5 cm apart. The segment of vessels complete with ligatures was transferred to Krebs solution for cannulation of the proximal end of the artery with a 2 cm length of polyethylene 100 tubing (inner diameter 0·034 inch; outer diameter 0·060 inch), drawn out to a suitable diameter at the tip. The distal end of the artery was similarly cannulated with polyethylene 100 tubing premoulded into a U-tube for delivery of effluent perfusate. The final length of the artery at room temperature in Krebs solution was roughly standardized at 3·5 cm since the length perfused has considerable bearing on sensitivity (de la Lande & Rand, 1965). The artery together with the vein was then transferred to a 25 ml organ bath and was perfused at 37° with Krebs bicarbonate solution (Umbreit, Burris & Stauffer, 1964), gassed with carbon dioxide in oxygen by a Watson Marlow flow inducer. Slight irregularities in the rate of delivery from this pump were effectively damped by running the pump at high speed and limiting the delivery to the artery by means of an adjustable clamp. Perfusion pressure was continuously monitored by an E & M linear transducer coupled to a pen recorder. The fluid was not recirculated.

Electrical stimulation, when used, was periarterial. Platinum electrodes were placed closely adjacent to and on either side of the proximal 0·5 cm of the preparation.

A Grass stimulator (S4K) was used to deliver rectangular pulses of 1 ms duration at a fixed voltage of 15 V at various frequencies.

Drugs. (−)-Noradrenaline (Winthrop Laboratories), vasopressin (Parke Davis & Co. Ltd, Pitressin), phentolamine mesylate and angiotensin II (Ciba Laboratories Ltd.), histamine acid phosphate and tyramine hydrochloride (Koch-Light Laboratories Ltd.), bradykinin triacetate (Sigma Chemical Co.), cocaine hydrochloride (Macfarlan Smith Ltd.), reserpine (Ciba), guanethidine sulphate (Ciba), amphetamine sulphate (May & Baker).

RESULTS

The relation between flow and perfusion pressure

Flow rates of 2 to 3 ml/min through the tail artery initially generated high perfusion pressures which did not exceed 70 mm Hg pressure. During the first 10–20 min the initial pressures invariably decreased steadily and stabilized within the range 15 to 30 mm Hg. Once stabilized at a given flow rate the arteries responded differently to short and to long periods of change in flow. When an increase in flow was maintained for no more than 3 min, the pressure rose during the greater flow but returned to the initial value when the resting flow was restored. If, however, the raised flow was maintained the pressure gradually fell and re-stabilized at a value close to that at which it had first stabilized. Fig. 1 A shows results from an experiment in which the flow rate was increased at 2 min intervals to raise the perfusion pressure step by step from 23 to 58 mm Hg. Thereafter, flow rate was decreased step by step. The results demonstrate that this procedure has decreased the tone of the tail artery. Fig. 1 A also shows that if this procedure is carried out with return to the initial flow rate between each step, tail arterial tone is almost unaffected.

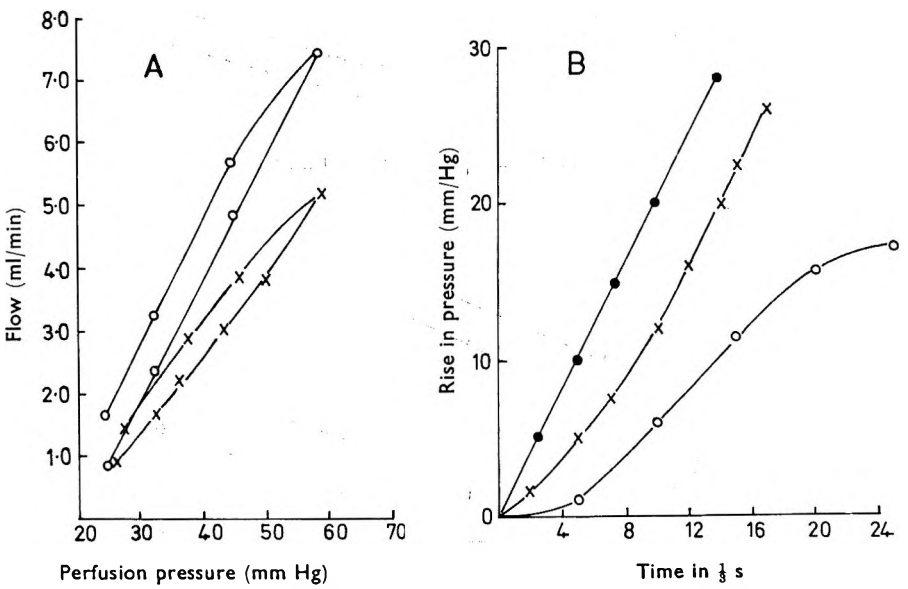

FIG. 1. A: the effects of increased perfusion pressure on flow through a perfused rat tail artery. B: comparison of the rise in pressure caused by 5 ng of noradrenaline in a low toned high flow preparation ○ – ○ and in a high toned low flow preparation × – ×, showing also the effects of distal arterial clamping ● – ●.

The relation between arterial tone and vasoconstrictor response

High initial flows produced high flow low toned preparations whereas low initial flows yielded high toned low flow preparations. All stabilized at pressure within the range of 15 to 30 mm Hg. Low toned high flow preparations usually responded to constrictor agents by reduction and not by cessation of flow (Fig. 1 B, open circles). High toned low flow preparations responded to vasoconstrictors by cessation of flow and the resulting rise in perfusion pressure was directly related to the duration of arrest. Fig. 1 B shows results from a single artery, clamped, and responding to 5 ng of noradrenaline either by occlusion during high tone low flow conditions or reduction the low tone high flow state. Sensitivity to noradrenaline was much enhanced by pre-selection of high tone low flow conditions.

Comparison of the effects of noradrenaline and electrical stimulation on the isolated tail artery of the rat

Electrical excitation caused constriction of the tail artery and hence a rise in perfusion pressure. When the voltage used produced maximal effect this rise of pressure was directly related to the log of the pulse frequency. Log frequency effect curves were found parallel to log dose-effect curves for (—)-noradrenaline. Both curves were similarly shifted to the right by phentolamine mesylate (Fig. 2A) and cocaine potentiated the effects of electrical stimulation and of (—)-noradrenaline similarly (Fig. 2 B, and Fig. 3). Phentolamine antagonism of the response to

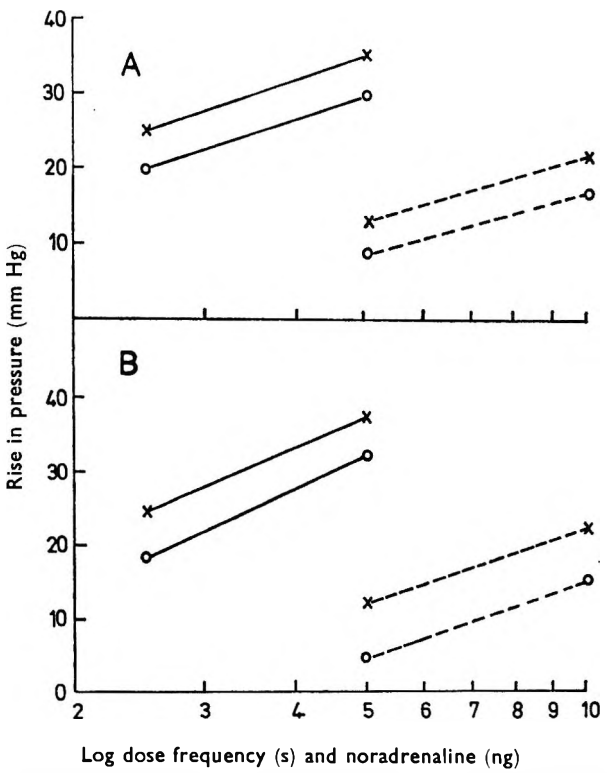

FIG. 2. Effects of the addition of 500 ng of phentolamine to the bath fluid (A) and of cocaine hydrochloride (B) perfused through the isolated rat tail artery, 3 μg/min, on the responses of the artery to noradrenaline (——) and to electrical stimulation (– – –). × Before, ○ after treatment.

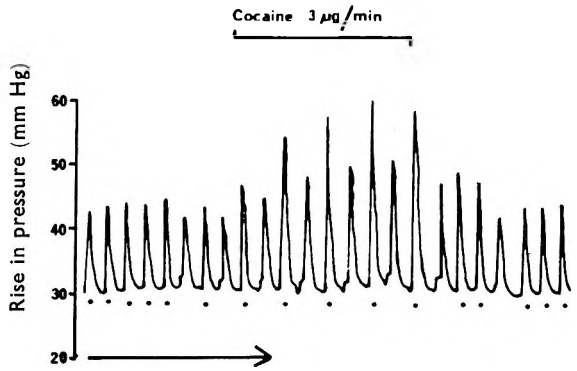

FIG. 3. Typical effects of cocaine hydrochloride, 3 μg/min, on the responses of a rat tail artery to electrical stimulation (●) and to the injection of 5 ng of noradrenaline.

periarterial stimulation was, however, more easily reversed than the responses to noradrenaline. Extraluminal concentrations of guanethidine up to 1·0 μg/ml often potentiated the effects of periarterial stimulation and produced the expected supersensitivity to noradrenaline (Fig. 4b). Concentrations of 1·5 to 2·0 μg/ml guanethidine regularly inhibited arterial responses to electrical stimulation and amphetamine sulphate 200 mg/ml reversed this inhibition (Fig. 4a). Higher concentrations of

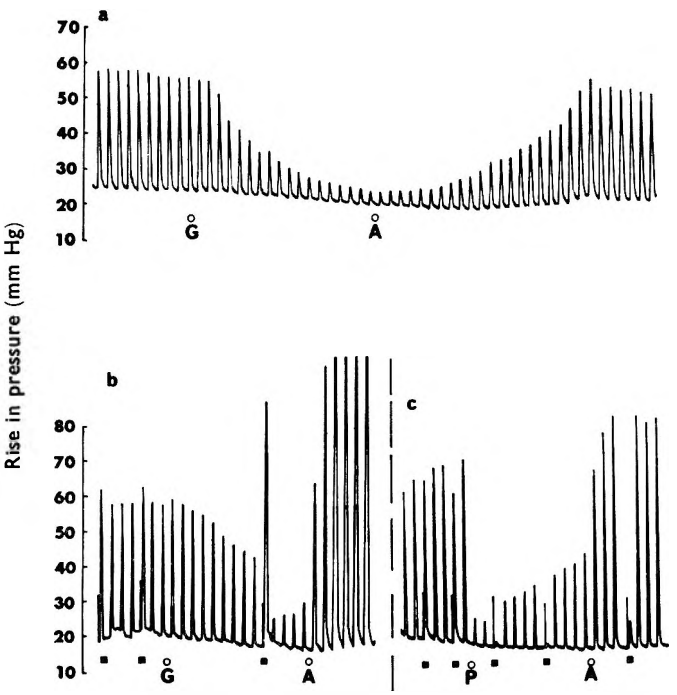

FIG. 4. (a) The effect of extraluminal doses of 1·5 μg/ml of guanethidine (G) followed by amphetamine (20 ng/ml) (A) on the responses of the perfused artery to electrical stimulation.

(b) The effect of extraluminal doses of guanethidine (1·5 μg/ml) (G) followed by 1·5 μg/ml of amphetamine (A) on the response of the perfused artery to electrical stimulation, 10/s, to 10 ng of noradrenaline (■).

(c) The response of a perfused artery to extraluminal doses of 20 ng/ml of phentolamine (P) followed by 600 ng/ml of amphetamine (A) during periarterial stimulation interrupted by injections of 10ng of noradrenaline (■).

amphetamine 400 mg/ml and upward, grossly enhanced the effects of periarterial stimuli in the absence of guanethidine and in the presence of phentolamine 20 mg/ml (Fig. 4b and c). Arteries taken from rats 24 h after intraperitoneal reserpine 5 mg/kg, showed greatly reduced responses to periarterial stimulation. Addition of pentolinium (250 μg) and atropine sulphate (0·5 μg) to the organ bath did not influence the responses of the preparation to electrical stimulation or to noradrenaline.

The threshold dose of noradrenaline, given as a single injection, was usually 0·5 ng at a flow rate of 2·5 ml/min. Responses to 5 ng noradrenaline at intervals of 3 min or to electrical stimulation delivered for 3 s at 5/s, every minute were maintained for 8 or more hours with no more than a 10% reduction in response. Over the whole of this period the base-line perfusion pressure rarely fell by more than 5 mm Hg.

The sensitivity of the isolated tail artery of the rat to various pressor agents

The isolated tail artery of the rat responded to electrical stimulation at intervals of 1 min and to single injections of noradrenaline at 3 min intervals for many hours without appreciable reduction in sensitivity. Mean log dose effect curves for the actions of noradrenaline, tyramine and electrical stimulation are shown in Fig. 5. These curves are calculated regression lines: the broken lines delineate the standard deviation of the slopes. Data for the effects of angiotensin and of vasopressin were less reliable since tachyphyllaxis was produced by both compounds. Arteries were initially very sensitive to angiotensin and usually responded to 2 ng, but tachyphyllaxis developed rapidly even when the doses used were minimally effective. The extent

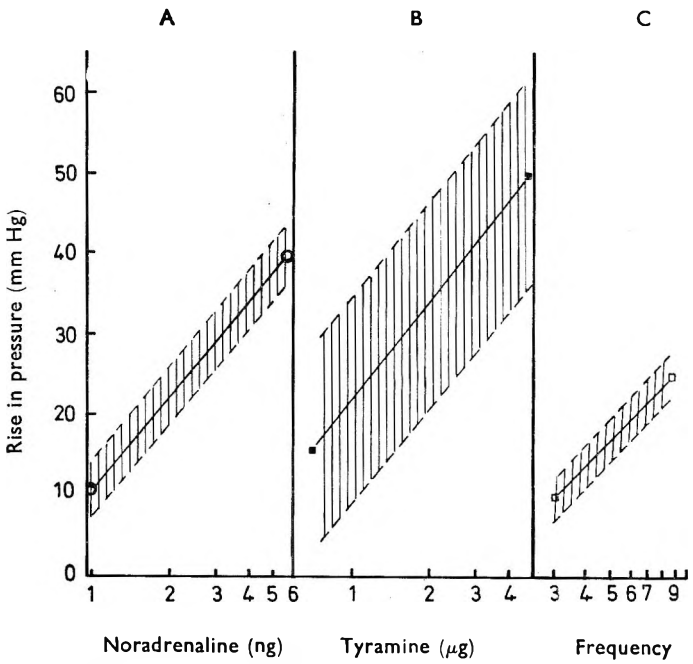

FIG. 5 Log dose effect curves for the actions of (−)-noradrenaline (A) tyramine (B) and periarterial stimulation (C) on the perfused tail artery of the rat. These curves are regression lines calculated from data supplied by 38, 9 and 23 arteries respectively. The shaded areas within broken lines depict the standard deviations of the slopes.

of the reduction in the response to a fixed dose, administered several times, was directly related to dose frequency. Tachyphyllaxis to vasopressin was less marked than to angiotensin but was extreme for bradykinin. The development of tachyphyllaxis to one pressor agent did not affect responses to any other.

The preparation was remarkably insensitive to histamine and regularly failed to respond to 40 μg.

DISCUSSION

An isolated perfused rat tail artery as described is very easy to set up and is exceptionally sensitive to catecholamines. The artery can either be used as a low-tone preparation where the response to vasoconstriction is a function of the extent to which the lumen is narrowed, or a high-tone preparation where the response is dependent on the period the lumen remains closed. Sensitivity is enhanced by preselection of high-tone low flow conditions, and a change in the slope of a log dose response relation can be artificially generated by selection of a flow rate which produces an arterial lumen obliterated by high doses and merely reduced by lower doses of a vasoconstrictor agent.

The perfused tail artery of the rat has been found to be very sensitive to periarterial stimulation. These effects of periarterial stimulation are in very large part mediated by the sympathetic nervous system since they are readily reduced to 10% of control values by phentolamine, guanethidine or prior treatment with reserpine, and are potentiated by cocaine. Postganglionic neurons are alone involved since the response to periarterial stimulation is unaffected by pentolinium and atropine. Amphetamine reversed the effects of guanethidine as would be expected from the work of Day & Rand (1963) who showed amphetamine to be a competitive antagonist of guanethidine. Observations made by these former workers do not, however, explain the gross potentiation caused in the responses of these arteries to periarterial stimulation which resulted from excessive concentrations of amphetamine. Day & Rand found that doses of dexamphetamine larger than those required to antagonize the blocking action of guanethidine abolished and did not potentiate the responses of the nictitating membrane, ileum or vas deferens to nerve stimulation.

Readily reproducible parallel effects of phentolamine on the dose-effect curves for noradrenaline and the frequency-effect curve for periarterial stimulation were found only when phentolamine mesylate was applied to the extra luminal surface of the artery. These observations contrasted with the rapid abolition of responses, even to 500 ng noradrenaline intra-arterially and more gradual reduction of the effects of periarterial stimulation by 10 ng/min phentolamine mesylate intraluminally.

Since the completion of this work, two papers by Hinke & Wilson (1962), have been encountered. These authors have examined the tail artery of the rat for its elastic properties and its responses to changes in the electrolyte composition of its environment.

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The influence of the monoamine oxidase inhibitor pargyline hydrochloride on the retention of dopamine in the isolated perfused spleen of the cat

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Much of the dopamine accumulated in the cat spleen after the intravenous infusion of this amine into the anaesthetized animal appears in the venous effluent when the spleen is subsequently isolated and perfused with a dopamine-free medium. Pre-treatment of the animals with the monoamine oxidase inhibitor pargyline hydrochloride has no effect on the rate of decline of the spleen content of dopamine resulting from perfusion, but appears to significantly reduce the amount of dopamine that can be removed from the spleen in this way. The significance of these findings is discussed in relation to a "false" neurochemical transmitter hypothesis for the antihypertensive effects of pargyline.

We have recently shown that perfusion, with a catecholamine-free medium, of cat isolated spleens containing relatively large amounts of endogenous dopamine caused the appearance of dopamine in the venous effluent (Street & Roberts, 1969). The amount of endogenous dopamine washed out declined exponentially with time, a feature which resembled the situation described by Iversen (1965) for the disappearance of adrenaline and noradrenaline from the rat heart following their accumulation by the $Uptake_2$ process. We considered our finding to be evidence for the occurrence of endogenous dopamine in $Uptake_2$ storage sites, which are presumably not "nerve releasable".

We are currently investigating the influence of monoamine oxidase inhibition on the accumulation of dopamine as a "false" neurochemical transmitter. During the course of some of these experiments it was found that when spleens from cats infused with dopamine were isolated and perfused with McEwen solution large amounts of dopamine appeared in the venous outflow.

We have been interested, therefore, in investigating the influence of monoamine oxidase inhibition on the disappearance of accumulated dopamine from the cat isolated perfused spleen.

