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Research Papers

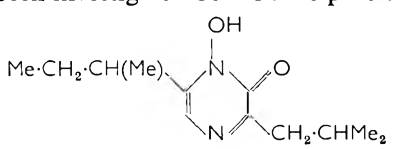
Some cyclic hydroxamic acids

R. T. COUTTS, D. NOBLE AND D. G. WIBBERLEY

The preparation of certain quinolines, quinazolines, quinoxalines, benzoxazines and benzothiazines containing the cyclic hydroxamic grouping is described. *In vitro* antibacterial activities showed that no compound had a broader spectrum of activity than the known 1,2-dihydro-1-hydroxy-2-oxoquinoline.

THE discovery of the high inhibitory *in vitro* activity of aspergillic acid (I) against both Gram-positive and Gram-negative organisms (White, 1940; White & Hill, 1943), together with the demonstration of a hydroxamic acid grouping in the molecule (Dutcher, 1947; Dunn, Gallagher, Newbold & Spring, 1948), stimulated a search for less toxic analogues. Despite the fact that over the last 20 years at least twelve different aromatic *N*-heterocyclic systems containing a hydroxamic acid grouping have been synthesised, the preparative difficulties have, in most instances, precluded the synthesis of more than a few examples of each particular system. The preparation of compounds closely related to the naturally occurring cyclic hydroxamic acids aspergillic acid (I), pulcherriminic acid (II) and iodinin (III) remains a moderately difficult task. The synthesis of bi- and tri-cyclic systems containing a hydroxamic group has, however, been facilitated by the discovery (Coutts & Wibberley, 1963) that reductive cyclisation of suitable *o*-nitro-esters with sodium borohydride and palladised charcoal yielded cyclic hydroxamic acids.

In view of the known superior antibacterial activity of 1,2-dihydro-1-hydroxy-2-oxoquinoline (IV; X–Y = CH = CH) (see Newbold & Spring, 1948) we have prepared a number of analogues of this compound which retain a bicyclic ring system but have other heteroatoms within the molecule. The influence of aromaticity on antibacterial activity has also been investigated both in the parent quinolines and in aza-analogues.



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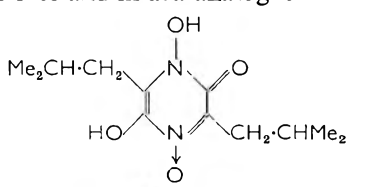

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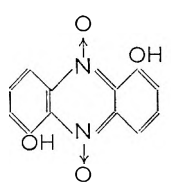

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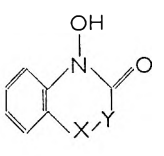

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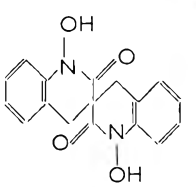

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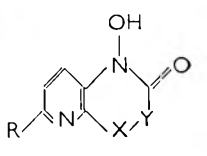

VI

From the School of Pharmacy, Sunderland Technical College, Sunderland, Co. Durham.

Ethyl β-(*o*-nitrophenyl)propionate gave a good yield of 1,2,3,4-tetrahydro-1-hydroxy-2-oxoquinoline (IV; X–Y = CH_2—CH_2) on treatment with sodium borohydride and palladised charcoal, and the precursor of this ester, ethyl *o*-nitrobenzylmalonate gave the related 3-ethoxycarbonyl-1,2,3,4-tetrahydro-1-hydroxy-2-oxoquinoline. The reduction of ethyl di(*o*-nitrobenzyl)malonate, a by-product in the synthesis of the *o*-nitrobenzylmalonate yielded the first spiro-cyclic hydroxamic acid, 3,3′-spirobi-(1,2,3,4-tetrahydro-1-hydroxy-2-oxoquinoline) (V) to be reported.

We have previously described (Coutts & Wibberley, 1963) the preparation of the benzothiazines (IV; X–Y = S–CH_2 and SO_2–CH_2), the antibacterial properties of which are recorded in Table 1. The closely related 3,4-dihydro-4-hydroxy-3-oxo-2*H*-1,4-benzoxazine (IV; X–Y = O–CH_2) was obtained from ethyl *o*-nitrophenoxyacetate in a higher yield than that found by Honkanen & Virtanen (1960), who reduced the ester with zinc and ammonium chloride. The isomeric 1,2-dihydro-1-hydroxy-2-oxo-4*H*-3,1-benzoxazine (IV; X–Y = CH_2–O) was similarly prepared from methyl *o*-nitrobenzyl carbonate.

The synthesis of 1,2,3,4-tetrahydro-1-hydroxy-2-oxoquinazoline (IV; X–Y = CH_2–NH) was accomplished from either methyl *o*-nitrobenzylcarbamate or from diethyl *o*-nitrobenzylidenebiscarbamate in only moderate yield, as also was the synthesis of 1,2-dihydro-1,4-dihydroxy-2-oxoquinazoline by the reduction of ethyl *o*-nitrobenzoylcarbamate. Oxidation of the former tetrahydroquinazoline to the fully aromatic 1,2-dihydro-1-hydroxy-2-oxoquinazoline (IV; X–Y = CH=N) was accomplished by passing a solution of the compound through an ion-exchange resin containing ferric ions. The isomeric 3,4-dihydro-3-hydroxy-4-oxoquinazoline was prepared by the method of Adachi (1957).

Ethyl *N*-*o*-nitrophenylglycine was best prepared by the interaction of glycine and 1,2-dinitrobenzene (cf Crowther, Curd, Davey & Stacey, 1949, for the synthesis of the 5-chloro-analogue). On reduction it gave the expected 1,2,3,4-tetrahydro-1-hydroxy-2-oxoquinoxaline (IV; X–Y = NH–CH_2), but when the nitrogen atmosphere maintained during reduction was removed and the reaction mixture stirred for several hours, atmospheric oxidation occurred to yield the corresponding 1,2-dihydroquinoxaline (IV; X–Y = N=CH). The latter was identical with the product obtained by the action of acetic anhydride on quinoxaline 1,4-dioxide (cf Elina, 1962). 1,2-Dihydro-1,3-dihydroxy-2-oxoquinoxaline was only obtained in very low yield by the reductive cyclisation of *N*-ethoxalyl-*o*-nitroaniline and was therefore prepared by the method of Tennant (1963).

Two examples of triazanaphthalenes containing the cyclic hydroxamic acid grouping were prepared. Reduction of 2-ethoxycarbonylmethylamino-3-nitropyridine yielded 1,2,3,4-tetrahydro-1-hydroxy-2-oxo-1,4,5-triazanaphthalene (VI; R=H, X–Y = NH–CH_2), and reduction of 2-ethoxalylamino-6-methyl-3-nitropyridine yielded 1,2-dihydro-1,3-dihydroxy-6-methyl-2-oxo-1,4,5-triazanaphthalene (VI; R=Me, X–Y = N=C(OH)).

All the cyclic hydroxamic acids gave wine-red or blue colours with aqueous ferric chloride solution and were soluble in sodium hydrogen

carbonate solution. Their infra-red spectra showed carbonyl and hydroxyl absorption.

ANTIBACTERIAL ACTIVITIES

The results of antibacterial screening tests are in Table 1. Previous reported figures for minimal inhibitory concentration of 1,2-dihydro-1-hydroxy-2-oxoquinoline (Newbold & Spring, 1948) were 2·0 and 1·0 mg/100 ml against *Staphylococcus aureus* and *Escherichia coli* respectively. None of the new compounds has an activity surpassing this although several compounds show more specific activity against *Staph. aureus*.

TABLE 1. MIC (MG/100 ML) OF CYCLIC HYDROXAMIC ACIDS

Compound	Test organisms				
	Staph. aureus	*B. subtilis*	*E. coli*	*S. typhi*	*P. vulgaris*
1,2,3,4-Tetrahydro-1-hydroxy-2-oxoquinoline	40	20	40	>40	>40
3-Ethoxycarbonyl-1,2,3,4-tetrahydro-1-hydroxy-2-oxoquinoline	40	>40	>40	>40	>40
3,3'-Spirobi-(1,2,3,4-tetrahydro-1-hydroxy-2-oxoquinoline)*	>40	>40	>40	>40	>40
3,4-Dihydro-4-hydroxy-3-oxo-1,4-benzothiazine[1]	>40	>40	>40	>40	>40
3,4-Dihydro-4-hydroxy-3-oxo-1,4-benzothiazine 1,1-dioxide[1]	>40	>40	>40	>40	>40
3,4-Dihydro-4-hydroxy-3-oxo-2*H*-1,4-benzoxazine	10	40	>40	>40	>40
1,2-Dihydro-1-hydroxy-2-oxo-4*H*-3,1-benzoxazine	40	>40	>40	>40	>40
1,2,3,4-Tetrahydro-1-hydroxy-2-oxoquinazoline	5	40	>40	>40	>40
1,2-Dihydro-1,4-dihydroxy-2-oxoquinazoline	>40	>40	>40	>40	>40
1,2-Dihydro-1-hydroxy-2-oxoquinazoline	20	40	>40	>40	>40
3,4-Dihydro-3-hydroxy-4-oxoquinazoline[2]	>40	>40	>40	>40	>40
1,2,3,4-Tetrahydro-1-hydroxy-2-oxoquinoxaline	20	>40	>40	>40	>40
1,2-Dihydro-1-hydroxy-2-oxoquinoxaline	20	>40	>40	>40	>40
1,2-Dihydro-1,3-dihydroxy-2-oxoquinoxaline[3]	>40	>40	>40	>40	>40
1,2,3,4-Tetrahydro-1-hydroxy-2-oxo-1,4,5-triazanaphthalene	5	40	>40	>40	>40
1,2-Dihydro-1,3-dihydroxy-methyl-2-oxo-1,4,5-triazanaphthalene	40	40	>40	>40	>40
1,2-Dihydro-1-hydroxy-2-oxoquinoline[4]	10	5	5	5	10

[1] Coutts and Wibberley, 1963.
[2] Adachi, 1957.
[3] Tennant, 1963.
[4] Ohta and Ochiai, 1962.
* Tested as a suspension in ethanol.

Experimental

General method of reductive cyclisation. A solution of the nitro-ester (0·1 mole) in dioxan was added over 5 min to a suspension of palladised charcoal (0·2 g) in 2% sodium hydroxide solution (20 ml) containing sodium borohydride (0·025 mole). A stream of nitrogen was passed through the stirred mixture for the stated time, the catalyst was removed by filtration, and the product isolated by suitable means from the acidified filtrate.

1,2,3,4-*Tetrahydro-1-hydroxy-2-oxoquinoline* (IV; X–Y = CH_2–CH_2). Reduction of ethyl β-(*o*-nitrophenyl)propionate (15 min) yielded, by extraction with chloroform, the *quinoline* (75%) as colourless plates (from ethanol), m.p. 117–118°. Found: C, 66·2; H, 5·7; N, 8·8. $C_9H_9NO_2$ requires C, 66·25; H, 5·5; N, 8·6%. ν_{max} 2,700–3,200 w (O–H), 1,690 s cm^{-1} (C=O).

3-*Ethoxycarbonyl*-1,2,3,4-*tetrahydro*-1-*hydroxy*-2-*oxoquinoline*. Reduction of ethyl *o*-nitrobenzylmalonate (30 min) yielded, by extraction with ether, the *ethoxycarbonylquinoline* (39%) as colourless needles (from ethanol), m.p. 137–139°. Found: C, 61·7; H, 5·8; N, 6·0. $C_{12}H_{13}NO_4$ requires C, 61·3; H, 5·5; N, 6·0%. ν_{max} 3,000–3,200 w (O–H) 1,715 s (ester C=O) 1,665 s cm^{-1} (ring C=O).

3,3′-*Spirobi*-(1,2,3,4-*tetrahydro*-1-*hydroxy*-2-*oxoquinoline*) (V). Reduction of ethyl di(*o*-nitrobenzyl)malonate (15 min) yielded, by filtration, the *spiro hydroxamic acid* (V) (76%). The solubility of this compound in organic solvents was extremely low, and purification was effected by repeated dissolution in alkali and precipitation with acid to constant m.p., followed by washing with hot acetic acid. Found: C, 65·45; H, 4·8; N, 8·7. $C_{17}H_{14}N_2O_4$ requires C, 65·8; H, 4·5; N, 9·0%. ν_{max}3,050–3,250 m (O–H), 1,650 s, 1,670 sh cm^{-1} (C=O).

3,4-*Dihydro*-4-*hydroxy*-3-*oxo*-2H-1,4-*benzoxazine* (IV; X–Y = O–CH_2). Reduction of ethyl *o*-nitrophenoxyacetate (15 min) yielded, on filtration, the *benzoxazine* (84%) as colourless needles (from aqueous ethanol), m.p. 156° with prior sublimation. Found: C, 58·5; H, 4·2; N, 8·5. Calc for $C_8H_7NO_3$: C, 58·2; H, 4·2; N, 8·5%. ν_{max} 2,550–3,250 m (O–H) 1,650 s, 1,680 s cm^{-1} split (C=O), Honkanen & Virtanen (1960) obtained the same product in 60% yield in a reduction with zinc and ammonium chloride.

Methyl o-*nitrobenzyl carbonate*. A solution of *o*-nitrobenzyl alcohol (7·3 g), methyl chloroformate (7·3 ml) and pyridine (3·8 ml) in chloroform (40 ml) was stirred at room temperature for 14 hr. The solution was washed with water, dried (Na_2SO_4) and distilled to yield the *carbonate* (64%), b.p. 132–134°/0·9 mm as colourless prisms (from ethanol), m.p. 46–47°. Found: C, 51·3; H, 4·35; N, 7·1. $C_9H_9NO_5$ requires C, 51·2; H, 4·3; N, 6·6%.

1,2-*Dihydro*-1-*hydroxy*-2-*oxo*-4*H*-3,1-*benzoxazine* (IV; X–Y = CH_2–O). Reduction of methyl *o*-nitrobenzyl carbonate (25 min) yielded, by extraction with chloroform, the *benzoxazine* (33%) as colourless prisms (from benzene), m.p. 127–128°. Found: C, 57·8; H, 4·2; N, 8·0. $C_8H_7NO_3$ requires C, 58·2; H, 4·2; N, 8·5%. ν_{max} 3,000–3,200 m (O–H), 1,690 s cm^{-1} (C=O).

Diethyl o-*nitrobenzylidenebiscarbamate*. *o*-Nitrobenzaldehyde (3·0 g) and ethyl carbamate (3·6 g) were melted together on a water-bath. Concentrated hydrochloric acid (0·2 ml) was added and the mixture heated for 30 min. The residue was washed with water and ethanol to leave the *carbamate* (5·1 g) as colourless needles (from 2-ethoxyethanol), m.p. 179–181°. Found: C, 50·6; H, 5·6; N, 13·7. $C_{13}H_{17}N_3O_6$ requires C, 50·2; H, 5·5; N, 13·5%.

Methyl o-*nitrobenzylcarbamate*. *o*-Nitrobenzylamine (0·7 g), methyl chloroformate (0·47 g), potassium carbonate (0·7 g) and ether (30 ml) were refluxed for 7 hr. Evaporation of the ethereal layer, after filtering off the inorganic material, gave the *carbamate* (0·76 g) as colourless needles (from ethanol), m.p. 84–85°. Found: C, 51·5; H, 4·75; N, 13·3. $C_9H_{10}N_2O_4$ requires C, 51·4; H, 4·8; N, 13·3%.

1,2,3,4-*Tetrahydro*-1-*hydroxy*-2-*oxoquinazoline* (IV; X–Y = CH_2–NH). Reduction of diethyl *o*-nitrobenzylidenebiscarbamate (2 hr) yielded, on concentration to low volume, the *quinazoline* (30%), m.p. 167–169° (from ethanol). Found: C, 58·7; H, 4·7; N, 16·3. $C_8H_8N_2O_2$ requires C, 58·5; H, 4·9; N, 17·1%. ν_{max} 3,350 w (free N–H), 3,050–3,300 w (bonded) O–H and/or NH), 1,665 s cm^{-1} (C=O). Reduction of methyl *o*-nitrobenzylcarbamate in the absence of dioxan, followed by extraction with ether, yielded the same quinazoline (m.p. and mixed m.p. 167–169°) (39%).

Ethyl o-*nitrobenzoylcarbamate*. *o*-Nitrobenzoyl chloride (70 g) was added over 15 min to ethyl carbamate (140 g) at 160° (oil-bath). The mixture was heated for a further 15 min at 160°, cooled, dissolved in ether, and the solution extracted with 5N sodium hydroxide. Acidification of the extract yielded the *carbamate* (15 g) as colourless needles (from aqueous acetic acid), m.p. 128–129°. Found: C, 50·55; H, 4·3; N, 11·9. $C_{10}H_{10}N_2O_5$ requires C, 50·4; H, 4·2; N, 11·8%.

1,2-*Dihydro*-1,4-*dihydroxy*-2-*oxoquinazoline*. Reduction of ethyl *o*-nitrobenzoylcarbamate in 10% sodium hydroxide solution (15 min) yielded, by filtration, the *quinazoline* (33%) as colourless needles (from acetic acid), m.p. 282° (decomp.). Found: C, 53·4; H, 3·2; N, 16·15. $C_8H_6N_2O_3$ requires C, 53·9; H, 3·4; N, 15·7%. ν_{max} 3,000–3,150 w (O–H), 1,690 s cm^{-1} (C=O).

1,2-*Dihydro*-1-*hydroxy*-2-*oxoquinazoline* (IV; X–Y = CH=N). Aqueous ferric chloride solution was passed through a strongly acidic cation-exchange resin until no more ferric ion was adsorbed. The column was washed thoroughly with water, and a solution of 1,2,3,4-tetrahydro-1-hydroxy-2-oxoquinazoline (0·8 g) in 2% sodium hydroxide solution (20 ml) passed through. The eluate was neutralised, evaporated to dryness and extracted into absolute ethanol. Evaporation of the extract yielded the *quinazoline* (0·15 g) as cream needles (from water), m.p. 212–213°. Found: C, 59·05; H, 3; N, 17·4. $C_8H_6N_2O_2$ requires C, 59·3; H, 3·7; N, 17·3%. ν_{max} 2,350–2,700 w (O–H), 1,665 s cm^{-1} (C=O).

1,2,3,4-*Tetrahydro*-1-*hydroxy*-2-*oxoquinoxaline* (IV; X–Y = NH–CH_2). Reduction of ethyl *N*-*o*-nitrophenylglycine (prepared in 80% yield from 1,2-dinitrobenzene by the method of Crowther, Curd, Davey & Stacey, 1949, for the 5-chloro analogue) in 5% sodium hydroxide solution yielded, by extraction with ether, the *quinoxaline* (66%) as colourless prisms (from ethanol), m.p. 156° (decomp.). Found: C, 58·35; H, 5·0; N, 17·0. $C_8H_8N_2O_2$ requires C, 58·5; H, 4·9; N, 17·1%. ν_{max} 3,200 m (N–H) 2,350–2,700 w (O–H), 1,685 s cm^{-1} (C=O).

1,2-*Dihydro*-1-*hydroxy*-2-*oxoquinoxaline* (IV; X–Y = N=CH). When the preceding reduction of ethyl *N*-*o*-nitrophenylglycine was carried out in the absence of nitrogen and the reaction mixture was stirred vigorously for a further 6 hr, atmospheric oxidation took place to yield, on acidification, the 1,2-*dihydroquinoxaline* (49%). Sublimation followed by crystallisation from ethanol gave cream needles, m.p. 210–211°. Found: C, 59·45; H, 3·9; N, 17·3. Calc for $C_8H_6N_2O_3$: C, 59·3; H, 3·7; N, 17·3%. ν_{max} 2,350–2,700 w (O–H), 1,645 s cm^{-1} (C=O). The action of acetic

anhydride on quinoxaline 1,4-dioxide gave the same hydroxamic acid, m.p., and mixed m.p. 210–211° in 18% yield. (Elina, 1962, states 23% yield, m.p. 208–209°).

1,2-*Dihydro*-1,3-*dihydroxy*-2-*oxoquinoxaline*. Reduction of *N*-ethoxalyl-*o*-nitroaniline (prepared by method of Reindel & Rosendahl, 1962, for 2-ethoxalylaminopyridine) (10 min) yielded the *quinoxaline* (15%), m.p. and mixed m.p. 292° (decomp.) with a sample prepared from 3-hydroxyquinoxaline 1-oxide by the method of Tennant, 1963. ν_{max} 2,500–3,200 w (O–H), 1,670 and 1,705 s cm^{-1} (C=O).

