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Mass spectra of derivatives of phenylalkylamines

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The mass spectra of 18 synthetic and naturally occurring phenylalkylamine derivatives and compounds having a similar pharmacological effect were taken and evaluated and possible fragmentation mechanisms for each are discussed.

THE mass spectrometer has proved useful for the identification and elucidation of the structure of small amounts of substances. We have been interested in the detection of catecholamines and similar transmitter substances in animal tissues and sympathetic ganglia and in body fluids, which after chromatographic separation are usually available in less than mg amounts.

The mass spectrometer appeared to be suitable for identifying these substances. To obtain meaningful results, we have run and evaluated the mass spectra of several substances of this kind. A problem is that they give only small or no molecular ions (Teeter, 1966). The mass spectra to be described should simplify the identification of compounds of this type. Transmitter substances usually contain as their basic structure a phenylalkylamine.

Experimental

All spectra were taken on a Hitachi-Perkin Elmer RMU-6D Mass Spectrometer with an electron beam energy of 70 eV using the direct heated inlet system.

Results and discussion

Amphetamine, methamphetamine (Beckett, Tucker & Moffat, 1967), phentermine, chlorphentermine and 2-dimethylamino-1-phenylpropane, e.g., undergo a common β-fission process. The alkylamine residue normally appears as the base peak. The molecular ion is less than 1%

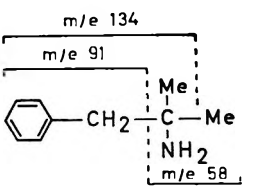

FIG. 1.

of the base peak or does not appear at all. In addition a peak at M-1 is present for all compounds when no further substituent is present on the benzene ring. This has also been observed with other aliphatic amines

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(Djerassi & Fenselau, 1965). The benzyl residue, which appears with an intensity between 30–50% of the base peak, fragments further as described by Beynon (1960), while the *p*-chlorobenzyl residue decomposes analogously to that described for *p*-chlorotoluene (Cornu & Massot, 1966). Fig. 1 shows the main fragmentation of phentermine.

When one or more OH groups are present on the benzene ring as with tyramine and dopamine, the molecular ion appears at an intensity of between 5 and 10% of the base peak which is the m/e 30 peak resulting from β-fission. A second important fragmentation involves the loss of COH from the phenolic portion (Beynon, 1960). A possible path is shown in Fig. 2 for tyramine.

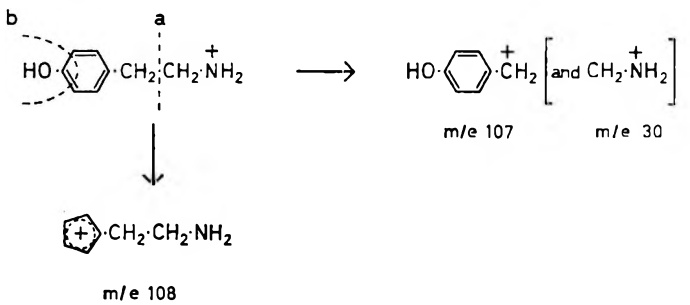


FIG. 2.

When an hydroxyl group is placed α to the benzene ring (β to the amino-group) as in norephedrine and ephedrine, no observable molecular ion is obtained. The base peak is m/e 44 or 58, respectively, resulting from β-fission. The benzylic residue also appears (m/e 107), however only to the extent of 1–2% of the base peak. Another interesting aspect is the presence of the benzoyl ion radical (m/e 105), as shown in Fig. 3 for ephedrine.

Some of the common transmitter substances possess hydroxyl groups both on the benzene ring and the carbon α to the benzene, e.g., noradrenaline, adrenaline, and isoprenaline. Among this type of compound

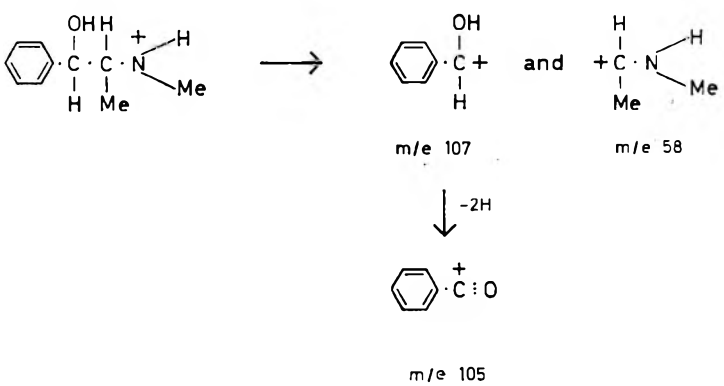


FIG. 3.

are norphenylephrine, ethyladrianol, and nylidrin, all of which undergo the usual β-fission process under electron bombardment. Norphenylephrine and ethyladrianol, besides giving a small molecular ion, have as their base peak m/e 30 and m/e 58 respectively. In addition, peaks corresponding to the loss of COH (from the phenolic portion) and peaks for the hydroxybenzoyl ion, as with norephedrine and ephedrine, can be observed. Because of the branched chain substituent on the nitrogen, nylidrin presents a more complex spectrum. Besides the usual β-fission, a fragmentation (A) α to the nitrogen and one (B) β to the non-phenolic benzene ring in the side chain occurs. It should be noted that the m/e 44 peak is the base peak as in the spectrum of isopropylamine (Cornu & Massot, 1966). Fig. 4 shows the proposed fragmentation for nylidrin.

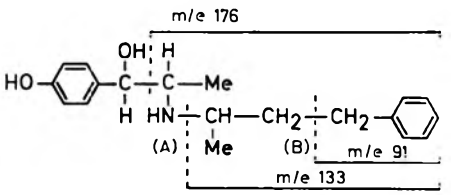


FIG. 4.

Noradrenaline, adrenaline, and isoprenaline with two phenolic hydroxyl groups all give an observable molecular ion. The peaks m/e 30 (noradrenaline), 44 (adrenaline), and 72 (isoprenaline) are again present.

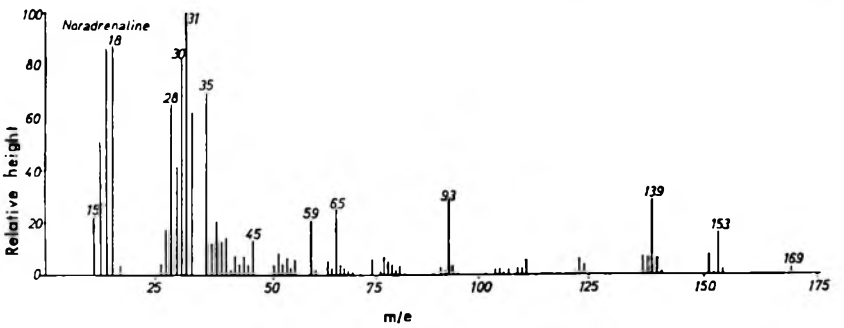


FIG. 5. Mass spectrum of noradrenaline.

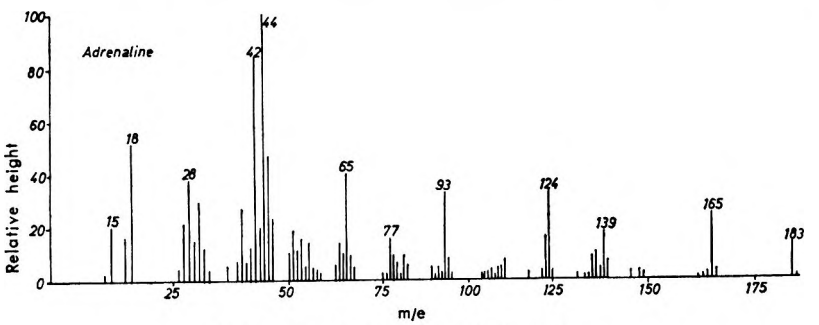
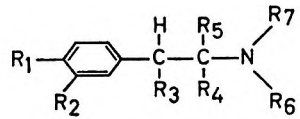


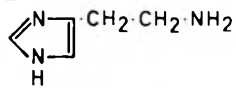
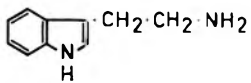
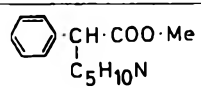
FIG. 6. Mass spectrum of adrenaline.

TABLE 1. STRUCTURAL FORMULAE AND M/E VALUES OF COMPOUNDS INVESTIGATED



Compound	R_1	R_2	R_3	R_4	R_5	R_6	R_7	M	100% peak	m/e (% relative height)
Amphetamine				Me				135	44	135 (0), 134 (1), 120 (3), 91 (20), 77 (3)
Methamphetamine				Me		Me		149	58	149 (<1), 148 (1), 134 (3), 91 (20), 77 (3)
Phentermine				Me	Me			149	58	149 (<1), 148 (<1), 134 (15), 117 (5), 91 (50), 77 (3)
Chlorphentermine	Cl			Me	Me			183	58	183 (0), 168 (5), 127 (2), 125 (10), 89 (7), 77 (2)
2-Dimethylamino-1-phenylpropane ..				Me		Me	Me	163	72	163 (1), 162 (2), 148 (10), 135 (5), 117 (8), 115 (8), 103 (5), 91 (60), 89 (7), 77 (50)
Tyramine	OH							137	30	137 (15), 120 (2), 109 (5), 108 (35), 107 (25), 91 (2), 77 (25)
Dopamine	OH	OH						153	30	153 (4), 124 (55), 123 (25), 105 (1), 77 (15)
Norephedrine			OH	Me				151	44	151 (0), 149 (1), 141 (1), 132 (1), 118 (1), 117 (1), 107 (5), 105 (5), 91 (5), 77 (15)
Ephedrine			OH	Me		Me		165	58	165 (0), 146 (1), 132 (2), 131 (2), 117 (3), 107 (10), 105 (10), 91 (10), 77 (70)
Norphenylephrine		OH	OH					153	30	153 (10), 124 (60), 123 (15), 121 (15), 107 (5), 95 (40), 77 (35)
Ethyladrianol		OH	OH			Me		181	58	181 (1), 162 (2), 152 (3), 138 (2), 121 (50), 107 (10), 95 (10), 93 (5), 77 (15)

TABLE 1—*continued*

Compound	R_1	R_2	R_3	R_4	R_5	R_6	R_7	M	100% peak	m/e (% relative height)
Noradrenaline	OH	OH	OH					169	31	169 (3), 153 (17), 151 (8), 139 (30), 137 (7), 124 (4), 123 (6), 111 (5), 93 (30), 77 (7)
Adrenaline	OH	OH	OH			Me		183	44	183 (13), 165 (25), 139 (18), 137 (10), 124 (35), 123 (15), 111 (7), 93 (30), 77 (15)
Isoprenaline	OH	OH	OH			$CHMe_2$		211	44	211 (0), 148 (1), 141 (2), 124 (20), 123 (15), 107 (4), 106 (3), 105 (4), 95 (3), 91 (2), 78 (5), 77 (4)
Nylidrin	OH		OH	Me		$CH<^{Me}_{CH_2CH_2Ph}$		299	44	299 (0, 1), 282 (1), 267 (1), 177 (5), 176 (25), 148 (2), 133 (2), 91 (50), 77 (5)
Histamine								111	82	111 (5), 83 (10), 82 (100), 81 (60), 77 (2)
Tryptamine								160	30	160 (10), 132 (20), 131 (90), 130 (90), 118 (10), 117 (10), 103 (10), 102 (10), 89 (5), 77 (50)
Methylphenidate								233	84	233 (0), 232 (1), 172 (2), 150 (2), 130 (3), 118 (5), 117 (5), 115 (4), 103 (2), 91 (40), 85 (45), 84 (100), 77 (20)

Noradrenaline and adrenaline also show the complements to these β-fission peaks, i.e. m/e 139 (10–15% of the base peak). This m/e 139 peak then fragments in at least two ways; in one, loss of 2 hydrogens gives the corresponding benzoyl ion which has appeared in every case where a hydroxyl group is present on the benzyl carbon; the second mode is the loss of CO (Reed, 1966) followed by a splitting off of water to give m/e 93. Also characteristic for these three catecholamines is the appearance of the m/e 124 peak, which could be derived by loss of water from the molecular ion and loss of CHN, C_2H_3N, or C_4H_7N, respectively. Figs 5 and 6 show the mass spectra of noradrenaline and adrenaline as representative examples.

Three chemically and pharmacologically similar compounds, histamine, tryptamine, and methylphenidate, were also subjected to mass spectrum analysis. With histamine the base peak m/e 82 was produced by β-fission with parallel rearrangement of one amine hydrogen to the imidazole ring. A second important process appears to be simple β-fission. Tryptamine undergoes the same two processes. The resulting peaks, m/e 131 and m/e 130, then fragment further as 3-methyl indole (Budzikiewicz, Djerassi & Williams, 1964). With methylphenidate the base peak is that at m/e 84 (). Two accompanying fragmentation modes are (i) the loss of protonated methylformate (fission of the C–C bond α to the carbonyl with abstraction of two hydrogens) and (ii) the formation of the methylbenzoate ion (m/e 150). This ion then decomposes in the normal fashion to give the benzyl ion. Table 1 shows the formulae of the compounds investigated and the characteristic m/e peaks in the mass spectrum of each along with the intensity of each expressed as percent of the largest peak (arbitrarily set at 100%).

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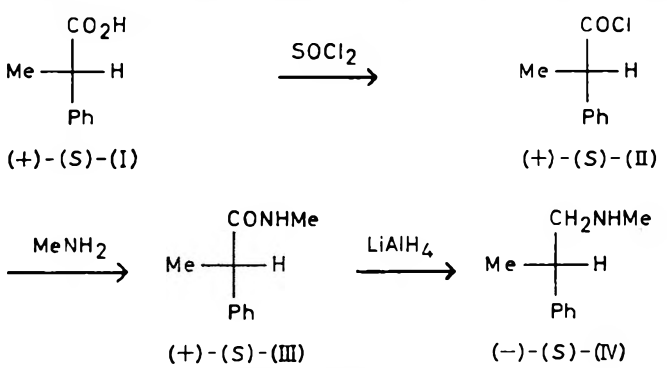
The assignment of absolute configurations to the individual enantiomorphs of phenylpropylmethylamine and pipradrol hydrochloride

R. J. HEMINGWAY

The configuration of the C-2 asymmetric centre of (−)-phenylpropylmethylamine (*N*-methyl-2-phenylpropylamine) has been related by a stereospecific route to that of the analogous centre of (+)-(*S*)-hydratropic acid. The configuration at the C-2 asymmetric centre of (−)-pipradrol (diphenylpiperid-2-ylmethanol) hydrochloride has been related by a stereospecific route to that of the analogous centre of (+)-(*R*)-pipecolic acid.

COMPOUNDS with the phenethylamine-(2-phenylethylamine) type structure, in general, exhibit an effect on the sympathetic nervous system. The classic paper of Barger & Dale (1910) showed that for those compounds with an asymmetric centre, the activity of one isomer is usually greater than that of its enantiomer. This was demonstrated more clearly by Gruber & Matthews (1953) who showed that (−)-adrenaline bitartrate and (−)-noradrenaline bitartrate were ten times more active than the (+)-isomers on dog intestine. Previously Pratesi, La Manna & others (1958, 1959) elucidated the absolute configuration of several of the important compounds exhibiting this effect. The aim of the present work is to relate the absolute configuration of (−)-phenylpropylmethylamine [(−)-*N*-methyl-2-phenylpropylamine] (IV) and (−)-pipradrol (diphenylpiperid-2-ylmethanol) hydrochloride (VII) to the analogous centres present in (+)-hydratropic acid (I) and (+)-pipecolic acid (V) respectively. It appears however that the pharmacology of the individual enantiomorphs of these two compounds has not as yet been investigated.

The stereospecific reaction sequences employed are shown below. (+)-Hydratropic acid (I) (obtained by resolution of racemic material by means of its strychnine salt) was converted to its (+)-*N*-methylamide



(+)-(S)-(I) (+)-(S)-(II) (+)-(S)-(III) (−)-(S)-(IV)

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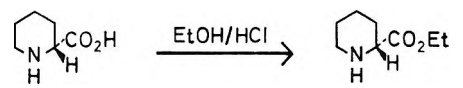
(III) via the (+)-acid chloride (II). Reduction of (+)-*N*-methylhydratropamide with lithium aluminium hydride afforded (−)-(*S*)-*N*-methyl-2-phenylpropylamine (IV) which was characterized as its hydrochloride. The sequence shown does not involve the asymmetric centre and hence the configuration of the (−)-*N*-methyl-2-phenylpropylamine will be the same as that of the starting (+)-hydratropic acid. The configuration of (+)-(*S*)-hydratropic acid has been established previously by Bernstein & Whitmore (1939) who related the (+)-acid to (+)-(*S*)-alanine and by Mislow & Heffler (1952) who, using the method of quasi-racemates, related (+)-hydratropamide derived from (+)-hydratropic acid, to (+)-(*S*)-α-chloro-α-phenylacetamide.

(+)-Pipecolic acid (V) [obtained by catalytic hydrogenation of picolinic acid and resolution of the resulting racemic acid with (+)-tartaric acid] was converted to its (+)-ethyl ester (VI) by a Fischer-Spier esterification. Reaction between this ester and phenyl magnesium bromide afforded diphenylpiperid-2-ylmethanol, which was converted to its (−)-(*R*)-hydrochloride (VII).



(+)-(*R*)-(V) (+)-(*R*)-(VI)

(−)-(*R*)-(VII)

The above sequence does not involve the asymmetric centre and hence the configuration of the (−)-diphenylpiperid-2-ylmethanol hydrochloride will be the same as that of the starting material, (+)-pipecolic acid. The configuration of (+)-(*R*)-pipecolic acid has been established previously by King, King & Warwick (1950), who prepared the (−)-acid by hydrogenation of (−)-baikiane, which they related to a derivative of (+)-(*S*)-aspartic acid.

Experimental

Hydratropic acid. Redistilled hydratropaldehyde (b.p. 95–98°/10 mm) (50 g) and silver nitrate (138 g) were dissolved in 50% aqueous ethanol (400 ml). Sodium hydroxide (52 g) in water (1,000 ml) was slowly added during 1·5 hr to the well-stirred mixture. The suspension was finally heated on a water bath for 1 hr, and the precipitate formed filtered off and washed with water and ether. The combined filtrate and washings were extracted with ether (1·5 litres), and the aqueous layer was acidified

with hydrochloric acid and re-extracted with ether (2·0 litres). This second ethereal extract was dried over anhydrous sodium sulphate, the ether removed by distillation, and the residual oil distilled, the fraction boiling between 110–112° at 1·2 mm being hydratropic acid (40 g) [Eliel & Freeman (1952) report b.p. 144–147°/11 mm].

Resolution of hydratropic acid. Hydratropic acid (35 g) and strychnine (78 g) were dissolved in 25% aqueous ethanol (350 ml). After storage at 0° for 18 hr, the solution deposited the strychnine salt (61 g), which was crystallized a further four times to yield strychnine (+)-hydratropate (4·5 g), $[\alpha]_D^{22}$ −30·2° (*c*, 1·46 in $CHCl_3$).

The rotation of the salt was unchanged on further recrystallization. The (+)-acid, liberated from the salt with dilute aqueous hydrochloric acid and extracted with chloroform, crystallized on removal of the solvent under vacuum and standing at 0° (1·2 g), m.p. 22°, $[\alpha]_D^{22}$ + 74·8° (*c*, 3·94 in $CHCl_3$). [Arcus & Kenyon (1939) report m.p. 29°, $[\alpha]_D$ + 74·8° (*c*, 3·06 in $CHCl_3$), Bakshi & Turner (1961) report m.p. 31·5–32°, $[\alpha]_D^{25}$ + 76·3° (*c*, 1·6, in $CHCl_3$).]

(+)-*Hydratropyl chloride.* (+)-Hydratropic acid (1·1 g) and thionyl chloride (1·1 g) were allowed to react at 50–60° for 1 hr. The product was distilled, (+)-hydratropyl chloride (b.p. 100–105°/0·5 mm) being collected as a colourless liquid (1·1 g), $[\alpha]_D^{22}$ + 106·1 (*c*, 7·5 in benzene). [Bakshi & Turner (1961) report b.p. 81–83°/10 mm, $[\alpha]_D^{22}$ + 101·5° (*c*, 2·44 in benzene).]

(+)-N-*Methylhydratropamide.* (+)-Hydratropyl chloride (1·1 g), dissolved in dry benzene (15 ml), was treated with dry methylamine gas for 10 min. The solvent was removed under vacuum and the residue, crystallized from water, gave (+)-*N*-methylhydratropamide as colourless plates, m.p. 80–81°, $[\alpha]_D^{22}$ + 53·2° (*c*, 2·63 in $CHCl_3$) [Carrington, Vassey & Waring (1953) report m.p. 82° for racemic material].

(−)-N-*Methyl-2-phenylpropylamine.* (+)-*N*-Methylhydratropamide (980 mg), in dry ether (40 ml), was refluxed for 5 hr with lithium aluminium hydride (500 mg). The reaction mixture was treated with dilute hydrochloric acid and the aqueous layer extracted with ether. The extracted aqueous liquid was made alkaline with dilute sodium hydroxide solution, saturated with sodium potassium tartrate and finally extracted with ether. The ether was removed from the extract on a water-bath and the residual oil distilled, the fraction boiling between 60–70° at 0·2 mm being (−)-*N*-methyl-2-phenylpropylamine (520 mg), $[\alpha]_D^{23}$ − 18·2° (*c*, 4·42 in EtOH).

The hydrochloride was precipitated from an ethereal solution of the free base by treating with dry hydrogen chloride. The hydrochloride crystallized from ethanol-ether, had m.p. 152° [Woodruff, Lambooy & Burt (1940) report m.p. 148–159° for racemic material].

Pipecolic acid. Picolinic acid (50 g) in water (150 ml) was shaken in the presence of hydrogen with platinum oxide (1·25 g), whereupon three moles of hydrogen were absorbed. The catalyst was removed by filtration and the solution treated with charcoal (2 g). After removal of the charcoal by filtration, the solvent was evaporated under reduced pressure

and the residue crystallized from methanol-ether to yield pipecolic acid as colourless needles, m.p. 262° (50 g). [Heilbron & Bunbury (1946) quote m.p. 264°.]

(+)-*Pipecolic acid.* Pipecolic acid (52 g) and (+)-tartaric acid (57 g) were dissolved in ethanol (1 litre), the solution boiled for 30 min and allowed to stand for 18 hr, whereupon the salt crystallized (57 g). The solid was crystallized three times from aqueous acetone to yield (+)-pipecolic acid hydrogen (+)-tartrate (19·5 g), $[\alpha]_D^{25}$ + 21·1° (*c*, 4·0 in H_2O). This salt, whose rotation was unchanged on further recrystallization, was dissolved in distilled water (250 ml), the solution passed through a column of Amberlite IR4b (OH^- form) resin (300 g), and the column washed with distilled water until the eluate was no longer alkaline. The total eluate was treated with charcoal (0·2 g) and filtered to remove the charcoal. The filtrate was evaporated under reduced pressure and the residue crystallized from aqueous acetone to yield (+)-pipecolic acid (7·0 g) as colourless needles, m.p. 280°, $[\alpha]_D^{23}$ + 24·6° (*c*, 3·35 in H_2O). [Heilbron & Bunbury (1946) quote m.p. 270°, $[\alpha]_D$ + 24·5°.]

(+)-*Ethyl pipecolate.* (+)-Pipecolic acid (6·5 g) was refluxed for 12 hr with ethanol (60 ml) containing hydrogen chloride (2·5 g). The reaction mixture was cooled on ice, treated with potassium carbonate to neutralize the acid, and the suspension filtered, the solid being washed with ethanol and ether. The solvent was removed under reduced pressure from the combined filtrate and washings and the residual oil distilled, (+)-ethyl pipecolate being collected as a colourless oil, b.p. 80–85°/1·0 mm (5·0 g), $[\alpha]_D^{23}$ + 10·0° (*c*, 4·08 in EtOH).

(−)-*Diphenylpiperid-2-ylmethanol hydrochloride.* A solution of (+)-ethyl pipecolate (4·8 g) in benzene (10 ml) was added to a Grignard reagent prepared from bromobenzene (12·0 g), magnesium (2·1 g) and ether (20 ml). The mixture was heated under reflux for 90 min and the product decomposed by pouring into a saturated aqueous solution of ammonium chloride (50 ml). The aqueous layer was made alkaline with aqueous sodium hydroxide (2N), the organic layer removed and the aqueous layer extracted with ether. The ethereal solution was extracted with hydrochloric acid (2N), and the aqueous layer made alkaline with sodium hydroxide solution (2N) and re-extracted with ether. The final ethereal extract was washed with water, dried over anhydrous sodium sulphate and the solvent evaporated off to yield a residual brown oil. The oil was distilled, the fraction boiling between 130–135° at 0·8 mm being crystallized from benzene-light petroleum (60–80°) to yield colourless needles of (−)-diphenylpiperid-2-ylmethanol (2·4 g), m.p. 71–72°.

The free base, dissolved in ethyl methyl ketone (30 ml), was treated with hydrogen chloride, and the precipitated solid was crystallized from ethyl methyl ketone to yield colourless needles (2·1 g), m.p. 306°, $[\alpha]_D^{25}$ − 64·3° (*c*, 1·74 in H_2O). [Tilford, Shelton & Campen (1948) report m.p. 308–309° for racemic material.]

Melting points were measured using an Electrothermal capillary melting point apparatus and were uncorrected.

Optical rotation measurements were taken using a Bellingham and Stanley Ltd. polarimeter. Probable limits of error involved in the measurement of $[\alpha]_D$ are about $\pm 5\%$.

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Kinetics of buccal absorption of amphetamines

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The buccal absorption of amphetamine, methylamphetamine and dimethylamphetamine in solutions at pH 8·16 and 9·18, was measured in man after 1, 2, 3, 4, 5 and 10 min. The recovery of the drugs from the buccal membrane after uptake was also measured by washing out the mouth for varying times with buffer solutions. An analogue computer model of the biological system was used and the kinetic parameters for the buccal absorption of the amphetamines were calculated.