EXPERIMENTAL

Methods

Male or female cats (2·4 to 3·5 kg) were anaesthetized with chloralose (8 ml/kg of a 1% w/v solution in 0·9% w/v saline) injected into a femoral venous cannula following induction with ether. Some of these cats were pre-treated with pargyline hydrochloride (50 mg/kg) injected subcutaneously 16 h before the experiment.

Dopamine (10 mg/kg) was infused intravenously at a constant rate over 45 min and when the sympathomimetic response had subsided (15 min after terminating the infusion in untreated cats and 90 min after terminating the infusion in pargyline-treated cats), portions of spleen (Dearnaley & Geffen, 1966) were isolated and perfused with McEwen solution (Thoenen, Hurlimann & Haefely, 1963). From some spleen portions venous effluent was collected at various times (up to 70 min) after the start of the perfusion, as 1 min samples in ice-cold centrifuge tubes containing 0·5 ml 2 N HCl, 0·2 ml 5% EDTA solution and a few mg ($\approx 2-5$) of ascorbic acid. Any red blood cells were removed by centrifugation and proteins were precipitated with perchloric acid at a final concentration of 0·4 N. Dopamine was isolated by modification of the method of Bertler, Carlsson & Rosengren (1958) using Dowex 50 WX-8 cation-exchange resin, 200–400 mesh, hydrogen form, dimensions 7 mm^2 $\times$ 25 mm, and was assayed by the method of Laverty & Sharman (1965). Other spleen portions were homogenized, before perfusion or after 30 min or 60 min perfusion, in 8–16 volumes (depending on the size of the spleen and the volume required) 0·5 N perchloric acid containing a few mg ascorbic acid for 1 min in an Ultra-Turrax homogenizer. EDTA (1 mg/ml) was added to the extracts and dopamine was isolated and assayed as described above. In some experiments phenoxybenzamine hydrochloride (3·4 μg/ml) was added to the perfusing solution.

RESULTS

Dopamine in the venous outflow

The rate at which dopamine (ng/min) was washed into the venous effluent from each spleen portion declined rapidly during the perfusion period (Fig. 1); when this rate was plotted on a log scale a straight line was obtained indicating an exponential decline with time. Experiments with different spleen portions yielded lines of identical slope; pre-treatment of the animals with pargyline or the addition of phenoxybenzamine to the perfusion fluid, or both, failed to modify this slope (Fig. 2).

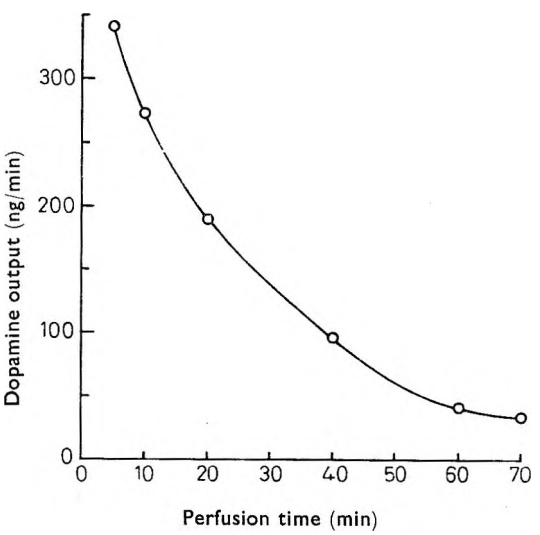

FIG. 1. The relation between the rate of output of dopamine (ng/min) and the time of perfusion of a cat isolated spleen containing dopamine accumulated during an *in vivo* infusion (10 mg/kg over 45 min).

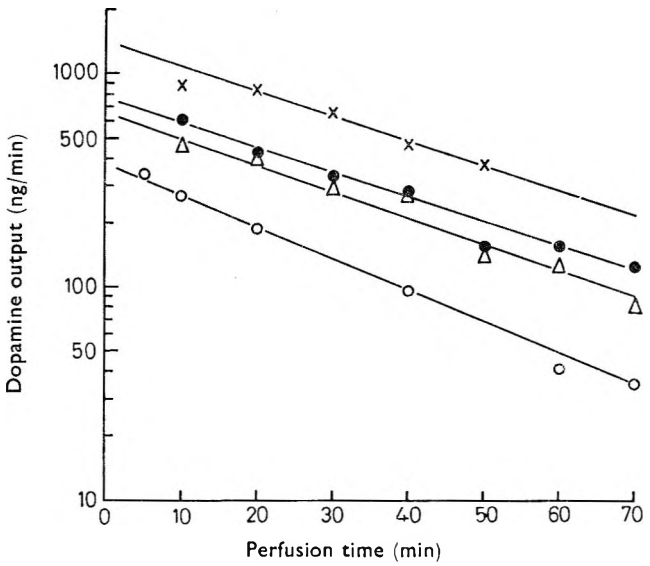

FIG. 2. The relation between the log rate of output of dopamine (ng/min) and the time of perfusion of cat isolated spleens containing dopamine accumulated during an *in vivo* infusion (10 mg/kg over 45 min). Open circles cat pre-treated with, and crosses, cat not pre-treated with, pargyline hydrochloride (50 mg/kg s.c. 16 h before experiment); triangles, cat not pre-treated with pargyline but phenoxybenzamine hydrochloride (3·4 μg/ml) added to the perfusing fluid; closed circles, cat pre-treated with pargyline and phenoxybenzamine added to the perfusing fluid.

Tissue content of dopamine

Perfusion of both untreated and pargyline-treated spleens reduced their dopamine (ng/μmol DNA-P) content (Table 1). Although these spleens contained similar concentrations of dopamine at the start of the perfusion, the amount remaining after 30 min perfusion of pargyline pre-treated spleens was considerably greater than that after similar perfusion of untreated spleens. Experiments after 60 min perfusion confirmed that dopamine was being removed less rapidly from the spleens in the presence of pargyline. In each case, the tissue concentration declined in an exponential

Table 1. *The influence of pargyline hydrochloride on the effect of perfusion with McEwen solution on the dopamine content of cat isolated spleen preparations containing dopamine accumulated during* in vivo *infusions.* Dopamine content in ng/μmol DNA-P. Pargyline hydrochloride (50 mg/kg) administered subcutaneously 16 h before the experiment and dopamine (10 mg/kg) infused intravenously over 45 min. Spleens removed 15 min (untreated cats) and 90 min (pargyline treated cats) after the termination of the dopamine infusions.

Pretreatment	Perfusion time (min)		
	0	30	60
Saline	124·24	44·08	23·56
	146·34	69·89	
	158·95	65·13	
	Mean 143·18	Mean 59·70	
Pargyline hydrochloride	134·63	71·13	63·69
	139·29	91·51	
	141·00	127·73	
	Mean 138·31	Mean 96·79	

manner since plots of the tissue content on a log scale against the perfusion time again resulted in the production of straight lines (Fig. 3). With untreated spleens the slope of this line was identical to that relating dopamine wash-out to perfusion time (Fig. 2), while with pargyline-treated spleens this slope was greatly reduced.

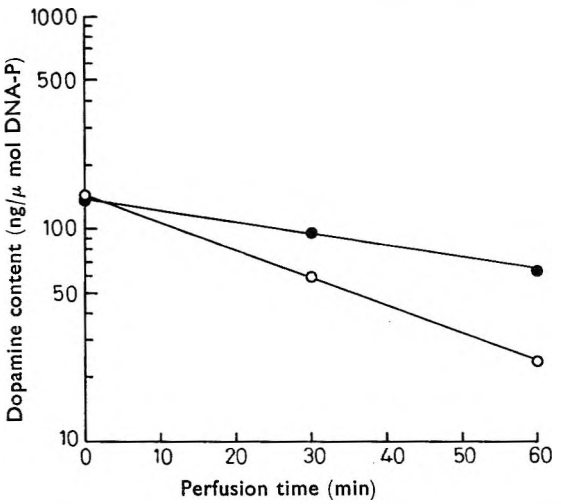

FIG. 3. The relation between the log dopamine content (ng/μmol DNA-P) and the time of perfusion of the cat isolated spleen containing dopamine accumulated during an *in vivo* infusion (10 mg/kg over 45 min). Closed circles, cat pre-treated with, and open circles, cats not pre-treated with, pargyline hydrochloride (50 mg/kg s.c. 16 h before the experiment). All points represent the means of 3 experiments except those after 60 min perfusion which are single observations (data from Table 1).

DISCUSSION

The exponential decline in the rate at which dopamine was washed out in these experiments is similar to that observed for noradrenaline and adrenaline by Iversen (1965) following the accumulation of high perfusion concentrations of both amines by the $Uptake_2$ process in rat isolated heart preparations. It seems not unreasonable, therefore, to assume that the large amount of infused dopamine in the present experiments is being accumulated into similar $Uptake_2$ storage sites in the cat spleen. We have already suggested, for similar reasons, that endogenous dopamine can occur in such sites (Street & Roberts, 1969).

Iversen (1965) observed many similarities between the kinetic and drug susceptibility properties of the $Uptake_2$ process and those of the mechanism by which accumulated amine disappeared during subsequent perfusion with a catecholamine-free medium. He found that phenoxybenzamine inhibited $Uptake_2$ in the rat heart and the experiments of Gillespie & Hamilton (1966) may be taken to indicate that a similar inhibition occurs in the cat spleen. In the present experiments, however, phenoxybenzamine appears not to have an inhibitory effect on the disappearance of accumulated dopamine since the rate constant for the wash-out process was unchanged; by the same token, there is presumably no re-uptake of amine during this wash-out process even in the absence of phenoxybenzamine.

We have found that the application of mathematical equations to the wash-out process simplifies our interpretation of the influence of pargyline in these experiments.

The decline in tissue content of dopamine is thus described by a simple exponential equation of the form:

$$x = ce^{-at} \text{ from which } \log x = \log c - at$$

where x is the tissue content at time t, c is the tissue content at the beginning of the perfusion (when t = 0) and a is the rate constant of the wash-out process.

The decline in the rate of wash-out of dopamine into the perfusing fluid is similarly described by an equation of the form

$$\frac{dy}{dt} = ace^{-at} \text{ from which } \log\frac{dy}{dt} = \log ac - at$$

where y is the total amount of dopamine washed into the perfusing fluid after time t.

Since log dy/dt plotted against t (Fig. 2) produces the same rate constant for untreated and pargyline-treated spleens it may be concluded that the wash-out process is not influenced by pargyline. The slopes of the lines from log x against t plots (Fig. 3) however were influenced by pargyline. The slope of this line from experiments with spleens from untreated cats was the same (i.e. equal to a) as that of the log dy/dt against t lines, and the disappearance of dopamine from such spleens is therefore in accord with the simple equations given above. With spleens from pargyline-treated cats, however, the slope of the log x against t line was much less than a so that the simple equations cannot apply. We feel that the most logical explanation of these findings is that although pargyline has no effect on the mechanism of release from $Uptake_2$ storage sites, the amount of dopamine which can be washed out of the tissue by this process is greatly reduced. Thus if p is assumed to represent a certain amount of dopamine which is not available to the wash-out mechanism, then a linear relation with slope a would only be observed between log (x-p) and t in that

$$\log (x - p) = \log (c - p) - at$$

Under these conditions

$$\log x = \log [(c - p)e^{-at} + p] \text{ and } \log\frac{dy}{dt} = \log a(c - p) - at$$

We would postulate, therefore, that the monoamine oxidase inhibitor pargyline hydrochloride changes the location of some of the dopamine accumulated in the cat spleen during an *in vivo* infusion so that it becomes resistant to wash-out. In support of the hypothesis that the antihypertensive effects of monoamine oxidase inhibitors might be associated with the accumulation of dopamine as a weakly sympathomimetic "false" neurochemical transmitter, it is tempting to suggest that this change might represent a subcellular re-distribution of dopamine from non-nerve releasable stores to nerve releasable stores.

Acknowledgement

We are grateful to Abbot Laboratories Ltd. and Smith, Kline & French Laboratories Ltd. for gifts of pargyline hydrochloride and phenoxybenzamine hydrochloride respectively.

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Effects of convulsant barbiturates on vascular smooth muscle

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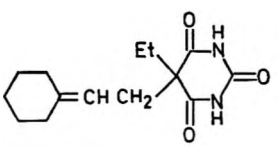
The convulsant barbiturate, 5-(2-cyclohexylidene-ethyl)-5-ethyl barbituric acid (CHEB), produces contraction in rabbit aortic strips. Contractions effected by either CHEB or tyramine were preceded by a lag time and both agents induced tachyphylaxis; however, cross-tachyphylaxis could not be demonstrated. Phenoxybenzamine and atropine failed to affect CHEB-induced responses, whereas pentobarbitone selectively blocked and also reversed CHEB contractions. Prevention, but not reversal, of tachyphylaxis was also accomplished with pentobarbitone. These results suggest that CHEB does not act through the release of noradrenaline or acetylcholine; nor does it exert an effect on the receptors for these amines or on those for histamine. Pentobarbitone, however, appears to compete with CHEB for common receptors. Another convulsant barbiturate, 5-ethyl-5-(dimethylbutyl)barbituric acid (DMBB), and its optical isomers were also examined. The racemic mixture had no contractile activity, but the (+)-isomer elicited CHEB-like effects. The (−)-isomer, on the other hand, was like pentobarbitone in that it antagonized both CHEB- and (+)-DMBB-induced contractions. These studies illustrate that convulsant barbiturates are able to stimulate vascular smooth muscle; therefore, it is suggested that the rabbit aortic strip may serve as an *in vitro* working model for study of the mechanism of action of these drugs in the central nervous system.

Convulsant barbiturates have usually been examined for their ability to produce central nervous system (CNS) excitation in contrast to the depression characteristically associated with hypnotic barbiturates. While studying the CNS activity of 5-(2-cyclohexylidene-ethyl)-5-ethyl barbituric acid (CHEB; I), a convulsant barbiturate, Downes, H. & Williams, J. K. (personal communication) observed that the drug produced a large increase in blood pressure in unanaesthetized spinal cats. We found it to induce a contraction on the rabbit aortic strip. This report describes the results of experiments designed to study some characteristic effects of CHEB on this preparation. In addition, a comparison of some of the actions of CHEB was made with another convulsant barbiturate, 5-ethyl-5-(dimethylbutyl)barbituric acid (DMBB; II), and its two optical isomers.



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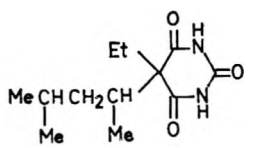

II

Recently, Perry, Downes & Karler (1969) reported that the convulsant activity associated with the racemic mixture of DMBB resides in the (+)-isomer which is a potent convulsant, whereas the (−)-isomer has primarily depressant activity. Like CHEB, the (+)-isomer of DMBB produces a contraction of the aortic strip. It is suggested that this smooth muscle preparation may serve as a working model for the study of the mechanism of action of the convulsant barbiturates on the CNS.

EXPERIMENTAL

Materials and methods

Aortic strips were obtained from 2·0–3·5 kg rabbits killed by rapid injection of air into an ear vein. Spiral strips, 3–4 mm in width and 20–30 mm in length, were prepared according to Furchgott (1960). The aortic segments were mounted vertically in jacketed 30 ml tissue baths maintained at 37·5°, and the tissues were bathed in Krebs bicarbonate solution gassed with 5% carbon dioxide in oxygen. The fluid in the bath was exchanged by overflow. Inactivation of catecholamines was retarded by the presence of the sodium salt of ethylenediamine tetra-acetic acid ($1{\cdot}0 \times 10^{-5}$M) in the Krebs bicarbonate solution.

A Grass stain gauge transducer (FT03C) and model 5 Polygraph were used to measure isometric contractions. Drug effects were recorded as mm of pen deflection at a sensitivity of 0·2 mV/cm and a chart speed of 0·25 mm/s.

The drugs used were: noradrenaline bitartrate (Sterling-Winthrop, New York, N.Y.); histamine dihydrochloride (Eastman Organic Chemicals, Rochester, N.Y.); acetylcholine bromide (Eastman Organic Chemicals, Rochester, N.Y.); tyramine hydrochloride (Mann Research Laboratories, New York, N.Y.); atropine sulphate (Mallinckrodt Chemicals, St. Louis, Mo.); sodium pentobarbitone (Robinson Laboratory Inc., San Francisco, Calif.); sodium phenobarbitone (Merck & Co.,

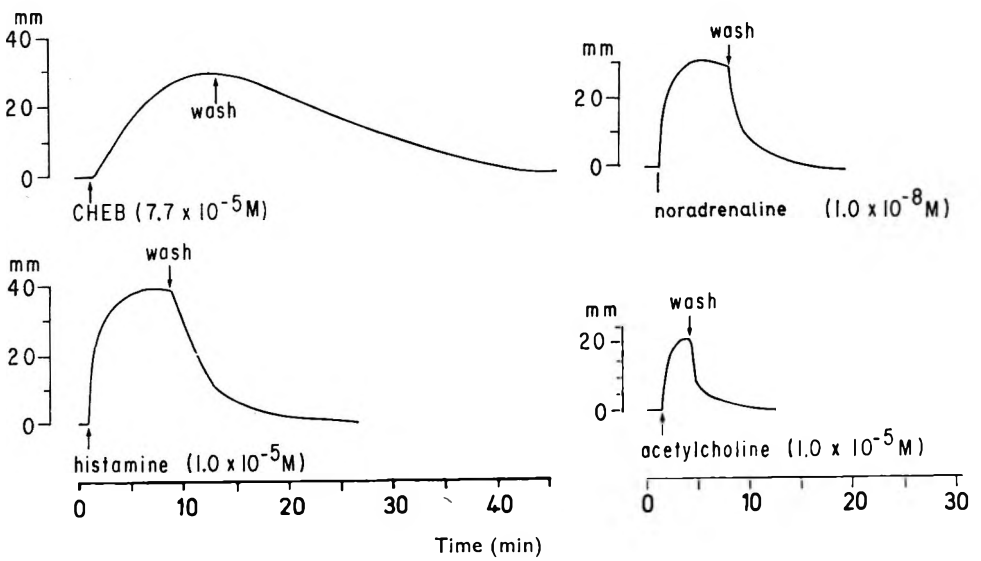

FIG. 1. Response of the aortic strip to CHEB and other agonists. Tissue bathed in a 37·5° Krebs bicarbonate solution. The responses are copies of original recordings with a compressed time scale. The bathing solution containing drug was replaced at the peak of contraction, as indicated by the term *wash* in each schematic response.

Rahway, N.J.); phenoxybenzamine hydrochloride (Smith Kline & French, Philadelphia, Pa.); the sodium salts of CHEB and DMBB, and of the two optical isomers of DMBB (all prepared in our laboratory). Phenoxybenzamine hydrochloride was first dissolved in a small amount of propylene glycol and final concentrations were made by diluting with isotonic NaCl solution. All other drugs were dissolved in isotonic NaCl solution; the sympathomimetic amines were similarly prepared and, in addition, were in 0·01M HCl. Drug solutions were added to the tissue bath in a volume of 0·5 ml or less.

RESULTS

Effect of CHEB on rabbit aortic strips. The addition of CHEB to the muscle bath caused a contraction of the aortic strip (Fig. 1). The onset of the effect and the recovery after wash out are slower than those of noradrenaline, histamine or acetylcholine. The (+)-isomer of DMBB displayed effects similar to those of CHEB; but the (−)-isomer did not produce contraction.