1,2,3,4-*Tetrahydro*-1-*hydroxy*-2-*oxo*-1,4,5-*triazanaphthalene* (VI; R=H, X–Y = NH–CH_2). Reduction of 2-ethoxycarbonylmethylamino-3-nitropyridine (Albert & Barlin, 1963) in 2% sodium hydroxide solution yielded directly the *triazanaphthalene* (46%) as colourless needles (from ethanol), m.p. 192° (decomp.). Found: C, 51·05; H, 4·45; N, 25·1. $C_7H_7N_3O_2$ requires C, 50·9; H, 4·2; N, 25·45%. ν_{max} 3,300 m (N–H), 2,300–2,700 w (O–H), 1,665 s cm^{-1} (C=O).

2-*Ethoxalylamino*-6-*methyl*-3-*nitropyridine*. 2-Amino-3-nitro-6-methylpyridine (11·25 g), pyridine (25 ml) and ethoxalyl chloride (10 g) were stirred together for 15 min. The mixture was poured into dilute hydrochloric acid and extracted with ether. The extract was evaporated to yield the *ester* (9·7 g) as orange needles (from ethanol), m.p. 106–107°. Found: C, 47·4; H, 4·3; N, 17·5. $C_{10}H_{11}N_3O_5$ requires C, 47·4; H, 4·35; N, 16·6%.

1,2-*Dihydro*-1,3-*dihydroxy*-6-*methyl*-2-*oxo*-1,4,5-*triazanaphthalene* (VI; R=Me, X–Y = N=C(OH)). Reduction of 2-ethoxalylamino-6-methyl-3-nitropyridine in sodium hydroxide solution (30 min) yielded, on concentration, the *triazanaphthalene* (38%) as colourless needles (from ethanol), m.p. 252–253° (decomp.). Found: C, 50·25; H, 3·7; N, 22·0. $C_8H_7N_3O_3$ requires C, 49·7; H, 3·6; N, 21·8%. ν_{max} 2,500–3,200 w (O–H), 1,680 s and 1,720 s cm^{-1} (C=O).

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Eserine and autonomic nervous control of guinea-pig vas deferens

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In the presence of eserine, the isolated preparation of guinea-pig vas deferens responds to electrical stimulation of the hypogastric nerve with enhanced contractions, which are no longer antagonised by adrenergic blocking agents but are strongly inhibited by atropine. Similarly, in preparations from reserpine-pretreated animals which have become unresponsive to the hypogastric stimulation, eserine elicits full-size responses which are almost completely abolished by atropine and not affected by adrenergic blocking drugs. Direct stimulation with acetylcholine contracts the vas deferens both of normal and reserpinised animals: the responses are antagonised by atropine and enhanced by eserine. It is concluded that there are grounds for inferring that eserine acts not by enhancing an adrenergic mechanism, but by uncovering a parasympathetic cholinergic component in the autonomic nervous control of the preparation.

IN agreement with previous observations (Boyd, Chang & Rand, 1960), Burn & Weetman (1963) advanced the hypothesis that the enhancing effect of eserine on the responses of the guinea-pig vas deferens to electrical stimulation of the hypogastric nerve is due to a reinforcement of a non-synaptic action of acetylcholine promoting a greater release of adrenergic transmitter.

In line with this assumption we made experiments to establish whether an analogous effect was exerted by eserine *in vivo* at the level of the peripheral sympathetic structures and which could thus account for its hypertensive action in the urethanised rat (Della Bella, Gandini & Preti, 1964). Whilst doing this work, some observations made on the isolated vas deferens aroused our interest and seemed worthwhile investigating more thoroughly so that the mechanism whereby eserine potentiates the responses of the preparation to electrical stimulation might be better understood, and additional information about the autonomic nervous control of the vas deferens could be collected.

Methods and materials

HYPOGASTRIC NERVE—VAS DEFERENS PREPARATION

Guinea-pigs weighing 400–500 g were used. The preparation was set up according to Huković (1961) in a 100 ml organ bath, containing Krebs solution gassed with 5% carbon dioxide and 95% oxygen, at 32°. The hypogastric nerve, about 3 cm, was placed on shielded platinum electrodes submerged in the bath, 2 cm from the vas deferens and connected to an electronic stimulator. Rectangular pulses, 200 of 1 msec duration, were applied at 2 min intervals, at the alternate frequency of 10 and 50 shocks/sec, from a constant voltage source at 2–3 V. Contractions were recorded by an isotonic writing lever (load 2 g) with a magnification of four times.

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For the experiments using direct chemical stimulation, the vas deferens was removed from the body without the hypogastric nerve, and the mesenteric investment was carefully stripped away so as to give a more sensitive preparation (Bentley & Sabine, 1963). In this work a 20-ml organ bath was used. Some preparations were made from reserpine-pretreated animals, which had received reserpine, 0·5 and 1 mg/kg intraperitoneally, for the 2 days previously as described by Hukovič (1961).

The following drugs were used: acetylcholine chloride, carbachol, acetyl-β-methylcholine chloride, noradrenaline, atropine sulphate, dihydroergotamine methansulphonate, dibenamine chloride, phenoxybenzamine chloride, phentolamine methansulphonate, veratrine, hexamethonium bromide, propantheline bromide, diphemanil methylsulphate. With the exception of noradrenaline and veratrine the concentrations in the text refer to the salts.

Results

INTERACTION BETWEEN ESERINE AND ATROPINE

In agreement with Boyd & others (1960) and Burn & Weetman (1963), we found that the addition of eserine to the bath at doses ranging from

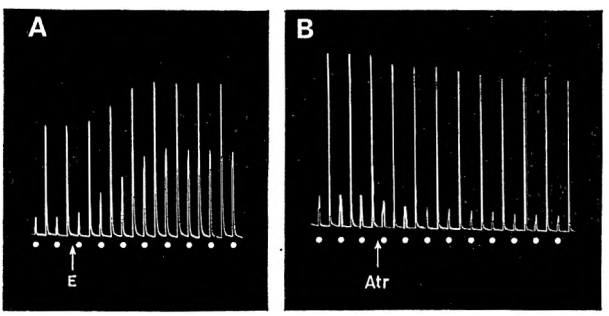

FIG. 1. Contractions of guinea-pig isolated vas deferens in response to hypogastric nerve electrical stimulation. Each stimulation consisted of 200 shocks, applied alternately at the frequency of 50 and 10 shocks/sec (at dots), every 2 min. A. Addition of eserine to the perfusion bath (at E, 2·5 μg/ml) evokes an immediate and progressive increase in the responses of the preparation, more evident at the low frequency stimulation. B. The responses appear scarcely affected by atropine (at Atr, 1 μg/ml): a slight reduction, more marked for the contractions at the low rate of stimulation, is observable.

0·5 to 5 μg/ml significantly enhanced the responses of the vas deferens to the electrical stimulation of the hypogastric nerve in 11 out of 14 preparations (Fig. 1A). Potentiation was most consistently seen with the 5 μg/ml dose and was more pronounced for responses to 10 than to 50 shocks/sec. Doses of eserine of 0·5–2 μg/ml elicited fewer potentiated responses. In contrast to the observations by Burn & Weetman (1963), only in two of the eleven experiments did we note a progressive decline of the contractions at high frequency.

The results obtained with atropine agreed with the previous findings: 0·05 to 2 μg/ml concentrations only slightly reduced the responses of the electrically-stimulated preparation, the inhibition concerning mainly the contractions at the low rate of stimulation (Fig. 1B).

When eserine was added to the bath in high doses, the presence of atropine slightly inhibited, but did not abolish, the typical potentiation

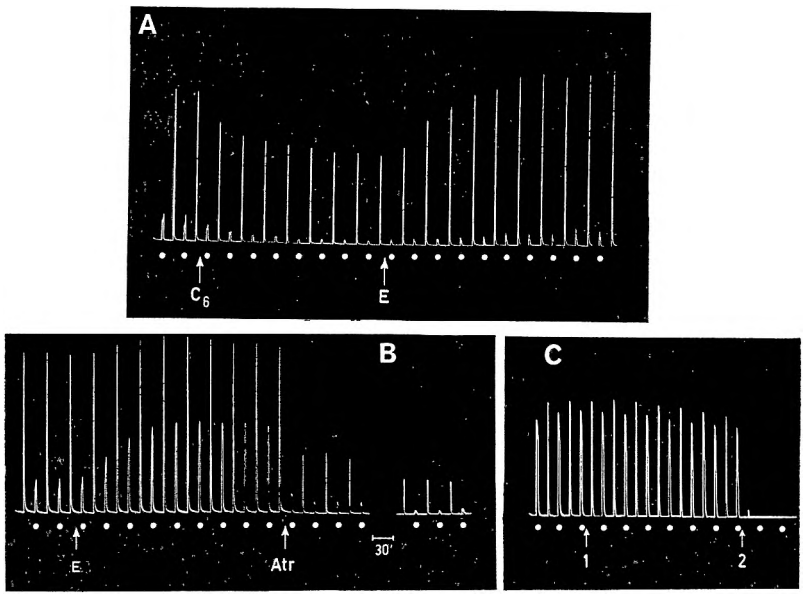

FIG. 2. Preparations and parameters as in Fig. 1. A. At C_6, hexamethonium, 10 μg/ml. At E, eserine, 3 μg/ml. Addition of eserine to a preparation partially blocked by hexamethonium does not exert the typical enhancing effect at the low frequency stimulation. The potentiation by eserine develops as usual for the responses at high frequency. B. At E, eserine, 5 μg/ml. At Atr, atropine, 0·25 μg/ml. Unlike the experiment in Fig. 1B, a small dose of atropine, given after the enhancing effect of eserine has developed, causes an immediate strong reduction of the responses. The inhibitory effect becomes progressively more intense, but even 30 min later the responses are not abolished. C. Eserine has been added to the bath at the concentration of 5 μg/ml 20 min previously. At 1, phentolamine, 0·5 μg/ml. At 2, veratrine, 1 μg/ml. Phentolamine leaves the height of contractions unimpaired while veratrine almost immediately abolishes the responses.

induced by eserine. On the contrary, doses as low as 0·5–2·5 μg/ml in the presence of atropine occasionally failed to exert any potentiating effect; at this dose level we obtained contrasting results from different experiments and enhancement, when present, varied from one preparation to another.

When the preparation was pretreated with hexamethonium, the eserine-potentiating effect appeared only at the higher frequency of stimulation (Fig. 2A).

When atropine was added to the bath after the enhancing effect of eserine had developed, different results were obtained: concentrations of

atropine as low as 0·05–0·5 μg/ml strongly reduced the potentiated responses of the preparation in all instances (Fig. 2B). Atropine mainly reduced the responses to the low-frequency stimulation. These were almost completely abolished; the high frequency responses declined to 10–25% of their previous height. Further addition of atropine, even up to 1–2 μg/ml did not abolish them.

Hexamethonium (10 μg/ml), given after the potentiating effect of eserine had developed, was responsible for a progressive reduction of the responses ranging between 20 and 50%; a partial block, more pronounced than that by atropine, was obtained with 0·2–0·5 μg/ml of diphemanil and propantheline, which are parasympatholytic drugs endowed with both atropine-like and ganglion blocking properties (Margolin, Doyle, Giblin, Makovsky, Spoerlein, Stephens, Berchtold, Belloff & Tislow, 1951; Johnson & Wood, 1954). Similarly, as already reported for normal preparations (Della Bella & Benelli, 1964), 1–2 μg/ml veratrine abolished the responses potentiated by eserine (Fig. 2C).

INTERACTION BETWEEN ESERINE AND ADRENERGIC BLOCKING DRUGS

The reported effects of pretreatment with adrenergic blocking drugs on the responses of the vas deferens to the electrical stimulation of the hypogastric nerve are not in agreement. Ohlin & Strömblad (1963) showed that the effect of the hypogastric stimulation is enhanced by dihydroergotamine and phenoxybenzamine; they excluded sensitisation due to the anticholinesterase activity of the drugs, as suggested by Boyd & others (1960), who described the inhibitory properties of other adrenergic blocking agents. Inhibitory effects by dihydroergotamine and phentolamine on the transmurally-stimulated preparation were clearly demonstrated by Birmingham & Wilson (1963). In our experience, dibenamine, phentolamine, phenoxybenzamine and dihydroergotamine, given at the concentrations of 0·05–0·1 μg/ml, reduced the responses of the preparation by 50–90%. The effect of higher doses was investigated only for dihydroergotamine and phenoxybenzamine: paradoxically, lower responses were sometimes obtained.

The addition of eserine, 2·5–5 μg/ml, to preparations blocked by an adrenergic blocking agent, evoked normally potentiated responses in seven of nine preparations. The responses were particularly evident at the lower rate of stimulation (Fig. 3A & B). Further addition of the adrenergic blocking agent at this point left the height of contractions unaffected (Fig. 3A): occasionally some enhancement was observed.

Analogous results were obtained when eserinised preparations were treated with adrenergic blocking agents: no modifications of the eserine-enhanced contractions were observed (Fig. 2C).

EFFECTS OF ESERINE ON VAS DEFERENS PREPARATIONS FROM RESERPINE-PRETREATED GUINEA-PIGS

Our findings on preparations from reserpine-pretreated animals were consistent with those of Huković (1961): after a few normal responses to electrical stimulation, the responsiveness of the preparation became

progressively less, without becoming completely abolished; 15–30 min after beginning stimulation, the height of contractions varied from 8 to 19% of the initial responses. In line with previous suggestions by Huković (1961), the phenomenon has been ascribed by Birmingham & Wilson (1963) to a progressive reduction of noradrenaline tissue stores.

Addition of eserine after the preparation had become almost unresponsive to the electrical stimulation, evoked immediately increasing

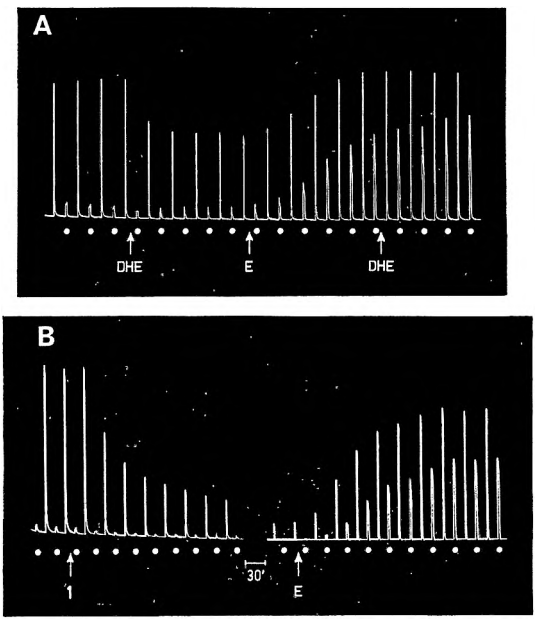

FIG. 3. Preparations and parameters as for Fig. 1. A. At DHE, dihydroergotamine, 0·5 μg/ml initially, then 10 μg/ml. At E, eserine, 2·5 μg/ml. Addition of eserine to a preparation partially blocked by dihydroergotamine evokes normally potentiated responses. A further higher dose of dihydroergotamine is completely ineffective on the eserine-potentiated contractions. B. At 1, dibenamine, 0·25 μg/ml. At E, eserine, 5 μg/ml. Addition of eserine to a preparation under prolonged block by dibenamine, evokes the typical enhanced responses.

contractions at both rates of stimulation (Fig. 4A & B): the enhancing effect of the drug developed progressively and lasted throughout the experiment, in the same manner observed in normal preparations.

Treatment with an adrenergic blocking drug did not impair the eserine enhancement but this was diminished by hexamethonium and abolished promptly by atropine (Fig. 4A & B).

PHARMACOLOGICAL ANALYSIS OF THE RESPONSES OF THE VAS DEFERENS TO NORADRENALINE AND ACETYLCHOLINE

We had observed previously that the responsiveness of the vas deferens to the chemical stimulation is greatly increased when the organ is carefully

isolated from the mesenteric investment without the hypogastric nerve. Similar observations were made by Bentley & Sabine (1963).

With this preparation, direct chemical stimulation was applied at 3–5 min intervals using noradrenaline and acetylcholine at concentrations ranging from 0·1–0·5 μg/ml. In all instances the preparation responded immediately with regular contractions, which reached a peak in a few sec and then rapidly declined.

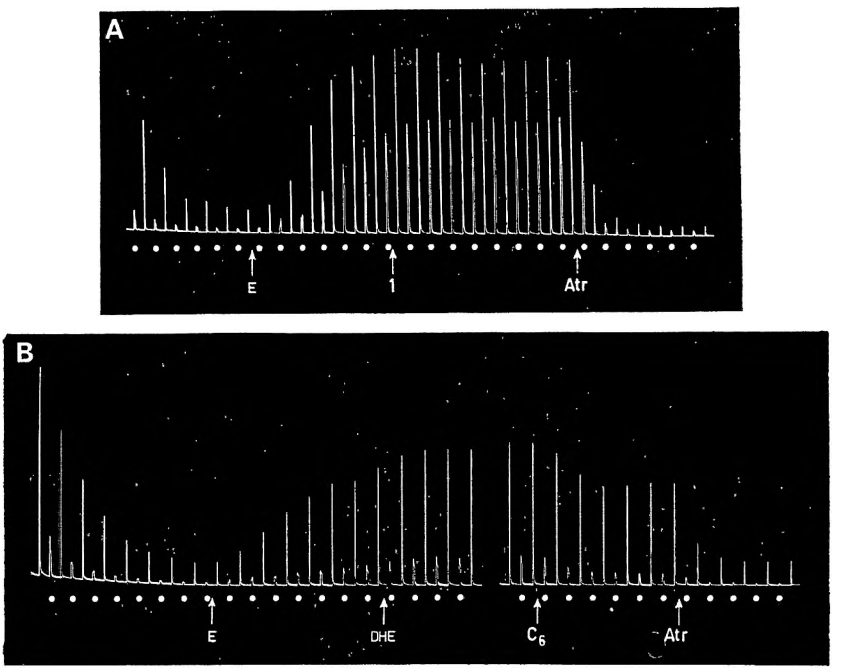

FIG. 4. Preparations and parameters as for Fig. 1. Preparation made from a reserpine-pretreated animal. A. At E, eserine, 2·5 μg/ml. At 1, phenoxybenzamine, 0·25 μg/ml. At Atr, atropine, 0·01 μg/ml. Note the initial spontaneous progressive reduction of the responses to the electrical stimulation. Addition of eserine then elicits enhanced responses which are unaffected by phenoxybenzamine and, on the contrary, are almost completely abolished by atropine. B. At E, eserine, 2·5 μg/ml. At DHE, dihydroergotamine, 1 μg/ml. The addition of eserine when maximal spontaneous reduction of the responses to the electrical stimulation has occurred, evokes strongly potentiated responses. No modification appears upon addition of dihydroergotamine. Note also the inhibitory effect of hexamethonium (at C_6, 5 μg/ml) and subsequently that of atropine (at Atr, 0·01 μg/ml).

Responses to noradrenaline. Noradrenaline-evoked contractions appeared to be abolished completely by the adrenergic blocking drugs tested. Fig. 5A illustrates the antagonistic effect of dihydroergotamine at the dose of 0·2 μg/ml. No modification was observed with atropine, eserine, hexamethonium and bretylium. Veratrine and occasionally bretylium caused some enhancement.

Responses to acetylcholine. Contractions in response to acetylcholine were effectively antagonised by atropine at 0·01 μg/ml: the inhibitory

effect was immediate and was slowly reversible (Fig. 5B). Some enhancement, which progressively disappeared over 3–4 responses, occurred upon addition of 0·25 μg/ml of eserine (Fig. 5C).