RECENTLY, the importance of examining the kinetics of drug transfer between aqueous and organic phases has been emphasized (Doluisio & Swintosky 1964; Perrin 1967). These authors have devised various *in vitro* systems which allow rate of partition studies to be made, but as with partition coefficient experiments, rate of partition is profoundly influenced by the nature of the organic phase. Since these *in vitro* systems are intended to be models for the behaviour of drugs in various physiological functions i.e., gastrointestinal absorption, the success of the interpretation of the behaviour of a drug will depend on the extent to which the organic phase chosen simulates *in vivo* lipid membranes.

To overcome this major disadvantage in currently available *in vitro* partition systems, the buccal absorption of drugs has been proposed as an *in vivo* model system for the study of drug transfer across physiological membranes (Beckett & Triggs, 1967). A description of the kinetics of the buccal absorption of three chemically related drugs, amphetamine, methylamphetamine and dimethylamphetamine is now presented.

EXPERIMENTAL—BUCCAL ABSORPTION MEASUREMENTS

Apparatus. Perkin-Elmer F11 Gas Chromatograph. Dynacap pH Meter.

Buffer solutions. Potassium hydrogen phthalate (0·05 M) pH 4·00. Sodium tetraborate (0·05 M) pH 9·18. Sörensens phosphate buffer pH 8·16.

Drug solutions. Solutions of the drugs amphetamine, methylamphetamine and dimethylamphetamine were prepared in the buffers of pH 8·16 and 9·18 such that 25 ml contained the equivalent of 1 mg drug base.

Buccal absorption measurements. Male volunteers aged 20–40, who produced only small volumes of saliva, were used. A drug solution (25 ml) was introduced into the subject's mouth for 1, 2, 3, 4, 5 and 10 min. After each time the solution was expelled, diluted to a suitable volume and analysed for drug content. (For detailed procedure see Beckett & Triggs, 1967, and also Beckett & Moffat, to be published, in which analyses of acids under conditions of varying saliva flow are reported).

Immediately after the solution had been expelled from the mouth after contact times of 5 and 10 min, 25 ml of pH 4·0 buffer was placed in the

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mouth, circulated for 1 min and expelled. This was repeated every min for a further 4 min. The expelled solutions were diluted and analysed.

Analysis. Drug content in the expelled solutions was determined by the gas-liquid chromatographic procedure described by Beckett & Triggs (1967).

RESULTS—BUCCAL ABSORPTION

The data points in Fig. 1 represent the experimentally determined amounts of drug remaining in the mouth after each contact time in a buffer pH of 8·16. The cumulative return of drug to the mouth after the wash-out procedure is also shown. In each case the absorption appears to level off after a contact time of approximately 5 min. Also about 50%

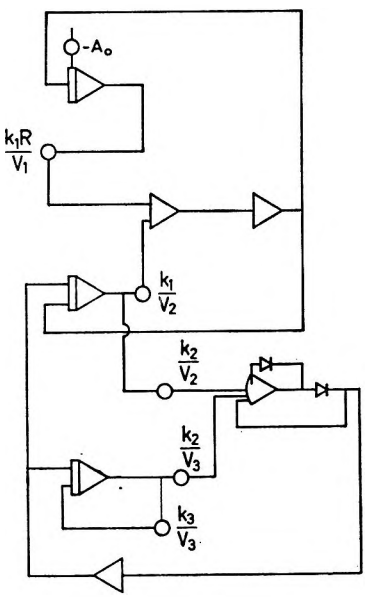

FIG. 1. Analogue computer program for the kinetics of buccal absorption of the amphetamines.

of the absorbed drug is returned to the mouth after washing out with the buffer of pH 4·0. The buccal absorption after contact times of 5 and 10 min using a buffer of pH 9·18 was: amphetamine 79·5 and 88·5%; methylamphetamine 68·5 and 80·5%; dimethylamphetamine 78·5 and 84·5%.

EXPERIMENTAL—MATHEMATICAL TREATMENT

Apparatus. Electronics Associates Ltd. TR-20R Analogue Computer.

Method. Inspection of the results of the buccal absorption experiments described above for the 'amphetamines', indicated that a simple three compartment model might mathematically simulate the physiological system. Although absorption appears to level off approximately 5 min

after placing the drugs in the mouth (see Fig. 1), less than 50% of the amount of the drug absorbed could be recovered by successive rinsing of the mouth after this period of time. This suggested that two absorption compartments were involved only one of which was in rapid equilibrium with buffer in the mouth. The model shown in Fig. 2 was therefore proposed to study the kinetics of the buccal absorption of the 'amphetamines.' The compartments were arranged such that transfer of drug between A and B was freely reversible, and movement from B to C depended on the apparent concentration difference in the compartments. Results indicated that reverse transfer from C to B was a very slow process compared to forward movement from B to C. In the total system this reverse process would be very difficult to estimate with any degree of confidence and therefore was considered to be zero for our purposes. A steady slow loss from compartment C was required to account for the slight absorption occurring between the 5 and 10 min contact times.

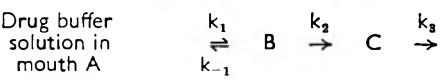

FIG. 2. Proposed kinetic model for the buccal absorption of the 'amphetamines'.

The following mathematical equations were used to describe the transfer of drug between the compartments:

$$\frac{dA}{dt} = -k_1\left(\frac{AR}{V_1} - \frac{B}{V_2}\right) \quad .. \quad .. \quad .. \quad .. \quad (1)$$

$$\frac{dB}{dt} = +k_1\left(\frac{AR}{V_1} - \frac{B}{V_2}\right) - k_2\left(\frac{B}{V'_2} - \frac{C}{V_3}\right) \quad .. \quad (2)$$

$$\frac{dC}{dt} = k_2\left(\frac{B}{V'_2} - \frac{C}{V_3}\right) - k_3\left(\frac{C}{V_3}\right) \quad .. \quad .. \quad .. \quad (3)$$

equations (2) and (3) apply when:

$$\frac{B}{V'_2} > \frac{C}{V_3}$$

when $\frac{B}{V'_2} \leqslant \frac{C}{V_3}$ the following differential equations were used to describe compartments A and B.

$$\frac{dB}{dt} = k_1\left(\frac{AR}{V_1} - \frac{B}{V_2}\right) \quad .. \quad .. \quad .. \quad (4)$$

$$\frac{dC}{dt} = -k_3\left(\frac{C}{V_3}\right) .. \quad .. \quad .. \quad .. \quad (5)$$

where: A, B and C = the total amount of drug in the respective compartments; V_1 = volume of buffer solution placed in the mouth (25 ml); R = % unionized drug/100 at the particular buffer pH; V_2 = apparent

volume of compartment B with respect to compartment A; V'_2 = apparent volume of compartment B with respect to compartment C; V_3 = apparent volume of compartment C; and k_1, k_2 and k_3 are the rate constants governing the transfer of drug between the compartments.

The equations (1–5) were programmed on the analogue computer as shown in Fig. 3.

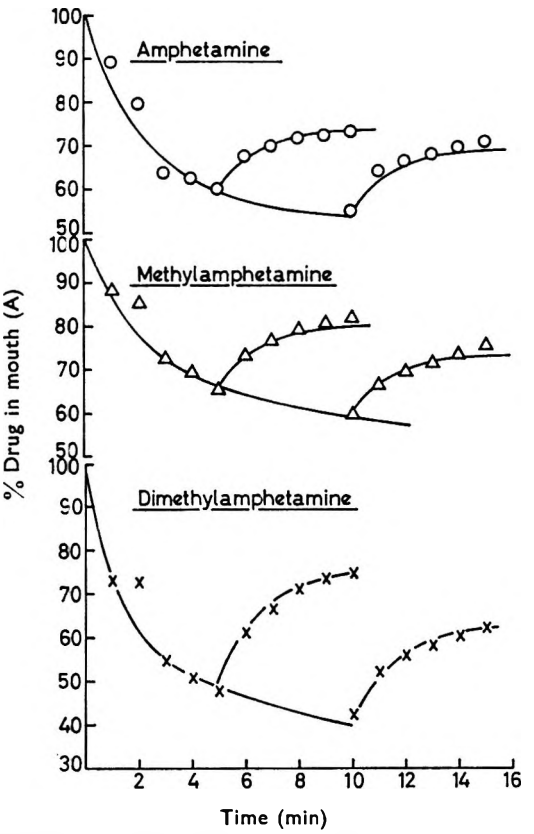

FIG. 3. Buccal absorption of some amphetamines. The points show the experimental data, the curves the computer calculations.

Conversion from equations 2 and 3 to equations 4 and 5 was made on the computer using a diode limiter; the term $k_2\left(\frac{B}{V'_2} - \frac{C}{V_3}\right)$ only exists on the computer when $\frac{B}{V'_2} > \frac{C}{V_3}$. The washout procedure was simulated on the computer by setting the value of the term k_1R/V_1 to zero.

Computer solutions for the equations (1–5) were obtained by systematically altering the potentiometers representing the constant parameters until good agreement was obtained between the computer calculations for

the amount of drug in compartment A and the experimental data for both absorption and wash-out procedures. For these calculations it was assumed that neither the rate constants nor the apparent volumes of the compartments changed appreciably during the course of each experiment. Since V_1 was known and R could be calculated for each drug at a particular buffer pH, the value of k_1 could be calculated from the potentiometer representing the term k_1R/V_1.

RESULTS—MATHEMATICAL TREATMENT

The continuous lines in Fig. 1 represent the computer-calculated amounts of drug in compartment A as a function of time at a buffer pH of 8·16; there is close agreement between the computer calculations and the experimental data points for both the absorption and wash-out procedures for all three drugs. The values of the constant parameters for each drug at a buffer pH of 8·16 are summarized in Table 1.

TABLE 1. KINETIC PARAMETERS FOR THE BUCCAL ABSORPTION OF THE AMPHETAMINES

Drug	Buffer pH	pK_a*	V_1(ml)	R	$\frac{k_1R}{V_1}$	k_1†	$\frac{k_1}{V_2}$	$\frac{k_2}{V'_2}$	$\frac{k_3}{V_3}$	$\frac{k_3}{V_3}$
Amphetamine	8·16	9·77	25	0·0240	0·2012	210·0	0·6515	4·292	2·485	0·0166
Methylamphetamine ..	8·16	9·87	25	0·0191	0·1740	227·5	0·6363	3·506	3·184	0·0920
Dimethylamphetamine	8·16	9·40	25	0·0544	0·3794	174·4	0·5794	3·000	7·343	0·2974

* Leffler, Spencer & Burger (1951).
† Units—ml min^{-1}.

Direct conversion of the parameter k_1R/V_1 from a buffer pH of 8·16 to one of 9·18 resulted in too rapid a predicted rate of absorption for all of the 'amphetamines', although the experimental and calculated amounts of the drugs absorbed after 10 min were in reasonably close agreement, i.e., for amphetamine the predicted amounts of the drug absorbed after 5 and 10 min were 80·0 and 89·0% respectively compared with the corresponding experimental values of 79·5 and 88·5%. Good agreement between calculated and experimental data at a buffer pH of 9·18 could be obtained for all the 'amphetamines' by making the value of the parameter k_1R/V_1 less than expected on the basis of the results at a pH of 8·16.

Discussion

Drugs may be classified in terms of their relative order of partitioning into a biological fluid by the 'Buccal Absorption Test' (see Beckett & Triggs, 1967). Our results indicate that the kinetics of the buccal absorption of drugs also may be useful in assigning numerical values to these relative partitioning properties.

Previous work (Beckett & Triggs, 1967) indicated that buccal absorption of the 'amphetamines' was related to the concentration of unionized drug in the mouth, i.e., as the buffer pH was made progressively more alkaline there were substantial increases in the amounts of the 'amphetamines'

absorbed in a fixed period of time. It was also found that optical isomers were absorbed to the same extent, and when more than one drug was placed in the mouth at the same time the same absorption occurred as when the drugs were placed in the mouth singly. This evidence indicated that buccal absorption involved passive diffusion of the unionized form of the drug from an aqueous phase to a lipid phase. Thus transfer from compartment A to B in the proposed model (see Fig. 2) involves a partitioning process and therefore the apparent volume (V_2) of compartment B will be a combination of the true volume and the partition coefficient of the drug between buffer and lipid. The nature of compartment C is unknown and therefore the apparent volume of B with respect to C was given as V'_2. Since it is possible to obtain good agreement between the computer calculations and the experimental data for the buccal absorption of the 'amphetamines' it is reasonable to assume that the proposed computer model is a valid mathematical description of the biological system.

The calculated parameters listed in Table 1 suggest that although more dimethylamphetamine than amphetamine and methylamphetamine is absorbed at any time, the rate constant for absorption (k_1) is less than for the latter two drugs. The more extensive absorption of dimethylamphetamine at a pH of 8·16 therefore results from the higher concentration of unionized drug in the buffer due to the pK_a differences between the drugs (see Table 1). This higher concentration of unionized moiety may result in association or reduced solubility of the dimethylamphetamine in the buffer and therefore the rate constant (k_1) for this drug may involve an availability (or activity) term. The failure to obtain good agreement between calculated and experimental absorption data on conversion of the parameter k_1R/V_1 from a pH of 8·16 to 9·18 indicates that, for all the 'amphetamines', availability or activity terms must be included in the calculations when the concentration of unionized drug is relatively high. Further experiments at various pH values are necessary to elucidate the relation between percentage of the 'amphetamines' which are unionized and the rate of buccal absorption.

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A note on the absorption of 3-methoxy-*N*-methylmorphinan hydrochloride from the rat stomach

G. FIESE AND J. H. PERRIN

(+)-3-Methoxy-*N*-methylmorphinan, a base with a pK_a of 7·97 is absorbed from the rat stomach at pH 2·0, in a manner which suggests passive diffusion rather than a specialized transport process to be the mechanism.

IT is widely believed as a result of the "pH partition hypothesis" (Brodie, 1964) that amines, other than quaternary ammonium derivatives, can be absorbed only from the small intestine where the pH is such that a significant fraction of the drug is in the non-ionized and lipid-soluble form. However, drugs of the morphinan class form salts which have considerable lipid solubility, a factor which is used in their chemical assay (Divatia & Biles, 1961; Hull & Biles, 1964). *In vitro* transfer into an organic phase and the disappearance of (+)-3-methoxy-*N*-methylmorphinan hydrochloride from a solution held in the rat stomach *in situ* have now been examined. Although the salt forms ion pairs of limited lipid solubility (Higuchi, Michaelis & others, 1967), it does contain the only anion of consequence in the stomach.

Experimental

MATERIALS

The (+)-3-Methoxy-*N*-methylmorphinan was an analytical sample of dextromethorphan base (Vick). Sodium chloride, potassium chloride, calcium chloride, hydrochloric acid, sodium borate, monosodium dihydrogen phosphate, amyl alcohol, and chloroform were reagent grade. Cyclohexane was spectroscopically pure. Tropaeolin 00 dye (sodium *p*-diphenylamineazobenzenesulphonate, Eastman) was recrystallized four times from water. Normal heptane (Phillips Petroleum, Oklahoma) was redistilled and the fraction collected at 93°.

PHOSPHOLIPID EXTRACTION

The yolks of 12 eggs were washed with 400 ml of acetone and the residue extracted with 1500 ml of a 5% solution of ether in ethanol. The extract was evaporated to dryness in a rotary evaporator and the residue dissolved in 400 ml of ether and precipitated with 4 volumes of acetone. This precipitation procedure was repeated three times, giving a final yield of approximately 12 g of phospholipid extract having an approximate composition of 4 choline containing lipids to 1 ethanolamine containing lipid (Lea, Rhodes, & Stoll, 1955). It was used without further purification.

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DISTRIBUTION EXPERIMENTS

The dextromethorphan was transferred from a 0·1 M chloride buffer of pH 2 through an organic layer into a 0·1 M phosphate buffer, pH 7·4, using a cell and techniques as previously described by Perrin (1967). Preliminary experiments showed that a 25% solution of cyclohexane in amyl alcohol (v/v) gave a suitable transfer rate, and various known quantities of phospholipid were dissolved in this solvent and also used as the lipid phase. In none of the experiments was any significant amount of drug found to have passed through the organic phase into the pH 7·4 phase. The experiments were made at 25° ± 0·5°. The two aqueous phases were assayed in a manner similar to that later described. The organic layer was not assayed.

In vivo EXPERIMENTS

Sprague-Dawley female rats weighing approximately 150 g were fasted for 24 hr in cages with wide mesh floors to reduce coprophagy, with water freely available. After weighing, the animals were anaesthetized with urethane (approximately 150 mg 100 g body weight). The stomach was exposed and ligated immediately adjacent to the cardiac sphincter, care being taken not to tie off any major blood vessels. A second ligature

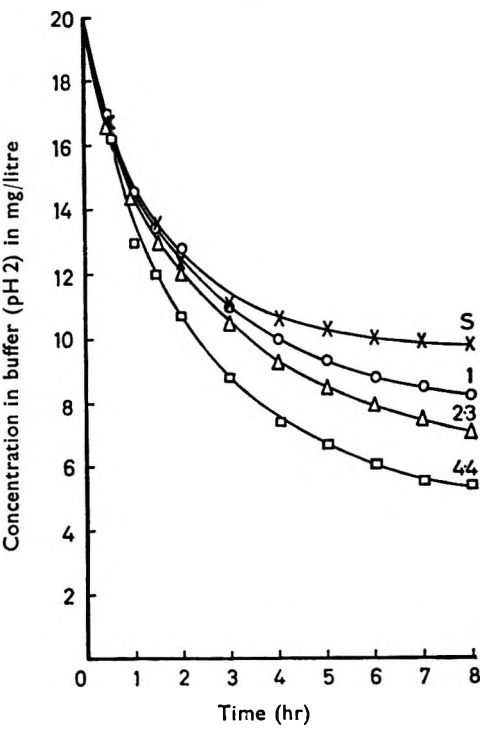

FIG. 1. Effect of phospholipid on the transfer of (+)-3-methoxy-*N*-methylmorphinan hydrochloride from pH 2·0 buffer into an organic phase of 75% amyl alcohol in cyclohexane. Solvent(S) and phospholipid concentrations are given on the curves.

was placed adjacent to the pyloric sphincter. A needle was introduced through the duodenum to project into the stomach via the pyloric sphincter before the ligature was finally secured after which, the drug was injected as a 3 ml dose in a pH 2·0 chloride buffer made isotonic (similar to Ringer solution) to prevent water absorption. The needle was then removed and the incision closed. After the desired time interval, the incision was opened, the stomach removed, and the animal killed. The contents of the stomach were removed with successive washings of normal saline to make a final volume of 25 ml after filtration through glass wool. Animals with food in their stomachs were rejected.

EXTRACTION AND ASSAY

To the saline washings was added 10 ml 0·1 M borate buffer pH 10 to give pH 9·5 approximately, and this aqueous phase was extracted with two 50 ml portions of normal heptane. The heptane was then extracted with 20 ml of a 0·1 M phosphate buffer pH 2·8. To 2 ml of this solution were added 2 ml of a pH 3·4, 0·1 M phosphate buffer, and 2 ml of a 4×10^{-4} tropaeolin 00 dye solution in water. The drug-tropaeolin

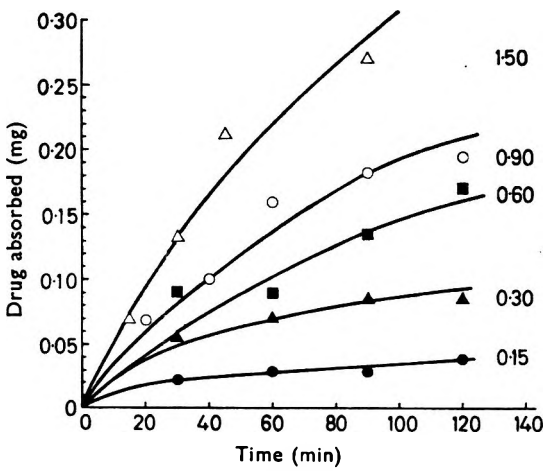

FIG. 2. Absorption of (+)-3-methoxy-*N*-methylmorphinan hydrochloride from rat stomach at pH 2. Figures on curves are doses in mg.

ion-pair was then extracted into 5 ml of chloroform, and the extinction measured at 410 mμ in a Cary 11 spectrophotometer. The washed stomachs were homogenized and extracted in a manner similar to that described and were found to contain little or no drug. Blank experiments in which 3 ml isotonic buffer containing no drug were given to the animals gave zero extinction when assayed in the above manner.

Results and conclusions

The effect of phospholipid on the rate of transfer of the drug from the pH 2·0 phase to the organic phase is shown in Fig. 1. The phospholipid

increases the rate and extent of transfer, but did not transfer the drug to the pH 7·4 phase. The drug clearly has an affinity for the phospholipid, a fact which we also observed in preliminary optical rotary dispersion investigations. We have also observed that the drug can penetrate a lecithin monolayer at a pH 2·0.

The *in vivo* investigations can be summarized by Fig. 2. Dextromethorphan has a pK_a of 7·97 which means that at pH 2 approximately one molecule in 10^6 is in the unionized state, suggesting that the drug transfer must involve the positively charged species. From Fig. 2 it can be seen that the extent of uptake increases in an almost regular manner with the dose level and does not become saturated. This suggests that absorption of this tertiary amine is by passive diffusion and not by the specialized transport process thought to be responsible for the transfer of quaternary amines. Shanker, Shore & others (1957), discussing the absorption of organic bases from the rat stomach and advancing the pH partition hypothesis, noted in preliminary experiments, that some absorption of dextromethorphan took place from a 0·1 M hydrochloric acid solution in the rat stomach, but they were unable to confirm this. The role of the anion in the absorption reported here is not clear and this is being investigated.

Acknowledgement. We would like to thank Dr. Eugene Gans of Vick Divisions Research and Development, Mount Vernon, New York, for the gift of the dextromethorphan base.

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Potentiation of ethanol by *Coprinus atramentarius* in mice

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Assessment of toxic manifestations and sleeping times in mice shows that the mushroom *Coprinus atramentarius* potentiates the action of ethanol when administered orally from 3 to 16 hr before a sub-lethal dose of ethanol. Administration of this mushroom 24 hr before, immediately after or 3 hr after the ethanol causes no potentiation. No similar effect was observed with *Coprinus comatus*.

THERE are about 75 North American species of *Coprinus*. One of the best known is *Coprinus atramentarius* (inky cap), a black-spored mushroom which is generally considered edible. Reports in the literature indicate a toxic reaction may result when this species is consumed with ethanol, and warn that ethanolic beverages should not be taken shortly before, during or after ingestion of the mushroom (Buck, 1961; Groves, 1962; Tyler, 1963). The related species *C. comatus* (shaggy mane) has also been incriminated (Zeitlmayr, 1955). On the other hand, Krieger (1911) and Child (1952) agree that *C. atramentarius* does not sensitize man to ethanol. Human idiosyncrasies, mode of preparation, and timing of consumption in relation to ethanol ingestion appear to influence the toxicity (Tyler, 1963). Attempts have been made to identify the toxic principle of *C. atramentarius*, and Simandl & Franc (1956) claim the isolation of disulfiram (Antabuse) which inhibits the *in vivo* oxidation of acetaldehyde. These findings have not been confirmed by others (List & Reith, 1960; Wier & Tyler, 1960). Four cases of poisoning attributed to inky caps and ethanol were recently reported by Reynolds & Lowe (1965).

We have investigated whether potentiation of ethanol by two *Coprinus spp.* could be demonstrated in mice.

Experimental

MUSHROOMS

Coprinus atramentarius (Bull. ex Fr.) Fr. (4·3 kg) and *C. comatus* (Mull. ex Fr.) S. F. Gray (0·9 kg) were collected on Ottawa lawns between August and October 1966. The samples were cleaned and frozen (−25°) within ½ hr of collection, freeze-dried within 5 days and powdered in a Wiley mill to pass a 100 mesh sieve. The dehydrated powder was stored at −25°. The average water content of a fresh sample of either species was 92%.

Sample preparation. Raw mushroom powder suspensions in a 0·25% aqueous tragacanth solution were freshly prepared. Cooked mushroom samples were obtained by reconstituting the mushroom powder with an appropriate amount of water and then boiling the mixture for ½ hr with stirring. After cooling, tragacanth was added. Animals were fed by stomach tube (2·4 or 2·5 ml suspension per 30 g mouse).

From the Research Laboratories, Food and Drug Directorate, Ottawa, Canada.

ETHANOL

Solutions of ethanol 30% w/v in water were administered by stomach tube. The doses selected were 5 or 6 g/kg. Lower doses did not affect the righting reflex and appreciably higher doses resulted in death.

ANIMALS

Non-fasted male albino mice (Quebec Farm) approximately 30 g were used. Their diet consisted of Master Fox Chow and water *ad lib.* All animals were randomly assigned to the experimental groups. They were observed for physiological symptoms in cages containing groups of 10. In the sleeping time experiment they were placed on tables with marked squares as soon as they fell asleep.

DEFINITIONS

Scores. The observed signs of ethanol intoxication were assigned scores, as follows: 4 death; 2 loss of righting reflex; 1 ataxia; ½ slight ataxia, other impairment (e.g., eyes closed, sedated); 0 normal. The total score in each experimental group was obtained by addition of the individual scores.

Induction time is the time interval between administration of ethanol and loss of righting reflex.

Sleeping time is the time interval between loss and return of righting reflex.

Results

The toxicity of raw mushroom suspensions was explored in preliminary experiments. Ingestion of very thick suspensions of *C. atramentarius* and *C. comatus*, up to 10·1 g/kg, had no visible effect other than sedation.