Effect of repeated exposure to CHEB. Repeated exposure of the aortic muscle preparation to CHEB produced tachyphylaxis (Fig. 2A). The (+)-isomer of DMBB caused a similar effect (Fig. 2B).

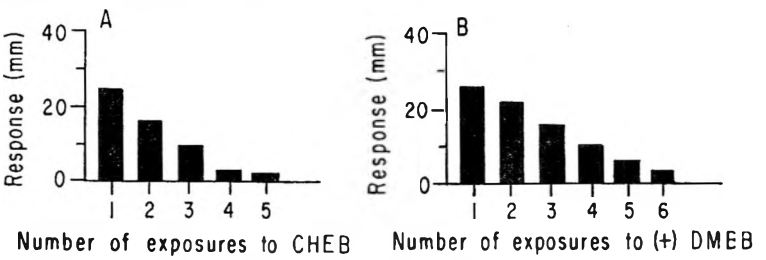

FIG. 2. (A) Effect of repeated exposures to the same concentration ($7·7 \times 10^{-5}$M) of CHEB. The figure illustrates data from a typical experiment. The bars of the histogram represent the magnitude of the peak responses.

(B) Effect of repeated exposures to the same concentration ($1·5 \times 10^{-4}$M) of the (+)-isomer of DMBB. The figure illustrates data from a typical experiment. The bars of the histogram represent the magnitude of the peak responses. In both A and B, each exposure was followed by a wash procedure.

Effect of pentobarbitone and phenobarbitone on the CHEB response. The prior addition of pentobarbitone ($5·5 \times 10^{-5}$M) to the tissue bath blocked the contraction produced by CHEB ($7·7 \times 10^{-5}$M) (Table 1); after washing pentobarbitone from the bath, a normal CHEB response could be elicited. Phenobarbitone was also able to block the CHEB-induced contraction, but in a concentration approximately ten times

Table 1. *Effect of pentobarbitone on the CHEB response*

Treatment (in order of exposure)	Concentration (M)	Response (mm)
Noradrenaline	$1·0 \times 10^{-7}$	31
Pentobarbitone + noradrenaline	$5·5 \times 10^{-5}$ $1·0 \pm 10^{-7}$	32
Pentobarbitone + CHEB	$5·5 \times 10^{-5}$ $7·7 \pm 10^{-5}$	0
CHEB	$7·7 \times 10^{-5}$	32
Noradrenaline	$1·0 \times 10^{-7}$	32

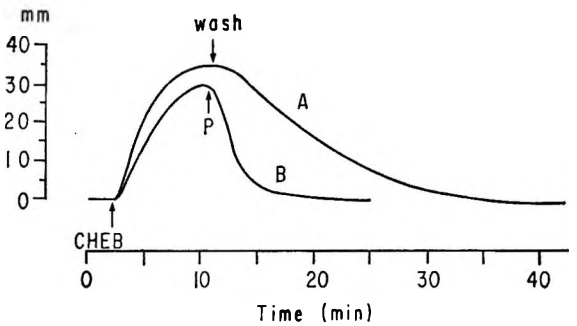

FIG. 3. Effect of (A) the wash and of (B) the addition of a blocking concentration of pentobarbitone ($5{\cdot}5 \times 10^{-5}$M) on the rate of relaxation of a CHEB-induced contraction (P = pentobarbitone). The different treatments were applied at the peak of response.

that of pentobarbitone. The blocking concentrations of both drugs did not influence the control response to noradrenaline.

The interaction between pentobarbitone and CHEB was observed in two other types of experiments. Fig. 3 shows the influence of pentobarbitone on the relaxation rate of CHEB-induced contraction. When pentobarbitone was added to the bath at the peak of a CHEB contraction, the muscle relaxed more rapidly than it did after washing out CHEB. The data in Fig. 4 illustrate another interaction between CHEB and pentobarbitone in which pentobarbitone not only blocked the CHEB contraction, but also protected the muscle against the effect of repeated exposures to CHEB. Fig. 4A shows that a CHEB response was unchanged even after several exposures of the muscle to CHEB in the presence of pentobarbitone; therefore, pentobarbitone blocked the development of tachyphylaxis. The results shown in Fig. 4B demonstrate that pentobarbitone could not reverse an existing CHEB-induced tachyphylaxis.

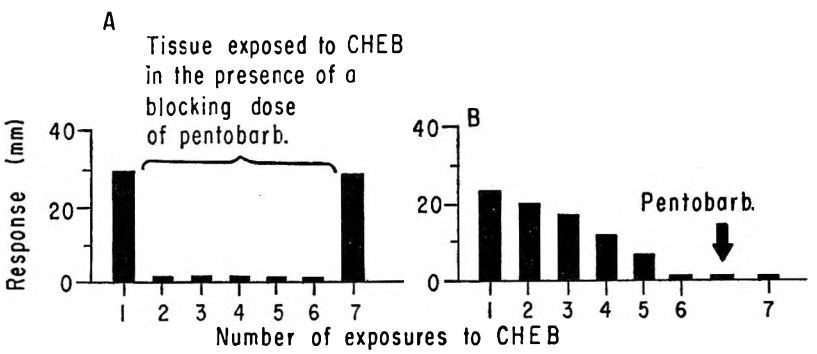

FIG. 4. The influence of pentobarbitone on the tissue response to CHEB. In (A), the initial and final responses were produced by CHEB alone in a concentration of $7{\cdot}7 \times 10^{-6}$. The intervening exposures to the same concentration of CHEB were made in the presence of a blocking dose of pentobarbitone ($5{\cdot}5 \times 10^{-5}$M). In (B), tachyphylaxis was produced by repeated exposures to CHEB. The arrow indicates the exposure of the muscle to pentobarbitone ($5{\cdot}5 \times 10^{-5}$M) for a 10-min period. In both (A) and (B), each exposure was followed by a wash procedure.

Pentobarbitone, previously placed in the tissue bath, also blocked the contraction produced by the (+)-isomer of DMBB; but doses reversing the CHEB contraction (Fig. 3) did not reverse the (+)-DMBB response.

Effect of noradrenaline depletion on the CHEB response. Tachyphylaxis to certain drugs has been explained on the basis of a depletion of transmitter substance. For example, the depletion of noradrenaline stores in tissue has been invoked as an explanation of tyramine-induced tachyphylaxis. To test the possibility that CHEB acts indirectly by stimulating the release of noradrenaline, the influence of tyramine-induced tachyphylaxis on the CHEB response was studied. No significant difference was found between the CHEB response after tyramine-induced tachyphylaxis and the control CHEB contraction at CHEB concentrations of $2{\cdot}0 \times 10^{-4}$–$5{\cdot}0 \times 10^{-5}$M. Furthermore, tissue rendered insensitive to CHEB responded normally to tyramine. Cross-tachyphylaxis was therefore absent.

Table 2. *Effect of atropine on the CHEB response*

Treatment	Concentration (M)	Control response (mm)	Response after atropine (1×10^{-5}M) (mm)
Acetylcholine	$1{\cdot}0 \times 10^{-5}$	24	0
Noradrenaline	$5{\cdot}0 \times 10^{-8}$	41	39
CHEB	$1{\cdot}0 \times 10^{-4}$	39	26

Effect of atropine on the CHEB response. The ability of atropine to block CHEB-induced contractions was examined in four experiments. Atropine was placed in the bath 5 min before an agonist and the results in Table 2 show that atropine, in a concentration that blocked an acetylcholine response, was ineffective against a contraction produced by either noradrenaline or CHEB. Although atropine appears to reduce the CHEB response in this experiment, the diminished second contraction can be explained by the occurrence of tachyphylaxis. Experiments of the type depicted in Fig. 2A demonstrated that a 10–40% reduction in the contractions produced by CHEB occurs with the second exposure of the tissue to the drug. Atropine did not produce a greater reduction in the CHEB responses than could be accounted for by tachyphylaxis; therefore, it did not exert any antagonism to CHEB.

Effect of phenoxybenzamine on the CHEB response. In three experiments, the influence of phenoxybenzamine on the contraction produced by CHEB and other agonists was studied. Phenoxybenzamine was placed in the muscle bath 45 min before the agonists. Table 3 shows that the α-receptor blocking drug blocked the noradrenaline contraction and greatly reduced the histamine response. On the other hand, it had little influence on an acetylcholine-induced contraction, and the CHEB response was not diminished more than expected from the occurrence of tachyphylaxis.

Table 3. *Effect of phenoxybenzamine on the CHEB response*

Treatment	Concentration (M)	Control response (mm)	Response after phenoxybenzamine ($1{\cdot}0 \times 10^{-8}$M) (mm)
Noradrenaline	$1{\cdot}0 \times 10^{-6}$	23	0
Acetylcholine	$1{\cdot}0 \times 10^{-4}$	6	5
Histamine	$1{\cdot}0 \times 10^{-5}$	28	10
CHEB	$7{\cdot}7 \times 10^{-5}$	12	10

DISCUSSION

CHEB initiated a contraction in the rabbit aortic strip after a brief lag time (30–60 s). This lag time is characteristic; it did not occur with the direct-acting agonists, noradrenaline, acetylcholine or histamine. However, tyramine, an indirect-acting agent, displayed a lag time comparable to that of CHEB. The similarity suggests that CHEB might exert its action on smooth muscle in a manner analogous to that of tyramine. The onset of contraction induced by the (+)-isomer of DMBB was also preceded by a lag time.

Burn (1959) reported that the vasoconstriction caused by thiopentone in normal rabbits could be prevented if the tissue stores of noradrenaline were first depleted by pretreatment with reserpine. It appears that the release of noradrenaline accounts for the vasoconstrictor activity of thiopentone. Furchgott (1963) has shown that tachyphylaxis to tyramine in rabbit aortic strips results from the depletion of releasable noradrenaline. Both CHEB and the (+)-isomer of DMBB produced tachyphylaxis, suggesting that they may also act through the release of a biologically active substance. Further study has since demonstrated that cross-tachyphylaxis exists between CHEB and the (+)-isomer of DMBB. The existence of cross-tachyphylaxis implies that these agents possess a similar mode of action, perhaps release of an active substance from a common pool. Tyramine also caused tachyphylaxis; however, cross-tachyphylaxis between CHEB and tyramine did not exist. Its absence indicates that CHEB and tyramine either do not release the same substance or, if the same substance is released, it must be from a different pool.

Furchgott (1954) observed that the blockade of α-adrenergic receptors also reduced the response to histamine and 5-hydroxytryptamine; therefore, response to an agent acting through the release of any of these substances or at their receptor level should be markedly reduced by phenoxybenzamine. In the present experiments, phenoxybenzamine did not exert any influence on the CHEB-induced contraction. On the basis of these findings, it appears that CHEB does not act through a release of noradrenaline, histamine or 5-hydroxytryptamine; nor does it act directly on the receptors for these biogenic amines. These data support the conclusion that CHEB and tyramine do not act by release of the same substance.

Because atropine failed to influence the CHEB effect, it is unlikely that the contractile effect is mediated either indirectly through the release of acetylcholine or directly at cholinergic sites. It was found that the acetylcholine-induced contraction can be selectively blocked by atropine without influencing the activity of either noradrenaline or CHEB.

Pentobarbitone appears to exert a specific antagonism towards the contractile activity of CHEB. In a concentration similar to that of CHEB, it has no effect on the response to noradrenaline, but blocked the response to CHEB. When this same dose of pentobarbitone was given at the peak of a CHEB-induced contraction, the muscle rapidly relaxed. These data suggest that pentobarbitone does not antagonize the action of CHEB by a physiological depression but possibly by a competition for common receptors.

Phenobarbitone can also block a CHEB-induced contraction, but the concentration required is ten times that of pentobarbitone. The difference in the blocking concentration may be explained on the basis of differences in lipid solubility; that is, in a methylene chloride:aqueous system, the partition coefficient of pentobarbitone is about ten times that of phenobarbitone.

When tissue was exposed to CHEB several times, tachyphylaxis developed. However, if the tissue was repeatedly exposed to CHEB in the presence of pentobarbitone and then to CHEB alone, the final CHEB response was equal in magnitude to the initial response. This prevention of tachyphylaxis lends further support to the conclusion that CHEB and pentobarbitone compete for a common receptor and that pentobarbitone is capable of antagonizing the interaction between CHEB and its receptor. Failure of pentobarbitone to reverse CHEB-induced tachyphylaxis indicates that CHEB probably produces contraction in smooth muscle through a chain of events, one of which is slowly reversible and responsible for the development of tachyphylaxis. Responsiveness to CHEB reappears only after time is allowed for this process to return to its original state.

Racemic DMBB, like CHEB, was reported to be convulsant in mice (Perry & others, 1969); however, unlike CHEB, it has no contractile effect on vascular smooth muscle. The *in vivo* studies established that the (+)-isomer of DMBB possesses the convulsant activity and that the (−)-isomer is depressant to the CNS. Our studies demonstrate that the (+)-isomer of DMBB exerted a CHEB-like effect on the aortic strips and the the (−)-isomer, like pentobarbitone, blocked contractions induced by both the (+)-isomer of DMBB and CHEB.

The close correlation between convulsant activity and the ability to induce vascular smooth muscle contraction suggests that the *in vitro* muscle preparation may serve as a working model for study of the mechanism of action of the convulsant barbiturates on the CNS.

Acknowledgements

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On the role of a central adrenergic mechanism in morphine analgesic action

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The influence of drugs interfering with brain monoamine metabolism on morphine analgesia of rats was estimated by the tail pressure method. Central adrenergic stimulation produced by apomorphine, cocaine, pyrogallol or amphetamine led to stereotyped behaviour and elevation of the pain threshold. Lower doses of these drugs potentiated morphine analgesic action. Reserpine, iproniazid and disulfiram weakened morphine analgesic action. α-Methyldopa increased morphine action and (±)-tryptophan did not influence it significantly.

Reserpine depletes brain catecholamine stores and antagonizes morphine analgesia (Schneider, 1954; Radouco-Thomas, Radouco-Thomas & LeBreton, 1957; Schaumann, 1958; Paeile & Munoz, 1966; Verri, Graeff & Corrado, 1967; Raevsky, 1969), so, too, does tetrabenazine (Takagi, Takashima & Kimura, 1964). On the other hand, Rudzik & Mennear (1965) believe that the antagonism of morphine by reserpine is not due to its action on catecholamine metabolism. A possible role of 5-hydroxytryptamine (5-HT) in morphine analgesia has also been considered (Medacović & Banić, 1964; Nićak, 1965). We now describe the relation between drugs which interfere with brain noradrenaline metabolism and morphine analgesic action.

EXPERIMENTAL

Analgesic activity of morphine was estimated by the change in pain threshold when mechanical pressure was applied to the tails of groups of ten white female rats, 150–220 g (Sangailo, 1962). The drugs, their doses, time and route of administration, are given in Table 1.

RESULTS AND DISCUSSION

The results are in Table 1 and illustrated in Figs. 1 and 2. We also found that apomorphine, 3–5 mg/kg, produced stereotypy and elevation in pain threshold and that amphetamine, 2 mg/kg, activated rats without producing any action on pain threshold, although at higher doses it produced stereotypy and raised the pain threshold. Also, α-methyldopa depressed the rats and elevated the pain threshold. Where the pain threshold increased, morphine was injected after the threshold had returned to the initial level.

Since Vogt's (1954) discovery of the depleting action of morphine on brain catecholamine, and Schneider's (1954) study of reserpine antagonism of morphine analgesia, the question of the significance of brain catecholamines in morphine action has attracted much attention (Radouco-Thomas & others, 1957; Schaumann, 1958; Takagi & others, 1964; Paeile & Munoz, 1966; Verri & others, 1967; Takagi & Nakama, 1966, 1968; Raevsky, 1969; Zakusov, 1969).

Table 1. *Doses, route and time of drug administration before morphine and its influence on morphine effect*

Drugs	Dose (mg/kg)	Route	Time before morphine (h)	Morphine HCl (mg/kg, s.c.)	Influence on morphine effect	
					Increase	Decrease
Pyrogallol	50	s.c.	1	2·5	+*	
Cocaine HCl	50	s.c.	1	2·5	+*	
(±)-Tryptophan	400	i.p.	4	2·5	+	
(±)-Tryptophan	400	i.p.	4	5·0	+	
α-Methyldopa	400	i.p.	23	2·5	+*	
α-Methyldopa	100	i.p.	4	4·0	+*	
Amphetamine	2	s.c.	1	2·5	+*	
Amphetamine	2	s.c.	1	4·0	+*	
Disulfiram	50	i.p.	2	2·5		—
Disulfiram	50	i.p.	2	4·0		—*
Iproniazid	100	i.p.	4	2·5		—*
Iproniazid	100	i.p.	8	5·0		—*
Reserpine	1	i.p.	8	5·0		—*

* Significant ($P < 0{\cdot}05$).

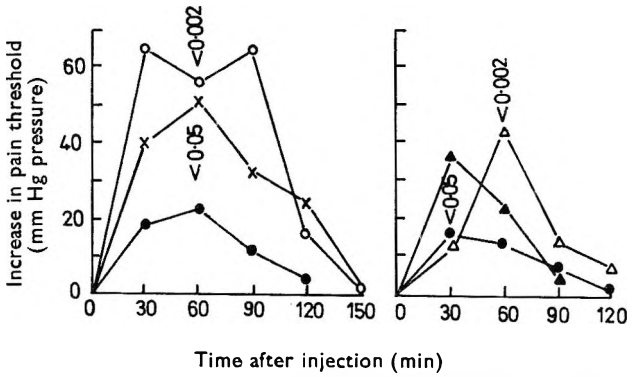

FIG. 1. Interaction of some drugs, interfering with brain noradrenaline metabolism and morphine. Each point represents the means of ten animals treated with morphine, 2·5 mg/kg alone (●) and morphine after pyrogallol (○), cocaine (×), α-methyldopa, 400 mg/kg (▲), amphetamine (△). Doses and routine are in Table 1.

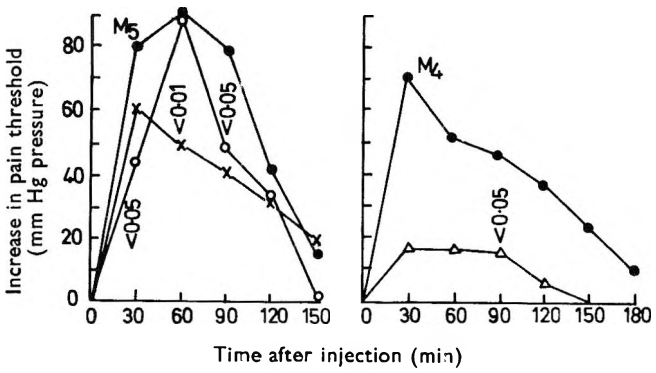

FIG. 2. Interaction of some drugs, interfering with brain noradrenaline metabolism and morphine. Morphine, 4 mg/kg (M_4) and 5 mg/kg (M_5) action on pain threshold and change in activity after reserpine (○), iproniazid (×) and disulfiram (△). Doses and routine are in Table 1.