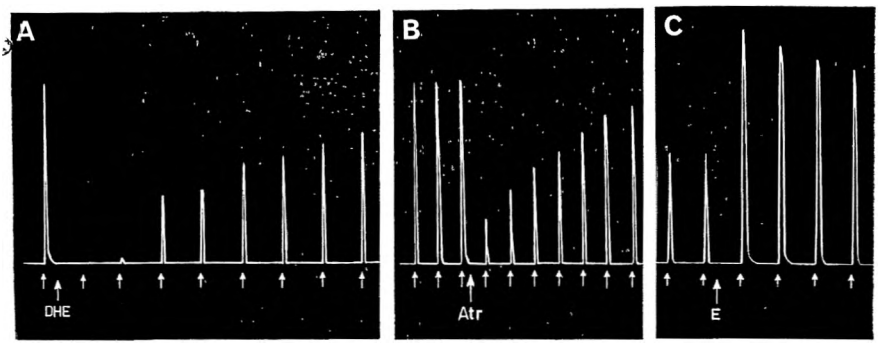

FIG. 5. Isolated, stripped vas deferens preparation. Recording of contractions in response to (A) noradrenaline, (B, C) acetylcholine (1 μg/ml at arrows). Time of contact, 45 sec. Interval between stimulations, 5 min. Note the pronounced and long-lasting inhibitory effect of dihydroergotamine (at DHE, 0·2 μg/ml) and the marked and slowly reversible inhibitory effect of atropine (at Atr, 0·01 μg/ml). Addition of eserine (at E, 0·25 μg/ml) is responsible for a strong and long-lasting potentiation of the responses.

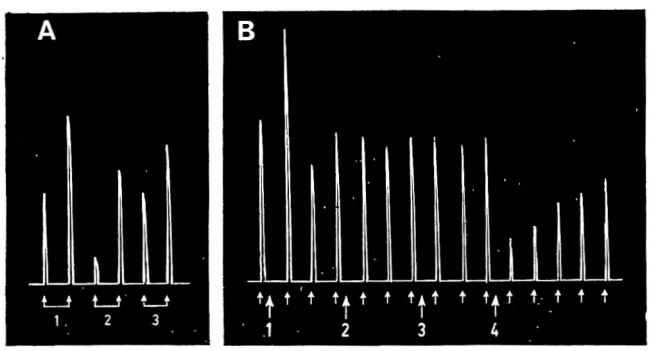

FIG. 6. Isolated, stripped vas deferens preparation. A. Recording of contractions in response to direct stimulation with two successive doses (0·05 and 0·1 μg/ml) of acetyl-β-methylcholine (at 1), carbachol (at 2) and acetylcholine (at 3). Time of contact for each drug, 45 sec. Then, thorough washing out of the preparation. Interval between stimulations, 5 min. B. Recording of contractions in response to acetylcholine (1 μg/ml at arrows). Time of contact, 45 sec. Interval between stimulations, 5 min. At 1, dihydroergotamine, at 2, phentolamine, at 3, dibenamine, at 4, phenoxybenzamine, all 2·5 μg/ml. Note the different influence of the same dose of four adrenergic blocking agents: phentolamine and dibenamine leave the responses of the preparation unimpaired, dihydroergotamine induces a clear but immediately reversible potentiation, phenoxybenzamine is responsible for a strong and prolonged reduction of the responses, similar to that induced by atropine.

Acetyl-β-methylcholine and carbachol were also tested on the preparation, which proved more responsive to acetyl-β-methylcholine than to carbachol (Fig. 6A), probably because of the stronger muscarinic properties of the former drug (Goodman & Gilman, 1955).

Amongst the adrenergic blocking agents tested, phentolamine and dibenamine proved practically ineffective towards the responses to acetylcholine; dihydroergotamine caused some potentiation, while phenoxybenzamine exhibited antagonistic properties at 2-4 μg/ml (Fig. 6B).

Hexamethonium, bretylium and guanethidine did not affect the responses of the preparation, occasionally, some potentiation was observed with veratrine.

Experiments on chemically-stimulated preparations of vas deferens from reserpinised animals, gave analogous results to those above.

Discussion

Our results may be summarised as follows:

(i) In contrast to the behaviour of the hypogastric nerve—vas deferens preparation towards atropine and adrenergic blocking agents, the responses after eserine treatment are strongly reduced by atropine and are not modified by adrenergic blocking agents.

(ii) Addition of eserine to a preparation in which the responses have been reduced by an adrenergic blocking agent elicits enhanced responses which are counteracted by atropine but not by further adrenergic blocking drug. Occasionally some enhancement is observed.

(iii) Addition of eserine to a reserpinised preparation, whose responses to electrical stimulation are significantly reduced because of the depletion of adrenergic mediator stores, evokes full-size responses which are unaffected by adrenergic blocking drugs but are abolished by atropine, as also observed by Schümann & Grobecker (1963).

(iv) The responses of the eserinised vas deferens to electrical stimulation are inhibited, although to different extents, by hexamethonium, veratrine, diphemanil and propantheline, which all share the property of affecting ganglionic transmission. The greater antagonistic activity exhibited by the two latter drugs may be accounted for by their also having atropine-like properties.

(v) The direct responses of the vas deferens to noradrenaline are not modified by atropine and eserine but are antagonised by the adrenergic blocking drugs tested.

(vi) The direct responses to acetylcholine of preparations of the vas deferens from normal or reserpinised animals are antagonised by atropine and enhanced by eserine. The finding that, under these experimental conditions and at the doses tested, no antagonism is present either with hexamethonium or veratrine seems to provide evidence for a direct action of acetylcholine on a muscarinic receptor. The greater responsiveness of the preparation to acetyl-β-methylcholine than to carbachol would also be consistent with this view. However, the possibility cannot be ruled out that under other experimental conditions and at higher doses, such as those adopted by Schümann & Grobecker (1963), acetylcholine

might elicit adrenergic responses mediated through either the ganglion or the chromaffin cells.

The analysis of the results provides grounds for thinking that the responses of the vas deferens to electrical stimulation, which are adrenergic under normal conditions, assume after eserinisation cholinergic parasympathetic-like features. Eserine therefore seems able to modify the responses of the preparation not only quantitatively, by promoting a greater release of adrenergic transmitter as postulated by Burn & Weetman (1963), but also qualitatively. Experiences on the reserpinised preparation strongly support this; less reliable, although consistent with our view, are data obtained with the adrenergic blocking agents, which proved far more effective against added noradrenaline than against sympathetic stimulation.

On the basis of the data obtained, we wonder whether a parasympathetic cholinergic mechanism may be playing a role in the nervous control of the vas deferens; if this is so, the problem arises as to why in the eserinised preparation atropine causes not only the disappearance of the potentiation, but also a marked reduction of the responses.

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The influence of diet on the toxicity of acetylsalicylic acid

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The toxicity of acetylsalicylic acid in rats is greater when animals are fed a high carbohydrate diet than when they receive a high protein diet. Magnesium deficiency increases this difference, particularly in pregnant animals. The maximum B.P. human dose of acetylsalicylic acid is well tolerated by pregnant rats fed on the high protein diet but one quarter of this amount produced foetal resorption and deaths in rats fed on the high carbohydrate diet. Acetylsalicylic acid is not teratogenic under the conditions used and appears to be unlikely to be teratogenic in man since its dose-toxicity curve is steep. It appears essential to report the composition of the diet in toxicity tests.

THIS paper describes the toxicity of acetylsalicylic acid in rats fed on diets of different protein levels with and without added magnesium. Magnesium was chosen as the trace metal to be omitted from the diet since the urinary free histamine levels in rats have recently been shown to be markedly elevated both in magnesium deficiency (Bois, Gascon & Beaulnes, 1963) and in pregnancy (West, 1960). Besides, magnesium influences the ability of other agents to produce coronary lesions (Olsen & Parker, 1964) and is essential for the maintenance and growth of the soft tissues (Martindale & Heaton, 1964). Both non-pregnant and pregnant animals have been used in the present work as it was considered possible that the stress of pregnancy might magnify some of the alterations in growth produced by various conditions. A preliminary note on some of the results has already been published (Brown & West, 1964). Other authors (for example, Obbink & Dalderup, 1964) have recently studied the effect of acetylsalicylic acid on foetal animals using one standard diet.

Experimental

Hooded Lister rats (150–200 g) of either sex were reared on standard diet (London Flour Millers No. 41B). Males were left with females for 3 days after which the sexes were separated and fed on test diets. These were either a high carbohydrate diet (sucrose 65%, casein 24%) or a high protein diet (casein 89%), with corn oil (5%) and the vitamin and salt mixture as used by Colby & Frye (1951). Drinking water was allowed *ad lib.* For the deficiency experiments, magnesium sulphate (600 mg/100 g diet) was omitted from the salt mixture in each diet, whilst for the drug experiments acetylsalicylic acid powder was mixed with the diet beforehand. Each rat consumed 14–16 g food per day when the diet was made up into a thick paste with water. With the mating regimen adopted, 70–80% of the females were successfully mated. Most of the pregnant animals were killed on the 20th day of gestation, and the number of live and dead foetuses, in addition to the resorption sites, were counted. Foetal mortality, represented by the proportion of dead foetuses and

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resorption sites to the total number of implantations, was then calculated for each dose of acetylsalicylic acid. A few pregnant animals were allowed to litter and to suckle their young until weaning time.

Results

Effect of diet on weight. The weight of both males and non-pregnant females continued to increase on both diets over a period of 90 days though it was always slower in the groups of animals on the high protein diet. The increase in weight of pregnant animals during the gestation period also was always less on the high protein diet (mean gain 16·6 g) than on the high carbohydrate diet (mean gain 45·3 g), and foetuses always weighed less at the 20th day of gestation. A similar result was found when rats were allowed to litter and suckle their young, the animals at weaning time being about 5 g less on the high protein diet than on the high carbohydrate diet (average weight 39 g).

Effect of diet on gastric ulceration produced by acetylsalicylic acid. When males and non-pregnant females were fed for 20 days on one of the two diets containing acetylsalicylic acid (500 mg/kg daily), the incidence of gastric ulceration was significantly greater ($P < 0{\cdot}01$) in those rats on the high carbohydrate diet (mean 45%) than on the high protein diet (mean 22%). In pregnant animals a similar result was obtained though the degree of ulceration was more extensive; in addition, no live foetuses were found in animals on either diet, although animals on the high protein diet continued to increase in weight during the gestation period (mean gain 10·0 g) and contained more foetuses (32 out of 96, or 33%), whereas those of the high carbohydrate diet steadily lost weight (mean loss 10·0 g) and contained less foetuses (6 out of 120, or 5%).

Effect of magnesium deficiency. When magnesium was omitted from the salt mixture in both diets, non-pregnant rats after a few days excreted large quantities of free histamine in the urine (estimated on the isolated guinea-pig ileum). At the same time, erythema of the ears developed and was particularly noticeable when the histamine excretion became maximal (after about 10 days). Continued feeding of the deficient diets resulted in excessive histamine excretion for about the next 10 days, after which the values returned to control levels. These results agree with those reported by Bois & others (1963). At this stage, many of the tissue mast cells in the subcutaneous connective tissue were grossly degranulated but not disrupted (stained with nuclear fast red), yet the dextran anaphylactoid reaction was still obtainable. When magnesium was restored to the diets for 15 days or more and then omitted for the next 30 days, the histamine excretion again increased, this process being repeatable many times (see Fig. 1). Replacement of the magnesium possibly enabled the store of histamine to be replenished and this was then released when magnesium was again withdrawn from the diets. It is now well known that magnesium is essential for the uptake of catecholamines and 5-hydroxytryptamine by adrenal medullary granules, and the same may be true for the uptake of histamine by mast cell granules.

Whereas the effects of magnesium deficiency were similar in non-pregnant rats fed on either diet, there was a marked difference in pregnant animals. Magnesium lack was so detrimental in animals on the high carbohydrate diet that there was no gain in weight during the gestation period (mean loss 3·4 g), and a large number of resorption sites and dead foetuses (116 out of 148, or 79%) were found when these rats were killed. On the high protein diet, however, magnesium deficiency did not markedly alter the gain in weight during pregnancy (mean gain 12·2 g) although at the 20th day of gestation the foetal mortality rate was 46% (see Tables 1 and 2). It is clear that magnesium deficiency in pregnant rats fed a high carbohydrate diet is more detrimental than in those on a high protein diet.

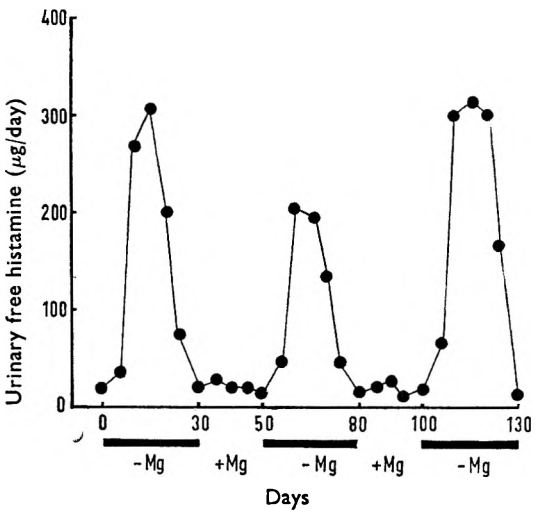

FIG. 1. Excretion of urinary free histamine (μg/day) by a non-pregnant rat when fed for 130 days the high carbohydrate diet in the presence and absence of magnesium. Note that the increase in histamine excretion occurs very soon after the deficient diet is fed but it is not maintained and does not re-occur until after magnesium is restored to the diet.

TABLE 1. TOXICITY OF ACETYLSALICYLIC ACID IN PREGNANT RATS FED A HIGH CARBOHYDRATE DIET WITH OR WITHOUT MAGNESIUM

Magnesium in diet	Acetylsalicylic acid in diet (mg/kg)	No. of rats	Mean weight gain (g) Day 6–20	No. of implants		No. of foetuses			No. of rescrption sites	Mortality (%)
				Total	Per litter	Total	Live	Dead		
Present ..	0	11	+45·3	95	8·6	94	94	0	1	1
	12·5	4	+44·9	43	10·8	39	35	4	4	19
	25	6	+30·7	83	13·8	71	49	22	12	41
	50	6	+41·9	74	12·3	64	49	15	10	34
	250	4	+31·0	46	11·5	23	10	13	23	80
	500	10	−10·0	120	12·0	6	0	6	114	100
Absent ..	0	14	− 3·4	148	10·6	48	32	16	100	79
	12·5	3	+ 3·0	32	10·7	14	4	10	18	38
	50	7	+10·6	72	10·3	57	1	56	15	99
	250	6	+ 0·7	60	10·0	5	2	3	55	96
	500**	11	−15·5	128	11·6	21	0	21	107	100

** At this dose, 4 other pregnant rats died after 12 days.

TABLE 2. TOXICITY OF ACETYLSALICYLIC ACID IN PREGNANT RATS FED A HIGH PROTEIN DIET WITH AND WITHOUT MAGNESIUM

Magnesium in diet	Acetylsalicylic acid in diet (mg/kg)	No. of rats	Mean weight gain (g) Day 6–20	No. of implants		No. of foetuses			No. of resorption sites	Mortality (%)
				Total	Per litter	Total	Live	Dead		
Present ..	0	10	+16·6	102	10·2	100	100	0	2	2
	50	9	+18·0	129	14·3	120	120	0	9	7
	250	8	+20·2	84	10·5	64	43	21	20	49
	500	9	+10·0	96	10·7	32	0	32	64	100
Absent ..	0	11	+12·2	99	9·0	83	52	31	16	46
	50	8	+10·6	93	11·7	78	58	20	15	38
	250	8	− 2·6	89	11·1	52	11	41	32	82
	500	7	− 7·1	60	8·6	10	0	10	50	100

Effect of diet and magnesium deficiency. The foetal toxicity of acetylsalicylic acid in pregnant rats on the two diets is shown in Tables 1 and 2. Although the weight gain was maintained when rats on the high carbohydrate diet received doses of acetylsalicylic acid up to 250 mg/kg, the foetal mortality rate steadily increased up to 80%. On the high protein diet, however, the toxic effect was less, 250 mg/kg yielding a 49% foetal mortality and 50 mg/kg showing no adverse effects. Thus the foetal toxicity of acetylsalicylic acid in animals on the high carbohydrate diet was much greater than in animals on the high protein diet (see Fig. 2).

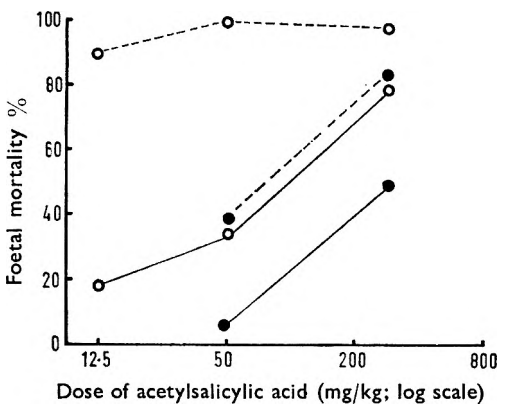

FIG. 2. Toxicity of acetylsalicylic acid in pregnant rats fed a high protein diet (●—●) or a high carbohydrate diet (○—○). The effects of magnesium deficiency are shown by the broken lines. Note that magnesium deficiency and the high carbohydrate diet markedly increase the toxicity.

When magnesium was omitted from the diet, the foetal toxicity of acetylsalicylic acid in pregnant rats again increased. Thus, a dose of 50 mg/kg killed 99% of foetuses when the high carbohydrate diet was used, and 250 mg/kg on the high protein diet produced over 80% mortality. It is interesting to note from Table 2 that a dose of 50 mg/kg acetylsalicylic acid did not increase the toxic effects of magnesium deficiency in pregnant

rats fed the high protein diet. Four out of 15 pregnant rats receiving the highest dose of acetylsalicylic acid mixed with the high carbohydrate magnesium-deficient diet died after about 12 days on this diet (see Table 1).

Discussion

This work was initiated during the testing of drugs for those most likely to exhibit teratogenic activity in man, but it was soon evident that dietary factors play important roles in the toxicity of drugs and this is seen also in non-pregnant animals. For example, the incidence of gastric ulceration, as a result of feeding acetylsalicylic acid to non-pregnant rats, was found to be much greater on the high carbohydrate diet than on the high protein diet. The result was similar in pregnant animals though here, in addition, the toxicity of acetylsalicylic acid to the foetus was increased. The highest dose of acetylsalicylic acid used in the present experiments (500 mg/kg or about 10 times the maximal B.P. human dose) produced death of all the foetuses though it was of interest that pregnant animals continued to increase in weight during gestation when fed the high protein diet but lost weight on the high carbohydrate diet.

When the daily dose of acetylsalicylic acid was reduced to 50 mg/kg (equivalent to 3 × 5 grain tablets four times a day in man), a relatively large number of dead foetuses and resorption sites were found in those animals fed the high carbohydrate diet. On the other hand, the foetal mortality rate in animals given this dose on the high protein diet remained well within the range of values found in control animals (maximum 10% in 82 rats studied). Only when the dose in the protein diet was increased five times did the toxic action of acetylsalicylic acid become prominent. It should be noted that the diet for optimal growth of the young rat has always been accepted as about 14% protein and 75% carbohydrate.

When the effects of magnesium deficiency were assessed, gastric ulceration occurred in nearly all the non-pregnant rats fed acetylsalicylic acid in the high carbohydrate diet but only in half of those on the high protein diet. In pregnant animals, even without acetylsalicylic acid, magnesium deficiency resulted in a foetal mortality on the high carbohydrate diet which was about twice that on the high protein diet, and when the acid was included, the mortality rates were correspondingly increased. Thus, magnesium plays an important role in the developing rat embryo; the full diet contained about 600 ppm magnesium whereas the deficient diet had only about one-tenth this amount. Magnesium deficiency has been extensively studied in young rapidly-growing animals by other workers who have reported skin lesions, hyperexcitability and convulsions, but in the present experiments these effects were not found. It is possible that the magnesium content of the deficient diet was higher than that in diets used by other workers or that the experiments were not carried on for a long enough period. Other factors such as the dietary cholesterol (Olson & Parker, 1964) may be involved. The importance of using foods containing adequate amounts of absorbable magnesium during pregnancy is emphasised.

This preliminary study thus shows that acetylsalicylic acid, when incorporated in a diet high in carbohydrate, is substantially more toxic to foetal rats than when it is given in a diet high in protein. The foetal toxicity also increases when magnesium is omitted from both diets. The toxicity of acetylsalicylic acid in the first week of pregnancy in rats is now being tested. Animal tests cannot prove a drug to be non-teratogenic to man, but it is useful to note that the steep slope of the dose-response curves of acetylsalicylic acid is similar to that of reserpine and unlike that of thalidomide (West, 1963). Consequently, acetylsalicylic acid appears to be less likely to produce congenital malformations in the young (and it did not in the present experiments), since a 10-fold increase in dose produces a large increase in toxicity. The present work, however, indicates that one of the important factors which must be taken into account in assessing the toxicity of a drug is the diet.