EXPERIMENT I

This was a preliminary investigation of the influence of the time between feeding mushroom suspensions and a sublethal dose of ethanol on toxic effects. Raw *C. atramentarius* and *C. comatus* (4·5 g/kg) were fed to groups of 10 mice. Ethanol (5 g/kg) was administered 3 hr before, immediately after, and 3, 6, 16 and 24 hr after mushroom feeding. Control groups of 10 animals were fed only ethanol, *C. atramentarius* or *C. comatus*. The animals were observed for 40 hr and their scores recorded. In the mushroom control groups the score was zero for the entire period. Scores of the ethanol control group were 11 (1 hr), 3·5 (3 hr) and zero after 4 hr. Ethanol + *C. atramentarius* groups, given the ethanol 3 or 6 hr after the mushroom, scored higher than the ethanol control group. Groups given the ethanol immediately or 16 hr after the mushroom had higher scores than the ethanol control group 4 hr after ethanol administration. Groups administered ethanol 3 hr before, or 24 hr after the mushroom meal had scores similar to the ethanol control group. Animals given *C. comatus* and ethanol showed only minor score differences from

the ethanol controls so further experimentation with this species was discontinued. No deaths occurred in this experiment; the main symptoms noted were ataxia, loss of righting reflex and sedation.

EXPERIMENT II

This was to assess that time interval between feeding of *C. atramentarius* and ethanol leading to the highest score. Also the action of cooked and raw mushroom was compared.

Raw and cooked *C. atramentarius* (8 g/kg), were administered alternately to groups of 10 mice, six groups receiving each preparation. Two groups (one fed raw and the other cooked mushroom) were used as controls and ethanol (5 g/kg) was administered to each of the other groups

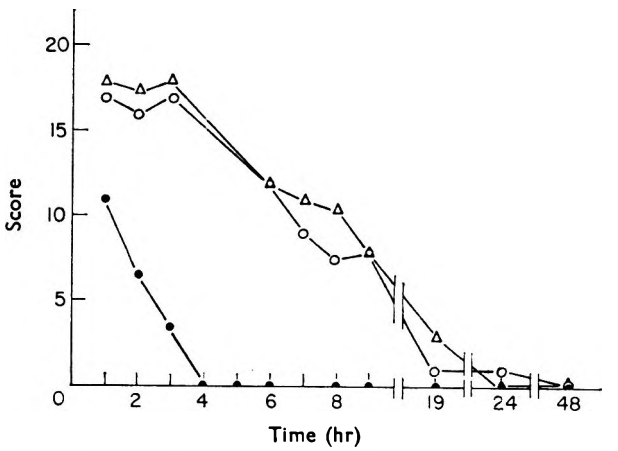

FIG. 1. Observed score vs. time (hr) after ethanol administration in mice given (●) ethanol only, (○) raw mushroom + ethanol (after 4 hr), and (△) cooked mushroom + ethanol (after 4 hr).

after 4, 6, 8, 10 and 12 hr, respectively. Another control group of 10 mice received ethanol only. The animals were observed for 48 hr and their scores recorded. The group dosed with ethanol 4 hr after the mushroom preparation showed the highest score. The scores of groups administered cooked and raw mushrooms were nearly identical (Fig. 1). Raw mushrooms and the 4 hr interval between mushroom and ethanol administration were therefore used in further work.

EXPERIMENT III

Sleeping times were measured with 120 animals in groups of 10. Six groups were given 6 g/kg of ethanol only. The other six groups were administered 8 g/kg of raw *C. atramentarius* preparation and, after 4 hr, 6 g/kg ethanol. Ethanol groups and the mushroom + ethanol groups were fed alternately. The elapsed time for feeding was 6 min per group. Results showing the sleeping time and the incidence of sleeping in both experimental groups are given in Table 1. The incidence of sleeping in

TABLE 1. SLEEPING TIME AND INCIDENCE OF SLEEPING AFTER ADMINISTRATION OF ETHANOL AND *C. atramentarius*

Treatments	Number of animals sleeping	% of animals sleeping	Deaths	Average induction time (min)	Average sleeping time (min)
Ethanol	19/60	32	0	30 (6–66)*	203 (39–670)
Mushroom (raw) + ethanol (4 hr later)	54/60	90	2†	28 (5–52)	394‡ (104–775)

* Figures in parentheses are ranges.
† One death before and one after regain of righting reflex.
‡ Animal which died before regain of righting reflex not included.

the mushroom ethanol-treated mice increased markedly over those given ethanol alone (P of χ^2 <0·005). Although the averages of the induction times in the two groups were close, the average sleeping time in the mushroom + ethanol group was nearly double that of the control group. An analysis of variance for log induction time and log sleeping time is

TABLE 2. ANALYSIS OF VARIANCE OF SLEEPING TIME

Source	d.f.	Mean squares	
		Induction time	Sleeping time
Treatments ..	1	0·020	1·523*
Within ..	70	0·086	0·051

* P <0·01.

given in Table 2. It shows that the sleeping times are significantly different for the two treatments (P <0·01), but that induction time is not.

Discussion

The time factor of the *C. atramentarius*-ethanol syndrome in man is controversial. Buck (1961) states that nitritoid symptoms are caused by this mushroom if ethanolic beverages are drunk before, during or after its ingestion. List & Reith (1960) who also cited older European literature on poisoning cases with *C. atramentarius*, reported toxic effects in one person after a 250 g (about 20 g dry weight) meal of cooked mushrooms and consumption of ethanolic beverages (approximately 28 ml ethanol) immediately after the meal; a glass of beer after 16 hr; and a glass of wine after 24 hr. The toxic effect was observed one day after the mushroom meal. However, two persons who ingested the same amount of raw mushroom and ethanol had no adverse effects. Further poisoning cases have been cited by Wier & Tyler (1960).

We found that a toxic reaction in mice could be demonstrated best when ethanol was given 3 to 6 hr after mushroom feeding. It was less manifest with simultaneous administration or with a 16 hr feeding interval. After a 24 hr interval or with reversal of the feeding order the toxic effect as practically absent.

Sleeping times have been used extensively in studies of the combined effects of ethanol and drugs in animals (Aston & Cullumbine, 1959;

Svedin, 1966) but to our knowledge no comparable data on the *Coprinus*-ethanol potentiation in animals have been reported. This reaction does not appear to be unique for *C. atramentarius* since *Boletus luridus* (Zeitlmayr, 1955) and Morels (Groves, 1964) have also been reported to exert this effect.

Acknowledgements. We wish to express our appreciation to the following: Dr. J. W. Groves, Department of Agriculture, Ottawa, for identification of the mushroom material, Dr. D. F. Bray for statistical services, Dr. S. Wiberg for helpful suggestions concerning the manuscript, Dr. H. C. Grice and Dr. Leo Levi for encouragement and Messrs. G. Belec, W. Skakum and G. Solomonraj for technical assistance.

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The hydrolysis of acetylsalicylic acid by liver microsomes

J. F. HOWES AND W. H. HUNTER

Pretreatment of rats and guinea-pigs with phenobarbitone or phenylbutazone leads to a decrease in the rate of hydrolysis of acetylsalicylic acid *in vitro* by liver microsomes, treatment with phenacetin does not.

HYDROLYSIS of acetylsalicylic acid *in vivo* is known to occur in serum (Augustinsson, 1948) and in liver and kidney (Ecobichon & Kalow, 1962). A comparative study of hydrolysis rates of acetylsalicylic acid in serum from rats, rabbits, guinea-pigs and man was reported by Morgan & Truitt (1965). The effect of treatment with other drugs upon the hydrolysis of acetylsalicylate has not been reported but Burns, Cucinell & others (1965) found that blood levels of aspirin did not alter as a result of its continued administration. We report the hydrolysis of acetylsalicylic acid in liver microsomes from rats and guinea-pigs pretreated with phenobarbitone, phenylbutazone or phenacetin.

Experimental

MATERIALS AND METHODS

Phenobarbitone sodium was administered intraperitoneally, as an aqueous solution, at 50 mg/kg to male Wistar rats and to guinea-pigs. Phenacetin and phenylbutazone at the same dose level were administered as suspensions in 0·2M Tris buffer (pH 7·6). Suspensions were prepared by ultrasonic disintegration of the solids in buffer solution. The animals were killed at suitable intervals after the administration of a single dose of drug or after the appropriate dose in the experiments involving multiple doses. Blood samples were obtained by severing the jugular vein immediately after killing the animals. The livers were removed, weighed and homogenized in a Waring-Blender (15 sec at low speed) with two volumes of isotonic potassium chloride solution. The homogenate was centrifuged at 9,000 *g* for 30 min to remove nuclei, mitochondria and other cell debris. The supernatant was centrifuged 1 hr at 140,000 *g* at 0–4°. The clear, soluble fraction was decanted from the microsomal pellet and this pellet resuspended in a volume of 0·2M Tris buffer equal to the volume of the discarded layer.

A 1·0 ml sample of the microsome suspension was diluted to 100 ml with distilled water and the protein content determined colorimetrically (Lowry, Rosebrough & others, 1951). Fifty ml of a solution of sodium carbonate 2% w/v and sodium tartrate (0·02% w/v) in 0·1N sodium hydroxide solution were added to 1 ml of copper sulphate solution (0·5% w/v $CuSO_4,5H_2O$) and 5·0 ml of this mixed solution were mixed with 1·0 ml

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of the microsome suspension. After 10 min, 0·5 ml of Folin and Ciocalteau reagent (5 ml BDH stock solution diluted to 12·0 ml) was added and the solutions allowed to stand 1 hr. The absorbance of the solutions was measured at 750 mμ. Solutions of bovine serum albumin (BDH) containing 100 μg/ml and 200 μg/ml were used, at the same time, to calibrate the absorbance readings.

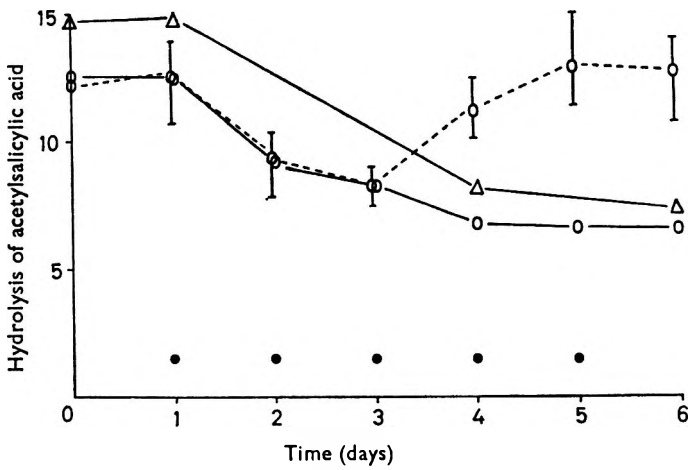

FIG. 1. Solid lines show the effect of phenobarbitone administration (days 1–5) on the hydrolysis of acetylsalicylic acid in microsomes of rat (O) and guinea-pig (Δ) liver. Broken line shows the effect of a single dose of 50 mg/kg on day 1 of phenobarbitone on the hydrolysis of acetylsalicylic acid in rat liver microsomes. Hydrolysis expressed as μg salicylate formed/mg protein/min. Each point is the mean of six experiments.

The microsomal suspensions were diluted with Tris buffer (0·2M) to contain 1 mg of protein per ml. These solutions were then allowed to stand for 30 min to allow the enzymes to re-associate after dilution. It was found that freshly diluted suspensions of microsomes had much lower activity in hydrolysing acetylsalicylate. The acetylsalicylic acid esterase activity of these preparations was then determined in the following way.

Cuvettes were prepared as follows: (1) containing 3·0 ml of 0·2M Tris buffer and used to set the base-line absorbance; (2) containing 0·1 ml of the microsomal suspension (1 mg protein/ml), 1·0 ml of 0·2M Tris buffer, 0·9 ml distilled water and 1·0 ml of acetylsalicylic acid solution (10^{-3}M) (this cuvette measured the rate of enzymic hydrolysis); (3) containing the same reagents as (2) but without the microsomal suspension, which was replaced by 0·1 ml of distilled water. This cuvette measured the slow spontaneous hydrolysis of acetylsalicylic acid in the buffer solution.

The absorbance of all three cuvettes at 300 mμ was determined in a Gilford-Unicam dual wavelength spectrophotometer at 37° over 30 min and the extent of enzymic hydrolysis measured by the difference between the absorbance of cuvette (2) and cuvette (3).

The pH of all solutions was checked before and after the incubation. The hydrolysis of acetylsalicylic acid in serum from treated and untreated animals was measured by the method of Morgan & Truitt (1965). Phenobarbitone, phenylbutazone or phenacetin at levels up to 5 μg/ml had no effect *in vitro* upon the rates of hydrolysis of acetylsalicylic acid by either microsomes or by serum.

Results

The effect of phenobarbitone treatment upon the hydrolysis of acetylsalicylic acid by microsomes from rat and guinea-pig liver is shown in Fig. 1. Fig. 1 also shows the effect of a single dose of phenobarbitone, in

TABLE 1. THE RATES OF HYDROLYSIS OF ACETYLSALICYLIC ACID BY RAT LIVER MICROSOMES, 48 HR AFTER INTRAPERITONEAL ADMINISTRATION OF A SINGLE DOSE OF VARIOUS DRUGS

Drug	Dose mg/kg	Rate of hydrolysis*
Control	—	12·4
Phenobarbitone	50	8·2
Phenylbutazone	"	6·7
Phenacetin	"	12·6

* Expressed as μg salicylate formed/mg protein/min.

rats, upon microsomal hydrolysis of acetylsalicylic acid and illustrates the recovery to normal levels after 3–4 days. Table 1 shows the effects of pretreatment with various drugs on the rate of hydrolysis of acetylsalicylate by microsomes of rat liver.

Discussion

The hydrolysis of acetylsalicylic acid in various tissues of experimental animals and of man has been extensively studied (for references see Morgan & Truitt, 1965) but the effect of drug treatment on this hydrolysis has not been reported. The administration of compounds that are oxidized in the liver is known to cause marked changes in the level of microsomal oxidizing enzymes but the effect upon other microsomal enzyme activities has not been so thoroughly studied. Remmer (1964) reported a slight increase in the hydrolysis of procaine by microsomes from dogs pretreated with phenobarbitone and a slight decrease in the case of rats similarly pretreated. Orrenius & Ericsson (1966) reported decreased levels of glucose 6-phosphatase, ATP-ase and NADH cytochrome C reductase; this decrease could be adequately explained by increased non-enzymic protein levels in the liver microsomes of treated animals.

The present work shows that even a single dose of phenobarbitone or phenylbutazone decreased the ability of liver microsomes to hydrolyse acetylsalicylic acid (Fig. 1). The activity is restored to normal levels in 3–4 days. On continued treatment with phenobarbitone a decreased level of hydrolysing activity is maintained so long as the administration of phenobarbitone is continued (Fig. 1). This decrease of 40–45% in

the capacity of the microsomes to hydrolyse acetylsalicylate is greater than that reported for any other hydrolytic enzyme activity in microsomes. Such a decrease could not be adequately explained by the synthesis of non-enzymic protein in the liver. The administration of phenobarbitone at 100 mg/kg produces a 50% increase in microsomal protein in the rat after five daily doses (Orrenius & Ernster, 1964). A single dose of 90 mg/kg gave a 5% increase in microsomal protein (Kato, 1963). The acetylsalicylic acid esterase activity of serum from treated and untreated rats was identical and the hydrolysis in serum therefore was not affected by the administration of phenobarbitone.

Our results indicate that the hydrolysis of acetylsalicylic acid in isolated liver microsomes is suppressed when phenobarbitone or phenylbutazone is administered but not when phenacetin is used. Both phenobarbitone and phenylbutazone produce significant increases in the levels of components of the oxidative metabolic system in liver microsomes whereas phenacetin does not (Conney, Davidson & others, 1960). The decrease in hydrolysis of acetylsalicylate appears therefore to be associated with gross increases in the drug oxidative activity.

The administration of so-called "inducing" drugs in combination with acetylsalicylic acid might therefore be expected to affect the rate of hydrolysis of acetylsalicylic acid in the liver. The effect is of particular interest since drugs such as phenylbutazone are often used in conjunction with acetylsalicylic acid treatment.

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Inhibition of amine uptake in the mouse heart by some new "thymoleptic" drugs

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A number of new and established thymoleptic drugs were given to mice. Their inhibitory action on the uptake of [^{3}H]metaraminol and [^{3}H]noradrenaline in the heart was investigated. Among the compounds tested a monomethylamino-derivative was in general 2–3 times more potent than the corresponding dimethylamino-derivative. The phthalan derivative 3,3-dimethyl-1-(3-methylaminopropyl)-1-phenylphthalan (Lu 3–010) was as efficient as protriptyline in all the tests performed. Changes in the substitution of the phthalan skeleton influenced the activity critically. As it is devoid of anticholinergic activity Lu 3–010 appears to be the most specific inhibitor of the amine transport mechanism of the adrenergic cell membrane found so far.

POTENTIATION of the effects of catecholamines and antagonism of the reserpine-induced syndrome have been recognized as characteristics of thymoleptic drugs (Haefely, Hürlimann & Thoenen, 1964; Sigg, 1959; Domenjoz & Theobald, 1959; Sulser, Watts & Brodie, 1962; Wilson & Tislow, 1962; Petersen, Lassen & others, 1966). It has also been claimed that this potentiation of the effect of the catecholamines is due to blockade of their inactivation (Schaeppi, 1960), specified further as inhibition of noradrenaline uptake into sympathetically innervated tissues (Axelrod, Hertting & Potter, 1962). This inhibition has been shown to be located in the amine transport mechanism at the level of the cell membrane, the "membrane pump" (Carlsson & Waldeck, 1965a; Malmfors, 1965). Previously we have tested the blocking action of some antidepressive and related agents using [^{3}H]-metaraminol (^{3}H–MA) as an indicator (Carlsson & Waldeck, 1965b). Since a new series of bicyclic "thymoleptics" (as judged by animal data) has become available (Petersen & others, 1966) it seemed to be of interest to include these compounds in our study.

Experimental

The present investigation was made on female mice. The general experimental and analytical procedure has been described elsewhere (Carlsson & Waldeck, 1963, 1965a). Other experimental details are given in the results section.

Results

The compounds tested were given intravenously (10 and sometimes 1 mg/kg) to mice 5 min before the intravenous injection of 0·02 mg/kg ^{3}H–MA, and the animals were killed 30 min thereafter. The hearts were removed and the level of ^{3}H–MA estimated. Animals which received only ^{3}H–MA served as controls.

The drugs are listed two by two, one dimethylamino- and one monomethylamino-derivative of each compound (Table 1). In all instances

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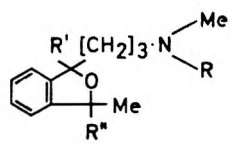
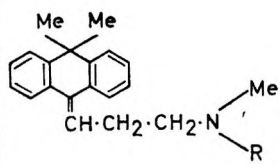
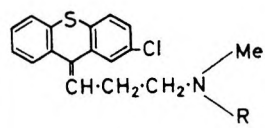
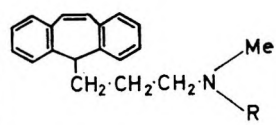
the monomethylamino-derivative was superior to its dimethylamino-analogue in preventing ^{3}H–MA uptake. The phthalan derivative 3,3-dimethyl-1-(3-methylaminopropyl)-1-phenylphthalan (Lu 3–010) proved to be the most efficient of the compounds tested. The corresponding compound lacking the phenyl group in position 1 (Lu 3–071) had a

TABLE 1. INHIBITION OF [^{3}H]METARAMINOL UPTAKE IN THE MOUSE HEART BY SOME THYMOLEPTIC DRUGS. The drugs were given intravenously to mice (grouped six by six) 5 min before the intravenous administration of 20 μg/kg [^{3}H]-metaraminol. After another 30 min the animals were killed, the hearts removed and their content of [^{3}H]metaraminol determined. The mean ±s.d. of 10 control groups which received [^{3}H]metaraminol only was 46·4 ±7·3 ng/g. In general, the values, calculated as per cent of the control values are the mean of 2 experimental groups.

Drug	R	R′	R″	Formula	Inhibition as % of control at 10 mg/kg	Inhibition as % of control at 1 mg/kg
Lu 3–009	Me	Ph	Me		15	63
Lu 3–010	H	Ph	Me		7	18
Lu 3–028	Me	H	Me		81	—
Lu 3–071	H	H	Me		64	—
Lu 3–092	Me	CN	Me		13	61
Lu 4–012	H	CN	Me		10	39
Lu 3–035	Me	Ph	H		38	—
Lu 3–051	H	Ph	H		12	—
Melitracene	Me				46	85
Litracene	H				14	44
Chorprothixene ..	Me				27	—
Demethylchlorprothixene ..	H				12	—
Protriptyline	H				9*	

* From Carlsson & Waldeck, 1965b.

much reduced activity, but the activity of the compound with a cyano-group replacing the phenyl group (Lu 4–012), however, was not appreciably less. A slightly lower potency than that of Lu 3–010 was observed in the compound in which one of the methyl groups in position 3 of Lu 3–010 was replaced by a hydrogen atom (Lu 3–051). The same structure-activity relations were observed for the corresponding dimethyl derivatives (Lu 3–009, Lu 3–028, Lu 3–092 and Lu 3–035).

In a subsequent experiment the dose-response relation of the blocking action of the most potent compounds in Table 1 was tested using [^{3}H]-noradrenaline (^{3}H–NA) as an indicator. For comparison, protriptyline was included in the test. The drugs were given in three different doses intravenously 5 min before the intravenous injection of 1 μg/kg of ^{3}H–NA. Controls were given ^{3}H–NA only. After another 30 min the animals were killed, the hearts removed and their content of ^{3}H–NA determined. In this test also Lu 3–010 appeared to be most potent and equal to protriptyline (Fig. 1). The three doses 0·5, 0·1 and 0·02 mg/kg blocked the

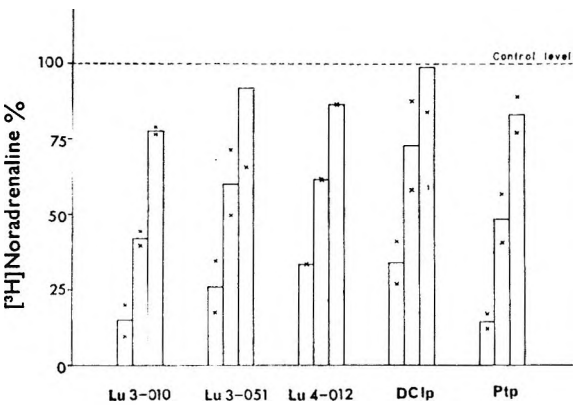


FIG. 1. Dose-response relationships for the inhibitory action of some thymoleptic drugs on the uptake of [^{3}H]noradrenaline in the mouse heart. The drugs were given intravenously to mice (grouped six by six) 5 min before the i.v. administration of 1 μg/kg [^{3}H]noradrenaline. After another 30 min the animals were killed and the content of [^{3}H]noradrenaline in the heart determined. Each substance was tested in 3 different doses, from the left: 0·5, 0·1 and 0·02 mg/kg. The mean $\pm$s.d. of 5 control groups which received [^{3}H]noradrenaline only was 2·61 $\pm$0·66 ng/g. The data are given in per cent of this control value. DClp = demethylchlorprothixene, Ptp = protriptyline.

^{3}H–NA uptake by 80, 40 and 15% respectively. The three drugs Lu 4–012, Lu 3–051, and Lu 3–092 appeared to be less potent but showed the same dose-response pattern.

The duration of the inhibitory action on the membrane pump was tested by giving the inhibitors intravenously (8–10 mg/kg) at different time intervals before the intravenous administration of ^{3}H–NA. In this series also, desipramine was included as a reference substance. Other experimental details were the same as in the previous experiment. The blockade of the ^{3}H–NA uptake induced by Lu 3–010 and protriptyline was prompt but appeared to be maximal for 1–2 hr only (Table 2). The blockade induced by desipramine disappeared even faster, because the ^{3}H–NA uptake was about 15 times higher when the drug was given 320 min rather than 5 min before the test amine. The compounds Lu 3–051 and demethylchlorprothixene appeared to be inefficient when given 320 min before the ^{3}H–NA although they caused a 90% blockade when given 20 min before.

Discussion

Metaraminol has proved to be a specific indicator of amine uptake at the level of the cell membrane of the adrenergic neuron (Carlsson & Waldeck, 1965a). It seems to be difficult, however, even with very potent inhibitors of the membrane pump, to reach ^{3}H–MA levels below 7% of the control value. This may in part be due to the metabolic stability of this compound, resulting in an extraneuronal retention of the amine. A high affinity for extraneuronal binding sites may also be a contributory factor. This is the reason why ^{3}H–NA was chosen for the study of dose-response relations and turn-over rates once the membrane-pump inhibiting qualities of a compound had been established by means of ^{3}H–MA.

TABLE 2. DURATION OF THE INHIBITORY ACTION OF SOME THYMOLEPTIC DRUGS ON THE [^{3}H]NORADRENALINE UPTAKE IN THE MOUSE HEART. The drugs were given intravenously to mice (grouped six by six) at various time intervals before the intravenous administration of 1 μg/kg [^{3}H]noradrenaline. After another 30 min the animals were killed and the content of [^{3}H]noradrenaline in the heart determined. The mean ±s.d. of 5 control groups which received [^{3}H]noradrenaline only was 2·30 ±0·94 ng/g. The data are single values given in per cent of the control value.

Drug	Dose mg/kg	Min drug given before			
		5	20	80	320
Lu 3-010	10	1	3	5	17
Lu 3-051	10	—	8	21	119
Demethylchlorprothixene ..	10	—	9	22	84
Protriptyline	8	2	2	3	6
Desipramine	10	2	4	10	37
		2	13	17	27

Of the compounds listed in Table 1, the monomethylamines in general showed 2–3 times higher activity in preventing ^{3}H–MA uptake than their corresponding dimethylamino-derivatives. This is in close agreement with previous data on other thymoleptics (Carlsson & Waldeck, 1965 a,b; Iversen, 1965). Further, changes in the substitution on the phthalan skeleton may influence the activity seriously (compare the activity of Lu 3–010 with that of Lu 3–071).