Inhibition of the re-uptake process by cocaine or depression of catechol-*O*-methyl transferase by pyrogallol (Fig. 1) or by apomorphine (Belenkii & others, 1966) produced stereotypy and elevated the pain threshold. In doses having no visible effect on behaviour (Table 1), these drugs potentiated the analgesic effect of morphine. It seems likely that the described effects follow the increase of noradrenaline at the receptor sites. It seems significant that iproniazid antagonized morphine analgesia, since inhibition of monoamine oxidase could inhibit release, reduce the rate of turnover or deplete the brain noradrenaline stores (see Glowinski & Baldessarini, 1966). The ability of amphetamine to potentiate morphine analgesia and evoke stereotype behaviour and elevation in the pain threshold after increasing the dose results from its direct and indirect sympathomimetic activity. Reserpine, by depleting noradrenaline from the brain, weakened morphine analgesic action significantly 30 and 90 min after morphine administration, while the maximum activity remained unchanged (Fig. 2).

Disulfiram, an inhibitor of dopamine-β-hydroxylase, inhibited morphine analgesia probably by depressing the noradrenaline formation from dopamine. α-Methyldopa increased the pain threshold. Morphine after the threshold returned to the initial level, caused a greater elevation of pain threshold than it did alone.

Tryptophan, a precursor of 5-HT, increased morphine analgesic action but not significantly.

From the results it may be concluded that central sympathetic activation can elevate the pain threshold. Drugs which increased noradrenaline concentration at the receptor sites potentiated, and the drugs which decreased it weakened, morphine analgesic action.

On the basis of the results obtained we support the idea that morphine analgesia is a result of liberation of noradrenaline from the brain stores. The direct action of morphine on the central adrenostructures may be kept in mind, but this mechanism seems to be less important.

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The determination of cellulase activity by viscometry

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The enzymatic activity of cellulase has been estimated by the decrease of viscosity of a hydroxyethylcellulose solution as a function of incubation time. This decrease is a function of the applied shear stress. It is proposed that the viscosity be measured in an ordinary Ostwald viscometer under conditions where the viscosity is Newtonian, that is, the decrease of viscosity by enzymatic activity is independent on the shear stress.

Methods for determining the activity of cellulase preparations have been recently discussed by Courtois & Bui Khac Diep (1965; 1967) who recommended the use of hydroxyethylcellulose as the most suitable substrate. It is accepted that the cellulose derivatives are attacked only if the degree of substitution is small, and these authors recommended an appropriate preparation procedure. The starting material is Whatman Cellulose Powder (CF II for chromatography), and this is hydroxylated at room temperature by ethylene oxide in alkaline solution.

Viscometry has been recommended for the assay of hydrolases and is most useful for the assay of enzymatically catalysed reactions consisting of simultaneously and successive breakdown of a polymeric molecule, which cannot be defined by a simple molecular scheme.

The enzyme response could be assayed by the rate of release of the reducing groups, as has been shown by Courtois & Bui Khac Diep (1965; 1967) the determination of the reducing saccharides liberated by hydrolysis of the β-1,4-linkages, and the quantitative determination of the glucose in the hydrolysate, lacked accuracy and reproducibility. The viscosities of the solutions were measured in a Haage Rotovisco apparatus. The decrease in viscosity of the solutions, being in direct relation to the amount of cellulase incubated, allowed for an easy assay in the initial period of breakdown of the proposed substrate.

Our aim has been to effect closer control of some physical factors involved and of the precision. For a control method a capillary viscometer of simple construction was thought to fit the laboratory needs and to give more dependable results.

Cellulose ethers can be considered as random coiled macromolecules (Ferry, 1961). The position of each segment (subunit) of the macromolecule relative to its neighbours gives rise to a configurational entropy, S, according to the Boltzmann formula:

$$S = k \ln P$$

where S = entropy, k = Boltzmann constant, P = number of configurations of the random coiled macromolecule.

Each linkage of such a subunit acts as a so called "entropy spring". By the action of viscous flow, the position of a segment relative to its neighbours changes, causing a change in configurational entropy. On deformation there will be some energy

storage. Further calculations indicate that such a solution behaves as a non-Newtonian fluid (Ferry, 1961).

Of the different devices for measuring viscosity, we considered the movement of concentric cylinders (Couette type) and the rate of flow through a capillary type viscometer (Phillipoff, 1942; Van Wazer, 1966).

The Haaze Rotovisco viscometer was recommended by Courtois & Bui Khac Diep (1967), and satisfactory results were obtained with collaborative tests made under the auspices of the F.I.P. Commission for standardization of pharmaceutical enzymes. But owing to the cost it seemed desirable also to develop a method using an ordinary capillary viscometer.

Methods of expressing the enzymatic activity

The enzymatic activity may be calculated by a formula like that of Courtois & Bui Khac Diep (1967).

$$\text{Enzymatic activity} = \frac{-1}{P} \cdot \frac{100 \times \left(\frac{d\eta}{dt}\right)}{\eta_0 - \eta_w}$$

where P = the amount of cellulase in the test solution (in mg), $d\eta/dt$ the decrease of viscosity η as a function of the incubation time t over the linear part of the $\eta - t$ curve, η_0 = the viscosity of the test solution at zero incubation time (blank value) and η_w = the viscosity of water.

Although using this formula no absolute calibration of the viscometer is necessary, the value of η_w is low relative to η_0 and it is difficult to determine accurately. Also this formula requires that $(d\eta/dt)/(\eta_0 - \eta_w)$ is constant, but it is also a function of the initial viscosity η_0, and experiment shows that if η_0 undergoes a small variation $\delta\eta_0$, due to the preparation or pipetting of the initial solution, $d\eta/dt$ is not affected, so the value of enzyme activity depends on the value of η_0, even though the value of $d\eta/dt$, which measures the enzymatic activity of the cellulase solution, remains reasonably constant. As this method is liable to errors which have nothing to do with the action of the enzyme itself, it seems more realistic to define enzyme activity as:

$$-\frac{1}{P}\frac{d\eta}{dt}$$

which gives more reliable results (η being expressed in $mNsm^{-2}$ and t in s). The hydroxycellulose sample must show a linear decrease of the viscosity due to the action of the enzyme.

EXPERIMENTAL

Apparatus

For the Rotovisko viscometer used, the cup was of the DMK type. The viscometer was calibrated with a heavy oil with a viscosity $\eta = 1{\cdot}62$ Nsm^{-2}, showing Newtonian behaviour.

The capillary viscometer used was of the Ostwald type and so constructed that the efflux time was about 30 s for the hydroxyethylcellulose solution. This viscometer was calibrated with an aqueous 50% glycerol solution, the viscosity of which was determined using another capillary viscometer with an efflux time for water at 25° of about 60 s ($\eta = 893{\cdot}7\,\mu$ Nsm^{-2}). In this way the short efflux time for the water in the measuring viscometer can be deduced. A fast efflux time for the measuring viscometer (30 s) has the advantage of minimizing the error on incubation time of the enzyme solution.

Preparation of hydroxyethylcellulose and its solutions

The hydroxyethylcellulose ether was prepared according to Courtois & Bui Khac Diep (1967). As enzymatic assay results may depend on the sample of hydroxyethylcellulose used (degree of polymerization, degree of substitution and homogeneity), samples showing a linear decrease in viscosity as a function of the incubation time were sought. Solutions were prepared by swelling a known amount of a sample in water over 12 to 20 h. After diluting with water to the mark, the whole solution was ultrasonicated or treated in a Servall mixer for 10 min. This solution was allowed to stand for 24 h to reach equilibrium.

Rheological behaviour of hydroxyethylcellulose solutions

To test the rheological behaviour of hydroxyethylcellulose solutions in the capillary viscometer, the pressure difference, Δp, was changed by an electromagnetic manostat.

Cellulase activity was assayed as follows: 2 ml of a suitable diluted cellulase solution, previously centrifuged, was added to a solution containing 16 ml of 2% hydroxyethylcellulose and 2 ml of buffer (0·45 ml 0·2 M Na_2HPO_4 and 0·55 ml 0·1 M citric acid). This test solution was kept at 25° in the viscometer and the viscosity measured every 2 min. Viscosity measurements were continued for 20 to 30 min and the curve of the efflux time as a function of the incubation time constructed. The incubation times were read exactly at the midpoint of the efflux time.

Blank value

It has been claimed that a blank test can be made after destroying the cellulase activity by boiling the solution for 30 min. We found that a residual activity remained. However by our different definition of cellulase activity the necessity for a blank value was eliminated.

The blank value could be obtained by taking 2 ml of water instead of the destroyed cellulase, or more conveniently by extrapolating the incubation time to zero. Interaction between the denatured cellulase and substrate did not alter viscosity values. The extrapolation of the efflux times to the initial incubation time avoids a supplementary pipetting of the hydroxyethylcellulose solution, which is best avoided, in view of the high viscosity of the solution.

RESULTS AND DISCUSSION

In Fig. 1A the viscosity (measured in the Rotovisko apparatus) as a function of the incubation time is shown for a given cellulase preparation. It appears that there is a linear relation, at least up to 25 min, between the apparent viscosity and the time of enzyme action. Since the viscosity of a hydroxyethylcellulose solution is a function of the rate of shear (or shear stress), it may be expected that the decrease of viscosity $-d\eta/dt$ to will also be dependent on the shear stress. This is indeed so as seen in Fig. 1B. It is also found that for higher values of shear stress that $d\eta/dt$ remains roughly constant.

The activities determined by the Rotovisko viscometer (at high shear stress) were markedly lower than those obtained by the capillary viscometer method.

The conditions for the assay of the enzymatic activity of a cellulase were also investigated with a modified Ostwald viscometer (Fig. 2).

The efflux times of the hydroxyethylcellulose were measured as a function of the applied external pressure (held rigorously constant). According to Poiseuille's law,

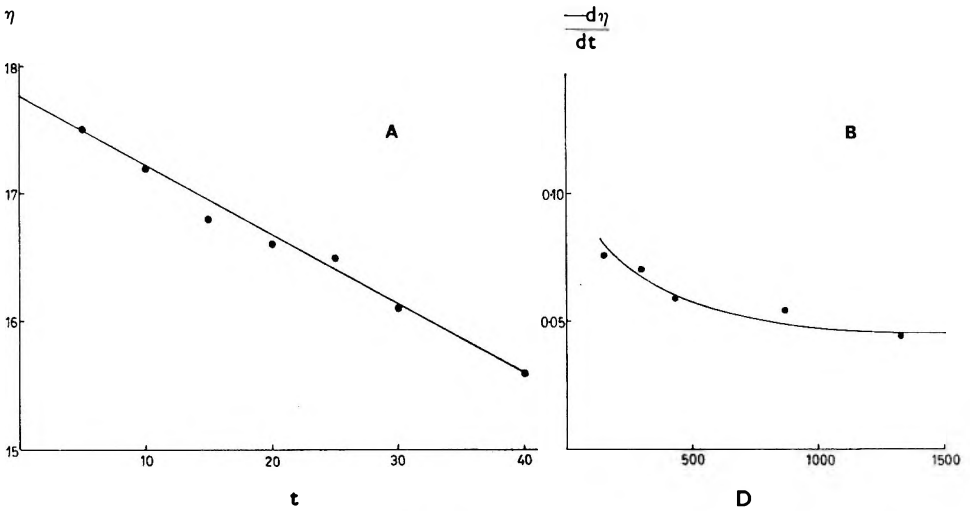

FIG. 1A. Decrease of viscosity (η, $mNsm^{-2}$) for a hydroxyethylcellulose solution, by cellulase as a function of incubation time (t in minutes) (D = 870, Rotovisco) (—$d\eta/dt$ = 0·9 pNm^{-2}).
B. Decrease of the viscosity (—$d\eta/dt$) for a given cellulase solution as a function of the rate of strain D (Rotovisco).

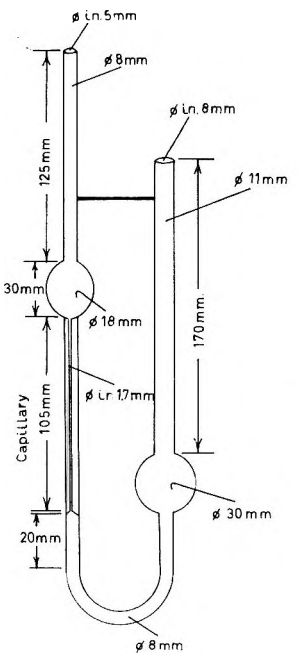

FIG. 2. Capillary viscosimeter for the assay of the cellulase activity. Efflux time for a 3% hydroxyethylcellulose solution ≈30 s.

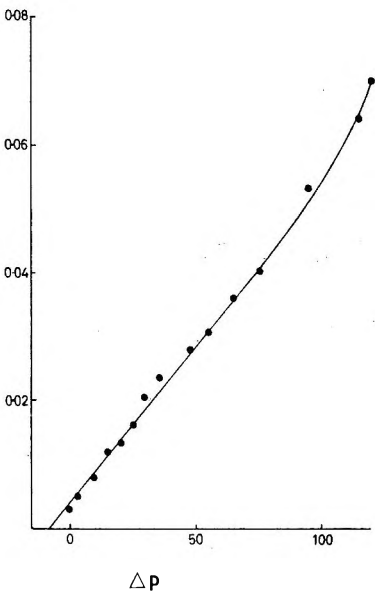

FIG. 3. Inverse of the elution time ($t^{-1}s$, ordinate) as a function of the applied pressure difference (Δp in mm Hg) for a 3% hydroxyethylcellulose solution.

for a Newtonian fluid a linear relation exists between the pressure difference Δp and t^{-1} (t = efflux time). For the results in Fig. 3 it appears that this linear relation holds for hydroxyethylcellulose solutions up to a pressure difference of about 50 mm Hg. Thus in this region the solution shows a Newtonian behaviour.

The pressure difference Δp, causing flow consists of two components: one the hydrodynamic pressure difference p_o, the other the applied pressure difference p, thus

$$\Delta p = p_o + p$$

From Fig. 3 it appears that the mean value of p_o is about 8 mm Hg.

The enzyme activity was measured for a cellulase sample with a 3% hydroxyethylcellulose solution. Since the elution time for this solution was about 35 s, it was easy to make measurements every 2 min. From Fig. 4 it appears that there is a linear decrease of the viscosity as a function of the incubation time. Deviations occur before 5 min and these points were not considered. Since pipetting the viscous solution is not very accurate, the initial viscosity of the test solutions may differ, however the decrease ($d\eta/dt$) remains constant. The results for three different runs were ($-d\eta/dt$) 472, 460, 488 mNm^{-2}. The accuracy of a determination is within 5%

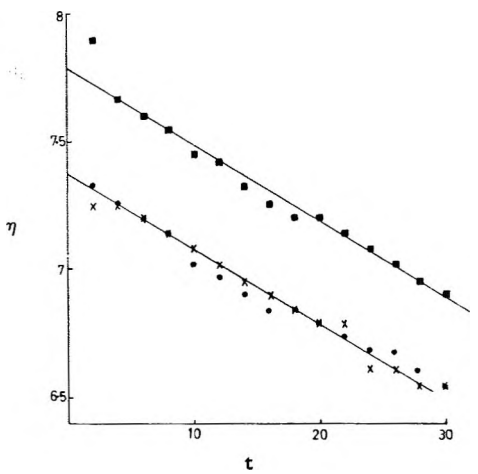

FIG. 4. Decrease of the viscosity (η, $mNsm^{-2}$) of a hydroxyethylcellulose solution by cellulase as a function of the incubation time (t min). Results of three separate runs.

Table 1. *Determination of the enzyme activity of cellulase. Effect of the amount cellulase added*

P (μg cellulase per 20 ml reaction solution)	$-d\eta/dt$ nNm^{-2}	Enzyme activity
100	800	8·00
100	800	8·00
100	892	8·92
100	733	7·33
100	783	7·83
100	967	9·67
200	1717	8·57
200	1667	8·33
200	1650	8·25
200	1700	8·50
200	1500	7·50
200	1717	8·57
300	2450	8·17
300	2550	8·50
400	3033	7·58
400	3017	7·54

We also varied the enzyme concentration. The enzyme activities were calculated by $-(1/P)(d\eta/dt)$, P being expressed in μg cellulase in the incubation solution of 20 cm^3 (Table 1). Units of enzyme activity are expressed as decrease of viscosity per μg enzyme in 20 ml solution.

Values for 5, 10, 15 and 20 μg cellulase/cm^3 of reaction solution were obtained using different substrates. They were most regular for a cellulase activity region of 8·20 to 8·80 units contained in 200 μg of enzyme preparation in the reaction mixture. For one substrate, 30 replicate assays gave a mean value of 8·36 with a standard deviation of $\pm$0·10.

Samples of one hydroxyethylcellulose solution were measured in 3 viscometers for an enzyme concentration of 10 μg/ml reaction mixture. The values are in Table 2.

Table 2. *Enzymatic cellulase determination for one hydroxyethylcellulose solution in 3 different Ostwald viscometers*

P (μg cellulase in 20 ml reaction solution)	Viscometer	$-d\eta/dt$ nNm^{-2}	Enzyme activity
200	1	1800	9·00
200	1	1650	8·25
200	1	1783	8·91
200	1	1717	8·58
200	2	1817	9·08
200	3	1800	9·00
200	3	1733	8·67

The optimal substrate concentrations were 2 to 3%. Enzymatic activity was not independent of the hydroxyethylcellulose concentration. In view of the complexity of the viscosity factors involved in such a system, this result is not surprising.

The method may be considered as a relative one and the unit definition has no absolute significance as the cellulase preparations are mostly mixtures of enzymes.

As a control procedure, the use of a simple capillary method is recommended and could easily be adapted for Pharmacopoeia purposes.

A difficulty remains in that the results for enzymatic activity are to some extent dependent on the sample of hydroxyethylcellulose used, even if it is prepared exactly according to recommendations. Some commercial samples examined did not show a linear relation between the decrease of viscosity with incubation time.

A further objective would be to relate the decrease of viscosity quantitatively with the breakdown of the macromolecule. But the calculation of the number of subunits split up per unit of time, or the reducing groups released, is still difficult to determine with accuracy.

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LETTERS TO THE EDITOR

The effect of butoxamine, *N*-isopropylmethoxamine and salbutamol (AH-3365) on melanophore β-adrenergic receptors

Recently, Goldman & Hadley (1969) demonstrated that the melanophores of the lizard, *Anolis carolinensis*, possess β-adrenergic receptors which mediate melanosome (melanin granule) dispersion within melanophores in response to adrenergic stimulation. Melanosome dispersion within melanophores is responsible for the normal darkening of skin in *Anolis*. Salbutamol (AH-3365) has been reported to be a new selective β-adrenergic receptor stimulant (Brittain, Farmer & others, 1968; Cullum, Farmer & others, 1969) which has a "considerably greater action on bronchial smooth muscle than other smooth muscles affected by β-stimulants" (Brittain & others, 1968). Similarly, the β-adrenergic blocking agents, *N*-isopropylmethoxamine and butoxamine, are selective in that they block "some but not all of the β-adrenergic responses to catecholamines" (Wilkenfeld & Levy, 1968). We have, therefore, tested these agents to determine whether they have any activity on the β-receptors of *Anolis* melanophores.