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Effects of anaphylaxis *in vivo* on the lipid and protein content of guinea-pig serum and extracellular fluid of lung tissue

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Determinations of total cholesterol, phospholipid and glyceride in serum samples obtained from guinea-pigs by cardiac puncture before and after anaphylaxis *in vivo* indicated reductions occurred in all three fractions after anaphylactic shock. The fall in glyceride was preceded by a rise. Anaphylaxis also induced an accumulation of lipid in oedema fluid in the lungs. These changes were reduced in animals protected from anaphylaxis by pretreatment with a combination of mepyramine and ethanolamine or mepyramine and hydrocortisone.

CHANGES in the lipid content of isolated sensitised guinea-pig lungs after anaphylaxis *in vitro* have been previously reported (Smith, 1962). Ethanolamine, which posssesses anti-anaphylactic activity (Smith, 1961) prevented some but not all of these changes. Similar changes in the lung lipids were noted *in vivo* by Goadby & Smith (1962) who showed that the exposure of sensitised guinea-pigs to an aerosol of antigen solution caused alterations in the lipid metabolism (see Smith, 1964). The major change was a substantial loss of phospholipid from the lungs. This could be prevented by pretreatment of the animals with hydrocortisone sodium hemisuccinate. A loss of phospholipid from isolated perfused guinea-pig lungs, after injection of histamine-releasing agents, has also been reported (Marquis & Smith, 1963). We have examined guinea-pig serum and the extracellular fluid in the lungs for changes which might indicate the route by which lipid is lost from guinea-pig lungs after anaphylaxis *in vivo*.

Experimental

EFFECTS OF ANAPHYLAXIS

Guinea-pigs of 200–350 g were fed on Diet 18 (Oxo), and received 50 mg of ascorbic acid each day in their drinking water. There were sensitised to egg albumin (BDH) by the intraperitoneal injection of 100 mg as a 5% solution in water. Three to five weeks after sensitisation, 3 ml of blood was removed by cardiac puncture from each of a group of 4 animals, and 5 min later each animal was exposed to an aerosol of 1% egg albumin until it experienced severe anaphylaxis (Herxheimer, 1952). A further 3 ml of blood was removed, and at a selected time interval after the shock the animal was killed by dislocation of the neck, and the heart and lungs excised and perfused through the pulmonary artery with aerated Tyrode solution at 37° to remove the blood (Brocklehurst, 1960). Perfusion was then stopped and the lungs left inflating for 30 sec to allow as much residual Tyrode's solution as possible to drip out of the pulmonary system. The lung lobes were then chopped into small pieces, centrifuged

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for 3 min at 2,000 rpm and the extracellular fluid, which collects below the lung tissue, aspirated. The lung tissue was centrifuged for another 2 min and any further fluid collected was added to that previously aspirated. The fluid from four sets of lungs was pooled and centrifuged at 2,500 rpm for 15 min to remove any tissue debris.

The blood samples were allowed to clot for 1 hr, after which the clot was broken, the samples centrifuged for 15 min at 2,500 rpm and the serum removed. These procedures were repeated with a group of four sensitised control animals which were not exposed to antigen. The same time interval, with an additional 2 min to compensate for the aerosolisation time of the shocked group, was allowed between blood sampling. The control lungs were similarly chopped, centrifuged, and the fluid removed. Using four control and four shocked groups, each of four animals, in this way, the cholesterol, phospholipid, glyceride and protein composition of serum and extracellular fluid was estimated immediately, 15, 30 and 60 min after anaphylactic shock.

EFFECTS OF PRETREATMENT WITH ETHANOLAMINE AND HYDROCORTISONE

A further four groups (I-IV) of four animals were exposed to 1% egg albumin and their collapse times determined (Smith, 1961). One week later the animals in Group I were given 2 mg/kg ethanolamine (as the hydrochloride) and 1 mg/kg mepyramine (as the maleate) by intramuscular injection. After 55 min, 3 ml of blood was removed by cardiac puncture from each animal and 5 min later the animal exposed to aerosolised antigen until either it had reached a state of collapse or had been exposed to antigen for a period of twenty times its previous collapse time. Fifteen min after the collapse point a further 3 ml of blood was removed, the animal was killed, the lungs removed and the extracellular fluid collected as described above. The same course was followed using Group II as treatment controls, except that this group was not exposed to antigen. The same time intervals between removal of blood samples were observed, substituting a period equal to the mean collapse time of Group I for the aerosolisation time. Hydrocortisone sodium hemisuccinate (50 mg/kg) was given to Group III and Group IV by intramuscular injection followed 17 hr later by 1 mg/kg mepyramine. The first blood sample was taken 55 min after the antihistamine. From this point the same procedure used for Group I was followed with Group III, while Group IV was used as hydrocortisone control in the same way that Group II was used as ethanolamine control. The amounts of cholesterol, phospholipid, glyceride and protein in the serum and extracellular fluid samples were then estimated.

BIOCHEMICAL ESTIMATIONS

Phospholipid in the serum and extracellular fluid was extracted with chloroform : methanol solution (Dawson, 1960) and estimated as inorganic phosphorus (Bartlett, 1959). The cholesterol and glyceride fractions were extracted by the procedure of Mendelsohn & Antonis (1960) and estimated by the methods of Hanel & Dam (1955) and Van

Handel & Zilversmit (1957) respectively. The protein composition was estimated by cellulose acetate membrane filter electrophoresis using the stain Ponceau S according to Kohn (1960).

Reagents. Hydrogen peroxide, a 30% solution, phosphorus free was kindly donated by Laporte Chemicals, Luton. Other reagents and solvents were analar grade, except zinc chloride which was reagent grade.

Results

LIPIDS IN SERUM

Although all the guinea-pigs used in the present study were of the same age and were maintained on the same diet, wide differences in the normal values for serum lipids were noted. The figures for cholesterol ranged from 25·7 to 188 mg % over 48 samples of control serum. The phospholipid range was 39·5 to 113·5 mg %, and that for glyceride was 27·4 to 111·0 mg %. Consequently, changes induced by anaphylaxis were

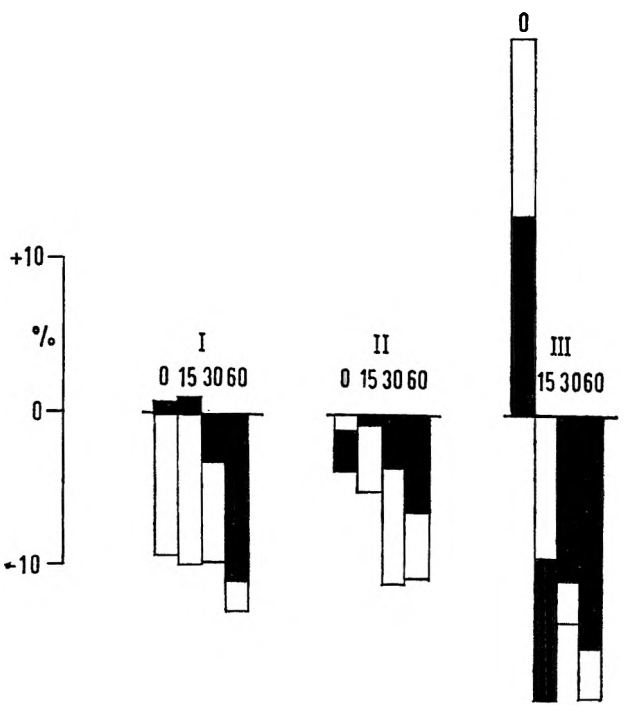

FIG. 1. The changes (%) in serum levels of cholesterol (I), phospholipid (II), and glyceride (III) in guinea-pigs immediately, 15, 30 and 60 min after anaphylactic shock (open columns) compared with those of control animals (solid columns). All columns originate from the base-line.

computed as percentage changes from the values determined for the control samples from each animal. The changes in serum lipid levels of animals exposed to aerosolised antigen are shown in Fig. 1, together with

the changes calculated from the two blood samples removed from the corresponding control groups of animals. Small but unequivocal decrements occurred in all three lipid fractions after anaphylaxis. These were similar but greater in magnitude than the changes occurring in control animals. Tests for statistical significance indicated that changes greater than 10% were statistically significant at $P = 0{\cdot}95$, except for the glyceride fraction where changes of around 20% were required for statistical significance at the same probability level.

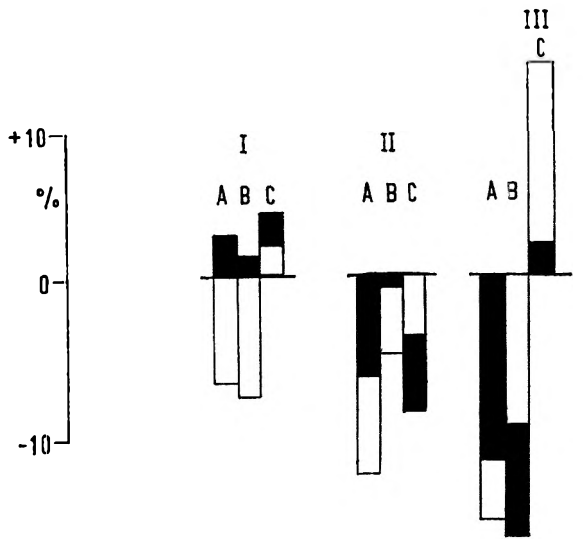

FIG. 2. The changes (%) in serum levels of cholesterol (I), phospholipid (II), and glyceride (III) in guinea-pigs after anaphylactic shock (B) compared with those in animals protected from anaphylaxis by pretreatment with mepyramine and ethanolamine (A) or mepyramine and hydrocortisone (C). The control responses in solid columns. All columns originate from the base-line.

The effect of pretreatment with ethanolamine plus mepyramine and hydrocortisone plus mepyramine upon the changes in serum lipid levels induced by anaphylactic shock can be noted from Fig. 2. The ethanolamine-treated animals still showed small decrements in cholesterol and glyceride similar to those in untreated animals with a somewhat greater loss of phospholipid. A loss of phospholipid still occurred after pretreatment with hydrocortisone but the cholesterol and glyceride fractions were slightly elevated.

LIPIDS IN EXTRACELLULAR FLUID OF THE LUNGS

In Table 1 the lipid present in extracellular lung fluid from animals exposed to aerosolised antigen and the corresponding control animals is recorded. The lipid content and volume of fluid collected were maximal 15 min after anaphylaxis, and fell to their lowest value 1 hr after shock. The lipid contents of the samples from the control groups were fairly

constant except for an increase in the glyceride content in the 15 min sample. From Table 2 it can be seen that protection from anaphylaxis using ethanolamine or hydrocortisone in combination with mepyramine reduced the volume of extracellular fluid and also its lipid content.

TABLE 1. THE VOLUME AND CHOLESTEROL, PHOSPHOLIPID, AND GLYCERIDE CONTENTS OF THE EXTRACELLULAR FLUID IN THE LUNGS OF GUINEA-PIGS AT VARIOUS TIMES AFTER ANAPHYLACTIC SHOCK COMPARED WITH THOSE OF CONTROL ANIMALS

Time min	Vol ml	Cholesterol		Phospholipid		Glyceride	
		mg/ml	Total	mg/ml	Total	mg/ml	Total
		Shock group					
0	1·5	0·28	0·42	3·02	4·53	0·47	0·70
15	2·2	0·29	0·62	3·23	6·95	0·32	0·69
30	1·3	0·38	0·59	3·54	4·61	0·42	0·55
60	1·0	0·15	0·15	2·63	2·63	0·20	0·20
		Control group					
0	0·5	0·24	0·12	4·50	2·25	0·24	0·12
15	0·9	0·24	0·22	2·65	2·39	0·50	0·45
30	0·7	0·26	0·18	3·61	2·53	0·34	0·24
60	0·6	0·39	0·23	6·50	3·90	0·53	0·32

TABLE 2. THE CHOLESTEROL, PHOSPHOLIPID, AND GLYCERIDE CONTENTS OF THE EXTRACELLULAR FLUID IN THE LUNGS OF ANIMALS AFTER ANAPHYLACTIC SHOCK COMPARED WITH THOSE OF ANIMALS PROTECTED FROM ANAPHYLAXIS BY TREATMENT BEFOREHAND WITH MEPYRAMINE AND ETHANOLAMINE OR WITH MEPYRAMINE AND HYDROCORTISONE

Group	Vol ml	Cholesterol		Phospholipid		Glyceride	
		mg/ml	Total	mg/ml	Total	mg/ml	Total
Mepyramine and ethanolamine							
Shock ..	1·45	0·19	0·28	2·78	4·00	0·18	0·25
Treated ..	0·95	0·23	0·22	3·60	3·42	0·23	0·22
Mepyramine and hydrocortisone							
Shock ..	1·70	0·24	0·40	3·64	6·19	0·26	0·44
Treated ..	0·70	0·20	0·14	5·11	3·57	0·27	0·19

TABLE 3. THE ALBUMIN AND GLOBULIN CONTENTS IN PERCENTAGES OF TOTAL PROTEIN OF THE EXTRACELLULAR FLUID IN GUINEA-PIG LUNGS AT VARIOUS TIMES AFTER ANAPHYLACTIC SHOCK

Time after shock in min	Shock group		Control group	
	Albumin %	Globulin %	Albumin %	Globulin %
0	35·5	64·5	33·7	66·3
15	34·1	65·9	34·3	65·7
30	37·6	62·4	32·2	67·8
60	32·4	67·5	31·3	68·7

PROTEIN IN EXTRACELLULAR FLUID AND SERUM

Table 3 shows that the relative proportions of albumin and globulin in extracellular fluid from the lungs of shocked animals and corresponding

controls showed no significant differences. The distribution pattern was observed to be diffuse and the bands on cellulose acetate were not easily distinguishable. Albumin appeared as a distinct band, often associated with a pre-albumin fraction, but all the globulin fractions tended to fuse into each other and form one diffuse band. A small rise in the proportion of albumin (statistically significant at $P = 0{\cdot}90$) can be observed 30 min after anaphylaxis. There were no noticeable changes in the protein composition of serum samples examined in these experiments. Since anaphylaxis had no pronounced effect on the protein content of extracellular fluid or serum, the effects of drug pretreatment were not investigated.

Discussion

These results show that anaphylaxis in guinea-pigs induces a fall in serum lipids; that observed for glyceride was preceded by a rise. These effects were also noted in control animals, indicating that the procedure used for collecting the blood samples (cardiac puncture) itself affected the serum lipid levels. A similar difficulty was experienced by Page, Pasternak & Burt (1931) whilst investigating the action of adrenaline on plasma lipids in the rabbit. Even so, the present results indicate that there is certainly no rise in serum lipids following anaphylaxis.

The lungs of guinea-pigs release large amounts of histamine in a vasoactive form as a result of anaphylaxis, and become oedematous in consequence. The isolation and biochemical estimation of the oedema fluid so formed presents technical difficulties. The procedure we used for recovering extracellular fluid from the lungs was devised in the belief that it represented oedema fluid diluted with Tyrode solution present in the pulmonary circulation. This belief is supported by the recovery of larger amounts of fluid from shocked compared with control lungs and also a small rise in its albumin content 30 min after anaphylaxis. The latter would be expected to follow an increase in lung capillary permeability since the molecular size of albumin is known to be smaller than that of the globulins, so that enrichment of tissue fluid with plasma protein would be expected to lead to an increase in albumin relative to globulin. The small extent of the albumin enrichment of extracellular fluid probably accounts for our failure to detect loss of albumin from serum. Anaphylaxis caused an increase in the lipid content of extracellular fluid followed by a return towards the control values. These changes suggest that lipid lost from lungs during anaphylaxis accumulates in oedema fluid in the lung and is then removed, presumably by lymphatic drainage. The reductions in volume of extracellular fluid and its lipid content observed in animals protected from fatal anaphylaxis by drug pretreatment are compatible with this interpretation.

The relationship between oedema fluid and phospholipid loss from lung tissue observed in these experiments is of interest in view of the previous findings of Kohler & Barbe (1954a,b). These workers reported the loss of total lipid from the lungs of rabbits following oedema induced by adrenaline and hypnotics. During anaphylaxis, therefore,

lipid loss from lung tissue may well be caused by the development of oedema in that organ.

The significance of the fall in serum lipids induced by anaphylaxis is unknown as yet. The fact that cardiac puncture itself induces changes essentially similar to those observed after anaphylaxis suggests that some "stress reaction" involving perhaps adrenaline- or ACTH-release might be operating (Page & others, 1931; Conn, Vogel, Louis & Fajans, 1950; Selye, 1950; Adlersberg, Shaefer & Drachman, 1951). The organ removing lipids from the serum under these experimental conditions is unknown. It might well be the liver. These tentative hypotheses invite early confirmation, but only in experiments in which adequate blood samples can be removed from guinea-pigs by a technique shown to be lacking in stimuli to the pituitary-adrenal axis. Otherwise, as in the present experiments, there remains the possibility that the technique of blood sampling may induce responses capable of modifying the subsequent anaphylaxis.

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Synthesis and antiprotozoal activity of some imidazoles derivatives

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The synthesis and *in vitro* antiprotozoal activity of a series of nitroimidazoles are described. A substituted phenyl group and, in many of the compounds, a *N*-substituent were also present: vinylogues of some of the compounds are described. Several of the nitroimidazoles exhibit very high *in vitro* activity against *Trichomonas vaginalis* and *T. foetus*, and good activity against *Histomonas meleagridis* and *Entamoeba histolytica*. Their antifungal action was lower than that of some of the un-nitrated imidazoles. The antiprotozoal activity of these compounds is discussed in relation to their chemical structure. 4-*p*-Acetamidophenyl-, 4-*p*-chlorophenyl- and 4-(3,4-dichlorophenyl)-1-methyl-5-nitroimidazole, and 4-*p*-chlorophenyl- and 4-(3,4-dichlorophenyl)-1-(2-hydroxyethyl)-5-nitroimidazole are the most active against *Trichomonas*.

THE failure of acinitrazole (2-acetamido-5-nitrothiazole; Tritheon) to fulfil its early promise (Cuckler, Kupferberg & Millman, 1955) as a clinically useful drug against *Trichomonas vaginalis* infections (Bushby & Copp, 1955) emphasises the need of further search for a trichomonacide for clinical and veterinary use. In such compounds as chloramphenicol, azomycin, acinitrazole and several nitrofurans the nitro-group attached to a benzene or heterocyclic ring is essential for their high chemotherapeutic efficiency. In view of the antifungal activity of some of the imidazole derivatives previously described (Ellis, Epstein, Fitzmaurice, Golberg & Lord, 1964), it was of interest to study the effect of nitration on their antiprotozoal activity. The new compounds prepared contain a nitro-group attached at either position 4 or 5 of the imidazole ring and a substituted phenyl group at the corresponding position 5 or 4. Many of the compounds also carry another substituent on the ring nitrogen atom.

The effect of the compounds described in this and the previous paper (Ellis & others, 1964) was studied on selected fungi and protozoa.

While the present work was in progress, reports on the use of metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole; Flagyl, 8823 R.P.] in the treatment of human trichomonad infections were published (Cosar & Julou, 1959; Sylvestre & Gallaiz, 1960; Bonzaine & Desranleau, 1960; Schnitzer, 1963).

For the preparation of the compounds studied, one of two standard methods of nitrating imidazoles was used. Either the imidazole nitrate was heated, with stirring, with concentrated sulphuric acid, or solid sodium nitrate was added to the imidazole base dissolved in sulphuric

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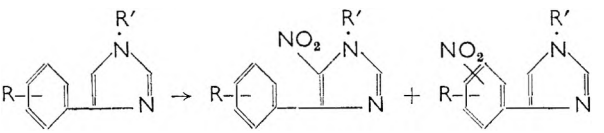
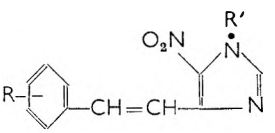
acid. Both methods gave a mixture of the nitroimidazole (I) and nitrophenyl (II) compounds, but the proportion varied with the substituent already on the benzene ring. Good yields of the almost colourless nitroimidazole were obtained only when the benzene ring carried a deactivating group. Separation of the isomers depended on the greater basicity of the yellow-coloured nitrophenyl isomer which remained in solution after dilution of the reaction mixture when the nitroimidazoles were precipitated. The structure of the nitrophenyl isomers was determined by oxidation with potassium permanganate to give a substituted benzoic acid. For example, 4-chloro-3-nitrobenzoic acid was obtained by the oxidation of the acid-soluble product of the nitration of 4(5)-*p*-chlorophenylimidazole. Nitroimidazoles unsubstituted at position 1 were



I II

alkylated by treatment with an alkyl sulphate to give the 1-alkyl-5-nitroimidazole. If an alkyl halide and potassium carbonate were used for alkylation, a mixture of the 4- and 5-nitroimidazoles was formed from which the pure 1-alkyl-4-nitroimidazole was obtained by fractional crystallization. Assignment of structures has been discussed by Ellis & others (1964). 5(4)-Nitro-4(5)-styrylimidazoles (III, R′ = H) analogous to the above phenylnitroimidazoles were readily prepared by condensing an aromatic aldehyde with 4(5)-methyl-5(4)-nitroimidazole or the 1-alkyl derivatives.