In the present study the activity of the most potent compound, Lu 3–010, did not appreciably differ from that of protriptyline in any of the tests. However, Petersen & others (1966) found Lu 3–010 about twice as active as protriptyline in the antagonism of reserpine and the potentiation of noradrenaline. As their test also included the adrenergic receptor mechanism, the discrepancy may reflect a higher sympatholytic activity of protriptyline than of Lu 3–010 (Petersen & others, 1966). In contrast to protriptyline, Lu 3–010 appears to be almost devoid of anticholinergic activity. It therefore appears to be the most specific inhibitor of the adrenergic membrane pump known so far.

Acknowledgements. For financial support and generous gifts of drugs I am indebted to H. Lundbeck & Co Ltd, Copenhagen. The skilful

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An investigation of the effects of angiotensin on the release of neurohumoral transmitters at motor, adrenergic and cholinergic nerve terminals

I. E. HUGHES

On the sciatic nerve-gastrocnemius-soleus muscle preparation of the cat, angiotensin (1–5 μg/kg, i.v.) potentiated the twitch response to maximal and submaximal stimulation of the sciatic nerve and produced partial reversal of an incomplete tubocurarine blockade. These actions could not be explained in terms of increased acetylcholine release since they were not seen in isolated motor nerve-striated muscle preparations and were probably secondary to the cardiovascular actions of angiotensin. Blockade of conduction in the postganglionic cholinergic nerves in the guinea-pig isolated ileum preparation by cooling or anoxia antagonized the response of this tissue to angiotensin. These procedures left the response to exogenous acetylcholine unchanged though they removed the cholinergic component of the response to angiotensin which is known to be present in this tissue. No evidence of increase in catecholamine output could be found in preparations of guinea-pig and rabbit vasa deferentia or rabbit duodenum responding to submaximal stimulation of their adrenergic nerves. It is concluded that angiotensin has no direct action on the stores of neurohumoral transmitter at motor, postganglionic cholinergic or postganglionic adrenergic nerves and that its known acetylcholine releasing action in the isolated ileum results from stimulation of the ganglia.

EARLY work on angiotensin indicated that the vasopressor actions of this peptide derived from a direct action on smooth muscle. More recent work has shown that release of neurohumoral transmitters may play an important role in the response of tissues to angiotensin. This release may be caused by central, ganglion stimulant, or direct action on the stores of neurohumoral transmitter found in the adrenal gland and at the postganglionic nerve terminal (Bickerton & Buckley, 1961; Robertson & Rubin, 1962; Feldberg & Lewis, 1964; Youmans, Davis & others, 1964; Lewis & Reit, 1965).

In the experiments reported in this paper the actions of angiotensin on the release of neurohumoral transmitters from motor, postganglionic cholinergic and post-ganglionic adrenergic nerves have been examined.

Experimental

Sciatic nerve-gastrocnemius-soleus muscle preparation of the cat. Cats of either sex, weighing from 1–3·6 kg were anaesthetized with chloralose (80 mg/kg i.p.), artificially respired and set up in a Brown-Schuster myograph to record twitch tension developed in the right gastrocnemius-soleus muscles in response to electrical stimulation of the distal end of the sectioned right sciatic nerve. Stimuli were applied through platinum hook electrodes from a Palmer H 44 square wave stimulator and twitch tension was measured isometrically by a Grass displacement transducer (FTO3C). Blood pressure was measured from the left femoral artery by an E. & M. linear core pressure transducer and all recordings were made

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on a Beckman RB Dynograph. Drugs were dissolved in saline (0·9% NaCl), injected into the left femoral vein, and washed in with 0·5 ml of saline. All animals were given 500 units/kg heparin injection B.P. as soon as the operative procedure was complete.

Phrenic nerve-diaphragm preparation. (Mogey, Trevan & Young, 1949). Preparations were suspended in modified Krebs solution (NaCl 6·92, KCl 0·35, $CaCl_2$ 0·21, $MgSO_4.7H_20$ 0·29, $NaHCO_3$ 2·1, KH_2PO_4 0·162, glucose 2·0 g/litre) at 37° and gassed with oxygen 95% carbon dioxide 5%. The response of the muscle to electrical stimulation of the phrenic nerve was recorded by the system described above.

Transmurally stimulated vas deferens and isolated rabbit duodenum preparations. These preparations were prepared as described by Clark & Hughes (1966).

Coaxially stimulated guinea-pig ileum. Short (2–3 cm) pieces of guinea-pig ileum taken from 5 cm above the ileocaecal junction were suspended in modified Tyrode solution (NaCl 8·0, KCl 0·2, $CaCl_2$ 0·15, $MgCl_2 \cdot 6H_2O$ 0·2, $NaHCO_3$ 1·0, $NaH_2PO_4 \cdot 2H_2O$ 0·05 glucose 2·0 g/litre) at 34° and gassed with oxygen 95%, carbon dioxide 5%. Coaxial stimulation was applied as described by Paton (1955) and longitudinal contractions were recorded isotonically on smoked paper by a frontal writing lever (load 1–2 g).

Anoxia was induced by replacing the oxygenated Tyrode solution by Tyrode solution made in freshly boiled distilled water which had been cooled under nitrogen. The arrangement of heating coils in the bath was such that the change-over from normal Tyrode to oxygen-free Tyrode solution could be accomplished by turning a three-way tap. Thus no break in dose schedule or alteration in temperature took place at the change-over. During the period of anoxia the bath was gassed with nitrogen 95%, carbon dioxide 5%. In all cases washing of the tissue was performed by overflow and agonists were added to the bath by pipette; in no case did the dose volume exceed 2% of the total bath volume.

The following drugs were used: acetylcholine chloride, atropine sulphate monohydrate, cinchocaine hydrochloride, heparin injection B.P., lignocaine hydrochloride monohydrate, morphine sulphate, procaine hydrochloride, (+)-tubocurarine chloride, val-5-hypertensin II asp-β-amide (Hypertensin, Ciba). All doses are expressed in terms of these salts.

No deterioration of dilute solutions of angiotensin took place over the period of the experiment when the solutions were stored in plastic vessels or in glass flasks treated with a silicone preparation.

Results

Sciatic nerve-gastrocnemius-soleus preparation of the cat. Injections of angiotensin (1·5 μg/kg) produced the expected rise in mean arterial pressure and potentiated the response of the gastrocnemius-soleus muscles to electrical stimulation of the sciatic nerve. Increase in twitch tension

was invariably observed in each of 5 preparations and occurred whether nerve stimulation was maximal or submaximal (Fig. 1a). This increase in twitch tension induced by angiotensin could be demonstrated repeatedly in the same preparation. Some degree of reversal of a partial tubocurarine blockade could also be demonstrated though this was not as marked as that caused by tetraethylammonium (TEA) (Fig. 1b). The angiotensin induced potentiation of the twitch response was of short duration and appeared related to the duration of the pressor response. The increase in twitch tension was however delayed by at least 40 sec after the blood pressure started to rise.

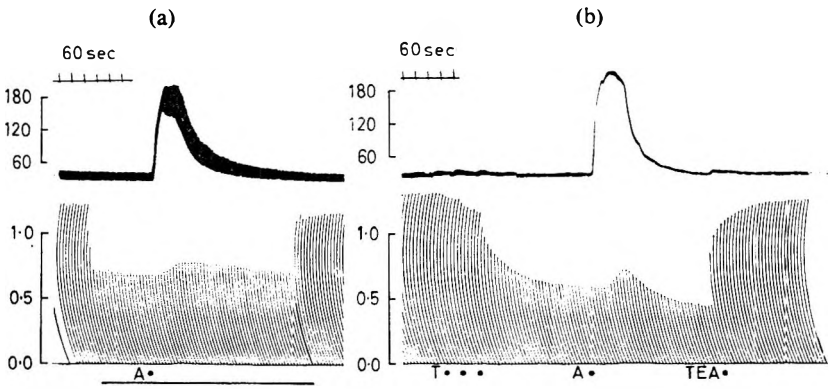

FIG. 1 (a). Cat, 2·05 kg, (b) cat 2·15 kg, under chloralose anaesthesia (80 mg/kg, i.p.). Upper record—femoral arterial blood pressure. Lower record—twitch tension developed in the gastrocnemius-soleus muscles in response to stimulation of the sciatic nerve at a rate of 3/min at 40 V 500 μsec duration in (a) and (b), and also submaximally in (a) at 2·8 V for 30 μsec duration applied as indicated by the black line. Angiotensin (A) (5 μg/kg, i.v.) was administered at the black dot in (a). In (b) its effect at the same dose with tetraethylammonium bromide (5 mg/kg, i.v. at TEA) on a partial tubocurarine blockade produced by 3 $\times$ 0·25 mg/kg tubocurarine (i.v.) at T.

Phrenic nerve-diaphragm preparations. In concentrations up to 5×10^{-6} g/ml, angiotensin showed no effect on the maximally or submaximally stimulated rat phrenic nerve-diaphragm preparation (Fig. 2a). Neither was any effect observed on the rate of onset (Fig. 2b) or rate of recovery from a partial tubocurarine blockade. TEA however produced a marked reversal of the partial tubocurarine blockade whether administered during the onset or recovery phases of the block.

In a single experiment on a phrenic nerve-diaphragm preparation taken from a cat, angiotensin produced no potentiation of the response to submaximal nervous stimulation in concentrations up to 1×10^{-8} g/ml.

Coaxially stimulated guinea-pig ileum. Various procedures were tried in an attempt to block the postganglionic nerve fibres in the ileum without affecting the response of the tissue to exogenous acetylcholine. The purpose was to discover whether the release of acetylcholine, known to be produced by angiotensin, results from a direct action of angiotensin on the nerve terminals or from the ganglion stimulant actions of angiotensin.

(*a*) *Effect of local anaesthetics.* Concentrations of procaine, lignocaine and cinchocaine which were just sufficient to block the response of the tissue to coaxial stimulation were found to antagonize the response to exogenous acetylcholine.

(*b*) *Effect of cooling.* Cooling is known to produce a failure of conduction in nerve fibres and was therefore used as an alternative procedure to block postganglionic nerve conduction. A maintained reduction in the temperature of the bath to 10° abolished the response to coaxial stimulation within 30 min. The response of the tissue to exogenous acetylcholine

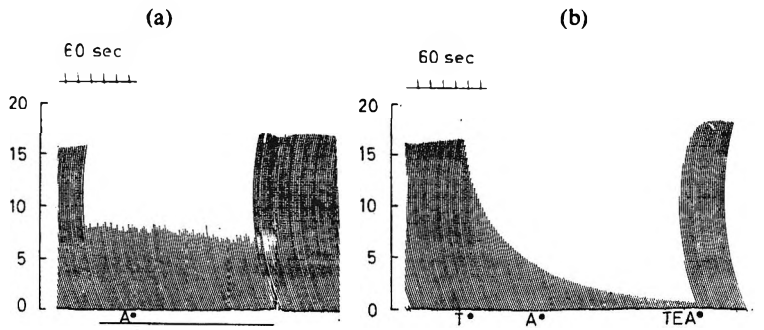

FIG. 2 (a) and (b). Rat phrenic nerve—diaphragm preparation suspended in Krebs solution at 37° responding to supramaximal stimulation (10 V, 200 μsec duration) of the phrenic nerve at a rate of 6/min in (a) and (b), also in (a) to submaximal stimulation (8 V, 30 μsec duration) applied as indicated by the black line. Angiotensin was administered to a final concentration of 5×10^{-6} g/ml at A. Its effect and that of tetraethylammonium bromide (1×10^{-4} g/ml at TEA) on a partial competitive neuromuscular blockade produced by a concentration of tubocurarine of 2×10^{-6} g/ml (at T) is shown in (b).

was unaffected by this procedure. When previously effective doses of angiotensin were administered to the cooled tissue no response was observed while angiotensin was in contact with the tissue. As soon as washout started however, a strong slow progressive contraction developed whether the angiotensin had been in contact with the tissue for a short or a long time (1 or 5 min). The contraction developing on washout lasted for several min despite further washing of the tissue.

(*c*) *Effect of anoxia.* Complete abolition of the response to coaxial stimulation was quickly achieved on supplying the tissue with oxygen-free Tyrode solution. Anoxia did not greatly affect the response of the tissue either to exogenous acetylcholine or to histamine but the response to angiotensin was markedly reduced. Ratios for equi-active doses of angiotensin before and during the period of anoxia were between 50 and 100 (6 experiments) although in one experiment the dose ratio was over 200 (Fig. 3). Response to coaxial stimulation returned to the control value in approximately 45 min after the oxygen supply was restored and tissue responses to histamine, acetylcholine and angiotensin had also returned to their control values after this time. Atropine (1×10^{-9} g/ml) was effective in antagonizing the response to angiotensin in experiments

not involving anoxia but failed to do so in each of two experiments where its action was tested during the period of anoxia.

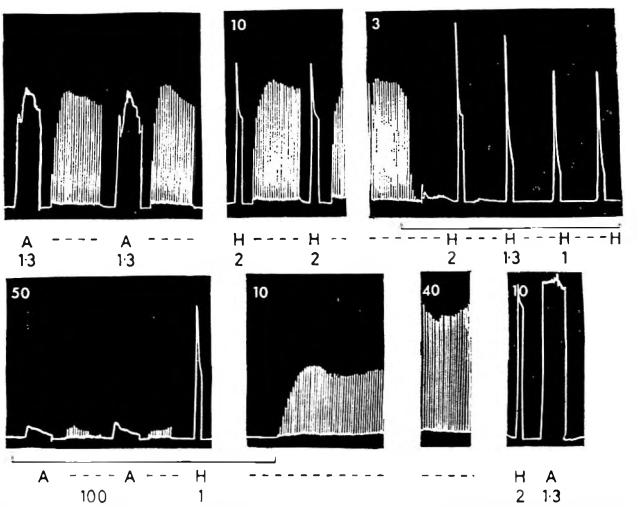

FIG. 3. Guinea-pig ileum preparation suspended in Tyrode solution at 34°. Showing the effect of anoxia (solid bars) on the response of the tissue to histamine (H), angiotensin (A) and coaxial stimulation (50 V, 1 msec duration, 6/min indicated by the broken bars). The numbers at the top of the records indicate the time (min) elapsed between the records. All concentrations are expressed in terms of μg/ml final bath concentration.

Transmurally stimulated vas deferens. In 6 experiments angiotensin, in concentrations up to 1×10^{-6} g/ml, had no effect on the response of guinea-pig or rabbit vasa deferentia to maximal or submaximal electrical stimulation (Fig. 4).

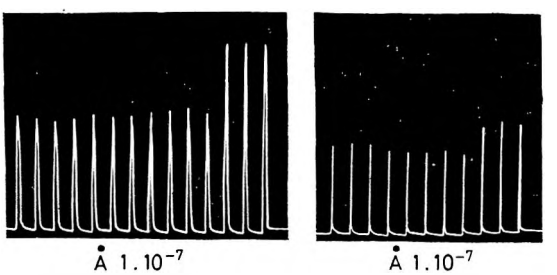

FIG. 4. Vas deferens preparation from the guinea-pig (left) and rabbit (right) suspended in Tyrode solution at 34° and responding to submaximal transmural stimulation (100 V, 400 μsec duration, 20/sec for 20 sec in every 5 min). For the last three responses on each record the repetition rate was raised to 50/sec angiotensin (Ang. 1×10^{-7} g/ml) was added to the bath as shown.

Isolated rabbit duodenum. The spontaneous pendular movements exhibited by this preparation are inhibited by stimulation of the periarterial nerves (Finkleman, 1930). Small doses of angiotensin (2–7·5

$\times 10^{-9}$ g/ml) caused a slight increase in the tone of the preparation but had little effect on the size of the spontaneous movements. These small doses of angiotensin did not influence the degree of inhibition of the pendular movements caused by submaximal stimulation of the periarterial nerves. In one experiment a slight decrease in the size of the inhibition was noted but on no occasion was an increase found (Fig. 5).

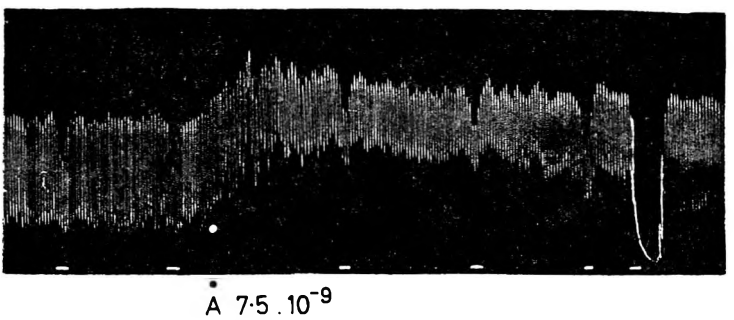

FIG. 5. Rabbit duodenum suspended in Tyrode solution at 36°. Showing the effect of angiotensin ($7{\cdot}5 \times 10^{-9}$ g/ml at A) on the response to submaximal stimulation of the periarterial nerves (40 V, 300 μsec duration, 10/sec for 20 sec) as indicated by the white bars. The repetition rate was raised to 50/sec before the last response.

Discussion

It has been established (Khairallah & Page, 1961: Robertson & Rubin, 1962) that acetylcholine contributes appreciably to the response of isolated rabbit and guinea-pig ilea to angiotensin. This release of acetylcholine by angiotensin could be the result of a direct action of angiotensin on the stores of acetylcholine at the postganglionic cholinergic nerve terminal. If this were so then it might be expected that a similar release might be shown at the neuromuscular junction. The potentiation of the twitch response of the gastrocnemius-soleus muscles to maximal and submaximal electrical stimulation of the sciatic nerve and the partial reversal of the competitive neuromuscular blockade which were induced by angiotensin could be accounted for in terms of an increased release of acetylcholine. If this explanation were correct then angiotensin would be expected to show a similar effect on isolated motor nerve-striated muscle preparations such as the rat phrenic nerve-diaphragm. However, angiotensin did not potentiate the effect of stimulation of the phrenic nerve in this isolated preparation. This difference in the *in vivo* and *in vitro* responses is unlikely to be due to a species variation as observations made on a single phrenic nerve-diaphragm preparation taken from a cat accorded with those in the rat.

It seems more likely that the potentiation of the effects of electrical stimulation of the sciatic nerve seen in the cat is due to a secondary effect of the action of angiotensin on the cardiovascular system. The large pressor response produced by angiotensin might be expected to change both pressure and flow in the limb. These changes in flow will affect

oxygenation and other factors in the tissue and may be responsible for the potentiation of the effects of stimulation of the sciatic nerve. In addition, angiotensin is known to release catecholamines from the adrenal gland (Felberg & Lewis, 1964: Robinson, 1965) and this is especially likely to occur with the high doses used in these experiments. It is well known that catecholamines are capable of facilitating the process of neuromuscular transmission (Bowman & Zaimis, 1958: Krnjević & Miledi, 1958) and the release of catecholamines may well contribute to the potentiation of the twitch response seen in these experiments. The delayed onset of the potentiation of the twitch response as compared with the pressor response may be due to the time required for angiotensin to release catecholamines from the adrenal gland into the general circulation and hence to the gastrocnemius and soleus muscles.

As no increase in the release of acetylcholine at the neuromuscular junction could be demonstrated, attempts were made to discover if the presence of ganglia and of functional postganglionic nerves was necessary for angiotensin to induce a release of acetylcholine in the guinea-pig ileum.

Blockade of conduction in postganglionic nerves by local anaesthetics was found to be accompanied by atropine-like actions. Local anaesthetics could not be used therefore to distinguish between acetylcholine released by a direct action on the store and acetylcholine released by nerve impulses induced in the postganglionic nerves.

Although the experiments involving cooling of the ileum were complicated by a washout phenomenon the cold preparation showed a great reduction in the response to angiotensin but not to acetylcholine. This reduction in the response was probably due to a blockade of conduction in the postganglionic nerve fibres which eliminated the ganglionic component of the response to angiotensin. Any release of acetylcholine produced by a direct action of angiotensin on the stores of acetylcholine would be unaffected by this procedure since the cold tissue responded well to exogenous acetylcholine. However, the release processes may have been affected by cooling.

The antagonism of the response to angiotensin produced by anoxia is probably also due to failure of conduction in the post-ganglionic nerves and the elimination of the ganglionic component of the response. The failure of atropine to antagonize the response to angiotensin during the period of anoxia indicates that the cholinergic component of the response has been removed by this treatment. The large ratio between equieffective doses before and during the period of anoxia indicates that, in normal ileum, a large proportion of the response to angiotensin is mediated by acetylcholine.

The suggestion that ganglion stimulation is responsible for the release of acetylcholine by angiotensin is supported by the findings of Godfraind, Kaba & Polster (1966). They found that there was no cholinergic component in the response of isolated strips of longitudinal muscle taken from guinea-pig gut. This preparation is known to contain postganglionic cholinergic nerves since electrical stimulation produces a response that is

completely abolished by lachesine (Rang, 1964). The absence of ganglia in the longitudinal muscle strip probably accounts for the lack of any cholinergically mediated action of angiotensin in this preparation.

The failure of hexamethonium and related compounds to antagonize the response of the guinea-pig ileum to angiotensin is not in conflict with this hypothesis. It has been shown in isolated ganglia that the ganglion stimulant action of angiotensin is insensitive to hexamethonium but is sensitive to morphine (Lewis & Reit, 1965, 1966; Trendelenburg, 1966). This drug is an effective antagonist of angiotensin on the guinea-pig ileum though at least some of the action will be due to the inhibition of the release of acetylcholine from the postganglionic nerve terminal which is produced by morphine (Schaumann, 1957).

No evidence was found that angiotensin increased catecholamine release when tested on preparations responding to electrical stimulation of adrenergic nerves. Thus angiotensin produced no increase in the tension developed by guinea-pig or rabbit vasa deferentia in response to maximal or submaximal transmural stimulation. Similarly, angiotensin produced no increase in the degree of inhibition produced by standard submaximal stimulation of the periarterial nerves to the rabbit duodenum preparation.

These results are in accord with the findings of Hertting & Suko (1966) who could find no increase in the output of tritiated noradrenaline in response to stimulation of nerve fibres in the presence and absence of angiotensin. The results obtained on the guinea-pig vas deferens preparation are however in contrast to those obtained by Benelli, Della Bella & Gandini (1964) who found that angiotensin produced a potentiation of the response of the guinea-pig vas deferens to submaximal stimulation of the hypogastric nerve. However, the preparation used by Benelli and his co-workers contains ganglia (Birmingham & Wilson, 1963) and this may account for the potentiation. In addition, unpublished experiments have revealed a variation in the response of the vas deferens to angiotensin depending on the ionic composition and temperature of the bathing fluid.

CONCLUSION

The experimental evidence presented supports the view that angiotensin has no direct effect on the stores of neurohumoral transmitter at the endings of motor, postganglionic cholinergic or postganglionic adrenergic nerves and that the release of transmitter which mediates the response of some tissues to angiotensin is a result of the known ganglion stimulant action of angiotensin.

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Actions of phencyclidine on the perfused rabbit ear

STELLA R. O'DONNELL* and JANET C. WANSTALL†

Phencyclidine potentiated the responses of the rabbit perfused ear and its isolated central artery to noradrenaline, adrenaline and periarterial nerve stimulation. The potentiation of nerve stimulation was more pronounced than the potentiation of exogenous noradrenaline or adrenaline. Where the access of noradrenaline was restricted to one surface of the artery only, phencyclidine caused a much greater potentiation of extraluminal than of intraluminal noradrenaline. A biphasic response to tyramine was observed, the secondary prolonged vasoconstrictor phase being blocked by phencyclidine. It is postulated that phencyclidine inhibits the uptake of both noradrenaline and tyramine by the adrenergic axons in the blood vessel wall in a manner similar to that suggested for cocaine.

THE vasopressor response produced by the anaesthetic agent, phencyclidine, arises from both direct and indirect sympathomimetic effects (Ilett, Jarrott & others, 1966). Phencyclidine also potentiates the pressor effects of noradrenaline and adrenaline and transiently reduces the pressor response to tyramine. Ilett & others (1966) speculated that phencyclidine might potentiate the effects of exogenous noradrenaline and adrenaline by inhibiting their uptake into the adrenergic axon. The antagonism of the effect of tyramine could result from phencyclidine also blocking the uptake of tyramine. Since cocaine can block the pressor effects of phencyclidine, it is possible that cocaine and phencyclidine compete for the same uptake site. Some cocaine-like properties of phencyclidine have been reported by Chen, Ensor & Bohner (1965) and we now describe experiments on rabbit isolated ear and its isolated central artery which support the hypothesis that the action of phencyclidine is cocaine-like.

Experimental

METHODS

Lop-eared rabbits, 2–3 kg, were used. Three kinds of preparation were used: 1. The isolated perfused central artery of the rabbit ear cannulated only at the proximal end as described by De la Lande & Rand (1965), i.e. single cannulated artery. 2. The isolated perfused central artery of the rabbit ear cannulated at both the distal and proximal ends (De la Lande, Cannell & Waterson, 1966), i.e. double cannulated artery. 3. The central artery of the rabbit ear perfused *in situ* with vascular changes recorded as described by De la Lande & Rand (1965), i.e. isolated perfused rabbit ear.

The central artery was perfused with Krebs bicarbonate solution at 37° by a roller pump at constant rate of flow (6–8 ml/min). Constrictions were recorded as an increase in perfusion pressure using either a Condon manometer or a Statham P23AC pressure transducer.

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Drugs were injected into the perfusion fluid immediately proximal to the preparation (intraluminal injection) or were added to the perfusion fluid (intraluminal perfusion) or were added to the bath fluid surrounding the isolated artery (extraluminal addition). Periarterial nerve stimulation was effected by bipolar platinum electrodes as described by De la Lande & Rand (1965).

Drugs and solution. Drugs used were (−)-adrenaline acid tartrate, (−)-noradrenaline acid tartrate, cocaine hydrochloride, phencyclidine hydrochloride, tyramine hydrochloride. Doses of noradrenaline and adrenaline are expressed as dose of free base and doses of tyramine, cocaine and phencyclidine as dose of salt.