Skins of *Anolis carolinensis* were removed and prepared (Goldman & Hadley, 1969). Responses to pharmacological stimulation were monitored as changes in light reflectance from the epidermal surface of skins as originally described for the frog skin bioassay for melanophore stimulating hormone, MSH (Shizume, Lerner & Fitzpatrick, 1954). An increase in reflectance indicated skin lightening resulting from melanosome aggregation whereas a decrease in reflectance indicated skin darkening caused by melanosome dispersion within melanophores. The preparation and method of adding pharmacological agents has been described (Goldman & Hadley, 1969).

Table 1. *Effect of salbutamol on melanophore β receptors*

Exp.	No. of animals	Treatment M	% Decrease in reflectance*
A	8	Isoprenaline 1×10^{-6}	16 ± 2·18
	8	Salbutamol 1×10^{-5}	5 ± 2·56
B	6	Isoprenaline 1×10^{-6}	25 ± 1·71
	7	Salbutamol 1×10^{-4}	10 ± 2·08

* Values are means ±s.e. Decrease in reflectance represents skin darkening. The results are the greatest response within 60 min after addition of salbutamol and isoprenaline. In both experiments the difference between the groups is significant ($P < 0.01$ in A; $P < 0.001$ in B) according to the student's *t*-test.

Salbutamol darkens the skins of *Anolis* but this response is less than the isoprenaline-induced darkening (Table 1). Even at a concentration of 10^{-4}M salbutamol darkened the skins much less than isoprenaline which was at a concentration of 10^{-6}M. These observations agree with the findings of Brittain & others (1968) and Cullum & others (1969) who found salbutamol (AH-3365) to be a selective stimulant of β-adrenergic receptors that was less potent *in vitro* than isoprenaline.

Using the β-adrenergic blocking agents, we found (Fig. 1) that even at concentrations of 10^{-4}M neither *N*-isopropylmethoxamine nor butoxamine inhibited darkening by isoprenaline. In fact, if anything, these agents seemed to slightly enhance the

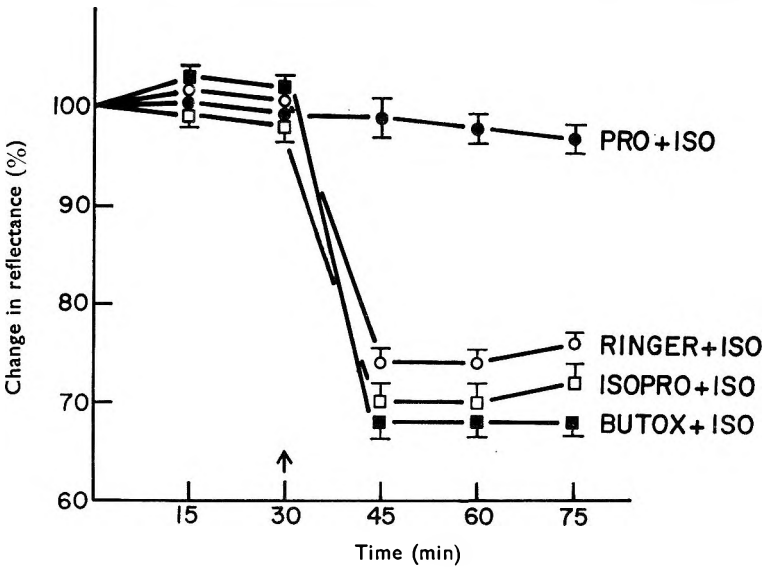

FIG. 1. Inhibition of isoprenaline-induced darkening by β-adrenergic blocking agents. Three groups of eight skins each were incubated in β-blocking agents ●, propranolol (PRO) 1×10^{-4}M; □, *N*-isopropylmethoxamine (ISOPRO) 1×10^{-4}M; ■, butoxamine (BUTOX) 1×10^{-4}M); while one group of eight skins remained in Ringer solution (○). After 30 min (arrow) isoprenaline (ISO, 1×10^{-6}M) was added to all of the groups. Each point on the graph represents the mean reflectance measurement from the eight skins per group. Vertical lines indicate the standard error of the mean.

darkening. Propranolol, however, completely blocked isoprenaline-induced darkening. Again, our experiments agree with the findings by Levy (1964, 1966) and Wilkenfeld & Levy (1968) that *N*-isopropylmethoxamine and butoxamine inhibit only some β-adrenergic receptor responses and are different, therefore, from other β-adrenergic blocking agents such as propranolol which inhibit all β-adrenergic receptor stimulation.

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Assay of acetylcholine using toad rectus abdominis muscle in the presence of hemicholinium-like substances

The presence of hemicholinium-3 (HC-3) has been reported to depress the guinea-pig ileum response to acetylcholine, and to a lesser extent, that of the frog rectus abdominis muscle (Prasad & MacLeod, 1966; Bertolini, Greggia & Ferrari, 1967). This action can sometimes prevent the satisfactory assay of acetylcholine in solutions containing hemicholinium-like substances. For example, when comparing the inhibitory activity of HC-3 and its *p*-terphenyl analogue TPHC-3 on acetylcholine biosynthesis by brain mince (Gardiner & Lee, 1969), the low acetylcholine content of some samples (4–10 nmol/g wt wet tissue) could not be detected on the usual eserinized rectus preparation in the presence of HC-3 or TPHC-3. Since ethanol is known to increase the sensitivity of the frog rectus abdominis to acetylcholine (Emmelin Nils, 1939), the following method of acetylcholine assay was adopted.

Modified Ringer solution of the following composition is used (g/litre): NaCl, 7·5; KCl, 0·15; $CaCl_2$, 0·2; $NaHPO_4$, 0·01; $NaHCO_3$, 0·2; glucose, 1; eserine salicylate, 0·01; and ethanol, 2% v/v; it is aerated continuously with 5% carbon dioxide in oxygen. One rectus abdominis muscle of the local toad (*Bufo melanostitus*) is suspended in a bath of 2 ml volume, and the preparation is used at room temperature.

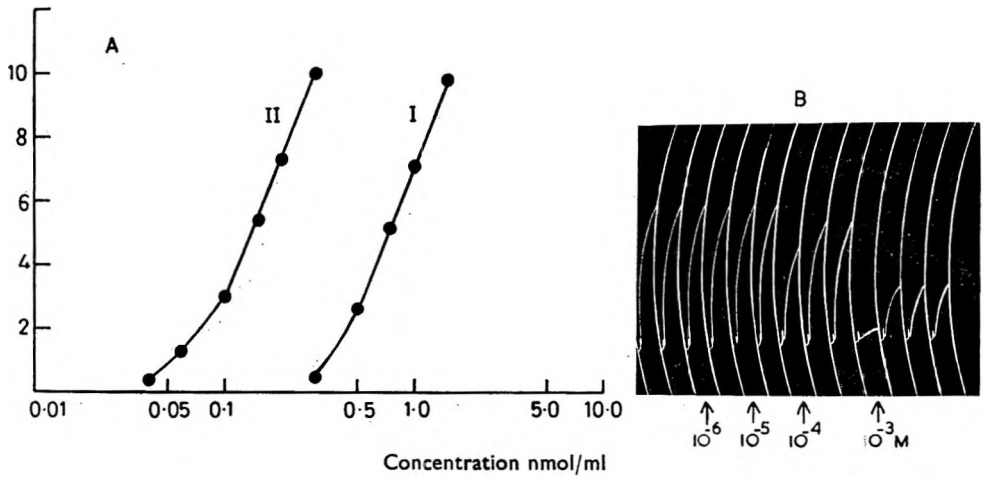

FIG. 1A. Concentration-effect curves of acetylcholine in 10^{-5}M HC-3. (I) In the modified Ringer solution without ethanol. (II) In the modified Ringer solution. Ordinate: recorded height of contraction (cm).

B. Contractions of a toad rectus abdominis muscle to 0·2 nmol/ml acetylcholine as recorded by a Gimbal lever having a magnification of 10 and exerting a 2·5 g tension on the muscle. Time cycle used was 3 min : 1 min exposure and 2 min rest period. At each arrow indicated HC-3 was added. HC-3 at concentration of 10^{-4}M and higher depressed the contractions.

Fig. 1A shows the presence of 2% ethanol in the Ringer, producing a useful increase in sensitivity of 5–6 fold.

The preparation remains stable for at least 4 h.

However, in the presence of higher concentration of HC-3 (10^{-4}M and above), the contractions of the muscle to acetylcholine are depressed (Fig. 1B), as reported by Bertolini & others (1967).

We wish to thank Prof. J. E. Gardiner for his advice and helpful criticism in this work.

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Potentiation of [^{3}H]noradrenaline accumulation in rat heart by angiotensin

There have been many reports in recent years that angiotensin interacts with the sympathetic nervous system to potentiate sympathetic activity. Conflicting reports based on experiments made on isolated tissues *in vitro* and on isolated perfused organs indicate that angiotensin inhibits (Palaič & Khairallah, 1967; Panisset & Bourdois, 1968) or has no effect upon (Thoenen, Hürlimann & Haefely, 1965; Hertting & Suko, 1966) the uptake of noradrenaline at peripheral nerve endings. Results are also conflicting for *in vivo* experiments on the effect of angiotensin on myocardial uptake of noradrenaline. Thus Buckley (1965) found no significant alteration in rat myocardial catecholamines at the end of 1 h of angiotensin infusion, but several investigators (Westfall & Peach, 1965; Peach & Ford, 1968) found an early increase in myocardial noradrenaline in the intact rabbit and cat under the influence of angiotensin, although this increase was attributed to an increase in plasma noradrenaline due to angiotensin-induced release of noradrenaline from adrenal and peripheral nerve endings (Peach & Ford, 1968).

It would appear that studies on angiotensin-noradrenaline interaction in animals with an intact nervous system may offer some advantage over *in vitro* studies, because Zimmerman (1962) has shown that vasoconstrictor response to angiotensin in the perfused hindquarters of dog is partly dependent on an intact sympathetic innervation.

Fifty experiments were made on Charles River CD male rats of approximately 200 g weight. Rats were anaesthetized with sodium pentobarbitone, which has been shown not to influence the uptake of [^{3}H]noradrenaline (^{3}H-NA) in the cat (Whitby, Axelrod & Weil-Malherbe, 1961) and has been used with the same rationale in the guinea-pig (Crout, 1964). Twenty-five control rats were injected via left lateral tail vein with 0·9% saline and 25 rats were injected with 0·1 μg angiotensin II amide (Hypertensin Ciba) in 0·9% saline. One min after the injection, 40 μCi ^{3}H-NA (6·6–8·45 Ci/mmol, New England Nuclear) was injected over a period of 10 s into the right lateral tail vein. Rats were killed by cervical dislocation exactly 1 min after the second injection and the heart was quickly dissected, killing and dissection time being standardized to 25 s. The left ventricle of each heart was trimmed and washed twice with cold saline and blotted on Whatman No. 1 filter paper. Each specimen was weighed and then ground to a fine consistency with 0·4N perchloric acid and sea sand in a mortar. Specimens were washed twice and the material centrifuged and the supernatant brought to 10 ml volume. A 1 ml aliquot was added to 14 ml of counting solution and total radioactivity was counted in a Packard

Tri-Carb liquid scintillation spectrometer. Total radioactivity was considered mainly to represent ^{3}H-NA, since it has been shown that in the guinea-pig heart the peak radioactivity at 1 min after injection of ^{3}H-NA represents 90–95% ^{3}H-NA (Crout, 1964). Control experiments and angiotensin experiments were alternated singly, and the two rats of each pair were maintained under identical conditions and were matched for equal weight. Accumulation of radioactivity in the control group of rats ($n = 25$) was $625{\cdot}6 \pm 18{\cdot}1$ nCi/g in the left ventricle, while the figure for the angiotensin-treated group ($n = 25$) was $745{\cdot}7 \pm 35{\cdot}8$ (means $\pm$ s.e.), $P < 0{\cdot}005$.

My findings are consistent with those of Westfall & Peach (1965) and Peach & Ford (1968) of increased accumulation of noradrenaline in the heart of the intact animal under the influence of angiotensin, and demonstrate further that this increase in accumulation occurs with noradrenaline from an exogenous source and that the process is not dependent upon release of the endogenous amine from adrenal and sympathetic nerve endings.

The findings in these experiments are consistent with, but not conclusive of, an increase in uptake of noradrenaline by innervated myocardium under the influence of angiotensin. One factor which must be considered but cannot be isolated in these experiments is the contribution of haemodynamic effects of angiotensin, since Hertting & Suko (1966) have demonstrated that the effect of angiotensin on contraction induced by nerve stimulation in the perfused cat spleen can be duplicated by reducing flow to the spleen. Changes in blood flow and distribution could result in altered accumulation of noradrenaline in a tissue. However, even if the increased accumulation in the myocardium were due to haemodynamic changes with no effect on uptake mechanism, an increased accumulation implies that more noradrenaline will be taken up by the nerve endings since it has been shown (Dengler, Spiegel & Titus, 1961, Iversen, 1963) that uptake of noradrenaline obeys Michaelis-Menten kinetics. Thus below saturation the rate of uptake would depend upon concentration of noradrenaline in the tissue surrounding the site of uptake.

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The effect of ethanol on the metabolism of amphetamine by the rat

In the course of an investigation into the effect of ethanol on the metabolism of amines we have studied the effect of pretreatment with ethanol on the metabolism of (±)-[2-^{14}C]amphetamine. Male Sprague-Dawley rats, 150 g, housed in metabolism cages received ethanol, 5 g/kg, as a 25% solution (v/v) by stomach tube followed 30 min later by (±)-[2-^{14}C]amphetamine sulphate (1·6 mCi/mmol), 5 mg/kg, intraperitoneally in 0·5 ml of isotonic saline. Control animals received an appropriate volume of isotonic saline by stomach tube and the same dose of (±)-[2-^{14}C]amphetamine sulphate. The pH of the urine was measured immediately after voiding, and the urine frozen. The urinary pH (5·5–6·8) was not altered by ethanol treatment. Urine was chromatographed in two dimensions on Whatman 3MM paper in n-butanol–acetic acid–water (4 : 1 : 1 v/v) followed by isopropanol–ammonia–water (8 : 1 : 1 v/v). The metabolites, located by autoradiography, were cut out and the radioactivity counted in a Packard Model 3375 liquid scintillation counter. Amphetamine, *p*-hydroxyamphetamine and *p*-hydroxyamphetamine glucuronide accounted for over 85% of the urinary radioactivity. Amphetamine and total *p*-hydroxyamphetamine were also determined by the method of Axelrod (1954) after treatment of the urine with β-glucuronidase. Amphetamine was further determined by isotope dilution and recrystallization to constant specific activity. The identification of *p*-hydroxyamphetamine glucuronide was confirmed by hydrolysis of the eluate from chromatograms with β-glucuronidase which converted it quantitatively to *p*-hydroxyamphetamine. Recovery of radioactivity in the urine in the first 24 h after dosing with amphetamine had a mean value of 77% for both control and ethanol-treated animals. The percentage of the urinary radioactivity present as amphetamine was greatly increased and that as free and conjugated *p*-hydroxyamphetamine greatly reduced by ethanol pretreatment: control and ethanol pretreated values being, respectively, 16·5 ± 5·28 and 65·4 ± 6·91 for amphetamine ($P < 0{\cdot}001$); 7·3 ± 3·60 and 2·4 ± 1·49 for *p*-hydroxyamphetamine ($P < 0{\cdot}005$); 61·8 ± 4·04 and 20·9 ± 5·99 for *p*-hydroxyamphetamine glucuronide ($P < 0{\cdot}001$) (means for 6 animals ± s.d.). The proportion of *p*-hydroxyamphetamine excreted in the free form (about 10% of the total *p*-hydroxyamphetamine) was the same in each group.

Ethanol pretreatment has a profound effect on the pattern of metabolism of noradrenaline, 5-hydroxytryptamine and tyramine (Davis, Brown & others, 1967, 1967a; Tacker, Creaven & McIsaac, 1969). However, these amines are deaminated by monoamine oxidase and the effect of ethanol is to increase the reduction of the intermediate aldehyde so formed. Amphetamine is metabolized in the rat largely by hydroxylation of the benzene ring (Axelrod 1954) so that no effect of ethanol on its metabolism would be expected. Whether the observed effect of ethanol on amphetamine metabolism is specific or is a general effect on aromatic hydroxylation, and whether the metabolic interaction of these two commonly abused drugs occurs also in man, is still unknown since the pattern of amphetamine metabolism in man is different from that in the rat (Dring, Smith & Williams, 1966).

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Observations on some actions of ergometrine, noradrenaline and dopamine on the guinea-pig vas deferens and on the rabbit jejunum

Some of the effects of dopamine may be brought about by an action on specific dopamine receptors as opposed to an action on α- or β-adrenergic receptors (McDonald & Goldberg, 1964; Eble, 1964; Rossum, 1965, 1966; Goldberg, Sonneville & McNay, 1968; Woodruff & Walker, 1969). In the brain of the snail, *Helix aspersa*, dopamine hyperpolarizes and inhibits some of the neurons by an action on specific dopamine receptors (Woodruff & Walker, 1969) and this action of dopamine is blocked by low concentrations of ergometrine, less effectively by α-blocking agents (Walker, Woodruff & others, 1968). Ergometrine has little or no α-blocking activity (Brown & Dale, 1935).

Cumulative concentration–effect curves were obtained for dopamine and for (—)-noradrenaline on the guinea-pig vas deferens, using the method of Rossum (1963). The mean pD_2 values were 4·6 for dopamine, and 5·6 for noradrenaline. Ergometrine maleate 10^{-6} to 5×10^{-6} M potentiated the response to noradrenaline (Fig. 1A), taking the form of an increase in the maximum effect obtainable, which rose to between 120% and 200% of control values. In Fig. 1A, there is also seen a shift to the left of the concentration–effect curve, but in other experiments in which ergometrine produced either no shift of the concentration–effect curve or a slight shift to the right, there was still seen an increase in the maximum response to noradrenaline. This action of ergometrine was reversed on washing. In contrast to its action on the noradrenaline response, ergometrine 2×10^{-6} to 10^{-5} M caused a decrease in the maximum effect of dopamine on the vas deferens. Over the lower concentration ranges of dopamine ergometrine caused potentiation of the response. Ergometrine alone had no effect on the vas deferens in concentrations up to 10^{-4} M.

Concentration–effect curves were obtained also on the isolated rabbit jejunum, using the method described by Rossum (1965). The mean pD_2 values obtained were 4·8 for dopamine and 7·0 for noradrenaline. In the presence of an amount of ergometrine (2×10^{-6} M), which itself had no effect on rhythmic activity, noradrenaline in concentrations of from 3×10^{-8} M to 10^{-6} M caused an increase in the amplitude of the spontaneous contractions instead of the usual decrease (Fig. 1B). Higher concentrations of noradrenaline in the presence of ergometrine caused the usual inhibition, with a small shift to the right of the concentration–effect curve, but with no change in the maximum effect obtainable (Fig. 1B). Similar results were obtained with dopamine as the agonist, with which ergometrine was less effective in reversing the inhibitory action of low concentrations, but caused a greater shift to the right of the concentration–effect curve. Ergometrine in concentrations greater than 10^{-5} M had a variable, but generally inhibitory, action on rhythmic activity.