III

Some representative syntheses are given in the experimental section and the melting-point and analysis of the compounds are listed in Table 1.

Experimental

Nitration of 4(5)-*p-chlorophenylimidazole.* 4(5)-*p*-Chlorophenylimidazole (75 g) was treated with 2N nitric acid (250 ml). The solid nitrate was filtered and dried and added carefully to concentrated sulphuric acid (750 ml) with stirring. The mixture was heated for 15 hr on a steam-bath, then cooled and poured on to ice (750 g) and water (2 litres). The precipitated yellow solid was filtered off, washed with cold water and then boiled with dilute hydrochloric acid and the suspension filtered. The colourless residue was 4(5)-p-*chlorophenyl*-5(4)-*nitroimidazole* (30 g), m.p. 285°. The filtrate was basified with aqueous ammonia to yield the yellow 4(5)-(4-chloro-3-nitrophenyl)imidazole, m.p. 245° (from ethanol). The filtrate

from the nitration mixture on basifying with aqueous ammonia precipitated yellow plates of 4(5)-(4-chloro-2-nitrophenyl)imidazole, m.p. 201° (from ethanol). The analyses for these two nitrophenyl compounds were given by Ellis & others (1964). Characterisation of the three nitro-compounds was effected by oxidizing 1 g of each with potassium permanganate (4 g) in water (50 ml) containing sodium carbonate (1 g) to give *p*-chloro, 4-chloro-3-nitro-, and 4-chloro-2-nitro-benzoic acids, respectively, which did not depress the melting-points of authentic specimens.

4-p-*Chlorophenyl*-1-*methyl*-5-*nitroimidazole.* 4(5)-*p*-Chlorophenyl-5(4)-nitroimidazole (1·0 g) and dimethyl sulphate (0·6 ml) were heated together on a steam-bath for 1 hr. Water (20 ml) was added, followed by an excess of sodium bicarbonate. The mixture was extracted three times with 50 ml portions of ether, the extracts were dried (Na_2SO_4), concentrated and then diluted with light petroleum (b.p. 40–60°) (50 ml) to give the pale yellow 4-p-*chlorophenyl*-1-*methyl*-5-*nitroimidazole*, m.p. 107–108° (from ether).

5-p-*Chlorophenyl*-1-*methyl*-4-*nitroimidazole.* 4(5)-*p*-Chlorophenyl-5(4)-nitroimidazole (3·7 g), methyl iodide (2·5 g) and potassium carbonate (1·4 g) were refluxed together in acetone (200 ml) for 4 hr. The solid residue was filtered off, and the filtrate was evaporated to dryness and washed with ether (50 ml). From the residue, colourless needles of 5-p-*chlorophenyl*-1-*methyl*-4-*nitroimidazole* (1·4 g), m.p. 238–240° (from acetone) were isolated. The ether washings contained 4-*p*-chlorophenyl-1-methyl-5-nitroimidazole (0·6 g), m.p. 105°.

4(5)-(3,4-*Dichlorophenyl*)-5(4)-*nitroimidazole.* 4(5)-(3,4-Dichlorophenyl)imidazole (1 g) was suspended in water (10 ml) and treated with 10% nitric acid solution until acid to Congo Red. The suspension of the sparingly soluble salt was evaporated to dryness *in vacuo* and the residue added with cooling to concentrated sulphuric acid (5 ml). After heating the solution for 1 hr at 100°, it was poured onto ice, whereupon 4(5)-(3,4-*dichlorophenyl*)-5(4)-*nitroimidazole* (0·4 g), m.p. 302–303° (from aqueous ethanol) was precipitated. Oxidation of a sample with alkaline potassium permanganate gave 3,4-dichlorobenzoic acid, m.p. 208°.

The acid filtrate, on basification with aqueous ammonia, gave the greenish-yellow 4(5)-(4,5-dichloro-2-nitrophenyl)imidazole (0·4 g), m.p. 225–226° (Ellis & others, 1964).

4-(3,4-*Dichlorophenyl*)-1-*methyl*-4- and -5-*nitroimidazole.* 4(5)-(3,4-Dichlorophenyl)-5(4)-nitroimidazole (5 g), potassium carbonate (1·5 g) and methyl iodide (1·3 g) were refluxed together in acetone (100 ml) for 72 hr. The reaction mixture was filtered, the acetone distilled off, the residual solid stirred with cold ether, and the solution filtered; the filtrate, after being decolorised, concentrated and diluted with light petroleum, yielded pale yellow needles of 4-(3,4-*dichlorophenyl*)-1-*methyl*-5-*nitroimidazole* (0·5 g), m.p. 134–135° (not depressed on admixture with the product of nitration of 4-(3,4-dichlorophenyl)-1-methylimidazole). The residue from the filtration of the ether solution was extracted with hot ethyl acetate. On cooling the extract, unchanged starting material (0·25 g),

m.p. 304–305°, was deposited. After removing this, the solution was decolorized, concentrated and diluted with light petroleum to precipitate the colourless 5-(3,4-*dichlorophenyl*)-1-*methyl*-4-*nitroimidazole* (1·7 g), m.p. 195–197°.

4-*p*-*Acetamidophenyl*-1-*methyl*-5-*nitroimidazole*. 4(5)-*p*-Acetamidophenyl-5(4)-nitroimidazole (Grant & Pyman, 1921) (10 g) was heated on a steam-bath with dimethyl sulphate (10 ml) for 2 hr. The excess of dimethyl sulphate was removed *in vacuo*, and the residue was dissolved in water and basified with aqueous ammonia. Chloroform extracts of this solution were decolorised and the solvent was removed *in vacuo* without heating, leaving golden-yellow leaflets of 4-p-*acetamidophenyl*-1-*methyl*-5-*nitroimidazole* (2·1 g), m.p. 214–215°.

4-*p*-*Aminophenyl*-1-*methyl*-5-*nitroimidazole*. Repetition of the above preparation, but with prolonged heating for 16 hr, gave *the aminophenyl compound* as orange-red crystals, m.p. 141–143° (4·0 g) [from ethyl acetate-light petroleum (b.p. 60–80°)].

4(5)-p-*Chlorophenyl*-2-*methyl*-5(4)-*nitroimidazole*. 4(5)-*p*-Chlorophenyl-2-methylimidazole (1·3 g) was suspended in water (5 ml) and concentrated nitric acid (0·4 ml) was added. The mixture was warmed, cooled and filtered. The solid nitrate was dissolved in cooled concentrated sulphuric acid (5 ml), and the blue solution was heated at 100° for 1 hr, cooled, poured into water (20 ml) and filtered. After warming the solid with dilute hydrochloric acid, filtering and washing with water, it gave the *nitroimidazole* (0·4 g), m.p. 263–266° (from ethanol).

Hydroxyethylation of 4(5)-(3,4-*dichlorophenyl*)-5(4)-*nitroimidazole*. A mixture of 4(5)-(3,4-dichlorophenyl)-5(4)-nitroimidazole (5 g), 2-bromoethanol (1·5 ml), acetone (100 ml) and potassium carbonate (1·5 g) was refluxed for 72 hr and filtered. The solvent was distilled off and the solid extracted with boiling ether (4 × 100 ml); the combined extracts, on standing, deposited crystals which were added to the ether-insoluve residue, which was the pale yellow 5-(3,4-*dichlorophenyl*)-1-(2-*hydroxyethyl*)-4-*nitroimidazole* (1·8 g), m.p. 177° (from aqueous ethanol). From the ether solution, 4-(3,4-*dichlorophenyl*)-1-(2-*hydroxyethyl*)-5-*nitroimidazole* (0·5 g), m.p. 102–103° (from ethyl acetate-light petroleum, b.p. 60–80°) was obtained as salmon-coloured crystals by dilution with light petroleum.

5-(3,4-*Dichlorostyryl*)-1-*methyl*-4-*nitroimidazole*. 3,4-Dichlorobenzaldehyde (0·9 g), 1,5-dimethyl-4-nitroimidazole (0·7 g) and piperidine (0·1 ml) were heated together at 150–160° for 2 hr. The liquid was cooled, stirred with ether and filtered. Extraction of the solid with boiling water yielded 5-(3,4-*dichlorostyryl*)-1-*methyl*-4-*nitroimidazole* (0·25 g), m.p. 199–201° (from ethyl acetate).

Biological methods

In vitro ASSAY OF ANTIPROTOZOAL ACTIVITY

The following pathogenic protozoa (obtained from the sources stated) were used as test organisms: *Trichomonas vaginalis* (T 70), Liverpool Public Health Laboratory; *T. foetus* (T 69, Belfast strain), Agricultural

Research Council, Weybridge; *T. gallinae* (T 80, Wilson strain), Agricultural Research Council Veterinary Laboratories, Midlothian; *Histomonas meleagridis* (Joyner strain), Agricultural Research Council, M.A.F.F. (Weybridge; *Entamoeba histolytica* (Strain DC), Liverpool School of Tropical Medicine. The culture media used were:

Trichomonas stock culture medium. The composition w/v of a modified transport medium (Stuart, 1954) was as follows: sodium glycerophosphate 1·0, calcium chloride 0·01, sodium thioglycollate 0·1, agar 0·2, and methylene blue 0·0002%. Final pH 7·4; 12 ml of this was sterilised in ½ oz. screw-capped bottles at 15 lb/in² for 15 min. Before inoculation, the colourless medium was supplemented with 10% v/v heat-inactivated bovine serum.

Trichomonas assay medium. The three trichomonas strains were grown in the following modification (designated T.V. medium) of Kupferberg's medium (Kupferberg, Johnson & Sprince, 1948): Difco Protease peptone No. 3, 10; Difco Tryptose peptone, 10; Kerfoot D-glucose, 10; sodium chloride, 5; L-cysteine hydrochloride, 1·0; ascorbic acid, 1·5 g; 'Panamede' (Paines & Byrne) desiccated liver extract, 0·5 g, and distilled water 1 litre. Supplements to the medium included 400 I.U./ml penicillin and 0·5 mg/ml streptomycin sulphate together with 10% v/v heat-inactivated bovine serum.

The T.V. medium was prepared by dissolving separately the peptones, sodium chloride, L-cysteine hydrochloride and liver extract in 100-ml portions of hot distilled water. The warm solutions were mixed and cooled to room temperature before adding the glucose and ascorbic acid. The clear filtrate obtained by gravity filtration through a fluted Whatman No. 1 paper was diluted to 1 litre before adding the antibiotics. The medium was adjusted to pH 6·2, prefiltered through a Pyrex (porosity grade 4) sterile sintered-glass filter before final sterilisation by filtration through an Oxoid membrane. The filtrate was stored in the dark at 3–5°, and used, within 2 weeks, for maintaining the trichomonad, and preparing the inocula and assay medium. In each case it was supplemented with 10% v/v heat-inactivated bovine serum immediately before use.

Histomonas and entamoeba stock culture medium. A diphasic medium maintained continuously at 37° was used for both organisms. Sterile serum slopes (4 ml) were prepared in 6 × ⅝ inch cotton-wool-plugged test tubes for the histomonas and in ½-oz screw-capped bottles for the entamoeba. Coagulation of the serum was effected by heating at 80° for 20 min and to the cooled slope it was necessary to add 5–10 mg of heat-sterilised Difco rice starch powder. The egg-white overlay liquid medium was prepared by aseptically separating the white of a medium sized egg, mixing with 200 ml of sterile Ringer-phosphate saline (*p*H 7·4), adding 2 ml of sterile 20% w/v aqueous D-glucose solution and mixing well by means of a magnetic stirrer. The medium was then stored at 3–5° and used within 10 days.

The final medium (Rees & Reardon, 1945) consisted of 4 ml of inspissated horse serum slant covered with 8 ml of diluted egg white plus

rice starch powder. For both organisms the stock culture was maintained by subculturing every two or three days; the overlay liquid medium was preconditioned by inoculating with the mixed "natural" bacterial associates of the respective strains 4–5 hr before inoculating with approximately 50,000 viable amoeboid cells.

MAINTENANCE OF STOCK CULTURES OF TRICHOMONAD STRAINS

A 24-hr old vigorously growing, bacteria-free trichomonad culture, the flagellate of which exhibited extremely rapid motility was used. A 10% v/v inoculum was added to 12-ml volumes each of the Stewart transport medium and the T.V. medium, and then stored at 3–5° for two or three days. The cultures were unimpaired by this storage and were then incubated at 37° for 24 hr to provide once again a vigorously growing culture which could be used for either a static culture to be stored in the refrigerator or provide an inoculum for assays to determine trichomonacidal activity of new compounds.

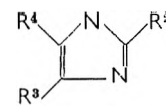
In vitro ASSAYS TO COMPARE THE ACTIVITIES OF NEW COMPOUNDS WITH STANDARD DRUGS

Trichomonacides. The minimum concentration of a compound which suppressed completely the growth of trichomonads at 37° over a period of 48 hr was determined. The end-points were ascertained by microscopic examination of the culture, death being indicated by morphological change from pear-shaped to rounded cells and complete cessation of motility. The standard drugs employed were (1) acinitrazole and (2) metronidazole. Nithiazide and dimetridazole were also included for comparison. Stock solutions (1% w/v) of standard and new compounds were prepared in either glycerol formal or liquid macrogol (Carbowax 300). Predilutions of the drugs were prepared in T.V. medium in wide-mouthed tubes, covering serial two-fold dilutions over the ranges 100–3 μg/ml, and 10–0·3 μg/ml; 5 ml portions of these dilutions were then transferred to $\frac{1}{4}$ oz screw-capped bottles.

The assay inoculum was prepared from a 24-hr old vigorous culture of the trichomonad strain in T.V. medium which was diluted in this medium to give approximately 500,000 viable protozoa per ml, 0·2 ml of which was added aseptically to each bottle so that each bottle received 100,000 trichomonads.

Amoebicides. Single-phase medium (Jones, 1946) containing 0·1% w/v autolysed yeast extract in buffered phosphate saline, pH 7·3, with rice powder supplement, along with 10% v/v heat-inactivated horse serum was used for screening new compounds. The Jones medium was preconditioned with the appropriate bacterial associates incubated for 4–5 hr at 37°, and two-fold serial dilutions of the drugs were prepared so that finally the medium plus rice powder in 5 ml portions were contained in $\frac{1}{4}$ oz screw-capped bottles. Each bottle then received 50,000 viable amoeboid cells as inoculum. After incubation for 48 hr at 37°, the sedimented layers in each bottle were examined microscopically and the minimum cidal concentration (MCC) was ascertained.

TABLE 1. ARYLNITROIMIDAZOLE DERIVATIVES



Compound No.	R^1	R^2	R^3	R^4	m.p. °C	Formula	Analysis: Found C	H	Cl or Br	N	Analysis: Required C	H	Cl or Br	N
1[a]	H	H	*p*-$NH_2{\cdot}C_6H_4$, HCl	NO_2	245–246	Grant and Pyman (1921)								
2	Me	H	*p*-$NH_2{\cdot}C_6H_4$	NO_2	141–143	$C_{10}H_{10}N_4O_2$	54·9	4·5	—	25·2	55·0	4·6	—	25·7
3	Me	H	*p*-Ac·NH·C_6H_4	NO_2	214–215	$C_{12}H_{12}N_4O_3$	55·2	4·9	—	21·9	55·4	4·6	—	21·5
4	Me	H	*p*-($Cl_2CH{\cdot}CO{\cdot}NH$), C_6H_4	NO_2	241–243	$C_{12}H_{10}Cl_2N_4O_3$	43·5	3·2	21·1	—	43·7	3·0	21·5	—
5[a]	H	H	*p*-Br·C_6H_4	NO_2	281–283	$C_9H_6BrN_3O_2$	40·5	2·4	—	15·5	40·3	2·2	—	15·7
6	Me	H	*p*-Br·C_6H_4	NO_2	99	$C_{10}H_8BrN_3O_2$	42·8	2·9	—	14·9	42·6	2·9	—	14·9
7	Me	H	NO_2	*p*-Br·C_6H_4	170	$C_{10}H_8BrN_3O_2$	42·8	2·8	—	14·9	42·6	2·9	—	14·9
8[a]	H	H	*p*-Cl·C_6H_4	NO_2	285	$C_9H_6ClN_3O_2$	48·6	3·1	15·4	18·2	48·4	2·7	15·7	18·8
9[a]	H	Me	*p*-Cl·C_6H_4	NO_2	263–266	$C_{10}H_8ClN_3O_2$	50·0	3·4	15·1	—	50·5	3·4	14·9	—
10	Me	H	*p*-Cl·C_6H_4	NO_2	107–108	$C_{10}H_8ClN_3O_2$	50·4	2·9	14·9	17·9	50·5	3·4	14·9	17·7
11	Me	H	NO_2	*p*-Cl·C_6H_4	238–240	$C_{10}H_8ClN_3O_2$	50·6	3·0	15·3	17·9	50·5	3·4	14·9	17·7
12	Me	Me	*p*-Cl·C_6H_4	NO_2	145–147	$C_{11}H_{10}ClN_3O_2$	52·1	4·1	14·4	16·8	52·5	4·0	14·1	16·7
13	Me	Me	NO_2	*p*-Cl·C_6H_4	200	$C_{11}H_{10}ClN_3O_2$	52·7	4·2	13·9	16·4	52·5	4·0	14·1	16·7
14	$CH_2{\cdot}CH_2{\cdot}CN$	H	*p*-Cl·C_6H_4	NO_2	105–106	$C_{12}H_9ClN_4O_2$	—	—	13·1	20·6	—	—	12·9	20·2
15	$CH_2{\cdot}CH_2{\cdot}CO{\cdot}OEt$	H	NO_2	*p*-Cl·C_6H_4	132–134	$C_{13}H_{12}ClN_3O_4$	50·4	4·0	11·7	—	50·1	3·9	11·5	—
16	$CH_2{\cdot}CH_2OH$	H	*p*-Cl·C_6H_4	NO_2	108–109	$C_{11}H_{10}ClN_3O_3$	49·4	3·7	13·5	16·0	49·3	3·8	13·3	15·7
17	$CH_2{\cdot}CH_2OH$	H	NO_2	*p*-Cl·C_6H_4	140–141	$C_{11}H_{10}ClN_3O_3$	49·1	3·8	13·9	15·8	49·4	3·8	13·3	15·7
18[a]	H	H	2-$CO_2H{\cdot}C_6H_3{\cdot}Cl$-H	NO_2	252–254	$C_{10}H_6ClN_3O_4$	44·9	2·4	—	15·4	44·9	2·3	—	15·7
19[a]	H	H	*p*-$SO_3H{\cdot}C_6H_4$	NO_2	>300	$C_9H_7N_3O_5S$	40·0	2·6	—	15·2	40·1	2·6	—	15·6
20[a]	H	H	3,4-Cl_2:C_6H_3	NO_2	302–303	$C_9H_5Cl_2N_3O_2$	42·4	2·1	—	—	41·9	1·9	—	—
21	Me	H	3,4-Cl_2:C_6H_3	NO_2	134–135	$C_{10}H_7Cl_2N_3O_2$	44·3	2·7	26·0	15·2	44·1	2·6	26·1	15·4
22	Me	H	NO_2	3,4-Cl_2:C_6H_3	195–197	$C_{10}H_7Cl_2N_3O_2$	43·8	2·5	25·7	15·5	44·1	2·6	26·1	15·4
23	Et	H	3,4-Cl_2:C_6H_3	NO_2	63–65	$C_{11}H_9Cl_2N_3O_2$	45·8	3·2	24·8	14·2	46·1	3·2	24·8	14·7
24	Et	H	NO_2	3,4-Cl_2:C_6H_3	175–176	$C_{11}H_9Cl_2N_3O_2$	45·9	3·2	24·9	14·4	46·1	3·2	24·8	14·7
25	Pr^n	H	3,4-Cl_2:C_6H_3	NO_2	49–51	$C_{12}H_{11}Cl_2N_3O_2$	48·8	4·0	23·8	13·7	48·0	3·7	23·7	14·0
26	Pr^n	H	NO_2	3,4-Cl_2:C_6H_3	165–167	$C_{12}H_{11}Cl_2N_3O_2$	48·6	3·5	23·1	13·8	48·0	3·7	23·7	14·0
27	$n\text{-}C_6H_{17}$	H	3,4-Cl_2:C_6H_3	NO_2	34–35	$C_{17}H_{21}Cl_2N_3O_2$	55·3	5·7	19·2	11·2	55·1	5·7	19·2	11·3
28	$n\text{-}C_6H_{17}$	H	NO_2	3,4-Cl_2:C_6H_3	89–90	$C_{17}H_{21}Cl_2N_3O_2$	54·2	5·7	19·2	11·4	55·1	5·7	19·2	11·3
29	$CH_2{\cdot}CO_2H$	H	NO_2	3,4-Cl_2:C_6H_3	225–226	$C_{11}H_7Cl_2N_3O_4{\cdot}H_2O$	39·7	3·0	21·0	—	39·5	2·7	21·3	—
30	$CH_2{\cdot}CO{\cdot}OEt$	H	3,4-Cl_2:C_6H_3	NO_2	113	$C_{13}H_{11}Cl_2N_3O_4$	45·6	3·3	20·8	11·8	45·3	3·2	20·6	12·2
31	$CH_2{\cdot}CO{\cdot}OEt$	H	NO_2	3,4-Cl_2:C_6H_3	139–140	$C_{13}H_{11}Cl_2N_3O_4$	45·1	3·4	20·7	12·2	45·3	3·2	20·6	12·2
32	$CH_2{\cdot}CO{\cdot}NH{\cdot}NH_2$	H	NO_2	3,4-Cl_2:C_6H_3	195–197	$C_{13}H_{12}Cl_2N_6O_3$	—	—	22·7	—	—	—	22·9	—
33	$CH_2{\cdot}CH_2OH$	H	3,4-Cl_2:C_6H_3	NO_2	102–103	$C_{11}H_9Cl_2N_3O_3$	43·3	2·8	24·2	14·3	43·7	3·0	23·5	13·9
34	$CH_2{\cdot}CH_2OH$	H	NO_2	3,4-Cl_2:C_6H_3	177	$C_{11}H_9Cl_2N_3O_3$	43·4	2·8	24·1	14·4	43·7	3·0	23·5	13·9
35	$CH_2{\cdot}CH_2OEt$	H	NO_2	3,4-Cl_2:C_6H_3	127–128	$C_{13}H_{13}Cl_2N_3O_3$	47·0	4·1	21·3	12·5	47·2	3·9	21·5	12·5
36	$CH_2{\cdot}CH_2{\cdot}NEt_2$	H	3,4-Cl_2:C_6H_3	NO_2	48–49	$C_{15}H_{18}Cl_2N_4O_2$	49·5	5·1	19·8	—	50·4	5·0	19·9	—
37	$CH_2{\cdot}CH_2{\cdot}NEt_2$, 2HCl	H	NO_2	3,4-Cl_2:C_6H_3	180–184	$C_{15}H_{20}Cl_4N_4O_2$	41·5	4·7	31·6	12·9	41·6	4·7	33·0	13·0
38	2-(pyrid-2-yl)ethyl, HCl	H	3,4-Cl_2:C_6H_3	NO_2	222–223	$C_{12}H_{11}Cl_2N_3O_4$	—	—	9·0[b]	—	—	—	8·9[b]	—
39	$CH_2{\cdot}CH(OH){\cdot}CH_2OH$	H	NO_2	3,4-Cl_2:C_6H_3	197–198	$C_{12}H_{11}Cl_2N_3O_4$	—	—	21·4	12·8	—	—	21·4	12·7
40[a]	H	H	2,4,5-Cl_3:C_6H_2	NO_2	207–208	$C_9H_4Cl_3N_3O_2$	37·6	1·8	—	14·8	37·0	1·4	—	14·4
41[a]	H	H	*p*-Cl·$C_6H_4{\cdot}CH$:CH	NO_2	315	$C_{11}H_8ClN_3O_2$	—	—	13·9	—	—	—	14·2	—
42[a]	H	H	3,4-Cl_2:$C_6H_3{\cdot}CH$:CH	NO_2	303–304	$C_{11}H_7Cl_2N_3O_2$	46·3	2·5	24·5	14·6	46·5	2·5	25·0	14·8
43	Me	H	3,4-Cl_2:$C_6H_3{\cdot}CH$:CH	NO_2	231–232	$C_{12}H_9Cl_2N_3O_2$	48·0	3·1	24·3	14·2	48·3	3·0	23·8	14·1
44	Me	H	NO_2	3,4-Cl_2:$C_6H_3{\cdot}CH$:CH	199–201	$C_{12}H_9Cl_2N_3O_2$	47·5	3·5	23·6	13·6	48·3	3·0	23·8	14·1