In experiments where noradrenaline was administered by intraluminal perfusion the Krebs perfusing fluid contained 200 μg/ml ascorbic acid.

Results

EFFECT OF PHENCYCLIDINE ON NORADRENALINE, ADRENALINE AND NERVE STIMULATION

Intraluminal injection of phencyclidine (0·5–1·5 mg) into the single cannulated artery preparation potentiated the intensity of the responses to periarterial nerve stimulation more than to intraluminal injections of noradrenaline and the duration of the potentiation was 30–40 min. The same

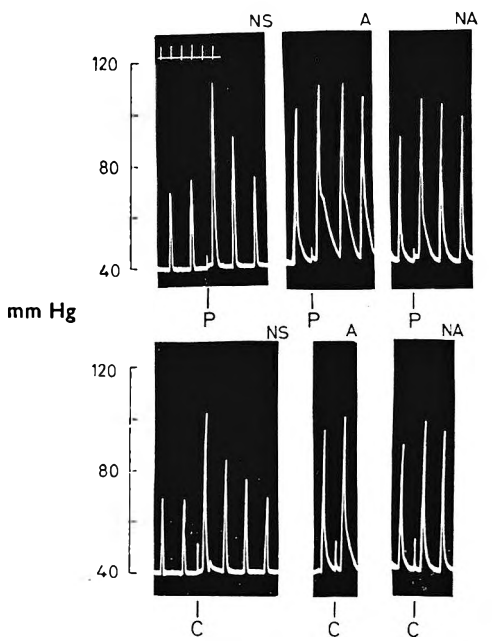

FIG. 1. Perfused rabbit ear. The effect of an injection of 25 μg of phencyclidine (P) and of 25 μg of cocaine (C) given 30 sec before nerve stimulation (NS, 40V, 1 msec, 20/sec for 10 sec); adrenaline (A, 5 ng); and noradrenaline (NA, 5 ng). Nerve stimulation is potentiated more than adrenaline or noradrenaline. Time in min.

effect could be produced by perfusing low concentrations of phencyclidine (5 μg/ml) through the artery for 15–20 min. The effect of phencyclidine was usually reversible 20–40 min after stopping the perfusion. In some experiments it was possible to find a concentration which would potentiate the responses to nerve stimulation without any effect on injected noradrenaline. Intraluminal perfusions of high concentrations of phencyclidine caused a reduction in the noradrenaline response whilst that to nerve stimulation was increased.

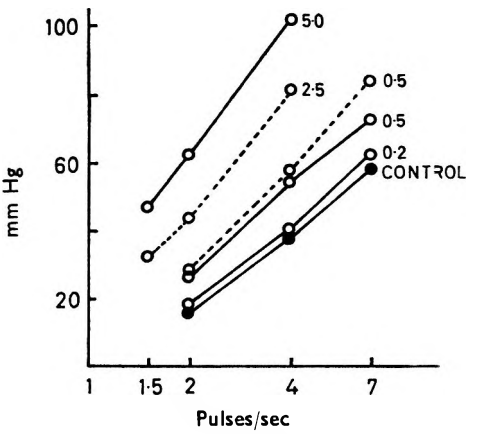

FIG. 2. Perfused rabbit ear. The effect of perfusions of phencyclidine ○——○ and cocaine ○ - - - ○ on the log frequency-response line to periarterial nerve stimulation (60 V, 0·05 msec, stimulated for 10 sec). Control responses are represented by ●——●. The doses of phencyclidine and cocaine are in μg/ml. Duration of perfusion was 14 min at flow rate of 8 ml/min.

A more marked potentiation of the intensity of the response to nerve stimulation than of that to an intraluminal injection of noradrenaline or adrenaline was also observed on the isolated perfused ear preparation. The effects of an intraluminal injection of 25 μg of phencyclidine and of 25 μg of cocaine are illustrated in Fig. 1.

Similar results were obtained if low concentrations of phencyclidine (0·5–5·0 μg/ml) were perfused through the ear for 14–20 min. The potentiating effect of phencyclidine on the responses to periarterial nerve stimulation increased with the concentration of phencyclidine and a parallel shift in the log frequency-response line occurred (Fig. 2). It was difficult to investigate the effects of larger concentrations of phencyclidine given by intraluminal perfusion since the resting perfusion pressure increased significantly and normal sensitivity to nerve stimulation and to noradrenaline did not readily return.

The relative potency of noradrenaline administered to the intraluminal and extraluminal surfaces of the double cannulated artery varied with the sensitivity of the preparation, but noradrenaline was always more active by intraluminal perfusion. When 10 μg/ml of phencyclidine was perfused intraluminally, the noradrenaline added extraluminally was potentiated much more than noradrenaline perfused intraluminally. The effect on

noradrenaline was easily reversed on reverting to normal Krebs solution (Fig. 3).

There was no increase in the response to single doses of noradrenaline administered by intraluminal injection. In some experiments continued perfusion of phencyclidine caused an increase in resting perfusion pressure.

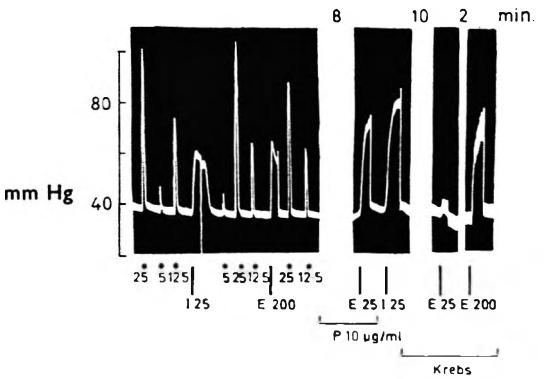

FIG. 3. Double cannulated artery. Unqualified figures refer to injections of noradrenaline (ng). An intraluminal perfusion of 25 ng/ml of noradrenaline (I25) gave the same response as 200 ng/ml bath fluid of noradrenaline added extraluminally (E25). After an intraluminal perfusion of 10 µg/ml of phencyclidine (P) for 15 min only 25 ng/ml of noradrenaline extraluminally (E25) was required to give a similar response to 25 ng/ml of noradrenaline intraluminally (I25). On reverting to normal Krebs solution the potentiation of extraluminal noradrenaline ceased.

EFFECT OF PHENCYCLIDINE ON TYRAMINE

The effect of phencyclidine on the response of the artery to tyramine was examined on the three kinds of preparation. The response to an intraluminal injection (10–30 µg) of tyramine was biphasic in both the

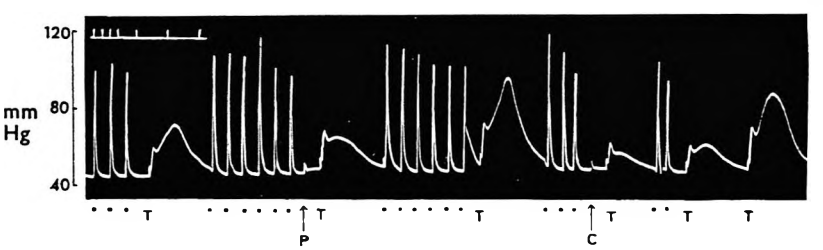

FIG. 4. Perfused rabbit ear. Unlabelled dots are injections of 10 ng noradrenaline. At T, 10 µg tyramine was injected. An injection of 25 µg phencyclidine (P) and of 25 µg cocaine (C) 30 sec before the tyramine reduced the secondary phase of the tyramine response. Time in min. During the tyramine response the drum speed was increased.

single cannulated artery and in the rabbit perfused ear preparation. There was an initial sharp vasoconstrictor response followed by a secondary more prolonged constriction. An intraluminal injection of either phencyclidine (25–50 µg) or cocaine (25–40 µg) 30 sec before the injection

of tyramine caused reduction or abolition of the secondary vasoconstriction but not the initial response (Fig. 4). The characteristic response to tyramine was restored as the phencyclidine or cocaine was washed out of the tissue by the perfusing Krebs solution. During most of these experiments injections of noradrenaline were made between doses of tyramine but the response to tyramine could be restored without the noradrenaline.

On the double cannulated artery, the response to an intraluminal injection of tyramine (30–60 μg) was a transient and reproducible constriction. Tyramine (2–3 μg/ml bath fluid) added to the extraluminal surface of the artery caused a more prolonged constriction which was not easily reproducible unless high doses of noradrenaline were added extraluminally to prevent tachyphylaxis. A perfusion of phencyclidine (10 μg/ml) for 20 min completely blocked the response to tyramine given extraluminally whereas that to intraluminal injection was unaffected.

Discussion

Uptake into storage sites in adrenergic nerves is now considered to play a major role in the termination of the response to noradrenaline released by nerve stimulation. Other sympathomimetic amines, including adrenaline, can also be taken up by adrenergic nerves (Iversen, 1965). Cocaine blocks this active uptake of noradrenaline (Whitby, Axelrod & Hertting, 1960; Iversen, 1965) and it has been suggested that this property of cocaine explains its potentiation of the effects of nerve stimulation and of noradrenaline *in vivo* (Trendelenburg, 1966). On the isolated ear central artery and the perfused whole ear of the rabbit, phencyclidine caused a greater potentiation of the effects of nerve stimulation than it did those of exogenous noradrenaline, an effect already reported for cocaine (De la Lande & others, 1966), and the degree of potentiation of nerve stimulation by phencyclidine and cocaine increased with increasing drug concentration.

On the double cannulated artery, an intraluminal perfusion of phencyclidine caused a marked potentiation of the effects of noradrenaline added to the extraluminal surface whilst having only a small effect on the intraluminal perfusion of noradrenaline. Since the adrenergic axons in the rabbit ear central artery are situated at the outer perimeter of the smooth muscle layer, the concentration of noradrenaline reaching the smooth muscle from the extraluminal surface will be reduced by uptake into the adrenergic nerve terminals (Gillespie, 1966; De la Lande & Waterson, 1967). The sensitizing action of phencyclidine is preferential for the extraluminal noradrenaline and can be explained if phencyclidine blocks the uptake mechanism of the adrenergic axon membrane, as has been described for cocaine on this preparation by De la Lande & Waterson (1967). Single intraluminal injections of noradrenaline on the double cannulated artery were not potentiated by phencyclidine although intraluminal injections into the single cannulated artery and the whole ear showed marked potentiation. If the differences between the three types

of preparation described are considered, this observation is consistent with the concept that the sensitizing action of phencyclidine is due to a block of uptake of noradrenaline acting from the extraluminal surface. In the single cannulated artery any drug injected intraluminally reaches the extraluminal surface, since the external bathing fluid is continuous with the perfusing fluid, and in the whole ear intraluminally injected drugs probably reach the extraluminal surface via the vascular system of the ear. If the artery is cannulated at both ends, noradrenaline injected intraluminally does not reach the extraluminal surface.

A biphasic response was obtained on injecting tyramine into the perfused rabbit ear or into the single cannulated artery. Phencyclidine blocked the secondary, more prolonged, phase of the vasoconstrictor response. Farmer (1966) observed a biphasic response of tyramine on the central artery and showed that the secondary phase was due to noradrenaline release since it was abolished by reserpine, sympathetic denervation or cocaine. Cocaine inhibits responses to tyramine by blocking uptake of tyramine into the adrenergic axon thus preventing release of endogenous noradrenaline (Furchgott, Kirpekar & others, 1963). Thus a block of the secondary phase of the tyramine response by phencyclidine would suggest that phencyclidine can block the uptake of tyramine. An intraluminal injection of tyramine caused what is possibly a direct sympathomimetic effect and an extraluminal injection the indirect effects (Waterson & De la Lande, personal communication). The observation that phencyclidine blocked only the extraluminal response to tyramine supports the concept that phencyclidine blocks the uptake of tyramine into the adrenergic nerve terminals. The similarity in the response to tyramine on the whole ear, and on the single cannulated preparation of the central artery, and also the responses observed when the artery is cannulated at both ends, can be explained by considering the surfaces of the artery to which the drug has access in the three kinds of preparation. In the first two preparations tyramine is in contact with both surfaces of the artery whereas in the double cannulated artery the drug has access only to the surface of the artery to which it is applied.

The rise in perfusion pressure observed after perfusions of phencyclidine or cocaine could be caused by indirect sympathomimetic effects of phencyclidine (Chen & others, 1965; Ilett & others, 1966) and cocaine (Teeters, Koppanyi & Cowan, 1963; Maengwyn-Davies & Koppanyi, 1966). Alternatively, the rise could be due to the drugs blocking the re-uptake of spontaneously released noradrenaline. Either mechanism would result in an increased concentration of noradrenaline in the region of the receptors.

Thus the observed effects of phencyclidine on the blood vessels of the rabbit ear can be ascribed to its ability to (*a*) block the uptake of exogenous noradrenaline, or noradrenaline released on nerve stimulation, into the adrenergic nerve terminals; (*b*) block the uptake of tyramine and thus prevent the release of noradrenaline by tyramine and (*c*) possibly release stored noradrenaline. In each of these respects its actions are similar to those previously reported by other workers for cocaine.

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A note on the subcellular distribution of adipose tissue noradrenaline

G. L. GESSA, S. CONGIU, P. F. SPANO AND C. MUSINU

The distribution of noradrenaline between the residue and high-speed supernatant of adipose tissue homogenates in rats, guinea-pigs and cats has been examined. Most of the noradrenaline is in the supernatant fraction; little is in the 100,000 *g* fraction.

THE presence of noradrenaline in the adipose tissue has been demonstrated by fluorimetric (Paoletti, Smith & others, 1961; Sidman, Perkins & Weiner, 1962) and histochemical methods (Dawkins, Duckett & Pearse, 1966). We have shown that the pattern of distribution of noradrenaline in adipose tissue differs among various animal species (Spano, Vargiu & others, 1967). In rats and in guinea-pigs, interscapular brown fat bodies are the richest in this amine, while in cats and dogs noradrenaline is concentrated in perirenal fat. We have now examined the subcellular localization of endogenous noradrenaline in adipose tissue in rats, guinea-pigs and cats.

Experimental

MATERIAL AND METHODS

Male Long-Evans rats (150–160 g) and guinea-pigs (350–450 g) were killed by neck fracture. Cats were killed with pentobarbitone. Soon after death, hearts, brains and interscapular adipose tissues were removed. When needed, organs from more than one animal were pooled and 1 g of each tissue was homogenized for 2 min with a Kontes glass homogenizer in 10 volumes of ice-cold 0·3M sucrose containing 100 μg/ml ascorbic acid and 1 mg/ml EDTA. All operations were on ice in a room refrigerated to 4°. The homogenates were centrifuged in a Spinco refrigerated ultracentrifuge at 100,000 *g* for 1 hr. After centrifugation each adipose tissue homogenate presented a particulate fraction, a fluid supernatant and, at the surface, a 1–2 mm thick film of fat.

Concentrated perchloric acid was added to the supernatant fluids to obtain a final normality of 0·4N. The particulate fractions of each tissue and the fatty films were taken up, by homogenization, in 0·4N perchloric acid. The noradrenaline from each fraction was extracted by a modification (Spano & others, 1967) of the method described by Brodie, Comer & others (1966) and assayed fluorimetrically according to the method of Chang (1964).

Results and discussion

Adipose tissue noradrenaline has a subcellular distribution different from that of other organs so far examined (Table 1). Most of the

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TABLE 1. SUBCELLULAR DISTRIBUTION OF ADIPOSE TISSUE NORADRENALINE IN COMPARATION WITH THAT OF HEART AND BRAIN

Species	Organ	% total noradrenaline			Total noradrenaline µg/g of tissue
		100,000 × g fraction	Fatty film	Supernatant	
Rat	Interscapular adipose tissue	16·2 ± 2·3	14·2 ± 1·0	69·5 ± 3·1	0·279 ± 0·02
,, ...	Heart	65·7 ± 4·2	—	34·3 ± 3·8	1·120 ± 0·12
,, ...	Brain	58·0 ± 3·0	—	42·0 ± 4·1	0·630 ± 0·13
Guinea-pig ..	Interscapular adipose tissue	6·7 ± 1·1	18·3 ± 2·4	74·0 ± 3·1	0·251 ± 0·09
,, ..	Brain	57·6 ± 7·1	—	42·0 ± 5·8	0·530 ± 0·19
Cat	Perirenal adipose tissue	5·2 ± 3·1	15·1 ± 2·8	79·7 ± 7·3	0·710 ± 0·17

Values for rats and guinea-pigs are means (± s.e.) from at least 10 experiments; values for cats are means (± s.e.) from 5 experiments.

amine occurs in the supernatant fractions (liquid supernatant and fatty layer).

A similar subcellular distribution of catecholamines, but less markedly in favour of the supernatant fractions, was described by Wurtman, Axelrod & Potter (1964) for rat uterus. However, unlike in the uterus, adipose tissue noradrenaline is contained in nerve terminals (Weiner, Perkins & Sidman, 1962).

There are several possible explanations of our finding. It was possible that adipose tissue contained synaptic vesicles with sedimentation characteristics differing from those of other adrenergic structures: this seemed not to be so, since the same results have been obtained varying homogenization media (0·25M sucrose containing 5×10^{-3} phosphate buffer, pH 7·4, and 10^{-3}M $MgCl_2$), times of centrifugation (up to 4 hr) and of homogenization (1 min). It is also possible that adipose tissue might contain some substance (U-factor?) (Lehninger & Remmert, 1959) which releases noradrenaline from the vesicles during the preparation of the samples. To check this, portions of adipose tissue and brain of the same weight were homogenized together, and the supernatant-particulate distribution of noradrenaline was assayed in the mixture. The results of these experiments showed that the noradrenaline distribution for one tissue is not influenced by the presence of the other.

TABLE 2. EFFECT OF TYRAMINE, GUANETHIDINE AND L-METARAMINOL ON NORADRENALINE CONTENT OF INTERSCAPULAR ADIPOSE TISSUE, IN RATS

Treatment mg/kg	Interval between treatment and death	Noradrenaline µg/g
—	—	0·279 ± 0·02
Tyramine 20 i.m.	4 hr	0·080 (0·008–0·092)
Guanethidine 15 i.p. ..	14 hr	0·000
(−)-Metaraminol 2·5 i.p ..	4 hr	0·002 (0·000–0·004)

It is interesting (Table 2) that noradrenaline levels of adipose tissue are depleted by drugs such as tyramine, guanethidine and metaraminol,

which are considered to release the amine by interfering with its uptake into granulated vesicles (Giachetti & Shore, 1966).

Acknowledgements. Metaraminol was kindly given by Merck, Sharp & Dohme, and guanethidine by CIBA.

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Potentiation of acetylcholinesterase by a series of quaternary ammonium compounds

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Eleven spiran, and two non-spiran, quaternary ammonium compounds have been examined and some of these compounds found to potentiate the hydrolysis of acetylcholine by acetylcholinesterase at high substrate concentrations. The experiments were made in three reaction media, each differing in its ionic composition. The results obtained in these media differ both quantitatively and qualitatively. The mechanism of the potentiation is discussed in terms of the effect of quaternary ammonium compounds on the deacetylation step of acetylcholine hydrolysis.

IN the presence of certain compounds, acetylcholinesterase hydrolyses some substrates at a faster rate than it does in the absence of these compounds. The earlier work was concerned mainly with the potentiation of acetylcholinesterase by both monovalent (Glick, 1941; Mendel & Rudney, 1945; Myers 1952) and divalent (Nachmansohn, 1940; Myers, 1952; Meer, 1953) inorganic cations. Wide discrepancies exist among these results, and Smallman & Wolfe (1954) were the first to recognize that the potentiation by inorganic salts was not estimated comparably by the manometric and titrimetric methods of determining enzyme activity. The differences were mainly a result of the different ionic composition of the reaction media employed in the two methods. The results of Smallman & Wolfe demonstrated that the potentiation of the hydrolytic activity of acetylcholinesterase by inorganic ions was greater when the control activity was estimated in a medium of low inorganic ion concentration than in one with a high ionic concentration. The presence of relatively high concentrations of inorganic ions is inevitable in the manometric method of analysis, but very low concentrations may be used in the titrimetric method.

Some organic molecules have also been shown to potentiate the cholinesterases, e.g. acetylcholinesterase is potentiated by certain aliphatic alcohols (Todrick, Fellowes & Rutland, 1951) and by tetraethylammonium and hexamethonium salts (Kensler & Elsner, 1951). In all of the work so far cited, potentiation was obtained at substrate concentrations higher than optimum, and only with substrates causing substrate inhibition.

We have examined the activities of eleven spiran, and two non-spiran, quaternary ammonium compounds in media with and without added inorganic ions. Spiran quaternary ammonium compounds (spirans) have the characteristic of being relatively rigid, and their size and the stereochemistry of substituents can be controlled (Thomas, 1961a, b).

Experimental

CHEMICAL

The general method used for the synthesis of the spirans was to react an α-ω-dihaloalkane (1 mole equiv.) with a cyclic amine (2 mole equiv.)

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in a suitable solvent. The total concentration of reactants was kept below 5% (w/v) of the total volume of reaction mixture to promote an intramolecular cyclization, giving the required spiran and amine hydrohalide. Two methods were used for the reaction: *Method A*, the reactants were refluxed in chloroform for a suitable time (6–24 hr); *Method B*, a solution of reactants in absolute ethanol or methanol was autoclaved ($\frac{1}{2}$–1 hr) at 125°. The spiran was then isolated by one of two methods.

Method (*a*). The reaction solution was distilled to dryness under reduced pressure on a water bath, the solid residue dissolved in water, sodium hydroxide (1 mole equiv.) added and the solution distilled to dryness as before. The resulting solid was extracted with chloroform in a Soxhlet extractor. The product either crystallized from chloroform or was obtained by reducing the volume of chloroform and adding dry ethyl methyl ketone.

Method (*b*). The reaction mixture was distilled to dryness under reduced pressure on a water bath and the spiran isolated by repeated crystallization of the residue from absolute ethanol or methanol.

Commercial samples of tetraethylammonium iodide (TEA, Hopkin & Williams) tetramethylammonium iodide (TMA, British Drug Houses) and acetylcholine perchlorate (British Drug Houses) were used.

All the quaternary compounds are hygroscopic, and were dried over P_2O_5 under reduced pressure overnight before use.

2,2′-Di-iododiethyl ether. 2,2′-Di-iododiethyl ether was prepared from 2,2′-dichlorodiethyl ether by the Finkelstein reaction according to the method of Gibson & Johnson (1930), b.p. 80–84°/3·5 mm.

A list of spirans prepared is given in Table 1 together with analytical data and physical constants.

ENZYMIC

Purified bovine erythrocyte acetylcholinesterase (Nutritional Biochemical Corp.) was used. The enzymic activity was determined by the pH stat method with a Radiometer (Copenhagen) Titrator (TTTlc) attached to a magnetic valve, which regulated the flow of titrant (sodium hydroxide 0·02 or 0·01N) into a 100 ml beaker immersed in a thermostatically controlled water bath at 37°. The solution was stirred continuously and nitrogen bubbled through it. A stock enzyme solution was made by dissolving 20 mg of enzyme in 50 ml of a dilute phosphate buffer [Na_2HPO_4 (M/30)—500 ml, KH_2PO_4 (M/15) to pH 7·4 at 37° (about 200 ml)].

The compounds were studied in three reaction media: *Medium A* contained, in addition to the phosphate buffer, NaCl (0·1M) and $MgCl_2$ (0·04M); *Medium B* contained the phosphate buffer only, with no added ions*; *Medium C* contained no phosphate buffer or inorganic ions. When the hydrolysis was examined in this medium the method of analysis was different from the one described above. A fully automated recording titrimetric method was used as reported by Thomas & Roufogalis (1967).

* The enzyme preparation contains 30·7 mg NaCl, 100 mg gelatin and 5 ml M sodium phosphate per 20,000 "units".

TABLE 1. SPIRAN QUATERNARY AMMONIUM COMPOUNDS PREPARED

No.	Molecular formula	Cyclic amine	α-ω-Dihaloalkane	Method (see exptl.)	Reflux time (hr)	Solvent for recryst.	m.p.[g]	Analysis							
								Calc.				Found			
								C	H	N	X[h]	C	H	N	X[h]
I	$C_{10}H_{20}NBr$	Piperidine	1,5-Dibromopentane	Aa	12	Chloroform[a]	304(d.)	51·3	8·6	6·0	34·1	51·3	8·5	6·1	33·9
II	$C_{12}H_{24}NBr$	*cis*-2,6-Dimethyl[f] piperidine	1,5-Dibromopentane	Ba	—	Ethylmethyl ketone	248–249	55·0	9·2	5·3	30·5	54·5	9·0	5·5	30·6
III	$C_{11}H_{22}NBr$	*cis*-2,6-Dimethyl piperidine	1,4-Dibromobutane	Aa	6	Ethylmethyl ketone-chloroform	269–270	53·2	8·9	5·6	32·2	53·1	8·9	5·5	32·2
IV	$C_9H_{18}NBr$	Piperidine	1,4-Dibromobutane	Aa	15	Chloroform-ethylmethyl ketone	248–250	49·1	8·2	6·4	36·3	48·7	8·3	6·3	36·8
V	$C_8H_{16}NBr$	Pyrrolidine	1,4-Dibromobutane	Aa	24	Ethylmethyl[b] ketone	250–252	46·6	7·8	6·8	38·8	46·4	8·2	6·9	38·4
VI	$C_9H_{18}ONBr$	Morpholine	1,5-Dibromopentane	Ba	—	Chloroform[a]	271–273	45·8	7·7	5·9	33·8	45·4	7·6	5·8	33·9
VII	$C_8H_{16}O_2NI$	Morpholine	2,2′-Diiododiethyl ether	Ab[c]	6	Ethanol	275–276	33·7	5·7	4·9	44·5	33·4	5·7	5·0	44·0
VIII	$C_{11}H_{22}ONBr$	2,6-Dimethyl[d,e] morpholine	1,5-Dibromopentane	Aa	19	Chloroform	314(d.)	50·0	8·4	5·3	30·3	49·8	8·3	5·6	30·7
IX	$C_{10}H_{20}ONBr$	2,6-Dimethyl morpholine	1,4-Dibromobutane	Aa	17	Chloroform-ethylmethyl ketone	325	48·0	8·1	5·6	32·0	48·1	8·1	5·9	32·1
X	$C_{10}H_{20}O_2NI$	2,6-Dimethyl morpholine	2,2′-Diiododiethyl ether	Ab	6	Ethanol	282–283	38·4	6·4	4·5		38·1	6·4	4·5	
XI	$C_{11}H_{22}ONI$	*cis*-2,6-Dimethyl piperidine	2,2′-Diiododiethyl ether	Aa	10	Chloroform	237	42·5	7·1	4·5		42·4	7·1	4·6	

(*a*) Crystallized out during Soxhlet extraction. (*b*) This compound is hygroscopic and was handled in a dry box. (*c*) This compound is unstable to prolonged heating and over P_2O_5/90°C. (*d*) Oxygen is numbered position one. (*e*) Isomers not separated. (*f*) See reference. (*g*) Melting points are uncorrected. (*h*) X = I or Br as appropriate.