One possible explanation of our observation on the rabbit jejunum is that ergometrine uncovers an excitatory action of noradrenaline, mediated through different receptors, perhaps also sympathomimetic. The mechanism of action of ergometrine on the vas deferens could possibly be similar to that suggested by Barnett, Greenhouse & Taber (1968) for other compounds on the rat vas deferens.

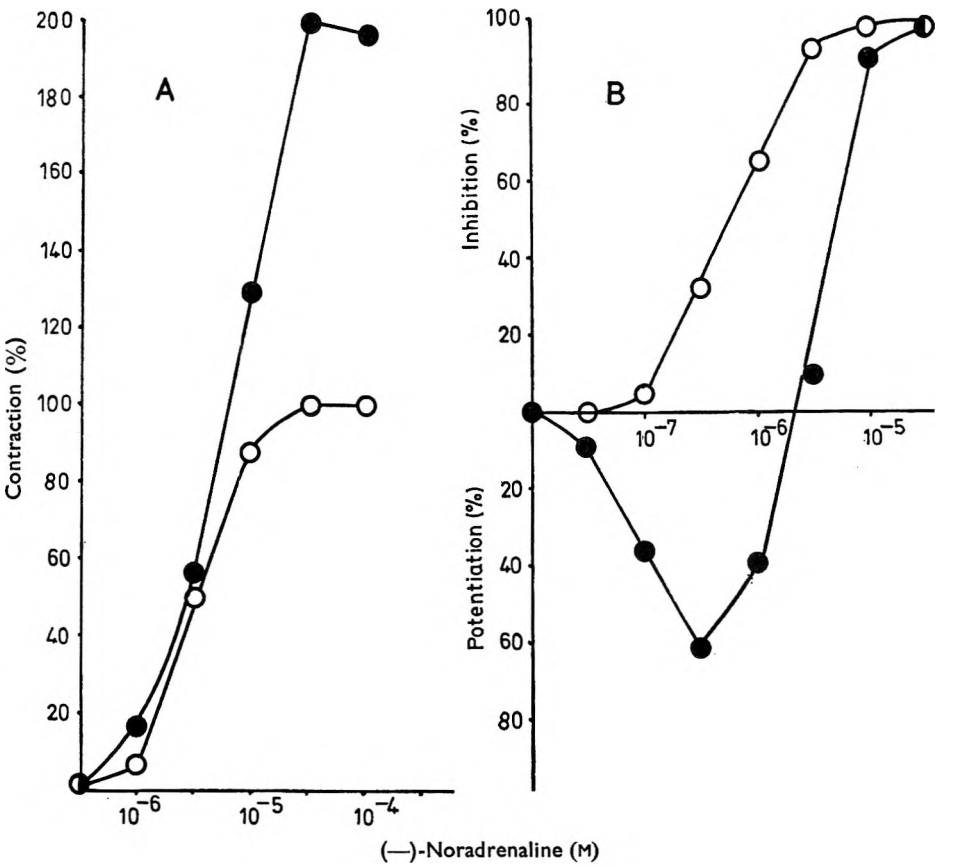

FIG. 1A. Cumulative concentration–effect curves for (−)-noradrenaline on the isolated guinea-pig vas deferens. ○—○ control curve. In the presence of 2×10^{-6} M ergometrine maleate, ●—●, there was a large increase in the maximum response.

B. Concentration–effect curves for (−)-noradrenaline on the isolated rabbit jejunum. ○—○ control curve. In the presence of 2×10^{-6}M ergometrine maleate, ●—●, there was a reversal of the effect of low concentrations of noradrenaline.

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A rapid method for the extraction of angiotensin II from blood

Published methods for the extraction of angiotensin from blood require large amounts of blood and usually elaborate methods of column chromatography for the separation of the hormone from other peptides (Skeggs, Lentz & others, 1967). Recent methods involving radioimmunoassay of angiotensin (Haber, Page & Jacoby, 1965; Page, Haber & Lagg, 1965; Goodfriend, Fasman & others, 1966; Boyd, Landon & Peart, 1967; Catt, Cain & Coghlan, 1967; Catt & Coghlan, 1967; Heffernan, Gillibrand & Prout, 1967; Valotton, Page & Haber, 1967) have greatly increased the sensitivity of measurement and they are also more specific than those using bioassay in the rat which cannot distinguish between angiotensin I and angiotensin II. It is suggested that the following method of extracting angiotensin II from blood would be useful as an adjunct to radioimmunoassay for measuring angiotensin II (and probably angiotensin I) or in conjunction with bioassay procedures using the rat colon (Regoli & Vane, 1964) or the whole rat.

The method to be described is essentially an adaptation of the extraction procedure of Kahn, Skeggs & others (1952) involving the extensive use of centrifuge tubes instead of large pieces of glassware, the substitution of s-butanol for n-butanol and of the final stage of column chromatography using alumina columns with a simple precipitation of angiotensin II from methanolic solution by diethyl ether. The precipitation of angiotensin II by diethyl ether was suggested by Morris & Robinson (1964). The four stages of the present extraction procedure are rapidly performed and the final residue dissolves readily in Krebs-Ringer or Tyrode solution.

Blood (3 ml) is added directly to 6 ml of methanol (AR) contained in round-bottomed centrifuge tubes, 15 ml capacity. The tubes are immediately stoppered with rubber bungs and shaken vigorously. They are then centrifuged at 1000 *g* for 5 min and the supernatant subsequently decanted into centrifuge tubes placed in a metal rack. The volume is then reduced to about 1 ml with a stream of air in a water bath at 40°. This takes 30 to 40 min.

If appreciably larger quantities of blood are taken (up to 50 ml) this initial stage is replaced by one in which the blood is added to four times its volume of laboratory ethanol contained in conical flasks which are stoppered and shaken. The precipitated material is then separated by filtration through Whatman No. 42 paper and the filtrates transferred to round-bottom flasks (250 or 500 ml capacity) and reduced in volume to about 3 ml *in vacuo* at 30° with a rotary evaporator. The material in the flasks is then transferred with Pasteur pipettes to the centrifuge tubes; 2·0 ml of a 1·0M solution of NaCl in 0·2N HCl is then pipetted into the flasks which are scrubbed with a test tube brush for about 1 min. The solution is then added to the first transfer. Another washing and transfer from the flask is then carried out.

When the initial extraction is made in tubes, acidified NaCl solution (5 ml) is added after the initial reduction in volume and 5 ml of diethyl ether added. (No additional acidified NaCl solution is required when the extraction has been carried out in flasks). The tubes are then stoppered and gently shaken for 2 to 3 min. After centrifuging at 100 *g* for 20 s (to resolve emulsions) the upper ethereal layer is removed with Pasteur pipettes and a further extraction then carried out. After the second lot of diethyl ether has been removed air is blown into the tubes for about 20 s to remove any residual ether.

Concentrated hydrochloric acid (0·2 ml) is then added; this is followed by NaCl to give a saturated solution. s-Butanol (chromatographic grade, 2 ml) is pipetted into the tubes which are stoppered and shaken for about 3 min. This is followed by centrifugation at 1000 *g* for 3 min. The upper (s-butanol) layers are then transferred

to centrifuge tubes with Pasteur pipettes. Second similar extractions with s-butanol are then made and the extracts added to the first ones. The tubes are then placed in a water bath at 45° and taken to dryness over about 30 min under a stream of air.

The residues are then partially dissolved in methanol (1·0 ml) and the tubes mechanically agitated for 15 to 20 s on a mechanical agitator (Rotamixer, Model S.N. 536) and then centrifuged at 1000 *g* for 5 min. The clear supernatant is transferred to other tubes with Pasteur pipettes. Diethyl ether (8 ml) is then added, the tubes stoppered and shaken for several seconds and stood in a refrigerator at 4° for 1 h. They are subsequently centrifuged for 2 min at 500 *g* and the methanol–diethyl ether supernatant discarded. The residues are then dried under a stream of air and dissolved in Krebs-Ringer solution (0·3 to 1·0 ml) before testing.

The extracts are free of potassium and contain about a mg of sodium. They are protein-free and contain several mg of peptide as determined by the Lowry reaction (Lowry, Rosebrough & others, 1951) when extracts are made from 50 ml of blood.

The rat colon bioassay method of Regoli & Vane (1964) was used to estimate the replication of the method and the losses of added angiotensin II at the different stages. The overall recovery when 20 ng of angiotensin was added to 50 ml of human venous blood was about 30%. Similar recoveries were observed when this amount of the hormone was added to either dog venous or arterial blood (10 ml). This is comparable with published methods for the extraction of angiotensin II from blood (Osborn, 1966). The replication was excellent, the standard deviation of the population being about $\pm 10\%$ of the mean. When angiotensin II (20 ng) was added before each of the four stages, it was found that most of the losses occurred at the first stage of methanolic (or ethanolic) precipitation. There were negligible losses of angiotensin with the diethyl ether and s-butanol extractions. About 20% was lost in the final stage of diethyl ether precipitation from methanol. There were small cumulative losses in the transfer procedures. Extracts of 50 ml of human venous blood containing no added angiotensin gave contractions of the rat colon only slightly greater than those with Krebs-Ringer solution.

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Metabolism of exogenous cortisol in the rat in various experimental conditions

Cortisol is one of several steroids known to be metabolized by liver microsomal enzymes. The ability to metabolize drugs is lower in very young and old animals (Kato, Vassanelli & others, 1964; Catz & Yaffe, 1967; Kalser, Forbes & Kunig, 1969) and is modified by some drugs like the barbiturates (Conney, 1967). There is also a diurnal rhythm in the activity and metabolism of drugs (Scheving, Vedral & Pauly, 1968; Radzialowski & Bousquet, 1968; Szeberenyi, Szalay & Garattini, 1969).

We now report the half-life of cortisol in the plasma of rats under several experimental conditions. Sprague-Dawley rats of either sex, 150–200 g, were used. The infant rats were 12–14 day old and weighed 45 $\pm$ 5 g. The animals were housed at constant temperature (22°) and humidity (60%) in groups of 4–5 animals per cage and kept on a standard diet (Alal 56, Milan). Cortisol hemisuccinate (kindly supplied by Ormonoterapia Richter, Milan) at a dose of 5 or 10 mg of cortisol/kg in 2 ml saline was injected into the tail vein of the animals which were then killed at different times by incision of the carotid arteries. Plasma was collected and tested for cortisol. Experiments were usually made in the morning.

Corticosterone was estimated spectrofluorimetrically (Guillemin, Clayton & others, 1959) and cortisol by the method of Stockham (1963). The daily variations observed in the endogenous corticosterone plasma concentrations of male and female rats are shown in Fig. 1. There is a diurnal rhythm with a fall in the morning and the highest values in the late afternoon.

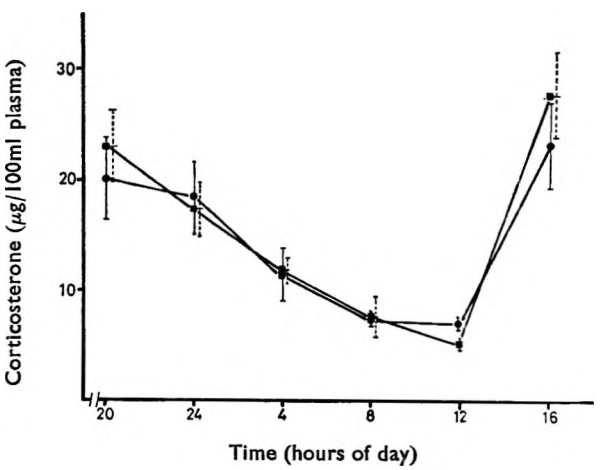

FIG. 1. Mean plasma levels of corticosterone in rats (145 $\pm$ 10 g) during the day. ■ — ■ female, ● — ● male rats.

The rate of disappearance of cortisol from plasma after an intravenous administration of 5 mg/kg at different times during the day is given in Table 1 for values at 9 a.m. and at 9 p.m. in animals previously fasted for 12 h. The half-life of cortisol in the evening is greater. Similar differences were obtained using animals fed *ad libitum*.

Table 1. *Plasma half-life of exogenous cortisol in several experimental conditions*

Treatment or experimental condition	Sex	Weight (g)	Dose of cortisol (mg/kg, i.v.)	Plasma half-life (min)	Significance *P*
Diurnal variation					
9 a.m.	Female	145 ± 10	5	10·7	<0·02
9 p.m.	,,	145 ± 10	5	16·4	
Age: Infant	Male	45 ± 5	10	20·4	<0·001
Adult	,,	180 ± 10	10	12·4	
Phenobarbitone acute ..	,,	200 ± 20	5	10·4	<0·05
Saline acute	,,	200 ± 20	5	20·2	
Phenobarbitone chronic	,,	220 ± 20	5	9·0	<0·02
Saline chronic	,,	220 ± 20	5	14·4	

Table 1 also shows the disappearance of intravenously given cortisol (10 mg/kg) from plasma of male young rats and infant rats. The disappearance of cortisol is more rapid in the young adult than in the infant rats. It also gives the results after an acute pretreatment of the animals with phenobarbitone (72 mg/kg, i.p.), 24–48 h before the injection of cortisol. Different slopes are evident after 24 h, but the most marked differences are after 48 h. In the chronic experiment, animals were treated with phenobarbitone (37·5 mg/kg, i.p.) twice a day for 6 days; 48 h after the last pretreatment they received cortisol. The half life in the plasma of cortisol in phenobarbitone-treated animals is significantly less than in controls (Table 1).

The daily variations in the endogenous levels of plasma corticosterone have been extensively studied and our data are in general agreement with those in the literature. According to Guillemin, Dear & Liebelt (1959) the peak in the adrenocortical secretion in the albino rat occurs at about 6 p.m. and it would reflect changes in the ACTH secretion by the pituitary (Perkoff, Eik-Nes & others, 1959; Galicich, Halberg & others, 1965).

Our experiments also show differences in the disappearance rate of injected cortisol at different times of the day. Concomitant with high endogenous corticosterone levels, the half-life of exogenous cortisol is longer. This would agree with the results of Radzialowski & Bousquet (1968) who showed a daily rhythm in hepatic drug-metabolizing enzymes in the rat and mouse that was abolished by adrenalectomy or by constant levels of plasma corticosterone. These results are further supported in man by our unpublished findings has exogenous cortisol that a longer half-life in the morning than in the afternoon. Thus, there appears to be a relation between the activity of the pituitary-adrenal axis and the activity of drug-metabolizing enzymes.

In early infancy, pituitary function is not well developed and the fluctuations in the level of plasma corticosterone are much less evident (Jailer, 1950; Schapiro, Geller & Eiduson, 1962; Leeman, 1963; Allen & Kendall, 1967). Drugs degraded by microsomal enzymes show a lower rate of metabolism at this time (Kato & others, 1964). We found in infant rats that the disappearance of intravenous cortisol is slower by comparison with that in adults and this difference is highly significant ($P < 0{\cdot}001$).

Steroid hormones are supposed to be endogenous substrates for drug-metabolizing enzymes present in liver microsomes, and substances stimulating the microsomal hydroxylation of drugs also stimulate the microsomal hydroxylation of steroids (Kuntzman, Jacobson & others, 1964). This is evident from the data of Conney, Jacobson & others (1965) who reported an altered cortisol metabolism after pre-treatment of guinea-pigs with phenobarbitone, with a major excretion of the 6-β-hydroxy-component. This correlates with the increased urinary output of the polar metabolite found in man after phenobarbitone by Werk, MacGee & Sholiton (1964) and Burstein & Klaiber (1965).

Recently, we have also found (unpublished) exogenous cortisol to have a faster disappearance rate in man after chronic treatment with phenobarbitone. Our results with animals agree with the above findings.

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Heats of reaction and stability of caffeine complexes

It is generally considered that the solubilization of caffeine in the presence of the sodium salts of aromatic acids is associated with the formation of complexes. Little is known about the stability constants and the enthalpies of formation of these complexes.

Since caffeine is self-associated in aqueous solutions, it was necessary to determine the stoichiometric ratio of complexes in a relatively low concentration range. For these determinations the lowering of vapour pressure was measured. The apparatus used* was similar to the osmometer model manufactured by Mechrolab Inc. The galvanometer readings are proportional to the change in resistance and depend on the activities of the dissolved substances. For the determination of the stoichiometric ratio of the caffeine complexes the continuous variation method of Job (1928) was used. The changes of resistance were first determined for a number of concentrations from 0·05 to 0·2 mol litre^{-1} of caffeine or sodium salt. These values were subtracted from those obtained for mixtures of constant molarity (0·2 mol litre^{-1}) of caffeine and the sodium salts of aromatic acids.

If we assume that complexation occurs in these systems, the number of individual particles decreases and consequently the scale readings decrease. Fig. 1A shows the differences in the scale readings between the additive (theoretical) values and the values obtained from the mixtures. The differences indicated a stoichiometric ratio of 1 : 1 for these particular examples of caffeine–Na-benzoate and caffeine–Na-salicylate. The decrease in the scale readings (or the number of particles) is greater for the caffeine–Na-salicylate complex than for caffeine–Na-benzoate, which is preliminary evidence for the greater stability of the salicylate complex.

In the next part of our investigation we determined the molar enthalpy of formation of a number of caffeine complexes by a calorimetric method. The calorimetric determinations were made with the LKB 8700-calorimetry-system (described by Sunner & Wadsö, 1959) at 25°.

The osmometric measurements indicated that, for a molar ratio of 1 : 10, only a small part of the caffeine was complexed. The determination of the molar heat of formation is possible only if a well-defined amount of caffeine is completely bound in the complex. Therefore we used a caffeine–salt ratio of 1 : 1000 in the first series of experiments and a ratio of 1 : 2000 in the second series. The self-association of caffeine can be neglected in the concentration range from *ca* 5·10^{-4} mol litre^{-1}.

The heat value Q, obtained after mixing a caffeine solution and a salt solution, is composed of the heat of reaction Q_1 and the heats of dilution of the caffeine solution Q_2 and the salt solution Q_3 (eqn 1).

$$Q = Q_1 - (Q_2 + Q_3) \quad .. \quad .. \quad .. \quad .. \quad (1)$$

Q_2 and Q_3 were measured when adding defined amounts of water to the caffeine and the salt solution so that the heat of reaction could be computed. The heats of reaction were then converted to the molar heats of formation by dividing by the number of moles.

In this manner the molar enthalpies of formation were determined for the caffeine–Na-benzoate complex. We found 16·65 kJ mol^{-1} for a molar ratio of 1 : 1000 and 16·40 kJ mol^{-1} for the ratio 1 : 2000. The difference is not significant. The good agreement of the values obtained for the different molar ratios demonstrates the formation of only 1 : 1 complexes in the concentration range used. The reported value of 13·8 kJ mol^{-1} of Higuchi & Zuck (1953) is in close agreement with our results.