[a] Where R^1 = H, R^3 and R^4 are interchangeable. [b] Ionic chlorine.

In vitro ASSAY OF ANTIFUNGAL ACTIVITY

The procedure used was the same as that already described by Ellis & others, 1964.

Results

Of the nitro-compounds described, five (compounds 6, 10, 21, 23 and 25, Table 1) showed antifungal activity at a concentration of 25 μg/ml or less. These compounds, and others which inhibited the growth of trichomonads at 1 μg/ml or less, are listed in Table 2. There was no

TABLE 2. ANTIPROTOZOAL ACTIVITY OF IMIDAZOLE DERIVATIVES AFTER 48 HR AT 28°

Compound No.	Minimum cidal concentration μg/ml			M.I.C. μg/ml
	Trichomonas	Histomonas	Entamoeba	Trichophyton
2	0·5		10	
3	0·075	1·5	2·5	
4	1	7·5	20	
6	0·1			25
7	1			
10	0·1	1·25	2·5	25
12	1		10	
16	0·05	1·5	1·5	
20	1			
21	0·1	2		20
23	0·5			10
25	1			10
30	1			
33	0·15			
34	1			
36	1			
42	0·5			
43	1			
44	1			
Metronidazole	0·3	2·5	2·5	
Acinitrazole	1	1	—	
*Nithiazide (Hepzide) ..	1	3	—	
†Dimetridazole (8595 R.P.) ..	0·2	0·5	2	

* 1-Ethyl-3-(5-nitro-2-thiazolyl)urea.
† 1,2-Dimethyl-5-nitroimidazole.

significant difference between the three species of trichomonads in their sensitivity to a drug.

Discussion

Microbiological tests on the nitro-compounds show them to have a pronounced difference in their spectrum of *in vitro* antimicrobial activity from that of their un-nitrated precursors (Ellis & others, 1964). Some of the latter possessed good antifungal action but no trichomonacidal properties. After nitration in position 4 or 5 of the imidazole ring, some of these compounds exhibited high inhibitory activity against several protozoa but their antifungal potency decreased; for example, on nitrating 4-(3,4-dichlorophenyl)-1-propylimidazole (compound 25), its antifungal activity fell from 4 to 10 μg/ml, while its trichomonacidal efficacy increased from a negligible value to 1 μg/ml. 4(5)-(2,4,5-Trichlorophenyl)imidazole inhibited the *in vitro* growth of *Trichophyton* species at 25 μg/ml and of trichomonads at 50 μg/ml, but nitration (to compound 40) changed these values to 100 and 2 μg/ml respectively. In general, the most potent trichomonacidal compounds showed little or no antifungal activity.

Alkylation of the ring nitrogen atom led to a considerable improvement

in trichomonacidal activity only if (*a*) the 5-nitro-isomer was formed and (*b*) the alkyl group was small. Thus, methylation of 4(5)-(3,4-dichlorophenyl)-5(4)-nitroimidazole (compound 20) produced the 1-methyl-5-nitro-derivative (compound 21), which was twenty times more potent than its 1-methyl-4-nitro-isomer (compound 22). On n-propylation of compound 20, however, the more active isomer (compound 25) was no better than the parent compound. Octylation (to compounds 27 and 28) destroyed the activity of compound 20. A similar effect was observed by Bushby & Copp (1955) when they acylated 2-amino-5-nitrothiazole with fatty acids of varying chain-length; the 2-octanoylamido-compound possessed only one-tenth of the activity of acinitriazoles.

Compounds which possessed water-solubilising groups attached to either the imidazole ring (compounds 16, 17, 29, 33, 34, 36, 37, 39) or the phenyl ring (compounds 1, 18, 19) exhibited poor *in vitro* anti-protozoal action. There was little or no difference in the activities of compounds with a chlorine or a bromine atom in the *p*-position of the benzene ring, or in the activity of a compound with a chlorine atom in the *p*-position and that of one with two chlorine atoms in positions 3 and 4. The high activity of the *p*-aminophenyl- and *p*-acetamidophenyl-imidazoles (compounds 1, 2, 3) parallels that of the corresponding nitrothiazole compounds (Bushby & Copp, 1955). Replacing the *p*-acetamido-group (compound 3) by a dichloroacetamido-substituent (compound 4) produced a decrease in activity which is not unexpected in view of the inactivity of chloramphenicol towards trichomonads (Schnitzer, 1963).

The vinylogous compounds (41–44) exhibited very good trichomonacidal activity but they differed from the phenylnitroimidazoles in two respects: first, alkylation of the ring nitrogen atom decreased potency slightly (compare compounds 42 and 43), in contrast to a ten-fold improvement between compounds 20 and 21. Secondly, the two positional isomers (43 and 44) were equally active, while the corresponding compounds 21 and 22 differed by a factor of twenty.

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Effect of various drugs on carrageenin-induced oedema in the rat hind paw

C. J. E. NIEMEGEERS, F. J. VERBRUGGEN AND P. A. J. JANSSEN

Local oedema in the rat hind paw is induced by subplantar injection of a 1% suspension of carrageenin. An assay procedure for the analysis of inhibitory effects of drugs on this inflammatory process is described. The results obtained with a single oral dose of 14 antirheumatic drugs (3 steroids and 11 non-steroids) and of 49 substances without established clinical antirheumatic value are reported. Only 8 compounds were found to be completely devoid of anti-carrageenin activity. Others were active at dose levels producing striking behavioural, autonomic or toxic effects. All clinically established antirheumatic substances were active in the carrageenin test at non-toxic doses producing no obvious behavioural or autonomic effects and data on dose-response relationship of these compounds are presented. It is concluded that the assay in its present form is an acceptable preliminary screening test for antirheumatic activity.

CARRAGEENIN is a sulphated polygalactose extracted from the marine alga *Chondrus crispus* (Irish sea moss). It is a complex mixture of at least 5 different polysaccharides. Its two main components have been designated kappa and lambda fractions (Smith, O'Neill & Perlin, 1955). Different authors found preparations of carrageenin to possess inflammatory properties in laboratory animals and the active fraction in inflammation has been identified as the lambda component (McCandless, 1962). Carrageenin-induced oedema in the hind paw of the rat, as an assay for anti-inflammatory drugs was introduced by Winter, Risley & Nuss (1962, 1963).

We describe the experimental details of the carrageenin-test in rats as used by us and report upon the inhibitory effects obtained with this procedure using a variety of well-known steroid and non-steroid antirheumatic drugs, and other pharmacodynamic agents without established clinical antirheumatic action.

Experimental

METHOD

A modification of the method described by Winter & others (1962) was used. Young male Wistar rats of 195 ± 10 g body weight were maintained in an air-conditioned room (temp. 22 ± 1°; relative humidity: 65 ± 15%). Food was withdrawn 16 hr before the start of the experiment. Tap water was withheld during the experiment only.

The carrageenin used is coded Seakem 402 AP,* it is a predominantly lambda carrageenin and, out of 10 commercially available carrageenins, it was found to be the most active in inducing inflammation (Atkinson, Jenkins, Tomich & Woollett, 1962). A 1% suspension in 0·9% saline was prepared 1 hr before each experimental session, and a volume of

From Janssen Pharmaceutica, N.V., Research Laboratoria, Beerse (Belgium).

* Obtained through the courtesy of J. T. Zolper and Murray H. Malin, Marine Colloids, Inc., Springfield, N.J.

0·05 ml was injected into the plantar side of both hind paws of the rats.

Drugs in aqueous solution or suspension (. . . 0·31, 0·63, 1·25 . . . 160 mg/kg) were administered by stomach tube in a volume of 1 ml/100 g body weight, followed immediately by tap water to a total of 5 ml/rat. Controls received 5 ml tap water only. This ensured uniform hydration in all rats and minimised the variability of oedematous responses in the paws (Winter & others, 1962).

Drugs or solvent were given 1 hr before the carrageenin treatment and the degree of the carrageenin-induced swelling of the hind paws was measured 3 hr after the carrageenin-treatment.

The apparatus for measuring foot volume was a commercially available electric antiphlogmeter developed by Kemper & Ameln (1959).

The degree of swelling is the ratio a/b, where "b" is the total volume of both hind paws before, and "a" the total volume of both hind paws after carrageenin treatment. Since it was found that there was a lack of correlation between the volume of both paws before and after carrageenin treatment ($\chi^2 = 0{\cdot}28$; $P > 0{\cdot}50$) the mean value "b" was calculated for 500 control experiments and preferred to the true "b" value obtained in each animal. In 500 control rats (1,000 paws) the mean ratio a/b was 2·0 (swelling equal to 100%). A ratio a/b $\leqslant 1{\cdot}5$ (swelling less than or equal to 50%) after drug administration was considered as a significant inhibitory effect of the drug. On this basis using different dose levels and 6 rats per dose level (2 groups of 3 rats on different days), the usual quantal assay procedure was employed. The dose producing a ratio of a/b $\leqslant 1{\cdot}5$ in 50% of the treated animals (ED 50 in mg/kg), the 95% confidence limits (LL and UL), the slope (S) and the slope function (fS) were determined by the graphic log-probit method of Litchfield & Wilcoxon (1949).

Results

CONTROL EXPERIMENTS

The degree of swelling of the carrageenin-injected paws was maximal 3 hr after injection and the mean increase in volume at that time was about 100% (a/b $\sim 2{\cdot}0$). The time-effect curve obtained in 15 rats (30 paws) is shown in Fig. 1. The 100% increase of the paw volume 3 hr after carrageenin injection remained fairly constant; in 500 control rats (1,000 paws), injected over a period of about one year, the mean increase was exactly 100% (mean ratio a/b = 2·0). As shown in Fig. 2 there was no remarkable difference between the right and the left paws of the rats and an increase less than or equal to 50% (a/b $\leqslant 1{\cdot}5$) was observed in only 3·1% of the control animals. Furthermore for 75% of the control animals the degree of swelling after carrageenin treatment was between 80% (a/b = 1·8) and 120% (a/b = 2·2) compared to the uninjected paws of the same animals (Fig. 2).

ANTI-INFLAMMATORY COMPOUNDS

The results obtained with a series of 14 so-called antirheumatic drugs (3 steroids and 11 non-steroids) in the carrageenin-induced oedema test

are shown in Table 1. No obvious behavioural or autonomic effects were observed with these drugs at the highest dose levels tested (Fig. 3). All clinically established antirheumatic drugs seem to possess anti-carrageenin activity.

TABLE 1. ANTI-CARRAGEENIN EFFECTIVENESS OF 14 ANTIRHEUMATIC DRUGS

Substances	ED 50	LL*	UL	S	fS	n
Indomethacin	2·2	1·2	3·8	1·67	1·41	30
Mefenamic acid	9·0	4·3	19	3·20	1·85	48
Flufenamic acid	10	4·8	21	4·32	3·25	36
Phenylbutazone	25	14	47	2·15	1·56	36
Amidopyrine	31	20	49	1·77	1·35	30
Phenacetin	57	39	83	1·60	1·22	30
Aspirin	72	50	104	1·39	1·20	24
Phenazone	87	53	142	1·87	1·58	24
Acetaminophen	88	53	145	1·56	1·52	18
Cinchophen	92	70	121	1·27	1·14	18
Sodium salicylate	98	65	148	1·69	1·24	18
Paramethasone	0·057	0·042	0·077	1·46	1·18	30
Hydrocortisone	30	16	57	3·10	1·96	36
Cortisone	40	28	57	1·37	1·16	24

* See page 811.

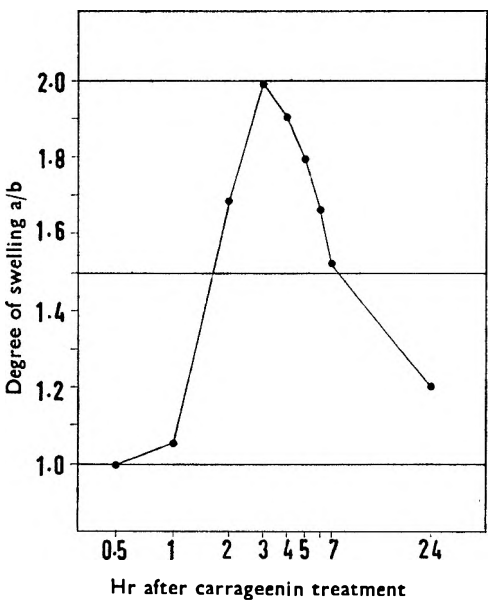

FIG. 1. Local carrageenin-induced oedema in the rat hind paw. Carrageenin 1% in 0·9% saline; 0·05 ml subplantar in both hind paws. Time effect curve obtained in 15 controls (30 paws).

OTHER DRUGS

To study the specificity of the carrageenin-test, 49 miscellaneous drugs of different pharmacological classes were administered orally at different

dose levels to groups of (2 × 3) rats. The results are summarised in Table 2.

Most of the active compounds of this group were found to antagonise the carrageenin-induced swelling reaction at dose levels producing overt

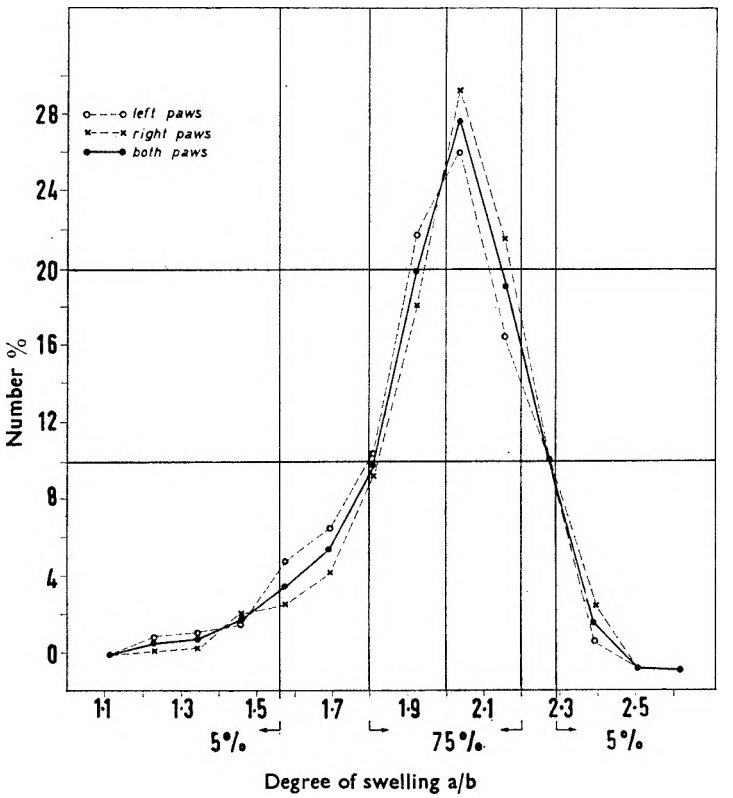

FIG. 2. Local carrageenin-induced oedema in the rat hind paw. Carrageenin 1% in 0·9% saline; 0·05 ml subplantar. Frequency distribution of the degree of swelling obtained in 500 control rats, i.e. 1000 paws.

behavioural changes or various autonomic effects such as mydriasis or both. Obviously anti-carrageenin activity is by no means a rare pharmacological property and as such insufficient evidence for potential usefulness in the treatment of rheumatic disorders.

Discussion

Oral administration was adopted for all compounds, thus largely avoiding the so-called "counter-irritant effect" a phenomenon described by Benitz & Hall (1963), defined by them as a competitive reaction to two or more equal or different stimuli at two or more different locations.