PROCEDURE

The quaternary ammonium compounds were incubated in the relevant media with 5·0 ml of the enzyme solution (stored at 4°) for 15 min at pH 7·4 and 37°. The volume of this mixture was (50–x) ml, where x is the volume of substrate solution. The substrate (x ml) was then added and the pH adjusted immediately to 7·4. The concentration of substrate used was one which preliminary investigations had shown to give near optimum potentiation with the compounds. This arbitrarily chosen concentration was different for each medium, but was kept constant for each particular medium throughout the investigation. The titrator was started, and the volume of sodium hydroxide consumed recorded every 2·5 min (or every 5 min) during a 15 min period of hydrolysis. The volume of sodium hydroxide used was plotted against time in min, and from the resulting graph the volume of sodium hydroxide consumed during 15 min was obtained and used as a measure of the rate of hydrolysis (when medium C was used the rate was the slope of the initial linear portion of the recorded plot). Since the experiments were made mainly at high substrate concentrations, the plots showed a linear relation.

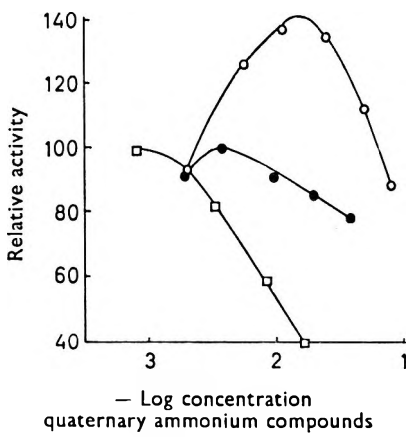

FIG. 1. Effect of quaternary ammonium compounds on the hydrolysis of acetylcholine in medium A. Acetylcholine concentration $3{\cdot}4 \times 10^{-2}$M. Activity in the absence of the compounds is arbitrarily taken as 100. Concentrations of quaternary ammonium compounds is given as g-mole/litre. ○–○, Tetraethylammonium iodide (TEA). □–□, *cis*-2,6-Dimethyl-1,1′-spirobipiperidinium bromide (II). ●–●, 1,1′-Spirobipiperidinium bromide (V).

Results

Figs 1 and 2 show representative examples of the results obtained in media A and B. In Table 2 are listed the I 50 (concentration causing 50% inhibition), the maximum potentiation obtained in each medium, and the concentration of compound which produced this maximum potentiation. I 50 values were determined at substrate concentrations at which potentiation did not occur.

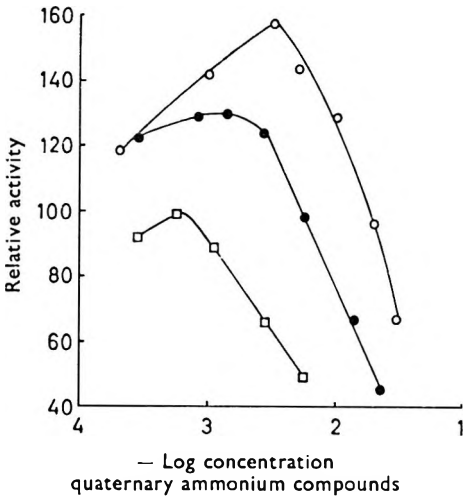

FIG. 2. Effect of quaternary ammonium compounds on the hydrolysis of acetylcholine in medium B. Acetylcholine concentration $3{\cdot}4 \times 10^{-3}$M. Activity in the absence of the compounds is arbitrarily taken as 100. Concentration of quaternary ammonium compounds given as g-mole/litre. ○-○, Tetraethylammonium iodide (TEA), ●-●, *cis*-2,6-Dimethyl-1,1′-spirobipiperidinium bromide (III) □-□, 1,1′-Spirobipiperidinium bromide (I).

Discussion

It is apparent from Table 2 that potentiation is influenced by the reaction medium used. The values obtained were some 20–60% higher in the media with no added inorganic ions (media B and C) than in medium A. Some of the compounds (IV, V and VI) which did not potentiate in medium A did so, though weakly, in media B and C.

INHIBITOR POTENCY AND POTENTIATION

There is an inverse relationship between I 50 and ability to potentiate among the series. The compounds which are the strongest inhibitors potentiate weakly or not at all, whereas those that are weaker inhibitors are the better potentiators. The exception is tetramethylammonium, which, although one of the weaker inhibitors, does not potentiate in medium B. It appears possible that this relation is a function of some physicochemical property of these quaternary ammonium compounds.

MECHANISM OF POTENTIATION

There is an inverse relationship between I 50 and ability to potentiate concentrations. This can be seen by comparison of the plots in Fig. 3 with those of Figs 1 and 2. Fig. 3 illustrates the effects of two of the compounds when examined at optimum substrate concentration. The hydrolysis rate does not rise above that of the control at any concentration of the compounds. Figs 1 and 2 show the results obtained above optimum substrate concentration. Under these conditions the hydrolysis

TABLE 2. POTENTIATION AND INHIBITION OF ACETYLCHOLINESTERASE BY QUATERNARY AMMONIUM COMPOUNDS. INHIBITION GIVEN AS I 50 IN G-MOLE/LITRE. POTENTIATION GIVEN AS PERCENTAGE INCREASE IN RATE OF HYDROLYSIS OF ACETYLCHOLINE

Compound number	Compound	I 50 × 10^3 Medium A [S] = $3{\cdot}4 \times 10^{-3}$M	Maximum potentiation (%) and concentration of quaternary ammonium compound which produce this potentiation in g-mole/litre × 10^3		
			Medium A [S] = $3{\cdot}4 \times 10^{-2}$M	Medium B [S] = $3{\cdot}4 \times 10^{-3}$M	Medium C [S] = $2{\cdot}34 \times 10^{-3}$M
I	1,1′-Spirobipiperidinium bromide	3·80	0%	0%	—
II	*cis*-2,6-Dimethyl-1,1′-spirobipiperidinium bromide	1·70	0%	—	—
III	*cis*-2,6-Dimethylspiro(piperidine-1,1′-pyrrolidinium) bromide	11·2	26% (6·2)	31% (1·59)	52% (1·3)
IV	Spiro(piperidine-1,1′-pyrrolidinium) bromide	8·41	—	0%	11% (5·0)
V	1,1′-Spirobipyrrolidinium bromide	7·25	0%	0%	19% (0·11)
VI	Spiro(piperidine-1,4′-morpholinium) bromide	26·4	0%	17% (4·68)	—
VII	4,4′-Spirobimorpholinium iodide	71[a]	[b]	37% (25)	—
VIII	2,6-Dimethylspiro(morpholine-4,1′-piperidinium) bromide	1·26	—	0%	—
IX	2,6-Dimethylspiro(morpholine-4,1′-pyrrolidinium) bromide	3·16	—	0%	—
X	2,6-Dimethyl-4,4′-spirobimorpholinium iodide	9·24	—	8·5% (0·10)	—
XI	*cis*-2,6-Dimethylspiro(piperidine-1,4′-morpholinium) iodide	20·0	—	25% (2·0)	—
XII	Tetraethylammonium iodide	21·2	40% (15)	57·5% (3·2)	76% (2·3)
XIII	Tetramethylammonium iodide	31·0	0%	0%	—

(*a*) Concentration producing 34·5% inhibition. (*b*) Concentration needed too high.

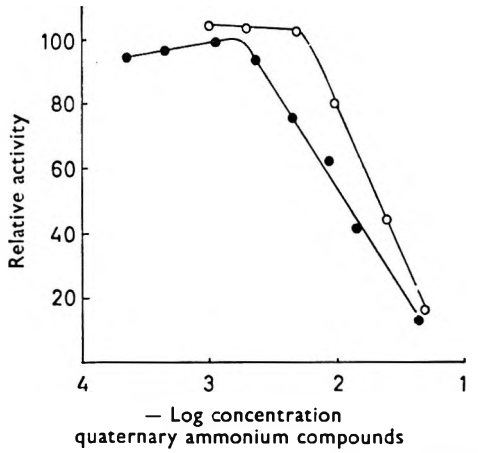

FIG. 3. Effect of quaternary ammonium compounds on the hydrolysis of acetylcholine in medium A. Acetylcholine concentration $3{\cdot}4 \times 10^{-3}$M. Activity in the absence of these compounds arbitrarily taken as 100. Concentration of quaternary ammonium compounds given as g-mole/litre. ○–○, Tetraethylammonium iodide (TEA). ●–●, *cis*-2,6-Dimethylspiro(piperidine-1,1′-pyrrolidinium) bromide (III).

rate in the presence of these (and some of the other) compounds is greater than the control hydrolysis rate in their absence. Potentiation occurs at low concentrations of the quaternary ammonium compounds, but at higher concentrations the hydrolysis rate is inhibited.

Acetylcholinesterase is inhibited by high concentrations of acetylcholine; this is generally considered to be due to the binding of a second molecule of acetylcholine on an enzyme substrate complex (Zeller & Bissegger, 1943; Bergmann, Wilson & Nachmansohn, 1950; Wilson & Cabib, 1956) to form a ternary complex. The presence of this second molecule inhibits hydrolysis. It follows that the most likely explanation for the observed potentiation at above optimum substrate concentrations

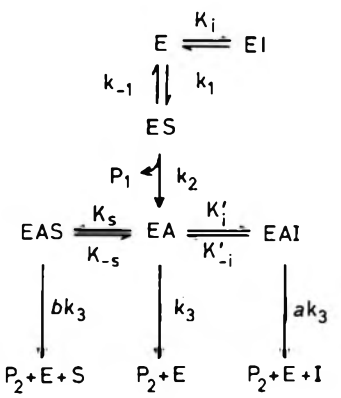

FIG. 4. Reaction scheme for substrate inhibition proposed by Krupka & Laidler in the presence of inhibitor.

is that the small quaternary ammonium compounds are bound to the binary complex, and so protect the enzyme against substrate inhibition. If this is so, then an explanation has to be found for the fact that only some of the compounds potentiate, and that the compounds potentiate at low concentrations but inhibit at high concentrations.

A general mechanism for the interaction of acetylcholine and inhibitors with acetylcholinesterase has been proposed by Krupka & Laidler (1961) (Fig. 4). In this scheme the substrate, S, can combine with the free enzyme, E, or with the acetyl enzyme, EA, but not with the Michaelis complex, ES, which does not have a free anionic site available for interaction. EAS, once formed, will break down to products at b times the rate of EA. The deacetylation of EA is considered to be the rate-determining step in acetylcholine hydrolysis (Wilson & Cabib, 1956). The value of b is generally considered to be 0·1 (Krupka, 1964).

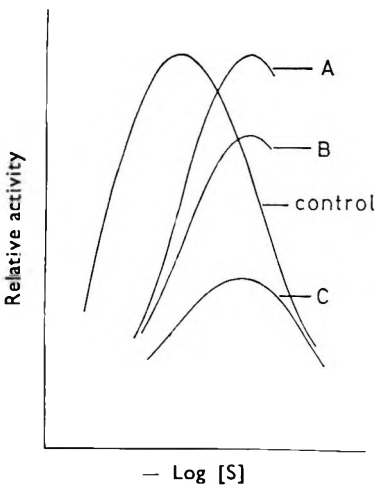

FIG. 5. Three classes of behaviour predicted by Krupka from the scheme in Fig. 4 (see discussion).

Likewise, a cationic compound, I, may react with the free enzyme to form the binary complex EI, or with the acetyl enzyme to give EAI. This ternary complex then may or may not deacetylate to give products, the rate of deacetylation being ak_3. Krupka (1965) considers that the value of a can vary between 0 and 1. When I completely blocks the deacetylation step $a = 0$, and if I has no effect on the deacetylation rate $a = 1$; a can also have intermediate values.

The reaction scheme in Fig. 4 shows that I can compete with S for interaction with EA*. If I blocks the deacetylation less than S, then, in the presence of I and at substrate concentrations where EAS can form, an apparent increase in enzyme activity would be predicted. This is

* That the compounds under consideration interact with EA is indicated by the non-competitive component in inhibition of acetylcholinesterase by tetramethylammonium and tetraethylammonium (Krupka, 1965).

what has, in fact, been observed to various degrees among the compounds under investigation (Table 2). The inhibition at high I concentrations is probably due to the high proportion of enzyme bound as EI.

Krupka (1963), in a theoretical treatment of the kinetics of this proposed scheme of inhibition, has predicted three classes of inhibitor behaviour, reproduced in Fig. 5. Class A behaviour results when the inhibitor competes with the substrate for acetylenzyme, the inhibitor having no effect on the deacetylation rate when bound. Class B behaviour arises when I partially blocks deacetylation, and class C when I completely blocks the deacetylation. These types of behaviour have been observed experimentally, and Fig. 6 shows the effect of III and TEA over a range of substrate concentrations, in a medium containing NaCl (0·1M) and $MgCl_2$ (0·04M). Under these conditions both III and TEA potentiate the rate of the acetylcholinesterase at high substrate concentrations, but the hydrolysis never rises above the optimum hydrolysis rate of the control. Krupka (1965) has calculated that the ternary complex EAI deacetylated at 0·83 times the rate of EA when I is TEA. TMA does not potentiate in either medium A or B, and the value of a found by Krupka (1965) for TMA in a medium similar to medium A was from 0·42 to 0·65.

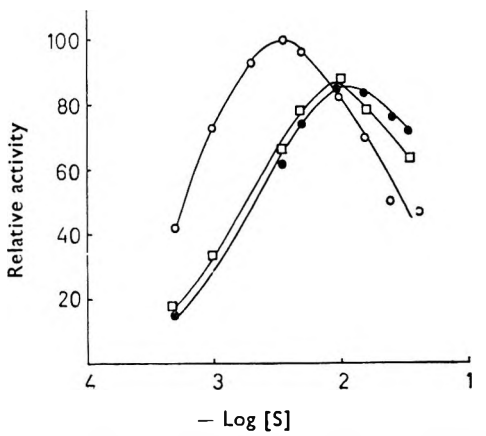

FIG. 6. Effect of quaternary ammonium compounds on the hydrolysis of acetylcholine over a range of substrate concentrations in medium A. Maximum activity of control arbitrarily taken as 100. Substrate concentration is given in g-mole/litre. ○–○, Control. ●–●, Tetraethylammonium iodide (TEA), $1{\cdot}45 \times 10^{-2}$M. □–□, *cis*-2,6-Dimethylspiro(piperidine-1,1′-pyrrolidinium) bromide (III), $6{\cdot}2 \times 10^{-3}$M.

Some of the compounds, in media B and C, where the enzyme has not been already potentiated by added inorganic ions, show a fourth class of behaviour (Fig. 7A, B). Again there is potentiation of the enzyme hydrolysis rate only at high substrate concentrations. But in these media the hydrolysis rises above that of the optimum rate of hydrolysis of the control. A possible explanation for this is that in media B and C the ternary complex EAI deacetylates at a faster rate than EA, that is, a is greater than 1. The possibility of this is being investigated.

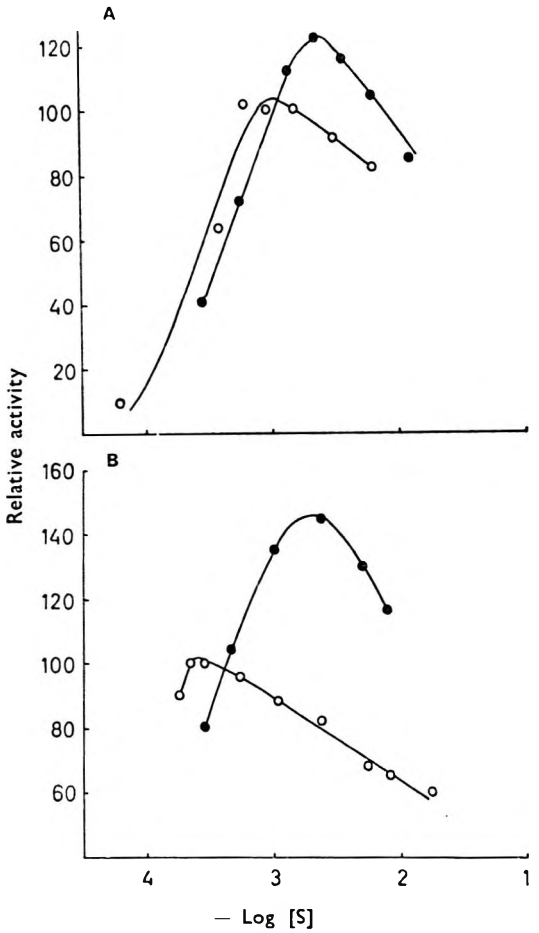

FIG. 7. Effect of quaternary ammonium compounds on the hydrolysis of acetylcholine over a range of substrate concentrations (A) in medium B; (B) in medium C. Maximum activity of control arbitrarily taken to be 100. Substrate concentration given in g-mole/litre. A. ○–○, Control. ●–●, *cis*-2,6-Dimethylspiro(piperidine-1,1′-pyrrolidinium) bromide (III), $1{\cdot}39 \times 10^{-3}$M. B. ○–○, Control. ●–●, Tetraethylammonium iodide (TEA), $2{\cdot}24 \times 10^{-3}$M.

STRUCTURE-ACTIVITY RELATIONS

Potentiation appears to be strongly structure dependent. There is no obvious relationship between potentiation and the size of the molecules, since all the compounds examined are a similar size. Thus the replacement of one methylene group in compound I by one oxygen atom does not significantly alter its size, but the change produces a potentiator VI— and the replacement of two methylene groups of compound I by two oxygen atoms (VII) has an even greater effect. Compound II does not potentiate, but the contraction of the unsubstituted ring by one carbon produces III, a strong potentiator. The features which appear to be

important for potentiator activity are bulk around the positive nitrogen (II, XI and TEA) and the presence of an oxygen atom (VI, VII, X, XI), although the presence of these features does not necessarily lead to potentiators (II, VIII, IX). Freedom of rotation of carbon-carbon, carbon-nitrogen bonds also appears to be important. Whereas TEA is the strongest potentiator of the series, when the rotation of the ethyl groups is restricted by cyclization, as in V, potentiation is greatly diminished. At the present time no satisfactory explanation can be given for the high degree of structural specificity exhibited by the series in relation to ability to potentiate acetylcholinesterase.

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Distribution of acetylcholinesterase, proteolipids and cholinergic receptor in cat brain

SIR,—Acetylcholinesterase-rich nerve-ending membranes isolated from the cerebral cortex have also the highest binding capacity for radioactive cholinergic blocking agents (De Robertis, Alberici & others, 1966). This binding capacity is not due to the lipids or glycolipids but probably to a proteolipid protein (De Robertis, Fiszer & Soto, 1967). Since myelin has the highest proteolipid content in the central nervous system but only a small binding capacity for dimethyl-[^{14}C]-(+)-tubocurarine, it was postulated that the cholinergic receptor may be a special type of proteolipid.

These results warranted the study of the content of proteolipids, acetylcholinesterase and of the binding capacity for dimethyl-[^{14}C]tubocurarine in the proteolipids extracted from different neuroanatomical regions of the brain of the cat including the retina (Table 1). Such regions were dissected in the cold room and homogenized in distilled water pH 7·4 to 10%. A total particulate was obtained by sedimenting at 100,000*g* for 30 min. Aliquots of this particulate were used for assay of total protein, and of the proteolipid protein extracted with chloroform–methanol (2:1) according to Folch & Lees (1951). Protein (Lowry, Rosebrough & others, 1951) acetylcholinesterase, (Ellman, Courtney & others, 1961) and the binding capacity for dimethyl-[^{14}C]tubocurarine (De Robertis, Azcurra & Fiszer, 1967) were estimated.

The results obtained are summarized in Table 1. To better correlate acetylcholinesterase activity with the binding capacity, ratios in which white matter was considered as 1·0 were used. It may be observed that there is a correlation of enzyme and the amount of dimethyl-[^{14}C]tubocurarine bound to the proteolipid protein. The parallelism is good in white matter, cerebral cortex, cerebellum and nucleus lentiformis but not as exact in corpora quadrigemina anterior and in nucleus caudatus, in which acetylcholinesterase is higher than the receptor capacity, and in retina in which this situation is reversed.

The content of proteolipid protein is 20–25 mg/g wet tissue in bovine white matter and in grey matter is 1/5 of that concentration (Folch & Lees, 1951). Amaducci (1960) studied the localization of proteolipids in numerous anatomical regions of the human brain. Our results in the cat are in general agreement although our figures for nucleus lentiformis and specially for corpora quadrigemina anterior are much higher. This may be due to species difference or to a wider inclusion of white areas in the sample.

The acetylcholinesterase activity has been examined by Nachmansohn (1939) and Burgen & Chipman (1951) in various regions of the CNS. Our results in

TABLE 1. CONTENT OF PROTEOLIPID PROTEIN, ACETYLCHOLINESTERASE (AChE) AND UPTAKE OF DIMETHYL-[^{14}C]TUBOCURARINE ([^{14}C]DMTC) IN THE CHLOROFORM–METHANOL (2:1) EXTRACT OF VARIOUS ANATOMICAL REGIONS OF THE CAT CNS

	Proteolipid protein mg/g tissue	AChE μM/hr ACh used/mg protein	[^{14}C]DMTC counts/min/mg proteolipid protein	Ratio AChE in X / AChE in *a*	[^{14}C]DMTC in X / [^{14}C]DMTC in *a*
White matter[a] ..	20·4	0·57	5,410	1·0	1·0
Cerebral cortex ..	7·9	2·30	36,160	4·0	6·7
Cerebellum ..	7·3	4·70	36,146	8·2	6·7
N. lentiformis ..	21·2	5·67	36,810	9·9	6·8
Corpora quadrigemina anterior	23·6	7·40	23,320	13·0	4·3
N. caudatus	12·3	23·40	43,540	41·0	8·0
Retina	7·5	3·90	51,177	6·8	9·5

the cat agree in general terms with those found in the rabbit, dog, ox and man by Nachmansohn (1959). In these species the concentration is highest in the caudatus and lentiformis nuclei. In the cat the highest acetylcholinesterase activity is the nucleus caudatus and this is followed by the corpora quadrigemina anterior and nucleus lentiformis.

The general relation between the enzyme and binding capacity indicates that both reside in the same cholinergic pathways in the CNS. Cell fractionation studies have demonstrated that the enzyme and the cholinergic receptor are concentrated in the same type of nerve-ending membrane (De Robertis & others, 1966). Such membranes comprise also the junctional complex formed by the synaptic membranes and associated structures. Recently, using a mild detergent, most of the limiting membrane of the ending was dissolved, together with the main proportion of acetylcholinesterase and other bound enzymes. With this technique the junctional complexes were isolated and shown to contain intact the binding capacity for the cholinergic blocking agents (De Robertis, Azcurra & Fiszer, 1967). This result suggested that the receptor protein has a postsynaptic localization while acetylcholinesterase has a wider distribution being mainly presynaptic. The enzyme and the receptor can also be dissociated by the chloroform-methanol treatment, which inhibits the enzyme without affecting the binding capacity. Some of these findings may explain the apparent lack of parallelism between the enzyme and the cholinergic receptor in certain neuroanatomical regions. Thus the predominance of acetylcholinesterase in the nucleus caudatus could be due to the presence of large amounts of the enzyme in presynaptic segments of the axons while in the retina the postsynaptic receptor protein may be proportionally higher. This is also confirmed by histochemical studies which show that the enzyme reaction is very strong and homogeneously distributed in the nucleus caudatus (see Gerebtzoff, 1959) while in the retina it is confined to the inner plexiform layer (Francis, 1953).

The fact that the retina has a very high choline-acetylase content (Hebb, 1957) is also in line with the high content of cholinergic receptor found in the present study.

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Interactions between monoamine oxidase inhibitors and sympathomimetic amines in the rat isolated vas deferens

SIR,—It has been assumed (Iversen, 1967) that monoamine oxidase inhibitors do not potentiate the effects of catecholamines administered exogenously or released by nerve stimulation. However, it has been shown (Bhargava, Kar & Parmar, 1963) that amphetamine, pheniprazine and tranylcypromine potentiate the responses of hypogastric nerve-vas deferens preparation elicited by electrical stimulation, and that monoamine oxidase inhibitors produce a large potentiation of the pharmacological actions of tyramine (Griesemer, Barsky & others, 1953; Goldberg & Sjoerdsma, 1959; Spano, 1966). Tranylcypromine and pheniprazine potentiate also the effects of catecholamines on the heart of reserpinized cat but not in the normal cat (Lee, Shin & Shideman, 1961).

In view of these conflicting reports, we have thought it worth while to study the interactions between some monoamine oxidase inhibitors and sympathomimetic amines in the rat isolated vas deferens preparation, using the technique of Laporte, Jané & Valdecasas (1966), since this is one of the most suitable preparations to assess *in vitro* noradrenaline supersensitivity (Cuenca & Valdecasas, 1965; Ursillo & Jacobson, 1965; Benvenutti, Bonaccorsi & Garattini, 1967). The monoamine oxidase inhibitors assayed were: pheniprazine, a hydrazine derivative, and tranylcypromine, amphetamine and pargyline, all non-hydrazine derivatives. Sympathomimetic agents used were a direct-acting amine, noradrenaline, and an indirect-acting amine, tyramine.