* Dampfdruckosmometer, manufactured by Dr. ing. Knauer, West-Berlin.

In Table 1 the molar enthalpies of formation for different caffeine–Na-salts are listed. The enthalpies vary between 15–29 kJ mol^{-1}, indicating weak, but specific interactions for the different sodium salts of the aromatic acids.

The stability constant for the caffeine–salt complexes can be expressed as:

$$K_c = \frac{[\text{caffeine–salt}]}{[\text{caffeine-caffeine–salt}]\,[\text{salt-caffeine–salt}]}$$

Since the starting concentrations are known, the complexed concentrations can be determined in the following way. With the aid of the molar enthalpies of formation the concentration of the complex for any concentration ratio of the complexing compounds can be computed by measuring the heat of reaction.

Table 1. *The molar enthalpies of formation and the K_c-values for the caffeine complexes of the sodium salts of the aromatic acids*

Salt	ΔH (kJ)	ΔH (kcal)	K_c-values (25°) for the concentration ratio caffeine/salt litre mol^{-1} 1:4	1:8	1:12	1:10	1:20	1:30	Mean value
Na-benzoate	16·64a	3·974	5·1	5·2	5·4	5·2	5·3	5·2	5·2
	16·39b	3·916							
Na-*o*-hydroxybenzoate	29·25a	6·986	13·2	13·8	13·8	13·1	13·6	13·7	13·5
	29·34b	7·008							
Na-*p*-hydroxybenzoate	27·27a	6·514	7·5	7·6	7·6	7·3	7·6	7·6	7·5
	27·38b	6·540							
Na-*o*-aminobenzoate ..	20·20a	4·826	8·3	4·3	4·4				4·3
	20·45b	4·888							
Na-*p*-aminobenzoate ..	21·83a	5·214	4·3	5·3	5·3	5·0	5·4	5·4	5·2
	21·82b	5·212							
Na-*m*-nitrobenzoate ..	15·56a	3·712	5·0	8·5	8·4	8·3	8·2		8·2
	15·00b	3·584							

a = molar ratio 1:1000, b = 1:2000.

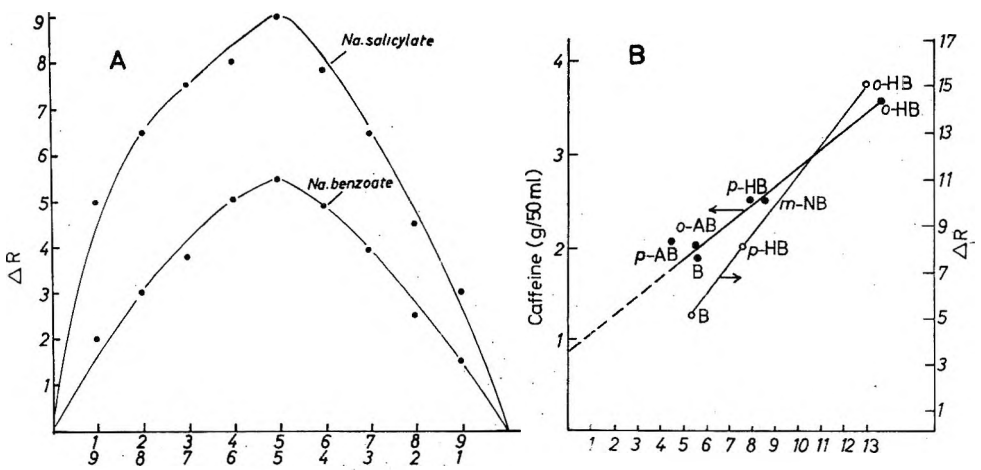

FIG. 1A. Differences in scale readings (45°) between theoretical and experimental values for caffeine-salt mixtures. Abscissa: Mol ratio salt (upper figures): caffeine (lower figures).

B. Relationship between (●—●) the ΔR values for equimolar mixtures of caffeine-salt and their K_c-values and ○—○ between the solubility of caffeine in 0·5M salt solutions (25°) and the K_c-values. Abscissa: K_c litre mol^{-1}.

A salt solution was added stepwise to a defined caffeine solution and after each addition the heat of reaction was determined. To eliminate the effect of the heats of dilution, the corrections were made as described above. In a second run the caffeine concentration was changed in order to produce a different ratio of caffeine–salt concentration.

The stability constants for the caffeine complexes (Table 1) are independent of the caffeine concentration, which is further evidence that the stoichiometric ratio 1 : 1 is correct.

The decrease in the number of free molecules detected by the isopiestic measurement for a given salt–caffeine mixture must be correlated with the stability of this complex. There is a linear relation between ΔR and the K_c-values (Fig. 1B), demonstrating the suitability of both methods for the determination of the stability of such systems.

Fig. 1B also shows that the amount of dissolved caffeine in a 0·5M salt solution is linearly dependent on the stability constants. Extrapolation to a stability constant of zero leads to the correct value of the solubility of caffeine in water. The results clearly show that the stability of the caffeine–salt complexes is the most important factor for the solubilization of caffeine by these substances.

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Toxicity of ethanol-barbiturate mixtures

It is frequently stated that ethanol and barbiturates potentiate each other. Wiberg, Coldwell & Trenholm (1969) have published observations which they are inclined to interpret as support for this view. In our opinion, however, their data should be interpreted differently. This can be demonstrated by plotting isobols (Loewe, 1953, 1957). These are lines on a combined dose diagram connecting those dose pairs which are equi-effective in producing a defined pharmacological effect.

Fig. 1 shows Wiberg, Coldwell and Trenholm's data on acute toxicity of ethanol-barbiturate mixtures in male rats, calculated from the original mortality figures kindly provided by the authors. In each case the line indicates no more than a simple additive effect; there is no suggestion of potentiation.

Their findings on the prolongation of sleeping time might be explained by potentiation but might equally well be due to summation. The fact that two inactive doses produce a marked effect when given in combination does not necessarily indicate potentiation; summation could produce the same result, especially if the log dose-response curves were steep as they appear to be for ethanol and the barbiturates. Unfortunately, the data are insufficient to enable isobols to be constructed. The authors have used threshold or subthreshold doses of barbiturates in combination with ineffective doses of ethanol. If they had tested combinations of half these doses, or, alternatively, recorded the effects of double doses of either drug alone, summation and potentiation might have been distinguished.

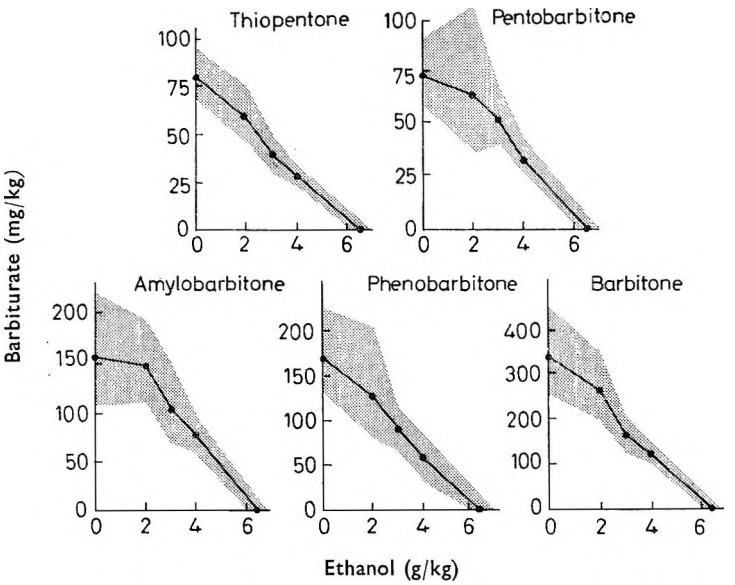

FIG. 1. Isobols showing combined lethal effects (LD50) of barbiturates and ethanol. Drug pairs were injected intraperitoneally at the same time. Horizontal axes: ethanol dose, g/kg. Vertical axes: barbiturate dose, mg/kg. Shaded areas represent 95% confidence limits.

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Activity of (+)-amphetamine at different environmental temperatures in three strains of mice

Halpern, Drudi-Baracco & Bessirard in 1962 suggested a correlation of (+)-amphetamine activity and the level of catecholamines in brain, and, more recently, several experimental findings confirmed and explained this view (Glowinski & Axelrod, 1965; Weissman, Koe & Tenen, 1966; Hanson, 1967; Sulser, Owens & others, 1968).

In relation to this and to previous results showing that (+)-amphetamine induces hyperthermia and toxicity to a decreasing extent in Albino Swiss, C_{57}Bl/6 and C_3H strains of mice (Dolfini, Garattini & Valzelli, 1969), we have investigated whether (+)-amphetamine depletes brain noradrenaline in a different way, in the three strains of mice. The effect of environmental temperature was considered as a variable able to influence this biochemical effect of (+)-amphetamine. This point seems to be of interest also in connection with the role attributed to noradrenaline in the central control of the thermoregulation (Feldberg & Myers, 1965; Beauvallet, Fugazza & Legrand, 1967; Simmonds & Iversen, 1969).

Male mice, 25 ± 2 g, were kept in sixes in Makrolon cages at a constant room temperature and fed a normal, balanced diet *ad libitum*. Albino Swiss mice were obtained from Alal (Milan), C_{57}Bl/6 and C_3H mice from the Jackson Laboratories (Bar Harbor, Maine). (+)-Amphetamine sulphate (Recordati, Milan) was dissolved in saline and injected intraperitoneally.

Table 1. *Effect of (+)-amphetamine on temperature and brain noradrenaline in three strains of mice kept at a room temperature of* 4°

Strain of mice	(+)-Amphetamine sulphate mg/kg, i.p.	Body temperature °C ± s.e.			Brain noradrenaline µg/g ± s.e.		
		C	T 30 min	T 120 min	C	T 30 min	T 120 min
Albino Swiss ..	10	37·4 ± 0·1	34·0 ± 0·2	32·8 ± 0·6	0·35 ± 0·01	0·32 ± 0·01	0·30 ± 0·02*
	30	37·8 ± 0·1	34·1 ± 0·6	32·1 ± 0·9	0·43 ± 0·01	0·43 ± 0·01	0·27 ± 0·03†
	45	37·7 ± 0·1	34·1 ± 0·6	25·6 ± 0·9	0·43 ± 0·02	0·37 ± 0·03	0·27 ± 0·02†
C_{57}Bl/6	10	36·7 ± 0·1	34·7 ± 0·3	32·5 ± 1·0	0·54 ± 0·02	0·52 ± 0·02	0·50 ± 0·03
	30	37·6 ± 0·2	33·9 ± 0·3	27·8 ± 0·8	0·54 ± 0·02	0·54 ± 0·01	0·46 ± 0·01*
	45	37·0 ± 0·1	33·0 ± 0·5	27·0 ± 0·9	0·60 ± 0·01	0·53 ± 0·02	0·46 ± 0·02†
C_3H/HeJ	10	37·3 ± 0·3	33·1 ± 0·2	31·9 ± 0·2	0·57 ± 0·02	0·53 ± 0·02	0·48 ± 0·02*
	30	37·1 ± 0·2	32·5 ± 0·4	20·2 ± 0·3	0·51 ± 0·02	0·44 ± 0·02	0·37 ± 0·01†
	45	38·1 ± 0·2	34·4 ± 0·2	22·4 ± 1·4	0·53 ± 0·02	0·48 ± 0·02	0·38 ± 0·02†

Controls (C) and treated (T) animals, 30 min and 120 min after (+)-amphetamine sulphate were in groups of at least 5 animals per point.

* $P = <0·05$.
† $P = <0·01$.

Table 2. *Effect of (+)-amphetamine on temperature and brain noradrenaline in three strains of mice kept at a room temperature of* 30°

Strain of mice	(+)-Amphetamine sulphate mg/kg, i.p.	Body temperature °C ± s.e.			Brain noradrenaline µg/g ± s.e.			Lethality %
		C	T 30 min	T120 min	C	T 30 min	T 120 min	
Albino Swiss ..	3·7	37·2 ± 0·1	40·2 ± 0·3	37·1 ± 0·3	0·42 ± 0·02	0·43 ± 0·02	0·36 ± 0·02*	0
	10	—	—	—	—	—	—	100
	15	—	—	—	—	—	—	100
C_{57}Bl/6 ..	3·7	36·8 ± 0·2	36·8 ± 0·1	35·3 ± 0·3	0·58 ± 0·01	0·56 ± 0·01	0·52 ± 0·02	0
	10	36·1 ± 0·2	41·0 ± 0·2	38·9 ± 0·9	0·55 ± 0·01	0·56 ± 0·01	0·40 ± 0·05†	33
	15	36·3 ± 0·2	40·6 ± 0·3	37·7 ± 0·8	0·54 ± 0·02	0·52 ± 0·03	0·40 ± 0·01†	33
C_3H/HeJ ..	3·7	37·7 ± 0·2	37·5 ± 0·2	36·2 ± 0·3	0·55 ± 0·02	0·56 ± 0·03	0·51 ± 0·02	0
	10	37·7 ± 0·3	39·3 ± 0·2	38·2 ± 0·3	0·53 ± 0·02	0·45 ± 0·01	0·44 ± 0·02*	0
	15	36·2 ± 0·1	39·4 ± 0·3	38·5 ± 0·5	0·55 ± 0·02	0·41 ± 0·02	0·36 ± 0·02†	0

Controls (C) and treated (T) animals, 30 min and 120 min after (+)-amphetamine sulphate were in groups of at least 5 animals per point.

* $P = <0·05$.
† $P = <0·01$.

Brain noradrenaline was measured spectrofluorometrically (Shore, 1959). Temperature was recorded electrically from the rectal cavity.

(+)-Amphetamine, 7·5 and 10 mg/kg at room temperature (22°), induced the typical hyperthermic response, differing significantly ($P < 0.05$) from control values after 30 min and accompanied by a release of brain noradrenaline significant at 120 min ($P < 0.01$) at 10 mg/kg amphetamine, only in Albino mice while in $C_{57}B1/6$ and C_3H mice there was no effect on either parameter.

The LD50 of (+)-amphetamine sulphate (mg/kg) was 13 for Albino Swiss mice, 54 for $C_{57}B1/6$ mice and 120 for C_3H mice.

If the mice were put in a cold room immediately after administering (+)-amphetamine, the hyperthermic response of the Albino Swiss mice was found to be completely blocked while the depletion of brain noradrenaline was not present at the lower dose.

The cold environment also protected against amphetamine toxicity as was found by Askew (1961), Fink & Larson (1962) and Hohn & Lasagna (1960). In this experimental condition, $C_{57}B1/6$ and C_3H mice showed release of brain noradrenaline (Table 1).

Finally, $C_{57}B1/6$ and C_3H mice kept at 30° immediately after the administration of amphetamine showed an increased temperature in parallel with a release of brain noradrenaline (Table 2).

Thus the hyperthermic response induced by amphetamine was found to be linked both to the strain of mice as well as to the influence of the environmental temperature. The decrease of brain noradrenaline was not related with the degree of hyperthermia, at least under the experimental conditions adopted.

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Interactions of methocarbamol with morphine and aspirin

Methocarbamol is a centrally acting skeletal muscle relaxant which, in clinically used doses, displays little or no sedative activity. Its locus of action in reducing skeletal muscle spasm is generally thought to be the spinal cord (Truitt & Little, 1958). However, its ability to potentiate barbiturate-induced sleeping times (Truitt & Little, 1958) suggests that it also produces effects at supraspinal levels. A number of clinical reports have indicated that in patients treated with methocarbamol, requirements for analgesic therapy are reduced (Hudgins, 1959; Feinman & Sherman, 1961; Truitt, Morgan & Nachman, 1961). While inhibition of skeletal muscle spasm may reduce pain in its own right, it was thought that methocarbamol might possess analgesic activity itself, or alternatively, might potentiate the analgesic activity of other drugs.

In the present experiments, the interactions of methocarbamol with morphine and aspirin have been studied in analgesic tests with mice. Behavioural effects were also noted and a gross assessment of motor reflexes was obtained by eliciting a withdrawal reflex from a hind paw.

Analgesic activity was assessed by the hot plate method (Eddy & Leimbach, 1953), and the abolition of writhing after the intraperitoneal injection of phenylquinone (Hendershot & Forsaith, 1959). Complete abolition of writhing for a period of 30 min after phenylquinone was taken as the end-point for analgesia. In both tests analgesic activity was measured 30 min after the injection of the drugs and ED50 values were calculated using the method of Litchfield & Wilcoxon (1949).

Morphine sulphate was dissolved in distilled water and injected subcutaneously. Aspirin was injected intraperitoneally as its sodium salt. This was prepared immediately before its injection by dissolving aspirin in an aqueous solution containing the calculated quantity of sodium citrate to form sodium acetylsalicylate. Methocarbamol was dissolved in a 25% (*v*/*v*) solution of polyethylene glycol 300 (Shell) in distilled water and injected intraperitoneally. Control experiments showed that the solvents themselves did not influence the effects obtained.

Table 1 shows ED50 values obtained in the two analgesic tests using methocarbamol, morphine and aspirin.

At the ED50 for methocarbamol, the mice were severely sedated. At doses of 150–200 mg/kg, i.p., sedation was apparent and breathing became laboured. At higher dose levels (200–300 mg/kg, i.p.) the animals became cyanotic, while at the highest dose of methocarbamol used (400 mg/kg, i.p.) approximately 50% of the mice lost their righting reflex. No loss of withdrawal reflexes was apparent at this high dose level. It would therefore appear that the "analgesia" produced by metho-

Table 1. ED50 *values by the hot plate and writhing tests*

Drug	ED50 (mg/kg) Hot plate		Writhing	
	ED50	No. of mice	ED50	No. of mice
Methocarbamol (i.p.)	218·5 (240·4–198·6)*	120	262 (284–240)	100
Morphine sulphate (s.c.)	8·0 (9·9–6·4)	160	0·85 (0·98–0·74)	120
Acetylsalicylic acid (i.p.)	>500	200	210 (237–186)	140
Morphine sulphate (s.c.) + methocarbamol (i.p.) (50 mg/kg)	4·2 (5·1–3·5)	150	0·51 (0·61–0·43)	150
Acetylsalicylic acid (i.p.) + methocarbamol (i.p.) (50 mg/kg)	>500	120	128 (154–106)	100

* The figures in parentheses are the 95% confidence limits.

carbamol at the ED50 level was non-specific and resulted from the general depressant action of the drug at this dose level.

Aspirin at doses up to 500 mg/kg, i.p., produced little change in behaviour and no abolition of withdrawal reflexes. At doses of 200 mg/kg, i.p., slight stimulant activity was apparent, while at the highest doses used a quietening effect was produced. No satisfactory ED50 value could be obtained using the hot plate analgesic test; this confirms previous reports (Collier, 1964).

Morphine in doses of 2·5–15 mg/kg, s.c., produced a sedative action in mice, while at higher doses (15–20 mg/kg, s.c.) stimulation, as evinced by ceaseless movements, was apparent. The stimulant action of high doses of morphine in mice has been noted previously by Schaumann (1958) and other workers. At doses greater than 5 mg/kg, s.c. of morphine the Straub tail effect was noted. No abolition of withdrawal reflexes occurred in the dose range of morphine used.