Only 8 compounds were found to be virtually devoid of anti-oedema

TABLE 2. ANTI-CARRAGEENIN EFFECTIVENESS OF 49 MISCELLANEOUS DRUGS, WITHOUT ESTABLISHED CLINICAL ANTIRHEUMATIC VALUE, BELONGING TO DIFFERENT PHARMACOLOGICAL CLASSES

Substances	Class	ED 50	LL	UL	S	fS	n
Diphenoxylate	Analgesics	2·5	1·6	4·0	1·52	1·25	30
Fentanyl		3·5	2·6	4·7	1·44	1·12	24
Dextromoramide		3·6	2·7	4·7	1·41	1·30	12
Piritramide (1)		17	14	21	1·21	1·08	18
Morphine		20	13	32	2·07	1·62	24
Codeine		40	22	72	1·70	1·45	30
Triperidol	Neuroleptics	4·2	2·7	6·5	1·74	1·44	24
Haloperidol		4·6	2·6	8·2	2·43	1·79	30
Reserpine		9·2	4·9	17	1·74	1·66	30
Chlorpromazine		14	9·4	21	1·67	1·43	18
Droperidol (2)		17	11	27	1·76	1·60	18
Fluphenazine		19	12	30	1·55	1·37	24
Spiroperidol		29	16	51	2·05	1·81	24
Pentobarbitone	Hypnotics	40	not linear				12
Phenobarbitone		>160	—	—	—	—	6
Diphenhydramine	Antihistamines	75	46	121	1·86	1·53	24
Pyrilamine		80	not linear				30
Cinnarizine		⩾160	—	—	—	—	6
Chlordiazepoxide	Tranquillising muscle relaxants	73	56	95	1·26	1·11	18
Meprobamate		>160	—	—	—	—	6
Hydroxyzine	Sedatives	160	—	—	—	—	6
Thalidomide		>160	—	—	—	—	6
Norimipramine	Antidepressants	42	not linear				30
Imipramine		70	not linear				30
Amitryptiline		92	57	149	1·86	1·80	18
Phencyclidine	Halucinogenics	7·1	4·2	12	1·94	1·46	30
Mescaline		67	52	86	1·25	1·11	18
Atropine	Anti-cholinergics	61	54	111	2·14	1·95	24
Benactyzine		160	—	—	—	—	6
Benzetimide (3)		⩾160	—	—	—	—	6
Tranylcypromine	MAO inhibitors	5·0	2·3	11	3·31	2·12	42
Iproniazid		94	49	182	2·32	2·23	24
Diphenylhydantoin	Anticonvulsant	>160	—	—	—	—	6
Cyclopenthiazide	Diuretics	65	40	105	1·84	1·77	18
Hydrochlorothiazide		68	31	145	3·93	2·66	36
Chlorothiazide		⩾160	—	—	—	—	6
Amphetamine	CNS-stimulants	1·8	1·0	3·2	2·07	1·75	36
Caffeine		66	not linear				30
Tryptamine		160	—	—	—	—	6
Apomorphine		>160	—	—	—	—	6
Aceperone (4)	Hypotensive drugs	26	18	37	1·58	1·37	18
Guanethidine		52	33	81	1·76	1·44	24
Acoxatrine (5)		80	60	107	1·30	1·17	18
Hexamethonium		>160	—	—	—	—	6
Cocaine	Local anaesthetics	40	19	84	1·94	1·72	36
Xylocaine		90	69	118	1·27	1·08	24
Procaine		>160	—	—	—	—	6
Prozapine (6)	Papaverine-like compounds	160	—	—	—	—	6
Papaverine		⩾160	—	—	—	—	6

Also known as (1) pirinitramide (Janssen, 1961); (2) dehydrobenzperidol (Janssen, Niemegeers, Schellekens, Verbruggen & Van Nueten, 1963); (3) dioxatrine (Niemegeers & Janssen, 1964); (4) acetabuton (Schaper, Jageneau, Xhonneux, 1962); (5) acetoxatrine (Schaper, Jageneau & Janssen, 1963); (6) hexadiphane (Gaussen, 1962).

properties at the highest dose levels tested (ED 50 >160 mg/kg oral). These were: apomorphine, diphenylhydantoin, hexamethonium, meprobamate, papaverine, phenobarbitone, procaine and thalidomide.

Three compounds possessed a slight inhibitory effect (ED 50 $\geqslant$160 mg/kg oral): chlorothiazide, cinnarizine and benzetimide (a/b $\leqslant$1·5 in 2/6 animals). For 4 other drugs—benactyzine, prozapine, hydroxyzine and tryptamine—a significant inhibitory effect was found in 50% of the animals at the 160 mg/kg dose level (ED 50 ~160 mg/kg oral; a/b $\leqslant$1·5 in 3/6 animals).

As seen in Table 1 all compounds of interest in the treatment of inflammation were found to be effective in the carrageenin-test at non-toxic

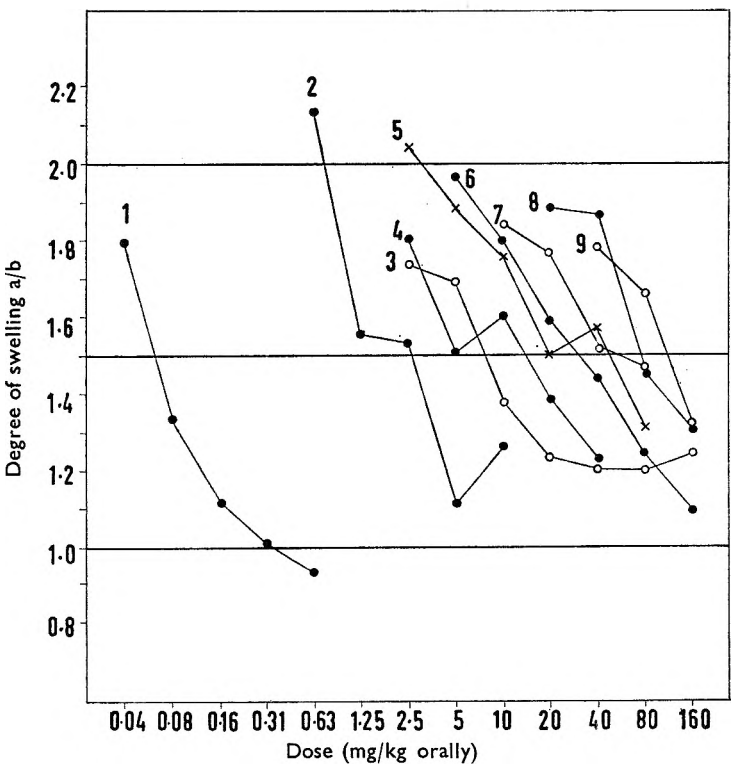

FIG. 3. Dose-effect curves for anti-carrageenin activity. Paramethasone (1), indomethacin (2), mefenamic acid (3), flufenamic acid (4), hydrocortisone (5), phenylbutazone (6), cortisone (7), acetylsalicylic acid (8), sodium salicylate (9).

dose levels. Satisfactory dose response curves (Fig. 3) were obtained with a relatively small number of rats.

It is concluded that the carrageenin-test in its present form is an acceptable screening assay for antirheumatic activity. Compounds showing effectiveness at low atoxic dose levels and producing no obvious behavioural or autonomic effects at relatively high (e.g. 4 times ED 50) dose levels merit further investigation as potentially useful antirheumatic drugs.

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Effect of phenol on the oxygen uptake of *Bacillus subtilis* spores

MURIEL LOOSEMORE AND A. D. RUSSELL

The effect of phenol on the oxygen uptake of *Bacillus subtilis* spores in glucose is described. All concentrations of phenol tested inhibited respiration, but a comparison with earlier work showed no correlation between this effect and death of the spores.

INVESTIGATIONS into the effect of phenol on the uptake of oxygen by micro-organisms have been made by Hugo (1956), who found that low phenol concentrations stimulated the rate of uptake of oxygen in *Escherichia coli* when glucose, mannitol or lactose was used as substrate, but not when succinate, lactate, pyruvate or acetate was the substrate, and by Chauhan, Rivers & Walters (1963), who showed that 0·05–0·2% of phenol progressively reduced uptake of oxygen by *Penicillium notatum* spores.

Loosemore & Russell (1963) have indicated that phenol concentrations as high as 2·5 and 5% have little lethal action on *Bacillus subtilis* spores, whereas, depending on the number of spores present, the minimum concentration of phenol required to inhibit growth in nutrient broth was 0·1–0·2%.

The present investigation was made to find out if metabolic processes were continuing during phenol treatment of the spores.

Experimental and results

All chemicals were of analytical reagent quality.

The organism was a laboratory strain of *Bacillus subtilis*. It was grown for 48 hr at 37° on the surface of nutrient agar (Oxoid, pH 7·4) in Roux flasks, washed from the surface with sterile water, and then washed twice with sterile water. The suspension was shaken with sterile glass beads to break up any clumps, heat-shocked at 75° for 20 min, and adjusted to a density of approximately 7×10^8 or 7×10^9 viable spores/ml.

Oxygen uptake was determined using the Warburg apparatus, by the method described by Umbreit, Burris & Stauffer (1959).

Effect of spore concentration on rate of oxygen uptake. It was necessary to determine first the optimum numbers of spores for measuring the rate of oxygen uptake over a period of time.

The results of this experiment are shown in Fig. 1, from which it is apparent that the higher spore inoculum gives an easily measurable response. Such an inoculum was therefore used in later experiments with phenol.

Effect of phenol on oxygen uptake. The effect of various phenol concentrations in duplicate on the respiration of the higher spore inoculum was investigated. Manometer readings (Fig. 2) were made at frequent intervals over several days to obtain some comparison with sporicidal tests (Loosemore & Russell, 1963).

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Discussion

With glucose as substrate, a heavy inoculum of *B. subtilis* spores is needed before oxygen uptake can be detected. Respiration of spores is

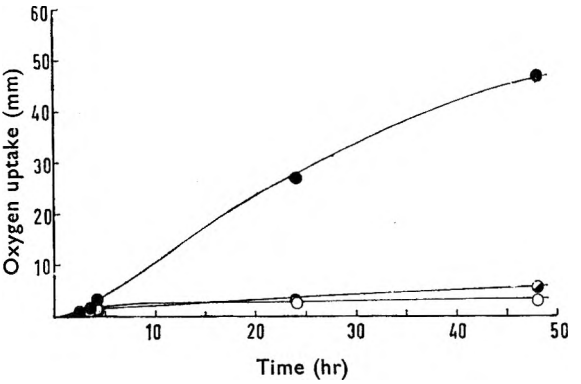

FIG. 1. Oxygen uptake of *B. subtilis* spores. ●—● Approx. 44×10^7 spores/ml, substrate glucose. ◒—◒ Approx. 44×10^6 spores/ml, substrate glucose. ○—○ Approx. 44×10^7 spores/ml, glucose absent.

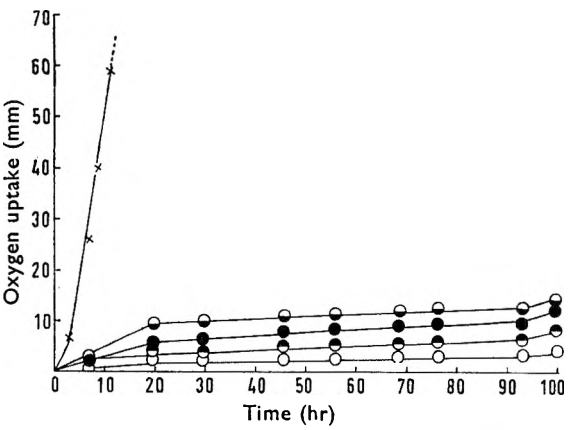

FIG. 2. Effect of phenol on the oxygen uptake of *B. subtilis* spores. ×—× Phenol absent. ●—● Phenol 0·5%. ◒—◒ Phenol 1·0%. ◓—◓ Phenol 2·5%. ○—○ Phenol 5·0%

greatly inhibited in the presence of phenol. The minimum concentration of phenol which inhibits germination and subsequent growth of approximately 10^7 spores of this organism in nutrient broth is about 0·2%. Preliminary experiments indicated that 0·25% phenol inhibited oxygen uptake, and it is therefore, apparent that such concentrations of the antibacterial agent can inhibit germination and subsequent growth, and prevent

respiration. However, there is no correlation of the inhibition of these processes and the sporicidal activity of phenol, which even in concentrations as high as 2·5 and 5% w/v, possesses little lethal effect against spores of this organism (Loosemore & Russell, 1963). It is interesting to note that Chauhan & others (1963) have concluded that measurement of oxygen uptake cannot be used for quantitative evaluation of fungicidal action.

Since glucose is a known germination stimulant, it is likely that the spores germinate in the absence of phenol. Phenol itself is known to inhibit a stage in the germination process (Lund, 1962).

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Anatomy of the leaves and young stem of *Mitragyna inermis* (O. Kuntze)

M. S. PILLAY

The anatomy and morphology of the leaves and young stem of *Mitragyna inermis* (O. Kuntze) is described and illustrated.

THE extract of leaves or that of the stem of *Mitragyna inermis* O. Kuntze (*M. Africana* Korth) is commonly used in herbal medicine in West Africa for a variety of ailments.

Although the constituents of the plant have been studied (Badger, Cook & Onley, 1950), no complete anatomical investigation appears to have been published, the morphology and anatomy of the plant are therefore described in this communication.

Mitragyna inermis is indigenous to the swampy savannah, and in Ghana is found growing both to the North and South of the Volta Region. The materials used in this investigation were leaves and young stems (twigs) collected in April and December, 1962, in the Volta Region from the Sogakope District. The identity of this species was confirmed by the Forestry Department, Kumasi, Ghana.

Macroscopy

The leaves have an opposite and decussate phyllotaxis and measure 2·5–12 cm long, and 1·5–8 cm wide. They are ovate, simple, petiolate and stipulate. The colour varies from reddish brown in the young to brownish green in the older leaves. Generally, the leaf apex is acuminate and rarely acute, with base rounded and symmetrical. The margin is entire. The upper surface is glabrous, while, on the lower surface trichomes are found mainly on the midrib and lateral veins. The midrib is prominent with 6–8 lateral veins leaving it at an angle of 40–50° and anastomosing near the margin. Venation is reticulately pinnate. Texture is thin and papery with odour slight and taste somewhat bitter.

The *petiole* is grooved and measures 6 mm–3 cm long, and 0·75–1·5 mm wide. The lower surface is rough due to the presence of short warty trichomes.

The *stipules* are in pairs. They measure 5 mm–2·5 cm long and 2–6 mm wide. They are oblong-lanceolate and reddish brown in colour (Fig. 1B). and are deciduous, having a thin and papery texture. They are odourless and the taste is slightly bitter. The lower or outer surface is pubescent while the upper and inner surface is glabrous except near the lower half of the stipule where dark brown elongated secretory glands are found (Fig. 1.C e.s.g.). These are arranged alternately in rows of 3–5. Each elongated secretory gland has a broad and flattened base with a tapering apex (Fig. 2.G). Between these glandular structures are long hair-like trichomes (Fig. 1.C. tr.) In the young stipule the secretion of

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the secretory glands is copious, milky white and sticky and coats the adjacent young leaflets.

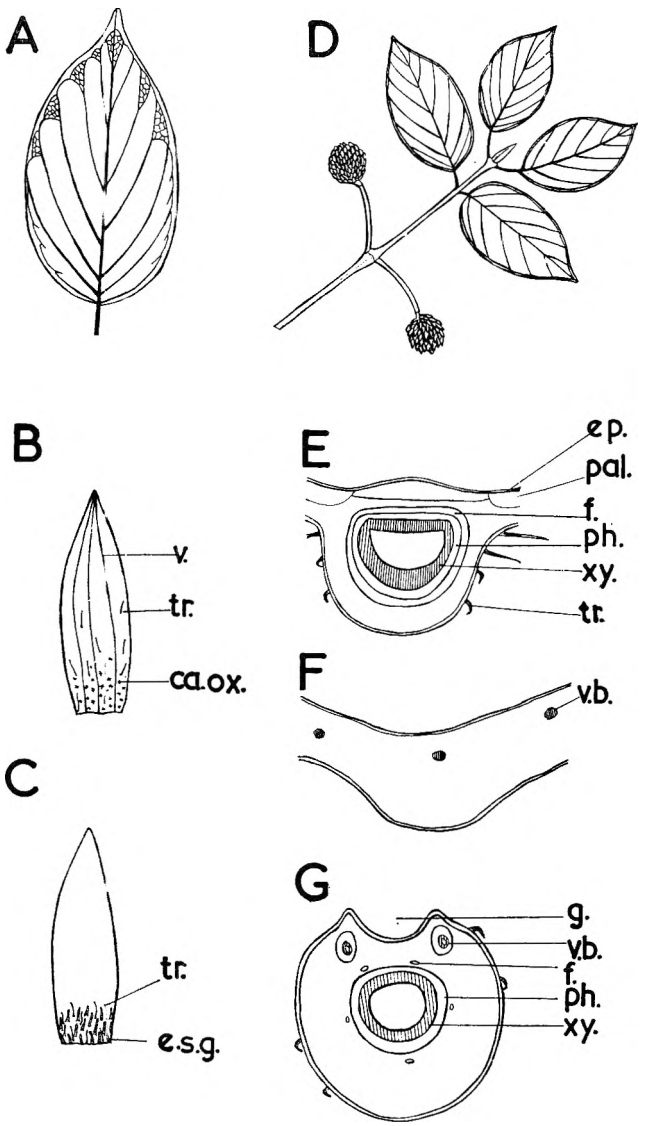

FIG. 1. Leaf × 1. B. Stipule (outer) × 1½. C. Stipule (inner) × 1½. D. Young leafy stem with fruit × ¼. E. T/S midrib of leaf. F.T/S stipule. G.T/S petiole all × 20. ca.ox., calcium oxalate; ep., epidermis; e.s.g., elongated secretory gland; f., pericyclic fibre; g., groove; ph., phloem; tr., trichome; v., vein; xy., xylem.

The *young stem* (twig) is rounded to semi-cylindrical with the outer surface reddish brown in colour. Slight longitudinal striations are present. A smooth transverse surface shows a narrow bark up to 15 mm wide with

a buff coloured radiate xylem. The greyish central pith is up to 2 mm in diameter. Branch scars are in pairs.

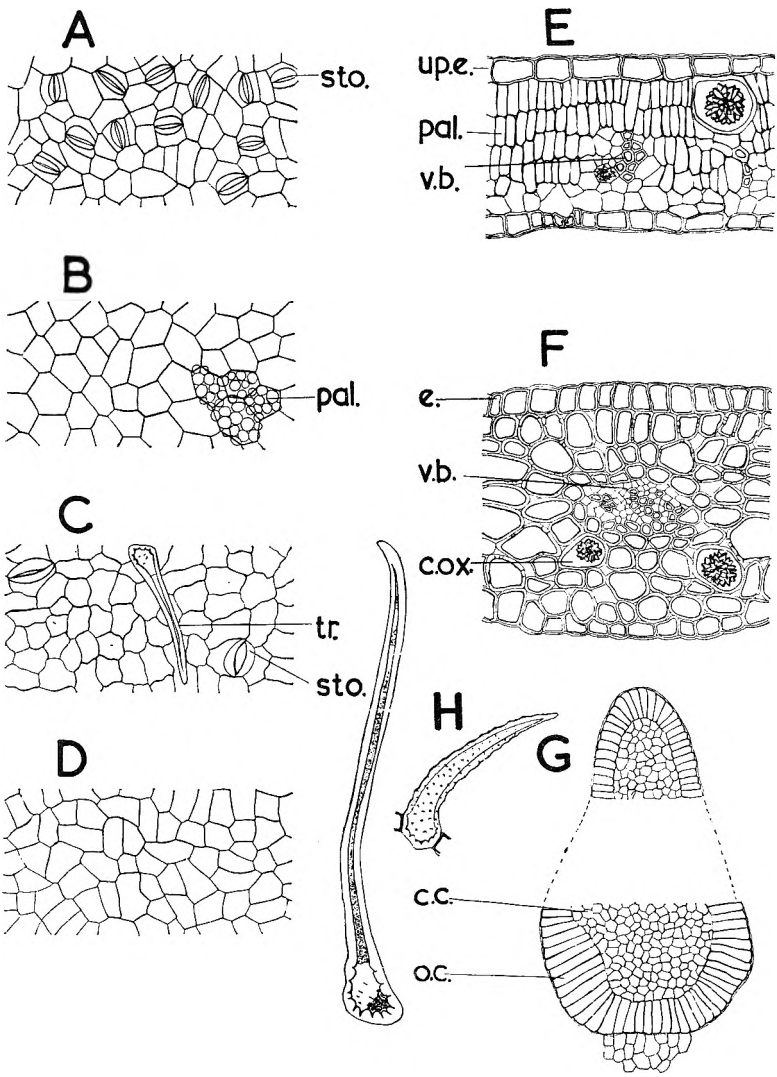

FIG. 2. A. Leaf, lower epidermis. B. Leaf, upper epidermis. C. Stipule, outer epidermis. D. Stipule inner epidermis. E.T/S leaf. F.T./S stipule through vein all × 160. G.T./S elongated secretory gland. H. Long hair-like trichome and warty trichome, × 160. c.ox., calcium oxalate; c.c., central cells; e., epidermis; pal., palisade; o.c., outer cells; sto., stomata; up.e., upper epidermis; v.b., vascular bundle.