Tranylcypromine and pheniprazine have no action on the rat isolated vas deferens in concentrations up to 1×10^{-6} g/ml. Stronger concentrations elicit small and irregular contractions, and an increase of the residual tonus. To see the same effects with amphetamine, higher concentrations must be used (1×10^{-4} g/ml).

After these apparently ineffective stimulations with tranylcypromine the sensitivity of the vas deferens to catecholamines increases markedly. This

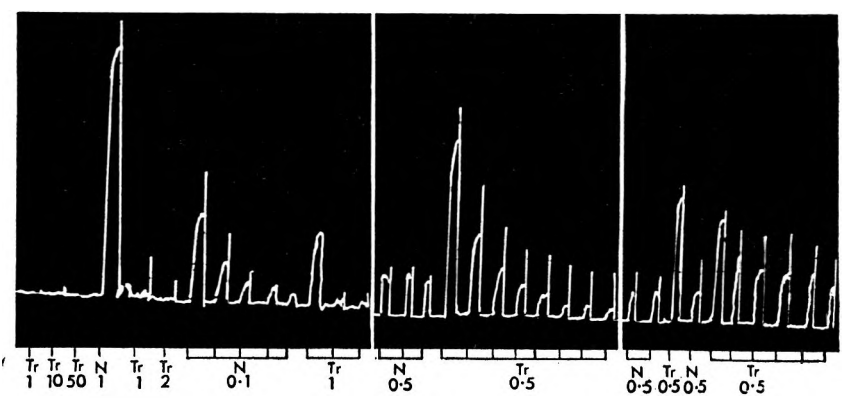

FIG. 1. The responses of isolated rat vas deferens to succesive doses of tranylcypromine (Tr) and noradrenaline (N), added to the bath at the points indicated. Drug concentrations μg/ml.

supersensitivity is highly transient and is observed in preparations from normal or reserpinized rats. But after stabilization of the responses of the preparation to catecholamines, the vas deferens becomes highly sensitive to tranylcypromine (Fig. 1): with concentrations of tranylcypromine completely ineffective before the stimulation with catecholamines, contractions of the same size and characteristics as the responses obtained with noradrenaline are elicited. This supersensitivity to tranylcypromine decreases but is long-lasting, and, when stabilization occurs, the sensitivity of the preparation to this monoamine oxidase inhibitor is markedly greater than before its previous stimulation with noradrenaline. These interactions are also seen in the reserpinized animal (1 mg/kg, 24 hr before). The same effects are seen with pheniprazine, but to a lesser extent. Supersensitivity of the vas deferens to amphetamine after its stimulation with noradrenaline also exists, but is quickly lost. In contrast, pargyline does not elicit any response of the vas deferens either before or after its stimulation with noradrenaline. However, the administration of repeated concentrations of pargyline potentiates about fivefold the effects of tyramine, while the responses to catecholamines are much less increased than is so with the other drugs tested. Potentiation of the effects of tyramine after tranylcypromine, amphetamine or pargyline is slight.

It has not been possible to reproduce these interactions between noradrenaline and tranylcypromine in other *in vitro* preparations of the same animal, like the isolated atria or the perfused renal artery (Garattini, Bonaccorsi & Laporte, unpublished). These results seem to confirm the uniqueness of the rat isolated vas deferens preparation for the study of adrenergic mechanisms, and to show that not all the monoamine inhibitors tested by us act in the same fashion on this preparation. It seems therefore that the direct or indirect effects elicited by these inhibitors on the rat vas deferens are not always a consequence of its biochemical action on the monoamine oxidase.

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The effect of imipramine of central 5-hydroxytryptamine neurons

SIR,—Ever since Sigg's (1959) discovery that imipramine is capable of potentiating the action of noradrenaline and of sympathetic nerve stimulation, the view has been favoured that a similar action on central noradrenaline is responsible for the antidepressive action of this drug. The peripheral potentiating action is probably due to blockade of an amine-concentrating mechanism located at the level of the cell membrane of the adrenergic neuron (Hillarp & Malmfors, 1964; Carlsson & Waldeck, 1965; Malmfors, 1965). The monomethyl derivatives of imipramine and related agents are more potent inhibitors of this mechanism than their parent compounds. We have investigated central noradrenaline neurons in this respect. The monomethyl derivatives protriptyline and desipramine proved to be active inhibitors of this mechanism in the central noradrenaline neurons (see Carlsson, Fuxe & others, 1966; Carlsson, Corrodi, Fuxe & Hökfelt, to be published). Later, however, imipramine was found to be surprisingly weak in this respect. This is in contrast to the good clinical antidepressive action of this drug. Evidence has recently been obtained that there exist also in the central 5-hydroxytryptamine (5-HT) neurons a reserpine-resistant uptake-concentration mechanism for amines. This mechanism appeared to be resistant to desipramine (Fuxe & Ungerstedt, 1967). We have now observed that imipramine is capable of blocking this mechanism in the 5-HT neurons.

Intraventricular injections of 5-HT (5 μg) were made into the lateral ventricle of rats pretreated with reserpine (12 hr before killing, 10 mg/kg, i.p.) and nialamide (500 mg/kg, i.p., 1 hr before killing). The rats were killed 30 min later (cf. Fuxe & Ungerstedt, 1966, 1967). Imipramine was given in doses of 20–30 mg/kg, intraperitoneally 15 min before the injection. There was a partial blockade of the reserpine-resistant accumulation of 5-HT in many 5-HT cell bodies, non-terminal axons and terminals lying close to the ventricles and the ventral part of the subarachnoidal space.

In another set of experiments rats pretreated with reserpine (10 mg/kg, i.p., 12 hr before killing) and nialamide (500 mg/kg, i.p., 1–6 hr before killing) were given imipramine intraperitoneally in a dose of 5 and 40 mg/kg respectively, 30–40 min before killing. In rats treated only with reserpine and nialamide there is a gradual accumulation of 5-HT in the 5-HT neurons which is maximal after about 4–6 hr, at which time the 5-HT neurons have a strong yellow fluorescence. Only in the highest dose (40 mg/kg) was there any clearcut effect of imipramine. With this dose there was a somewhat less marked accumulation of 5-HT compared to controls in all parts of the 5-HT neurons.

Systemically administered α-methyl-*m*-tyramine and related compounds are capable of reducing brain catecholamine levels, probably by displacement. This action is blocked by protriptyline and desipramine, less so by imipramine (Carlsson, Corrodi & Fuxe, unpublished data; see Carlsson, 1967). Continued work has shown that compounds of this class can also displace central 5-HT. This effect is blocked virtually completely by imipramine in doses of 25 to 50 mg/kg. Thus, the displacing agent alone caused a decrease of 5-HT in mouse brain from 0·40 to 0·18 μg/g. In animals treated with imipramine 30 min before the displacing agent the 5-HT level was 0·37 μg/g.

All these results taken together indicate that imipramine *in vivo* selectively blocks the reserpine-resistant uptake-concentration mechanism at the level of the nerve cell membrane in central 5-HT neurons. This action may be of importance for its antidepressive properties.

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An intravenous catheter for chronic use

SIR,—The use of conscious animals in experiments extending over weeks or months often necessitates the intravenous injection of fluids. Several indwelling catheters terminating in closures attached to the skin have been described but generally these require special machined parts (Khouri, Gregg & Rayford, 1965; Davis, 1966; Goetz & Hanis, 1967). We have designed and used a catheter which is easy to construct and whose components are usually found in research laboratories.

Fig. 1 is a diagram of a section through the catheter and skin-button connector. The connector is built around a No. 1 Record-fitting syringe needle. The needle mount (D) is first cut down to 1 cm in length and filed to an approximately cylindrical external contour. Next, the tapered mount hole is enlarged and a 1 ml serum closure cap (A) inserted. The cap is first cut as in the insert of Fig. 1 along the dotted lines. The closure skirt is then everted over the needle mount and covered with a short length (1 cm) of heat-shrinkable polyethylene tubing.

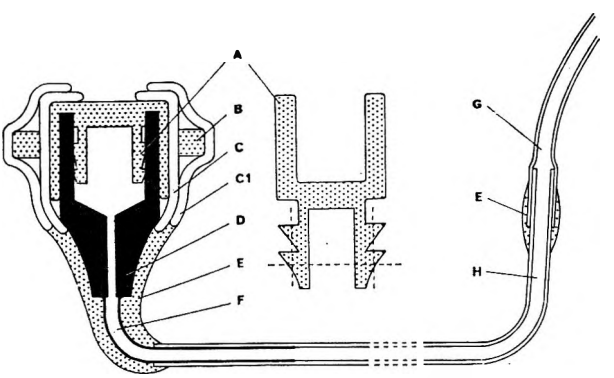

FIG. 1. Diagram of a section through the skin button connector and catheter to show the method of assembly.
A. Serum closure cap. B. Thick-walled rubber tubing. C and C_1, Heat shrinkable polyethylene tubing. D. Needle mount. E. Silicone rubber F. Syringe needle. G. Silicone rubber tubing. H. Teflon tubing.

Careful application of a small gas flame to this tube shrinks it so that it takes up the form shown in the diagram (C), holding the closure cap firmly in position. A narrow ring (2-3 mm) made from thick walled rubber tubing of suitable diameter (B) is pushed over the shrunken tube. Heat shrinkage of a second, wider bore polyethylene tube (C_1), over this ring produces a rounded ledge around the circumference of the button.

In our experience, Teflon tubing is too resilient for direct venous catheterization. Silicone rubber tubing, although ideal for this purpose, is very flexible and may kink along a subcutaneous pathway in a free moving animal. The catheter we have designed makes use of the properties of both Teflon and silicone rubber. The portion of the catheter (H) between the skin button and the vein is a Teflon tube (i.d. 1/32 in). This is pushed over the shortened syringe needle (F) and shaped in a gas flame to fit the expected contours of the subcutaneous pathway. The last centimetre is arranged to lie parallel with the vessel when *in situ*. The free end of the Teflon is prepared for bonding with an etching fluid (Tetra Etch) and inserted 5 mm into a 3–4 cm length (G) of silicone rubber tubing (i.d. 1/32 in). Cold curing silicone rubber (E) is used to strengthen this junction and to coat the exposed base of the needle mount. The completed catheter and termination button may be sterilized by the usual methods and filled by piercing the rubber cap with a fine syringe needle attached to a syringe of saline.

The operative procedure we use for the chronic catheterization of the jugular vein of a cat or rabbit is as follows. The catheter is pulled subcutaneously from a small x-shaped incision between the shoulder blades so that the rubber tube emerges near the mobilized vein. The vein is catheterized so that the rubber/Teflon junction just enters the vessel. Sutures are tied around the vein over this junction and the Teflon is secured to subcutaneous tissue. The skin button is secured in place by sutures which are passed through the outer polyethylene tube (C_1) and the rubber ring (B). Care is taken that the inner polyethylene tube (C) is not punctured by the suture needle. In use, a fine syringe needle attached to a length of fine polythene tube is pushed through the rubber diaphragm of the skin button. Intravenous injections may be made as often as necessary through this tube without further disturbance of the animal.

We have found that these catheters, implanted in the jugular veins of cats and rabbits, have remained patent over periods of several months with little attention other than occasional flushing with heparin/saline. These experiments were never terminated because of failure of the indwelling venous catheter.

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The manufacturers of the components used in the venous catheter are: 1 ml serum closures (Suba seal), Freeman & Co. Ltd., Steincross, Barnsley. Heat shrinkable polyethylene tubing, Hellermann Electric Ltd., Crawley, Sussex. Teflon tubing, X-Lon Products Ltd., London, S.E.11. Silicone rubber tubing and Silastic Elastomer 382 (Dow Corning), Down Bros. & Mayer & Phelps, Mitcham, Surrey. Tetra-Etch, W. L. Gore & Associates Inc., Newark, Delaware, U.S.A.

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The influence of xanthinol niacinate on ADP and adrenaline-induced platelet aggregation

SIR,—Xanthinol niacinate (7-[2-hydroxy-3-(*N*-2-hydroxyethyl-*N*-methylamino)-propyl]-1,3-dimethylxanthine-pyridin-3-carboxylate) has been reported by Balkuv, Akman & Ulutin (1966) to inhibit ADP induced platelet aggregation.

We have measured *in vitro* the inhibitory effect of this drug on platelet aggregation induced both by ADP and adrenaline using platelet rich plasma prepared according to Born & Cross (1964) and our own modification of O'Brien's (1964) method for the study of platelet aggregation, mentioned elsewhere by Ryšánek, Švehla & others (1967). Xanthinol niacinate was taken in quantities of 15, 10, 5, and 1 mg/2 ml of incubation mixture, i.e., in 750, 500, 250 and 50 mg % concentrations. Concentrations lower than 50 mg % were not examined since this was the lowest strength to give statistically significant results.

Table 1 shows that xanthinol niacinate significantly inhibited ADP induced platelet aggregation even in a concentration of 50 mg %.

TABLE 1. INHIBITION OF ADP-INDUCED PLATELET AGGREGATION BY XANTHINOL NIACINATE (Xn)

Xn conc.	n	Inhib. %	s.d.	t	P
750 mg % ..	8	97·5	3	88	<0·001
500 mg % ..	8	91·1	13	18	<0·001
250 mg % ..	9	45·7	30	4·3	<0·01
50 mg % ..	11	11·2	14	2·5	<0·05

Table 2 shows the inhibition of adrenaline induced platelet aggregation by xanthinol niacinate. The inhibitory effect was less marked here. This was due to a wider variation in standard deviation, though the resulting mean inhibition values did not differ significantly from those with ADP induced aggregation.

TABLE 2. INHIBITION OF ADRENALINE-INDUCED PLATELET AGGREGATION BY XANTHINOL NIACINATE (Xn)

Xn conc.	n	Inhib. %	s.d.	t	P
750 mg % ..	9	90	12·5	20	<0·001
500 mg % ..	8	84	21·5	10	<0·001
250 mg % ..	9	62	25	7	<0·01
50 mg % ..	9	13	22	1·6	>0·05

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Comparison of the effect of various sympathicolytics on thrombocyte aggregation

SIR—According to O'Brien (1963) the aggregation of thrombocytes induced by adrenaline and noradrenaline is mediated by specific receptors in thrombocytes themselves because phentolamine blocks this aggregation.

There is also a relation between the action of catecholamines on thrombocyte aggregation and their stimulating effect upon the smooth musculature of the nictitating membrane of the cat (O'Brien, 1964). If we assume that thrombocyte receptors possess the properties of adrenergic α-receptors, a contradiction exists in the fact that adrenaline-induced aggregation is also inhibited by dichloroisoprenaline which has good β-receptor blocking properties.

We have now compared quantitatively the inhibitory effect of phentolamine and tolazoline with that of some β-receptor blocking drugs.

In our experiments we used heparinized platelet rich human plasma, prepared according to Born & Cross (1963). We determined the inhibitory effect of sympathicolytics using our own modification of O'Brien's method (Ryšánek, Švehla & others, 1966). As a measure of 100% aggregation we took the drop in extinction in the plasma 2 min after adding either adrenaline in a concentration of 5×10^{-5}M, or ADP in a concentration of 5×10^{-4}M. We exposed thrombocytes to the action of the examined substances for 10 min. We expressed the degree of inhibition in per cent as the reciprocal of aggregation.

The drop in extinction was measured continuously after the addition of adrenaline during 120 sec, and then the samples were centrifuged for 60 sec at 25 rev/min. After centrifugation the drop in extinction was measured once more. We chose the time of 120 sec to be able to observe the first phase of the aggregating effect of adrenaline. The results of the measurement after aggregation proved more suitable for evaluation. The drop in extinction is given in per cent of the original value. For the evaluation of the mode of inhibition we used Lineweaver's & Burk's method (1934).

TABLE 1. INHIBITION OF THROMBOCYTES AGGREGATION BY SYMPATHICOLYTIC DRUGS

Drug	Aggregation induced by adrenaline			Aggregation induced by ADP		
	Conc. M 5×	Inhibition % (± s.d.)	Significance of inhibition P	Conc. M 5×	Inhibition % (± s.d.)	Significance of inhibition P<
Phentolamine	10^{-6}	98·25 (22·19) (8)*	<0·001			
Tolazoline	10^{-6}	95·19 (10·63) (8)	<0·001			
Pronethalol	10^{-4}	69·18 (17·22) (8)	<0·001	10^{-4}	43·55 (25·77) (15)*	<0·001
	10^{-5}	2·35 (11·27) (8)	<0·5	10^{-5}	6·21 (10·98) (8)	<0·2
	10^{-6}	8·32 (12·83) (8)	<0·2			
Isopropylmethoxamine	10^{-4}	67·05 (16·15) (8)	<0·001	10^{-4}	7·68 (8·73) (10)	<0·05
	10^{-5}	13·86 (18·87) (8)	<0·1	10^{-5}	0·24 (4·18) (8)	<0·9
	10^{-6}	1·23 (7·08) (8)	<0·6			
Propranolol	10^{-4}	95·50 (3·72) (9)	<0·001	10^{-4}	92·79 (2·78) (8)	<0·001
	10^{-5}	5·81 (13·58) (10)	<0·3	10^{-5}	−11·11 (19·70) (9)	<0·2

* No. of experiments

From our results (Table 1) it follows that among the investigated compounds phentolamine and tolazoline appeared the most potent, inhibiting adrenaline-induced thrombocyte aggregation 100% even at 5×10^{-6}M. β-Receptor blocking agents, pronethalol, isopropylmethoxamine, and propranolol, had a significant inhibitory effect only in 5×10^{-4}M concentrations. The inhibitory effect of phentolamine and tolazoline appeared when a concentration ten times lower than that of adrenaline was used. On the other hand the β-receptor blocking drugs were active only in a concentration ten times higher than that of adrenaline in the incubation mixture.

β-Receptor blocking agents at 1×10^{-4}M inhibited the second phase of adrenaline-induced aggregation only. These findings by themselves indicate that the inhibitory action of the β-receptor blocking drugs is probably a non-specific one.

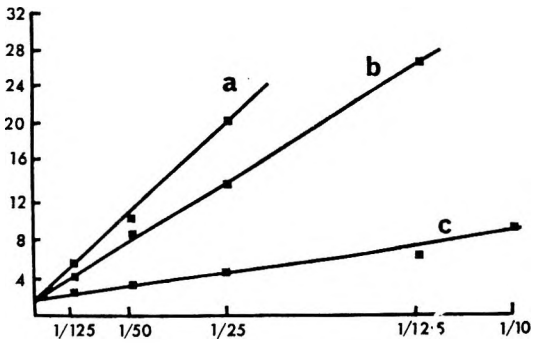

FIG. 1. Interference of phentolamine in different concentrations with adrenaline induced platelet aggregation. Abscissa: Reciprocal values of adrenaline concentration departing from the molar concentration $1 \times 10^{-}$. Ordinate: Reciprocal values of the degree of inhibition expressed in % of drop in extinction. phentolamine concentration, a, 5×10^{-8}M; b, 5×10^{-9}M. c, control group.

The finding of a competitive inhibition of the first phase of adrenaline-induced thrombocyte aggregation by phentolamine (Fig. 1) appears to us acceptable evidence that phentolamine inhibits the adrenaline receptors in thrombocytes. A similar competitive inhibition is produced by tolazoline.

This competitive inhibition was not seen with the β-receptor blocking agents even in high concentrations.

β-Receptor blocking agents also inhibited significantly at 5×10^{-4}M the ADP-induced thrombocyte aggregation. The most potent was propranolol (Table 1). The least effective was isopropylmethoxamine. These drugs accelerated the disaggregation of thrombocytes aggregated by ADP and thus in this sense they resembled desipramine (Mills & Roberts, 1966).

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Inhibition by disulfiram of the accelerated turnover of catecholamines in the adrenal glands of exercised rats

SIR,—Disulfiram is a potent inhibitor *in vitro* and *in vivo* of dopamine β-hydroxylase, the enzyme that catalyzes the final step in noradrenaline synthesis (Goldstein, Anagnoste & Lauber, 1964; Musacchio, Kopin & Snyder, 1964; Goldstein, Lauber & McKereghan, 1965). Disulfiram lowers heart and brain noradrenaline levels to a greater extent in cold-exposed rats than in rats kept at room temperature (Goldstein & Nakajima, 1966). This supports the view that a more rapid turnover of catecholamines occurs in brain and heart of cold-exposed animals. The accelerated catecholamine turnover in brain and heart can also be blocked with α-methyltyrosine (Gordon, Spector & others, 1966). α-Methyltyrosine inhibits tyrosine hydroxylase, the first and possibly rate-limiting enzyme in catecholamine synthesis. Using this inhibitor, an increased turnover of adrenal adrenaline was found to occur in rats forced to exercise (Gordon & others, 1966).

The results of Goldstein and Nakajima and of Gordon & others, suggest that disulfiram would lower adrenaline levels in the adrenal glands of exercised rats, although inhibitors of catecholamine synthesis produce little change in adrenal adrenaline in normal rats because of its slow turnover. We report the results of our experiments with disulfiram on the level of adrenaline in the adrenals of exercised rats.

Male Sprague-Dawley rats of approximately 160 g were injected intraperitoneally with disulfiram in a suspension of 5% acacia. The rats were then placed in a wood and screenwire drum (39 cm diameter) that rotated at approximately 8 rev/min, requiring the animals to run to remain upright. After 3 hr, the rats were decapitated and the adrenal glands quickly removed and frozen on dry ice. Adrenaline levels were measured fluorimetrically (Shore & Olin, 1958), the oxidation step being made at pH 5 so that noradrenaline was also included. Experiments in our laboratory have shown that noradrenaline comprises less than 10% of the total adrenal catecholamine as measured by this method; hence, the results are expressed as adrenaline. They are shown as means and standard errors of determinations in Table 1.

TABLE 1. ADRENALINE AND NORADRENALINE LEVELS IN THE ADRENAL GLANDS OF RATS (4 rats/group)

Treatment	Adrenaline + noradrenaline	
	μg/mg tissue	μg/adrenal pair
Control	1·05 ± 0·13	22·6 ± 0·5
Disulfiram	1·15 ± 0·08	21·9 ± 0·4
Exercise	1·10 ± 0·08	21·2 ± 1·0
Disulfiram + exercise	0·47 ± 0·06*	9·9 ± 0·5**

* Differed from saline controls, $P < 0{\cdot}01$.
** Differed from saline controls, $P < 0{\cdot}001$.

Disulfiram, at the 200 mg/kg dose, did not produce any effect in control rats during the 3 hr period studied. Likewise, exercise alone had no effect. Gordon & others (1966) found slight depletion of adrenal adrenaline after 3 hr of exercise in rats but our exercise conditions are probably less severe. However, the combination of exercise and disulfiram treatment caused a fall in adrenal adrenaline of more than 50% (Table 1).

These results are in agreement with those of Gordon & others (1966) in showing an increased turnover of adrenal catecholamines in exercised rats and demonstrate that this accelerated synthesis can be blocked by inhibiting an enzyme

other than tyrosine hydroxylase. Our results also show that disulfiram can inhibit noradrenaline and adrenaline synthesis in the adrenal glands as it does in the heart and brain.

In another experiment, rats were treated with 400 mg/kg of disulfiram intraperitoneally—the dose used by Goldstein & Nakajima to block brain and heart catecholamine turnover in cold-exposed rats. None of the treated rats survived the 3 hr of forced exercise. Goldstein & Nakajima mentioned that disulfiram-treated animals were sensitive to cold exposure. Our experiment shows that they are also very sensitive to exercise. Doses up to 1 g/kg of disulfiram were not lethal for up to three days in rats that were not exercised.

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Increased uptake of noradrenaline in the rat submaxillary gland during sympathetic nerve stimulation

SIR,—Uptake of noradrenaline in various organs innervated by adrenergic nerves has been studied extensively by a number of authors. However, it is not known whether this uptake mechanism is a stationary process or if there are factors which regulate the uptake of the neurotransmitter according to physiological state of the adrenergic neurons. This kind of information is important from the viewpoint that the reuptake of noradrenaline is regarded as the major pathway of the nerve impulse-released amine (Hertting & Axelrod, 1961; Rosell, Kopin & Axelrod, 1963; Gillespie & Kirpekar, 1965) and that the need for replacement of noradrenaline in the nerve endings as well as the amount of noradrenaline released would be increased when nerve activity increases. Gillis (1963) has shown that stimulation of the cardio-accelerator nerves caused an increased retention of noradrenaline by cat atria but not by ventricles while Blakeley & Brown (1964) observed a decreased uptake by the cat perfused spleen during nerve stimulation. The present paper shows that the uptake mechanism is enhanced on increasing the sympathetic activity in the submaxillary gland of rats.

Long Evans rats of either sex, weighing 250–300 g, were anaesthetized with intravenous injection of chloralose, 60 mg/kg. The rats were prepared for the stimulation of the sympathetic nerve to submaxillary gland according to the procedure described by Sedvall & Kopin (1967). The cervical sympathetic trunk and vagus nerve of one side were ligated and freed from the carotid artery and cut at the level of the clavicle. The vagus nerve was freed from the superior cervical ganglion and cut to interrupt afferent vagal impulses. The sympathetic nerve trunk was stimulated intermittently (10 sec each min) with rectangular pulses (5–7 V, 5 msec duration) at a frequency of 20/sec. The effectiveness of electrical stimulation was confirmed by salivation and wide opening of the eye

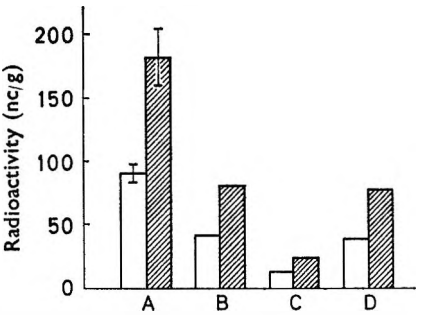

FIG. 1. [3H]Noradrenaline, [3H]normetanephrine and acid metabolites in the control (open columns) and stimulated (hatched columns) submaxillary glands of rats. A. Total. B. Noradrenaline. C. Normetanephrine. D. Acids.

of the stimulated side. The contralateral submaxillary glands served as control. (±)[^{3}H]noradrenaline (20 μc in 2 ml, 1400 mc/mM) was slowly infused into left femoral vein during a 30 min-period. At the end of infusion, electrical stimulation was interrupted. After resting for another 30 min the animals were killed and both submaxillary glands were rapidly excised and washed in cold Krebs solution. The glands were homogenized in 5 ml of chilled 0·4 N perchloric acid. [^{3}H]noradrenaline and its metabolites were isolated as described by Whitby, Axelrod & Weil-Malherbe (1961) and counted in a liquid scintillation spectrometer.