In the presence of 50 mg/kg of methocarbamol, a dose which produced no behavioural or "analgesic" actions itself, the analgesic ED50 values for both morphine and aspirin were significantly reduced (Table 1). This may, in part, explain the reduced requirements for analgesic therapy in patients treated concurrently with methocarbamol and analgesics.

The potentiation of the analgesic action of aspirin in the presence of methocarbamol was not associated with any noticeable behavioural effects other than those seen with aspirin alone. However, in the presence of methocarbamol, the stimulant action of morphine was apparent at lower doses (5–10 mg/kg) than those required to produce this effect in animals treated with morphine alone. In animals treated with methocarbamol (50 mg/kg, i.p.) the Straub response to morphine (1–20 mg/kg, s.c.) was absent, and at doses of morphine greater than 3 mg/kg, s.c., withdrawal responses were abolished in more than 50% of the animals, an effect which was absent at the highest doses of morphine and methocarbamol used in the experiments when the drugs were given alone.

Both morphine and methocarbamol are known to block polysynaptic reflex activity in the spinal cord (Martin, 1963; Truitt & Little, 1958). Summation or mutual enhancement of this effect with the two drugs might explain the abolition of the withdrawal reflexes and of the Straub tail response which is thought to be of spinal reflex origin (Liemdorfer, 1948; Bibley, Salem & Grossman, 1960).

The ability of methocarbamol to potentiate the analgesic actions of both aspirin and morphine and to enhance the actions of the latter drug as a stimulant and spinal reflex depressant, suggests that specific interactions at the cellular level are unlikely. A major pathway involved in the metabolism and excretion of morphine, aspirin and methocarbamol is their conjugation with glucuronic acid. Competition between methocarbamol and aspirin or morphine for gluconuride formation might therefore be expected to enhance blood levels of the drugs, and this could explain the actions observed in the present experiments. This possibility is supported by the findings of Truitt, Morgan & Nachman (1961) who showed that plasma salicylate and methocarbamol levels in patients were significantly raised when the drugs were given in combination rather than separately.

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Studies on the subcellular distribution of [^{3}H]reserpine

It is well established that reserpine can deplete tissues of their catecholamine content. Since existing chemical methods could not detect reserpine in the tissues at a time when the amine content was still quite low, a "hit and run" or irreversible damage hypothesis was formulated to explain reserpine's mechanism of action. However, the development of [^{3}H]reserpine enabled workers (Shepard, Tsein & others, 1958; Maggiolo & Haley, 1964) to demonstrate trace quantities for up to five days post injection. Evidence is available indicating that the depletion induced by reserpine *in vivo* is, in all probability, due to a blockade of amine uptake by the adrenergic nerve granules. (Bertler, Hillarp & Rosengren, 1961; Stitzel & Lundborg, 1967). It is possible, therefore, that the subcellular site of action of reserpine may coincide with a similar subcellular binding of this depleting agent. The present experiments were conducted to test this hypothesis.

Male Swiss-Webster mice, 18–20 g, were injected with [^{3}H]reserpine, 600 μg/kg (approximately 155 μCi/kg) intravenously. The animals were killed by a blow on the head either 15 or 60 min post injection, the hearts removed and homogenized in an ice bath with a Teflon pestle, in 0·25M sucrose containing 0·005M phosphate buffer, pH 7·4 and 0·001 M $MgCl_2$. Nuclear and mitochondrial fractions were obtained by centrifuging in the cold for 10 min at 600 and 8000 *g*, respectively. The 8000 *g* supernatant was then spun at 105,000 *g* for 60 min in a Spinco model L ultracentrifuge to provide a microsomal and a high speed supernatant fraction. Each fraction was analysed for its [^{3}H]reserpine content by a modification of the method of Manara (1967). All sediments were resuspended twice in 10 volumes of acetone. The acetone was evaporated under a stream of nitrogen and the dried material was redissolved in chloroform. Ten volumes of chloroform were added to the high speed supernatant fraction, shaken for 15 min and then centrifuged at 600 *g* for 10 min to

Table 1. *Subcellular distribution of* [^{3}H] *reserpine in the mouse heart at* 15 *and* 60 *min periods after injection*

Fraction	15 min*	60 min*	% decrease
Nuclear	385 ± 23	143 ± 10	64
Mitochondrial	86 ± 12	30 ± 2	65
Microsomal	49 ± 3	19 ± 1	61
Supernatant	17 ± 3	8 ± 2	55

* Each value represents the mean content of [^{3}H]reserpine (ng/g) ± s.e. The means are based upon at least 10 experiments.

Table 2. *A comparison of the reserpine and lipid content of each subcellular fraction*

Subcellular fraction	Reserpine content % 15 min	60 min	Lipid content %
Nuclear	71	71	77
Mitochondrial	16	15	12
Microsomal	10	9	8
Supernatant	3	4	4

break any emulsion formed. Aliquots of all the chloroform extracts were chromatographed on silica gel thin-layer plates to separate reserpine from its major metabolites.

[^{3}H]Reserpine was found in all subcellular fractions within 15 min after its intravenous administration (Table 1). The concentration was highest in the nuclear fraction> mitochondrial> microsomal> supernatant. The microsomal fraction, i.e. that fraction containing the catecholamine storage granules, did not have an unusually high proportion of [^{3}H]reserpine.

Although no unique pattern of distribution was apparent 15 min after injection, it was felt that study of the disposition of [^{3}H]reserpine at two time periods might unmask a specific binding. The results of this experiment are presented in Tables 1 and 2. There was a rapid decrease in [^{3}H]reserpine content in all fractions between 15 and 60 min. The rate of decline, however, was similar in all fractions, and therefore the percentage distribution of [^{3}H]reserpine was identical at both time intervals. Thus reserpine does not appear to have a specific affinity for the amine storage particles. Independently of these studies, Alpers & Shore (1969) also reported an inability to localize [^{3}H]reserpine in a single subcellular compartment.

The observation that some redistribution of [^{3}H]reserpine (Alpers & Shore, 1969; Wagner & Stitzel, unpublished observations), but not [^{3}H]noradrenaline (Stitzel & Lundborg, 1967), occurs during homogenization prompted us to examine the role physical-chemical factors play in determining reserpine's localization. From the evidence in Table 2 it appears that *in vivo* distribution of [^{3}H]reserpine may be accounted for solely on the basis of its high lipid solubility since its disposition closely parallels the total lipid content of each fraction.

In summary the physiological disposition of [^{3}H]reserpine in sympathetically innervated tissue appears to be primarily determined by its high lipid solubility. Subcellular distribution studies emphasized that reserpine is not uniquely associated with the noradrenaline-containing storage granules. However, the large amount of reserpine bound to lipid may mask a smaller more specific binding.

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The quantitation of metyrapone and its reduced derivative in urine

We have previously shown (Sprunt, Browning & Hannah, 1968) that the principal metabolite of metyrapone [(2-methyl-1,2-di(3-pyridyl)propan-1-one] in man is reduced metyrapone [2-methyl-1,2-di(3-pyridyl)propan-1-ol]. We now report a method for quantitating metyrapone and reduced metyrapone in urine, using gas-liquid chromatography.

The samples of urine were obtained from 9 patients undergoing a metyrapone test (Liddle, Estep & others, 1959). 24-h urines were collected on the day before metyrapone administration (Day 0), the day of metyrapone administration (Day 1), and the two days following metyrapone (Days 2 and 3). Metyrapone was administered orally in a dose of 750 mg every 4 h, the total dose being 4·5 g.

The urines were stored at −10° until the analysis could be done. The pH of the 24-h urines was adjusted to 7·0, and then 5 ml aliquots were taken. These were extracted with 2 × 10 ml methylene chloride to obtain free metyrapone and free

Table 1. *The mean values of metyrapone and reduced metyrapone recovered from the urine of* 9 *patients undergoing the metyrapone test. Metyrapone was administered orally on Day* 1 *in a dose of* 750 *mg every* 4 *h (total dose* 4·5 *g).*

	Day 1				Day 2			
	Metyrapone (mg)		Reduced metyrapone (mg)		Metyrapone (mg)		Reduced metyrapone (mg)	
	Free	Conj.	Free	Conj.	Free	Conj.	Free	Conj.
Mean	19·2	185·9	98·7	729·9	2·7	30·6	41·4	754·5
± s.e.	3·8	9·4	7·9	11·4	0·9	5·4	10·2	14·8

reduced metyrapone. Following this extraction, the pH of the urine was adjusted to 5·0 and 1 ml of 0·5M acetate buffer added. The urine was then incubated with β-glucuronidase (prepared from the visceral hump of the limpet, *Patella vulgata*) for 36 h at 37°. The pH of the urine was re-adjusted to 7·0 before again extracting with methylene chloride (2 × 10 ml). This extract contained metyrapone and reduced metyrapone which had been excreted as the glucuronides.

The methylene chloride extracts were taken to dryness before acetylation with 0·1 ml acetic anhydride and 0·1 ml pyridine, overnight at 37°. Metyrapone remains unchanged, but reduced metyrapone acetylates to a more stable derivative. After removal of the pyridine, the extracts were taken up in chloroform, and SU 9055 [3-(1,2,3,4-tetrahydro-1-oxo-2-naphthyl)pyridine] was added as internal standard.

A Pye Series 104 chromatograph was used. A 7 foot $\frac{1}{4}$ inch o.d. silicanized glass column was packed with 100/120 mesh Gas Chrom Q coated with 3% XE-60 (Applied Sciences). The gas flow rates were nitrogen 40 ml/min, hydrogen 45 ml/min, and air 600 ml/min measured at room temperature. The operating temperature of the column was 215°.

5 μl samples were injected into the chromatograph. In the range 1–5 μg, the ratio of the peak heights of metyrapone and the acetylated reduced metyrapone to those of the standard, SU 9055, were linear. The retention times under the stated conditions were metyrapone 2·3 min, acetylated reduced metyrapone 4·2 min, and SU 9055 5·7 min. The recoveries of metyrapone and reduced metyrapone added to urines were 94·2% ± 9·8 (s.d.) and 93·9% ± 7·3 respectively.

In Table 1 are shown the mean values for metyrapone and reduced metyrapone measured in the urine of the 9 patients undergoing the metyrapone test. Both compounds were excreted mainly as the glucuronides. Reduced metyrapone occurred in appreciably larger amounts than metyrapone, particularly on Day 2 when little change was seen in the level of reduced metyrapone, but the level of metyrapone fell markedly. Small but significant quantities (108 mg $\pm$ 14·9 s.e.) of reduced metyrapone could still be detected on Day 3. The mean quantity of metyrapone plus reduced metyrapone recovered from the urine on Days 1–3 was 1·97 g $\pm$ 0·13 s.e.

Reduced metyrapone is quantitatively an important metabolite of metyrapone. It is not an inactive metabolite, being as potent an inhibitor of ox and human adrenal 11β-hydroxylase as metyrapone (Sprunt & Hannah, 1968).

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Alterations in noradrenaline turnover in the peripheral sympathetic neurons induced by stress

It has long been known that injected noradrenaline can be taken up and concentrated in peripheral sympathetic nerves (Whitby, Axelrod & Weil-Malherbe, 1961; Hamberger, Malmfors & others, 1964). Furthermore, noradrenaline incorporation is greatest in tissues with rich sympathetic innervation (Kopin, 1966). In addition to the normal endogenous noradrenaline content, the uptake of noradrenaline into tissues after intravenous injection is related to the proportion of cardiac output delivered to the tissue. Endogenous transmitter and exogenously given tritiated noradrenaline (^{3}H-NA) can be released by nerve impulses which, together with other evidence, indicate that the latter is present in the same store as the endogenous catecholamine (Iversen, 1967). Thus, the disappearance rate and the impulse flow seems to be correlated. I now report the influence of various stress conditions, which might increase sympathetic tone, on the disappearance rate of administered ^{3}H-NA.

Male albino rats were injected with ^{3}H-NA 1 μg/kg, i.v., 3 h before stress was induced. The animals were stressed for 3 h and then killed by exsanguination under light chloroform anaesthesia. Various peripheral organs were dissected and homogenized with an "Ultra-Turrax" apparatus in ice cold 0·4 N perchloric acid. After centrifugation, filtration and neutralization, the samples were passed through Dowex 50 cation-exchange columns from which the noradrenaline was eluted (Carlsson & Waldeck, 1963). The ^{3}H-NA content was measured by liquid scintillation counting technique (Waldeck, 1968).

Three different kinds of stress were investigated, (1) cold stress, (2) stress induced by electric shock, (3) immobilization stress. For (1) the rats were shaved the day before the experiment under light ether anaesthesia. They were maintained at an environmental temperature of +3–4° for 3 h. The rectal temperature was +32–35° at

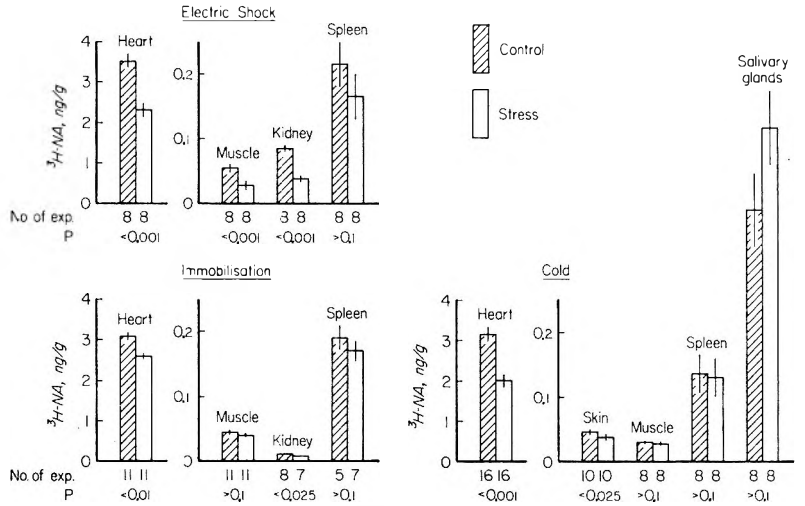

FIG. 1. The effect of different stress conditions on the disappearance rate of ^{3}H-NA from tissues. All values are 6 h after the i.v. injection of ^{3}H-NA. The stress was induced between 3 and 6 h after the injection. The vertical bars on top of the columns indicate s.e. *P* values are calculated by analysis of variance (q × q equatorial test, Winer, 1962).

death. The animals had a hunched back posture during the experiment. For (2) the shocks were given in commercially-available rat behavioural chambers containing a grid floor connected to a stimulator. Each rat was stressed for six periods of 10 min altering with rest periods of 20 min for a total of 3 h. During the 10 min stimulation period, each animal received 400 shocks of 1·6 mA intensity and 0·5 s duration. For (3) the rats were in small wire cages (Corrodi, Fuxe & Hökfelt, 1967) for 3 h.

Table 1. *Content of* ^{3}H-NA, ng/g, *in various rat tissues* 3 *and* 6 *h after injection of* ^{3}H-NA (1 mg/kg, i.v.). Values are means ± s.e.

	3 h	6 h	*P* value	Statistical test used
Heart	4·72 ± 0·40 n = 6	3·72 ± 0·35 n = 8	>0·10	analysis of variance
Spleen	0·33 ± 0·022 n = 6	0·24 ± 0·021 n = 4	<0·05	*t*-test
Kidney	0·18 ± 0·018 n = 6	0·11 ± 0·016 n = 8	<0·005	analysis of variance
Muscle	0·049 ± 0·0381 n = 6	0·045 ± 0·0330 n = 8	>0·10	analysis of variance

Table 2. ^{3}H-NA *in various rat tissues in control and electroshock stressed animals.* Comparison is made between untreated animals and animals pretreated with phenoxybenzamine (10 mg/kg, i.p.) and propranolol (10 mg/kg, i.p.). Values are means ± s.e., the number of animals being 8 in all cases; s.e. values are calculated by analysis of variance (p × q equatorial test, Winer, 1962).

	No drug			Phenoxybenzamine + propranolol		
	Control ng/g	Stress ng/g	Stress as % of control	Control ng/g	Stress ng/g	Stress as % of control
Heart	3·51 ± 0·183	2·30 ± 0·183	65·0 ± 7·38	2·32 ± 0·133	1·61 ± 0·133	69 ± 8·1
Kidney	0·08 ± 0·005	0·04 ± 0·005	44·0 ± 8·33	0·07 ± 0·008	0·04 ± 0·008	59 ± 15·3
Spleen	0·22 ± 0·035	0·17 ± 0·035	77 ± 22·28	0·11 ± 0·008	0·66 ± 0·008	55 ± 10·3
Muscle	0·05 ± 0·008	0·03 ± 0·008	50 ± 20·37	0·02 ± 0·002	0·01 ± 0·002	75 ± 17·7

In control experiments there was only a slight decrease of the ^{3}H-NA content in heart, spleen, kidney and muscle of the hind leg, during the period 3–6 h after administration of ^{3}H-NA (Table 1). In the cold stress experiments there was a decrease in the ^{3}H-NA content of the heart ($P < 0.001$) and skin ($P < 0.025$) compared to the corresponding organs of control animals, but there was no accelerated disappearance of ^{3}H-NA in the muscle of the hind leg, kidney and spleen ($P > 0.1$). In the electric shock experiment there was an accelerated disappearance of ^{3}H-NA in the heart, kidney and muscle of the hind leg ($P < 0.001$) but not in the spleen ($P > 0.1$). In the immobilization stress experiments, it was possible to show a somewhat accelerated disappearance of ^{3}H-NA from the heart ($P < 0.01$) and kidney ($P < 0.025$) but not from the spleen and muscle of the hind leg (Fig. 1).

These experiments show that stress factors can increase the disappearance rate of ^{3}H-NA from tissues which, however, may be influenced differently by various types of stress.

The accelerated ^{3}H-NA turnover seen in many of the organs investigated might reflect an increased impulse flow in the sympathetic nerves evoked by the different stress factors. However, other factors that could influence the disappearance rate of the given ^{3}H-NA must be kept in mind. For example, it is known that a decrease in blood flow caused by vasoconstriction may diminish the elimination of the released transmitter via the blood (Rossell, Kopin & Axelrod, 1963). Conversely, blood flow may be increased by activation of the tissue causing an increased disappearance rate of noradrenaline (Carlsson, Folkow & Häggendal, 1964). Experiments were designed to minimize the circulatory effects of vascular reflexes after activation of the sympathetic nervous system. Therefore, animals were given an α- and a β-receptor blocking agent (phenoxybenzamine, 10 mg/kg; propranolol, 10 mg/kg). After this treatment the animals showed a pronounced ptosis. The treatment accelerated the disappearance of ^{3}H-NA from tissues. Moreover, the stress factor investigated (electric shocks) still caused an acceleration of disappearance of ^{3}H-NA as in the experiments with untreated animals (see Table 2). It is thus likely that stress, at least that induced by electric shocks, increases noradrenaline turnover in the peripheral adrenergic system by increasing nerve impulse flow.

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Letters to the Editor