Table 1 shows that the intermittent stimulation of the cervical sympathetic trunk caused an increase of the radioactivity in the ipsolateral submaxillary glands by about 100% (23–163%). Analysis of the metabolites indicates that there was proportional increase of [^{3}H]noradrenaline as well as [^{3}H]normetanephrine and acid metabolites (Fig. 1). Desmethylimipramine (10 mg/kg; i.p.) abolished this increased uptake of [^{3}H]noradrenaline while the salivation evoked by the sympathetic stimulation as well as the resting uptake in the control submaxillary glands was not changed. This observation indicates that the increase of [^{3}H]noradrenaline uptake in the glands by nerve stimulation is not the result consequent to changes in blood flow or salivary secretion induced by sympathetic stimulation. Since both sides of submaxillary glands were exposed to the same concentration of [^{3}H]noradrenaline, it may be concluded that nerve impulses enhance the uptake mechanism of the nerve endings. The extent of enhancement may be actually more than we observed because a 30 min period of intermittent stimulation decreased respectively about 15% of endogenous noradrenaline and 30% of [^{3}H]noradrenaline in the submaxillary glands.

TABLE 1. EFFECT OF SYMPATHETIC NERVE STIMULATION ON THE UPTAKE [^{3}H]NORADRENALINE IN THE RAT SUBMAXILLARY GLAND

No. of expt.	Radioactivity in the submaxillary gland (mμc/g)		S−C	(S−C)/C
	Control (C)	Stimulated (S)		
1	65·8	80·6	14·8	0·23
2	116·6	184·9	68·3	0·59
3	90·3	220·5	130·2	1·36
4	80·2	211·3	131·1	1·63
5	90·6	234·4	143·8	1·58
6	97·4	160·9	63·5	0·65
Mean	90·2	182·1	91·9	1·01
Standard error	7·03	23·1	20·6	0·23

Whether this increased uptake caused by nerve activity is mediated by a mechanism similar to that of resting uptake is not known. The different effects of desmethylimipramine are suggestive of different mechanisms involved. A similar inference has been made by Geffen (1965) and by Boullin, Costa & Brodie (1967), although in their experiments desmethylimipramine and cocaine, in contrast to phenoxybenzamine, appear not to inhibit the nerve impulse-induced uptake. It remains to be elucidated whether the mechanism of nerve impulse-induced uptake of infused noradrenaline shown in the present experiment is similar to that shown by Geffen (1965) and Boullin & others (1967) for the uptake of nerve impulse-released amine.

Several factors which accompany nerve activity might account for the increased uptake. These are: increased permeability or active transport of noradrenaline in the nerve endings; increased noradrenaline concentration around the nerve endings, which consequently increases the extent of exchange of the amine; decrease of the store of available noradrenaline on repetitive stimulation and so increasing uptake, and an increased normetanephrine concentration due to increased release of noradrenaline. Since normetanephrine is known to enhance the uptake of noradrenaline in submaxillary glands of rats (Iversen, Fischer & Axelrod, 1966) and part of the impulse-released noradrenaline is metabolized to normetanephrine (Hertting & Axelrod, 1961), it follows that normetanephrine formed at sympathetic nerve terminals may be the mediator of the increased uptake. It is interesting that at the adrenergic nerve endings there exists such a feed-back system for the conservation of noradrenaline stores and for the disposition of the released neurotransmitter. We have also found that in the isolated vas deferens preparation of rats, transmural stimulation also caused an increase of the uptake of [^{3}H]noradrenaline. The reason why in the cat perfused spleen, sympathetic stimulation caused an impairment of uptake mechanism (Blakeley & Brown, 1964) is not known. It is interesting to note that the submaxillary gland and vas deferens are the only organs in which Iversen & others (1966) have found increased uptake of [^{3}H]noradrenaline by normetanephrine in rats.

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The origin of epileptiform seizures caused by oil of *Artemisia caerulescens* L.

SIR,—Vodopivec & Vatovec (1967) have recently shown that the oil of marine wormwood (*Artemisia caerulescens* L.) AC produced in rats, cats, rabbits and dogs, typical epileptiform seizures. These begin with salivation, followed by severe tonic-clonic convulsions especially in the region of the neck, limbs and trunk. The convulsions last from 10 to 60 sec and recur after a remission and sometimes the animal loses consciousness.

We have investigated oil of wormwood in white mice of either sex weighing about 20 g administered 40 mg/kg of an aqueous emulsion of the oil intraperitoneally. Epileptic seizures occurred as early as the 2nd or 3rd min, lasted about 30 sec and recurred at intervals of 1 to 3 min. In the interval between seizures the animals had difficulty in breathing, showed signs of fear and assumed unnatural dog-like postures. From 20 to 30% of the animals died; the surviving mice calmed down after about 1 hr.

Fifteen min before receiving the aqueous emulsion of oil of wormwood, the mice were given one of several substances (Table 1). Decortication was done in one group of animals by a method shown us by M. Taschler. The part of the cortex of the cerebrum which can be assumed to have motor function was first removed by a vacuum extractor under anaesthesia and the animals allowed to recover. The oil was extracted according to the method described by Vatovec, Vodopivec & Bohinc (1967).

TABLE 1. EFFECT OF SUBSTANCE P, MEPHENESIN, γ-AMINOBENZOIC ACID, PHENYTOIN, PHENACEMID, PHENOBARBITONE OR DECORTICATION OF THE CEREBELLUM ON THE BEHAVIOUR OF MICE GIVEN EMULSIFIED OIL OF WORMWOOD

	Substance P 4000 and 8000 units/kg (purity 1 mg = 13 units	Mephenesin 100 mg/kg	γ-Amino-benzoic acid 100 mg/kg	Phenytoin 200 mg/kg	Phenacemid 600 mg/kg	Pheno-barbitone 25 mg/kg	Decortica-tion of cerebrum
Oil of wormwood 40 mg/kg	−	−	−	+	+	+	+
n	16	12	10	16	16	10	9
P	0·05	0·05	0·05	0·05	0·05	0·05	

− Does not protect.
\+ Protects.
P is significance determined according to Bross, J. (1952), Sequential med. plans, Biometrics, 8, 188.

Phenytoin, phenacemid, or phenobarbitone and decortication prevented epileptic seizures, while substance P, mephenesin and γ-aminobenzoic acid produced no effect against oil of wormwood.

Decortication also prevents the action of oil of wormwood so that the site of its action is in the cortex. Mephenesin, which affects the polysynaptic reflexes in the spinal medulla, substance P which usually prevents leptazol spasm and γ-aminobenzoic acid which usually inhibits the synapses in the central nervous system were without effect.

The epileptiform seizures provoked by oil of wormwood may prove useful in examining new antiepileptic substances.

Acknowledgement. We are indebted to Professor Vatovec, Head of the Institute of Physiology and Pharmacology, School of Biotechnics, University of Ljubljana, for supplying the oil of *Artemisia caerulescens.*

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Comparison of tolazoline and thymoxamine on skin temperature in man

SIR,—Tolazoline hydrochloride (Priscol) is an α-adrenergic receptor blocking drug which is used clinically by oral and parenteral administration in the treatment of peripheral vascular disease. Thymoxamine (Opilon), a thymoxyalkylamine derivative, is a more recently discovered α-receptor blocking drug (Birmingham & Szolcsanyi, 1965, Foster, 1966). Its action in man has been demonstrated in the pupil, by prevention of ephedrine and phenylephrine mydriasis and reversal of hydroxyamphetamine mydriasis when applied to the conjunctival sac in the form of eye drops (Turner & Sneddon, 1967).

Variations in skin temperature may be used to measure changes in skin blood flow induced by α-receptor blocking drugs and rubifacients, and an investigation was, therefore, made to compare the effects of tolazoline and thymoxamine on skin temperature in man.

The same 10 subjects (5 men and 5 women, aged from 15 to 48 years) took part in three tests viz. comparisons between the two drugs and between each drug and a placebo.

Both tolazoline and thymoxamine were made up into 10% ointments in a water-free cetomacrogol base, the base alone serving as the control placebo.

TABLE 1. CHANGE IN FOREARM SKIN TEMPERATURE °C INDUCED BY THYMOXAMINE, TOLAZOLINE AND A PLACEBO IN 10 SUBJECTS

Treatment	Skin temperature °C					
	Before	After	Difference between treatments	s.e.	t	P
Thymoxamine .. v Placebo	33·34 33·51	33·85 33·55	0·47	0·152	3·12	<0·02
Tolazoline .. .. v Placebo	33·13 33·02	33·19 33·06	0·02	0·062		n.s.
Thymoxamine .. v Tolazoline	32·23 32·38	32·49 32·49	0·14	0·081	1·72	n.s.

Skin temperatures were measured by means of a copper-constantan thermocouple (voltage output 40 V/°C) fixed to the volar surface of each forearm and connected to Grass polygraph Model 7P1-preamplifiers calibrated within a range of 24–40°. When stable recordings were obtained (usually within 2 min), a thin smear of the respective ointments was applied under the thermocouples but was not rubbed in; the choice of side for active or control preparation was arbitrary. Temperatures were again monitored and a reading taken after 10 min.

Although air-movement in the room was minimized during these tests, they were conducted over a period of some weeks, during which the ambient temperature ranged between 18 and 24·5°; however, this temperature range obtained in tests of both drugs.

Changes in mean forearm temperatures in response to treatments are shown in Table 1. Thymoxamine produced a very significant ($P < 0.02$) increase in skin temperature when compared with the placebo base, but although its effect was greater than tolazoline in a direct comparison this did not reach statistical significance. When tolazoline was compared with the placebo, no significant effect was observed. In one subject, both tolazoline and thymoxamine produced an erythema in the treated area, with an increase of 0·8° with tolazoline and 0·4° with thymoxamine.

The principal nervous control of skin blood flow is mediated through α-adrenergic receptors, stimulation of which leads to cutaneous vasoconstriction. It is reasonable, therefore, that specific α-receptor blockade should lead to a reduction in vasoconstrictor activity with an increase both in cutaneous blood flow and in skin temperature. This investigation has demonstrated that thymoxamine 10% in a cetomacrogol base produces a significant increase in skin temperature compared with inactive base, and also that it appeared more effective than tolazoline 10% prepared in the same base in which preliminary uncontrolled experiments had shown tolazoline to be most effective. However, further investigations might show a better vehicle for its activity.

While it is probable that this effect of thymoxamine is specifically due to α-receptor blockade, other modes of action cannot be excluded. Tolazoline has histamine-like actions in animals, including stimulation of gastric secretion and peripheral vasodilatation (Nickerson, 1949), and it is possible that its cutaneous effects are due to this as well as its α-blocking activity. Further studies including pre-treatment with antihistamine drugs might elucidate this further. Thymoxamine is a weak competitive antagonist of histamine (Birmingham & Szolcsanyi, 1965) and thus it is unlikely that its peripheral vascular action is due to histamine-receptor activity. It is also possible that both drugs, like phentolamine (Taylor, Sutherland, & others, 1965), have direct smooth-muscle relaxing properties unrelated to adrenergic or histamine-receptor activity. Whatever the mechanism of action, however, the increase in skin temperature produced by topical administration of thymoxamine suggests that further studies of this drug are indicated both in normal subjects using different bases to determine the best vehicle for its activity, and in patients with ischaemic disease of the skin to assess its therapeutic value.

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A new principle for the fluorimetric determination of α-methylnoradrenaline

SIR,—α-Methyldopa, a drug widely used in the treatment of hypertension, is converted *in vivo* to α-methylnoradrenaline which displaces the endogenous noradrenaline (Carlsson & Lindqvist, 1962). The absolute configuration of this α-methylnoradrenaline appears to be 1*R*:2*S*, i.e., the (−)-*erythro* form (Lindmar & Muscholl, 1965). Although there are methods for the determination of α-methylnoradrenaline (Lindmar & Muscholl, 1965; Schümann, Grobecker & Schmidt, 1965), there is a need for a more sensitive method. It is also of importance to make a differential estimation of α-methylnoradrenaline and noradrenaline since these two compounds are not easily separated by cation-exchange chromatography.

Recently it has been shown that the *erythro*-α-methylnoradrenaline can be converted to the *threo* form in an acid medium (Hallhagen & Waldeck, 1968). Further it has been observed by Muscholl (personal communication) that the *threo* form treated according to the trihydroxyindole-method (Bertler, Carlsson & Rosengren, 1958) yields a fluorescence of the same order of magnitude as noradrenaline whereas the fluorescence obtained from *erythro*-α-methylnoradrenaline under the same experimental conditions is much lower.

In the present investigation 0·2–2·0 μg of the *erythro* form was added to 5 ml of 2N hydrochloric acid and the samples were then heated in a boiling water bath under a reflux condenser for 30 min. Non-heated samples were run in parallel. The samples were then neutralized to pH 6·5 by 5N potassium carbonate. One ml of the neutral sample was taken for fluorimetric determination (Bertler & others 1958). Fig. 1 shows that there is a constant ratio between the fluorescence and the concentration and that the method is sensitive down to 20–30 ng/ml sample.

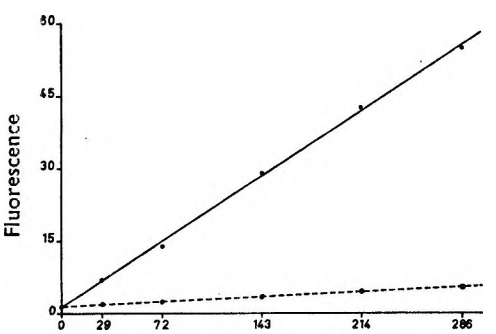

FIG. 1. Fluorescence intensity at various concentrations of *erythro*- α-methylnoradrenaline when treated according to the trihydroxyindole method with and without previous boiling in an acid medium (see text). Activating and fluorescence peaks: 400 and 510 mμ, respectively. —•— Boiled samples. — —•— — Unboiled samples.

In another experiment equal amounts (2 μg) of *threo*- and *erythro* α-methylnoradrenaline and noradrenaline were treated as described above. The fluorescence intensities of the samples were then compared. The mean of the unboiled samples of the *threo*-form was set to 100 (Table 1). When not boiled, *threo*-α-methylnoradrenaline showed a fluorescence intensity several times higher than its diastereoisomer. The boiling procedure, however, caused about 30%

decrease of the fluorescence obtained. In contrast, *erythro*-α-methylnoradrenaline after boiling gained a fluorescence of the same intensity as the boiled *threo* form. It thus appears that under the present conditions an equilibrium between the two diastereoisomers is reached. The fluorescence from noradrenaline was but slightly reduced after boiling in an acid medium (Table 1). So it would be possible to make differential estimations of *erythro*-α-methylnoradrenaline and noradrenaline.

TABLE 1. THE RELATIVE FLUORESCENCE INTENSITY OF *threo*-α-METHYLNORADRENALINE (α-Me-NA), *erythro*-α-Me-NA AND NORADRENALINE (NA) WHEN TREATED ACCORDING TO THE TRIHYDROXYINDOLE METHOD WITH AND WITHOUT PREVIOUS BOILING IN AN ACID MEDIUM (SEE TEXT). ACTIVATING AND FLUORESCENCE PEAKS: 400 AND 510 mμ, RESPECTIVELY

	threo-α-Me-NA	*erythro*-α-Me-NA	NA
Unboiled ..	100 ± 5	6 ± 1	103 ± 5
Boiled	69 ± 1	69 ± 2	92 ± 2

Mean ± s.e.m. of 3–5 determinations.

Experiments are now in progress to apply this principle on eluates from tissue extracts.

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Inhibition of ethanol-induced glycogenolysis in brain and liver by adrenergic β-blockade

SIR,—Ethanol is known to increase lipolysis in adipose tissue (Brodie, Butler & others, 1961; Estler & Ammon, 1967; Ammon, Estler & others, 1966) and glycogenolysis in brain and liver (Ammon, Estler & Heim, 1965; Ammon, Estler & Heim, 1966; Estler & Ammon, 1965). The increased lipolysis is supposed to be due to the action of catecholamines which are released from the adrenal medulla by ethanol (Klingman & McC. Goodall, 1957; Abelin, Herren & Berli, 1958; Perman, 1961; Wartburg, Berli & Aebi, 1961) because it can be prevented by adrenalectomy (Mallov & Gierke, 1957), α-adrenergic blocking agents (Brodie & others, 1961) and β-adrenergic blocking agents (Estler & Ammon, 1967). Since catecholamines increase glycogenolysis by stimulating the adenylcyclase system, which activates not only the hormone-sensitive lipase in adipose tissue but also phosphorylase, we examined whether the glycogenolytic action of ethanol in brain and liver is mediated by catecholamines which

stimulate the adrenergic β-receptors. Female white NMRI mice, kept at 24° and fed with a standard diet (Altromin, Altromin G.m.b.H. Lage/Lippe, Germany) were given 50 μg/g of the β-blocking agent Kö 592 [1-(isopropyl-amino)-3-(*m*-toloxy)-2-propanol HCl] subcutaneously 30 min before the intravenous injection of 1·5 mg/g ethanol. At 10 and 30 min after the ethanol injection the animals were killed by immersion in liquid air and brain and liver tissue were prepared frozen. The glycogen content was estimated (Kemp & Kits van Heijningen, 1954).

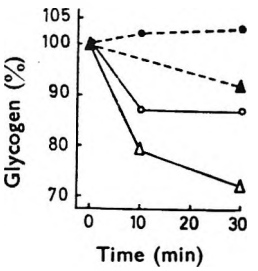

FIG. 1. Glycogen in brain (circles: 100% = 5·1 μmole glucose/g) and liver (triangles: 100% = 352 μmole glucose/g) of female white mice. —— after i.v. injection of 1·5 mg/g ethanol. – – – after i.v. injection of 1·5 mg/g ethanol in animals which had received 50 μg/g Kö 592 30 min before the ethanol. 100% = control value; ○ = $P \leqq 0{\cdot}05$.

The intravenous injection of ethanol was followed by a decrease of glycogen in brain and liver (Fig. 1). But neither in the brain nor in the liver was there a significant decrease of glycogen after ethanol when the animals were pretreated with Kö 592.

It seems that, similarly to lipolysis in adipose tissue, the enhanced glycogenolysis in brain and liver which follows the administration of ethanol is due to the action of catecholamines, which it releases. These results are in accordance with earlier findings from this laboratory (Estler & Ammon 1965) that ethanol-induced glycogenolysis in the brain could be prevented by adrenalectomy.

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Effects of diethyldithiocarbamate on the conditioned avoidance response of the rat

SIR,—Sodium diethyldithiocarbamate, the active metabolite of disulfiram, is a potent inhibitor of dopamine-β-hydroxylase *in vitro* (Goldstein, Anagnoste & others, 1964; Collins, 1965) and *in vivo* (Carlsson, Lindqvist & others, 1966). A single injection of the metabolite causes a decrease in brain noradrenaline content and a relatively small increase in brain dopamine. Compounds which effect the metabolism and distribution of catecholamines in brain such as reserpine, dopa and α-methyltyrosine also effect the maintenance of a conditioned avoidance response (Seiden & Carlsson, 1963, 1964; Moore, 1966), and evidence has been presented that the maintenance of a conditioned avoidance response requires dopamine or noradrenaline, or both (Seiden & Peterson, 1968). We have now measured the effects of diethyldithiocarbamate on the conditioned avoidance response.

TABLE 1. EFFECTS OF SODIUM DIETHYLDITHIOCARBAMATE ON CONDITIONED AVOIDANCE RESPONSES IN GROUPS OF 4 RATS

Dose mg/kg s.c.		Mean ± s.e. (%) responses at hr after injection			
		Normal	6 hr	24 hr	48 hr
125	(% avoid)	90·0 ± 1·8	92·5 ± 2·8	97·5 ± 2·2	96·2 ± 1·1
	(escape failure)	0	0	0	0
	(latency)	6·2 ± 0·9	6·5 ± 0·8	4·9 ± 0·5	5·3 ± 0·3
250	(% avoid)	95·0 ± 1·8	47·5 ± 13·3*	96·2 ± 1·1	96·2 ± 2·0
	(escape failure)	0	15 ± 11·6	0	0
	(latency)	5·7 ± 0·9	14·8 ± 3·6*	5·7 ± 0·5	5·4 ± 1·0
500	(% avoid)	96·2 ± 1·1	18·8 ± 7·2*	78·8 ± 18·4	96·2 ± 2·0
	(escape failure)	0	38·8 ± 20·4	11·2 ± 9·6	0
	(latency)	5·6 ± 0·6	21·8 ± 3·0*	10·0 ± 4·3	5·8 ± 0·6

* $P < 0.05$ Willcoxon Rank Test.

Male, albino rats (Holtzman Sprague-Dawley, 280–320 g) were conditioned (Seiden & Carlsson, 1963) in two modular testing units (12 in × 8 in × 7½ in) placed end to end, with an opening between them (4 in × 4 in). The conditioned stimulus was a buzzer; the unconditioned stimulus was an intermittent electrical shock delivered through a scrambler to the grid floor of the chamber (current 0·5 mA, duration 0·5 sec, frequency 1/2·5 sec). The conditioned-unconditioned stimulus interval was 15 sec. During training and testing, three types of responses were scored: if the rat crossed in response to the conditioned stimulus alone, an "avoidance response" was scored; if the cross occurred during the time when both the conditioned and unconditioned responses were being presented, an "escape response" was scored; if the rat failed to cross after receiving 6 shocks, an "escape failure" was scored. The latency is defined as the time (sec), required for the cross to occur. Forty trials were given to each rat daily (inter-trial time 45 sec) until a criterion of at least 90% avoidance responding was reached in the first or second block of 20 trials. When this occurred, an additional 20 trials were given the next day (overtraining session). Sodium diethyldithiocarbamate was injected 6 hr before testing, on the day after overtraining.

The performance of the conditioned avoidance response of rats 6 hr after treatment with 125 mg/kg of diethyldithiocarbamate did not differ significantly from their pre-injection performance. However, rats injected with either 250 mg/kg or 500 mg/kg showed depression of avoidance responding (50 and

80%, respectively) as well as an increase in the latency of the response (160 and 290%, respectively). At 24 and 48 hr after diethyldithiocarbamate injection no effects on the conditioned avoidance response were observed (Table 1).

Since the metabolite causes depletion of brain noradrenaline, it is possible that the effects of diethyldithiocarbamate on the conditioned avoidance response may be due to its effect on this catecholamine; however, it also has effects on other copper-containing enzymes, and has been shown to be a copper chelator (Frieden, 1962). Further work is necessary to establish a preference between the alternatives.

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Performance of the Ferranti-Shirley viscometer with automatic flow curve recorder unit

SIR,—A common method of investigating the rheological properties of materials which exhibit time dependent flow characteristics such as thixotropy is to use a rotational viscometer of, for example, the cone and plate type (see e.g. Boylan 1966, Talman, Davies & Rowan, 1967, Barry & Shotton, 1967). The Ferranti-Shirley viscometer (McKennell 1954, 1956, 1960, Van Wazer, Lyons & others, 1963) is designed so that when used in its automatic mode it is intended that the cone is accelerated at a uniform rate up to a preset maximum speed and then decelerated to zero speed at the same rate. A plot of torque on the cone against speed of the cone may then be displayed as a hysteresis loop on an X-Y plotter. This loop is then used to characterize the rheological properties of the system (Green, 1949, Green & Weltmann, 1946, Weltmann, 1960).

We have made a simple test on our instrument to check if acceleration and deceleration were constant (Barry, 1967). The sweep time was set at 600 sec, the indicator unit was switched to "check speed" and the maximum rev/min to 100, and the cone was set revolving; at the same time a stop watch (0·1 sec divisions) was started. The time was noted at every ten divisions of the speed scale and thus the time required for each increment of 10 rev/min obtained. The results are shown in Table 1.

Similar results were obtained using an alternative procedure. The cone rev/min was displayed as the Y coordinate on the X-Y plotter and the distance moved by the pen up this axis in equal increments of time (60 sec) was measured. Similar results were obtained with the same limits of reproducibility.

If the control unit was functioning linearly, each increment of 10 rev/min would require 60 sec; considerable deviation occurs even after allowing for the manufacturer's tolerance of 2% on all readings. However, the repeatability from run to run for the time required for any set increment was of the order of 2% except at the extreme ends of the scale. This repeatability was maintained over a period of six months.

TABLE 1. PERFORMANCE OF FERRANTI-SHIRLEY VISCOMETER IN AUTOMATIC MODE. Time required for each 10 division increment in cone speed.

Cone speed increment (revolutions per min)	Time required for each 10 division increment in cone speed (Theoretical time = 60 sec)	
	Upcurve	Downcurve
0–10	62·5	67·6
10–20	53·9	61·2
20–30	56·2	61·6
30–40	58·3	61·8
40–50	59·4	61·0
50–60	58·8	58·2
60–70	60·0	57·5
70–80	61·5	56·8
80–90	65·0	57·7
90–100	65·5	55·7

The characterization of the rheology of time dependent materials by means of hysteresis loops is at best a semiquantitative method in which the conditions of testing are chosen by the experimenter. When the Ferranti-Shirley viscometer is used in automatic mode simply to apply a standardized testing procedure the performance is adequate. However, when comparing results obtained with an instrument on different occasions or results from different instruments, the variation in the rate of increase or decrease in shear rates should be known.

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November 17, 1967

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Contents

Letters to the Editor

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