In the description that follows, the symbols R, T, and L signify measurements taken in radial, tangential, and longitudinal planes respectively. Although a wide variety of material was used, these values cannot be regarded as absolute.

Microscopy

LAMINA (Figs 1–3)

The transverse section through the *midrib* shows that the palisade cells do not extend over the meristele. The xylem is lignified and forms a half cylinder surrounded externally by phloem tissue. (Fig. 1.E., ph; Fig. 3.A., ex.ph.), and thick walled pericyclic fibres (Fig. 1.E., f.). The central core is made up internal phloem, parenchyma and moderately thin walled fibres. Phloem is made up of sieve cells which are restricted to groups. Generally, the cells of the parenchyma have thin pitted walls, but, occasionally some have reticulate thickening (Fig. 3.A., r.p.). The xylem is made up of vessels, tracheids, and parenchyma all of which are strongly lignified. Vessels may have alternately arranged bordered pits, spiral or annular thickening. Isolated vessel elements measure R and T, 12–40 μ, L, 100 μ —1·5 mm. The parenchyma around the xylem vessels is composed of rectangular to longitudinally elongated cells with pitted walls. These measure R and T, 9–20 μ; L, 9–75 μ.

Within the epidermis is a collenchymatous zone 5–15 rows in radial depth. These cells have pitted walls up to 7 μ thick, and measure R and T, 15–35 μ; L, 35–230 μ. Large intercellular spaces are often present between these cells. The collenchyma and parenchyma may contain large cluster crystals of calcium oxalate or reddish brown material not easily removed with chloral hydrate solution. Pericyclic fibres are thick walled and non-lignified, and measure from 250 μ–4·5 mm long and 7–24 μ wide. Occassionally, some may be branched.

The *upper epidermis* of the lamina (Fig. 2B) consists of a single layer of polygonal tabular cells covered with a thin cuticle. The anticlinal walls are usually straight, and stomata and trichomes are absent. The mesophyll is clearly differentiated into a broad palisade layer made up of 3–5 rows of thin-walled cells, which occupies up to three quarters of the width of the mesophyll (Fig. 2E). The upper palisade cells are much more elongated than the lowermost ones. The spongy mesophyll is from 1–3 cells wide; large calcium oxalate crystals in idioblast cells are present; these are conspicuous and mainly found near the vascular bundles and sometimes between palisade cells; they are similar in size and shape to those of the midrib.

On the *lower epidermis* (Fig. 2A), stomata are found in abundance, and these are of the paracytic or rubiaceous type. The trichomes are found mainly along the veins and are of two distinct types. There are the thick walled hair-like curved trichomes each with a broad basal foot (Fig. 2.H). These may be unicellular, but are often divided into compartments by very thin septa; trichomes of 2–14 compartments have been found; they measure from 100–1020 μ long and 12–50 μ wide at the base. They are found in greater abundance than the short unicellular thick-walled conical, warty appressed trichomes (Fig. 2.H). These ranged from 10–165 μ long and 10–25 μ wide at their base. Both kinds of trichomes contained reddish brown material.

The palisade ratio is 7·5–10–14; stomatal index, 11·75–15·1–17·6, and vein islet number 16–20–26.

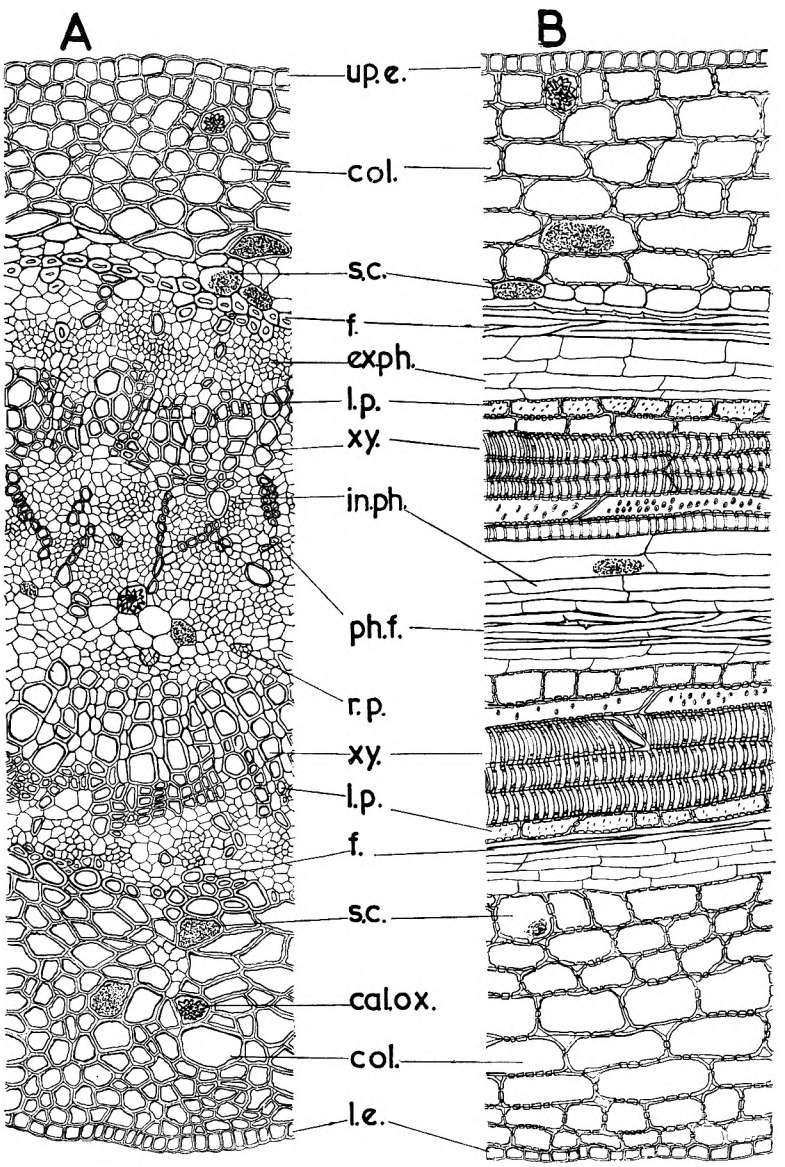

FIG. 3. A.T/S through midrib. B.L/S through midrib both × 160. cal.ox., calcium oxalate; col., collenchyma; f., pericyclic fibre; in.ph., internal phloem; l.e. lower epidermis; l.p., lignified parenchyma; ex.ph., external phloem; ph.f., phloem fibre; r.p., reticulate parenchyma; s.c., secretory cell., xy., xylem.

The *petiole* in transverse section resembles the midrib except that there are fewer pericyclic fibres around the vascular bundle. In addition to

the main bundle, there are two accessory bundles surrounded by sheath parenchyma (Fig. 1.G v.b.). The epidermal trichomes are of the thick walled warty type as described for the lamina.

The epidermal cells of both leaf and petiole contain a dense reddish brown substance not easily removed by chloral hydrate solution.

STIPULE

The inner or upper epidermis (Fig. 2.D) has no stomata, and is covered by a thin cuticle. Long hair-like trichomes are found in abundance mainly towards the base of the stipule and between the elongated secretory glands (Fig. 1C, tr.). The outer or lower epidermal cells are polygonal in surface view, except over the veins where they are elongated in the direction of the main veins; both stomata (paracytic type) and trichomes are present (Fig. 2C, sto.; tr.).

Below the epidermis is fairly thick-walled pitted parenchyma, composed of rectangular to longitudinally elongated cells (Fig. 2F). Both epidermal and parenchymatous cells are filled with a dense reddish brown substance. Large calcium oxalate crystals are found in the cells along the veins. The vascular bundle, like that of the midrib of the leaf, consists of vessels, tracheids, fibres, and phloem cells. Both fibres and vessels are lignified. In macerated material examined, vessel-elements up to 825 μ long and 4–10 μ wide have been measured. Isolated fibres measured up to 1200 μ long having blunt to contorted ends.

The elongated secretory glands (Fig. 2G) have a broad base and tapering apex. They measure 500–1150 μ in length and 130–400 μ in width at their basal end. They consist of a multicellular core of thin walled isodiametric parenchyma cells (Fig. 2G, c.c.) covered with a palisade-like layer of cells. The parenchyma contains in certain cells small solitary cluster crystals of calcium oxalate which measure from 6–18 μ in diameter. Starch is absent from leaf, petiole and stipule.

YOUNG STEM (TWIG) (Fig. 4)

The outer protective layer of the stem is made up of cork, the number of rows depending on the age of the stem. From specimens examined, the range was from 4–18 rows of radially arranged cells. The sections examined had cork cells of two types, that is cork cells tangentially and radially elongated cells (Fig. 4. ck and c'k).

All cork cells are thin walled suberised and measured R and T, 25–75 μ; L, 35–165 μ long. The *cortex* is made up of collenchyma and parenchyma. Most cortical cells contained reddish brown secretion while some have large cluster crystals of calcium oxalate. The *pericycle* is made up of thick unlignified fibres each with a small lumen. Isolated pericycle fibres measured R and T, 7·30 μ; L, 300 μ–8·1 mm long. The *secondary phloem* is made up of parenchyma, fibres, sieve cells, and is traversed by uniseriate medullary rays. Medullary ray cells have contents which are similar to those of the secretion cells of the cortex. No starch was found to be present. The cambial zone is well marked and consists of 2–3 rows of thin walled cells.

The vessels of the *secondary xylem* have mostly bordered pits while some reticulately thickened vessels and a few spiral vessels occur in the

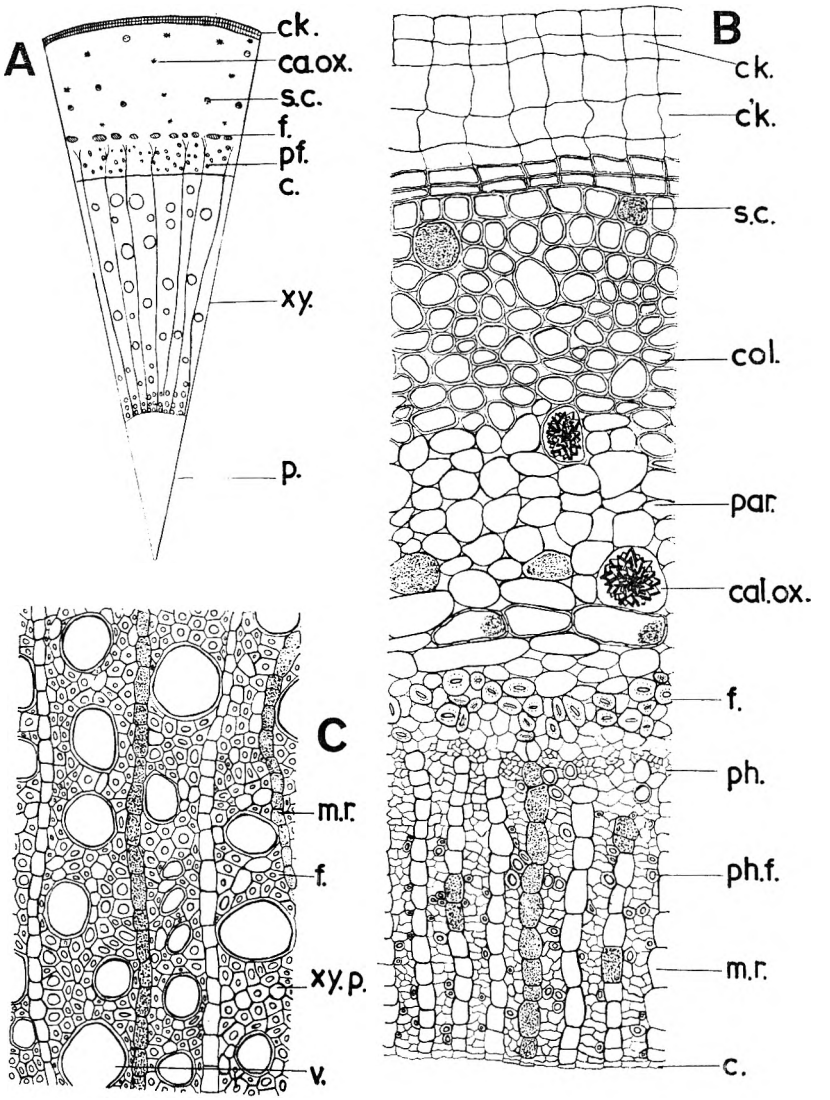

FIG. 4. A. T/S young stem (twig) × 17. B.T/S young stem bark. C. T/S section of xylem, both × 160. c., cambium; ca.ox., calcium oxalate; col., collenchyma; ck & c'k., cork cells; f., pericyclic fibres; ph., phloem; ph.f., phloem fibres; m.r., medullary rays; s.c., secretory cells; xy., xylem; xy.p., xylem parenchyma; v., vessel.

primary xylem. Bordered pitted vessels have tapering projections sometimes up to 325 μ long. Isolated vessel-elements measure R and T, 15–75 μ; L, 115 μ–1·05 mm long.

The *medullary rays* are heterogeneous, consisting of a central core of horizontally elongated cells, bordered on either side by erect vertically elongated cells. Cells of both types are lignified and pitted. Xylem fibres are thick walled with tapering to contorted ends. Isolated fibres measure R and T, 9–30 μ; L, 280 μ–3·06 mm. Both xylem parenchyma and medullary ray cells contain brown contents. Calcium oxalate crystals are absent.

The *pith* is made up of thin pitted walled parenchyma cells many of which contain reddish brown contents.

POWERED LEAF AND STEM

The features which are common to both leaf and stem powders are mainly the warty trichomes, unlignified pericyclic fibres, lignified fibres, bordered pitted vessels, cells containing reddish brown secretion or calcium oxalate. The main distinguishing features between leaf and stem powders are the long hair-like trichomes and fragements of the elongated secretory glands both of which are only found in leaf.

Discussion

The structure of the leaf, stipule and stem of *M. inermis* is typically that of the family *Rubiaceae*. The macroscopical and microscopical characters of this species can, however, readily be distinguished from that of *M. stipulosa*, and *M. ciliata* (Shellard & Shadan, 1963). The principal distinguishing feature is the presence of both pericyclic, and phloem fibres in *M. inermis*. Fibres are, however, reported absent from leaf and petiole of *M. stipulosa* and *M. ciliata*.

In *M. inermis* there are two distinct types of trichomes, the long hair-like type and the conical warty appressed trichomes. Finally, the calcium oxalate cluster crystals of *M. inermis* are much larger than those of *M. stipulosa* and *M. ciliata*.

Acknowledgement. I wish to thank Professor G. E. Trease of Nottingham University for commenting on this work, Professor A. N. Tackie for his useful suggestions and Mr. E. Enti for identifying samples of leaves and stems of the material examined.

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Letters to the Editor

Changes in sensitivity to noradrenaline in rats pretreated with reserpine

SIR,—Pretreatment with reserpine increases the sensitivity of the cardiovascular system of the cat to the actions of noradrenaline (Bein, 1953; Burn & Rand, 1958; Fleming & Trendelenburg, 1961). Similar observations were made using the anaesthetised dog and the rabbit isolated atria (Maxwell, Povalski & Plummer, 1959; Macmillan, 1959). Recently Bhagat & Shidemann (1963) and Bhagat, Booker & West (1964) did not observe increased sensitivity to noradrenaline in isolated atria of rats pretreated with reserpine and suggested that the sensitising action of reserpine varied according to the animal species and organ used. On the other hand, we have now found reserpine pretreatment to potentiate the pressor action of noradrenaline in the rat. The conflicting results would not therefore seem to be explained by the use of different species; rather that the explanation lies with the method used to demonstrate noradrenaline sensitivity.

Male rats, Sprague-Dawley, 350–450 g, were treated with a single intraperitoneal injection of reserpine (Serpasil, Ciba). 48 to 168 hr later the animals were anaesthetised with ethylurethane, 1·25 g/kg, i.p., and the carotid artery cannulated. Arterial blood pressure was recorded via the cannula by a pressure transducer (Statham P23 AC) and displayed on an ink-writing oscillograph (Grass Polygraph). Heparin was given intravenously (5 mg/kg). Noradrenaline was injected into the jugular vein of alternate animals as a logarithmic series of either progressively increasing or progressively decreasing doses. Sufficient time was taken between successive doses of noradrenaline to allow for complete recovery.

TABLE 1. EFFECT OF RESERPINE PRETREATMENT ON PRESSOR RESPONSES OF ANAESTHETISED RATS TO NORADRENALINE

Treatment	mg/kg i.p.	Time after reserpine (hr)	No. of exp.	Mean response (mm Hg ± s.e.) to noradrenaline base (μg) 0·25	0·5	1	2
Saline ..	—	—	15	35·7 ± 3·4	47·7 ± 4·6	61·3 ± 5·3	83·0 ± 6·1
Reserpine ..	5	48	15	39·8 ± 3·1	53·1 ± 4·1	78·5 ± 7·5	94·2 ± 7·4
Reserpine ..	5	72	15	42·2 ± 4·7	60·6 ± 5·4	87·6* ± 8·3	109·7† ± 6·5
Reserpine ..	5	96	13	43·6 ± 4·3	59·5 ± 6	75·1 ± 5·2	98·6 ± 5·8
Reserpine ..	5	168	10	41·0 ± 7·3	49·2 ± 6·3	68·6 ± 6·7	85·5 ± 6·3
Reserpine ..	2·5	72	17	56·5† ± 5·6	67·5* ± 5·4	94·6† ± 5·6	115·5† ± 6·6
Reserpine ..	2·5	96	7	35·0 ± 2·3	50·5 ± 5·4	73·7 ± 6·2	90·1 ± 7·8

* $P < 0.05$. † $P < 0.01$.

Table 1 shows that a single injection of 5 mg/kg of reserpine induced a moderate increase of sensitivity to noradrenaline within 48 hr. Maximal increase in sensitivity was achieved by 72 hr and then declined slowly; 7 days after reserpinisation the responses to noradrenaline had returned to normal. A smaller dose of reserpine (2·5 mg/kg) also produced an increase in sensitivity to noradrenaline in 72 hr; however, the increase in sensitivity was larger but declined more rapidly. Basal blood pressure and body temperature of reserpinised rats were not significantly different from those of controls and the

increased sensitivity was not due to non-specific factors like reduced food or water intake which occurs in reserpinised animals.

Studies in this laboratory (Giachetti & Montanari, unpublished observations) showed that the concentration of heart noradrenaline in rats treated with 5 mg/kg of reserpine is 5% of controls at 24 hr, 18% at 48 hr, and 25% at 72 hr, and so the increased sensitivity to the pressor action of noradrenaline is not directly related to the decreased concentration of the amine in the heart. These last results agree with those reported by Trendelenburg & Weiner (1962). The time elapsing after the administration of reserpine seems more important for the development of increased sensitivity. Similar conclusions were previously reached for the pressor action of noradrenaline in the cat by Fleming & Trendelenburg (1961). In our view the potentiation of noradrenaline by reserpine cannot be explained on the basis of an impairment of the uptake mechanism for the amine. Giachetti & Montanari (unpublished) have also shown that although treatment with reserpine (5 mg/kg, i.p.) reduced the uptake of injected tritiated noradrenaline into the rat heart, the effect was short-lived. The capacity of the rat heart to take up and bind the tritiated amine fully recovered within 48 hr of reserpine treatment.

The cardiovascular system of the rat can be made more sensitive to noradrenaline by reserpine. Rats need a larger dose of the alkaloid than do other animal species for the potentiation of noradrenaline effects, but this applies also to other actions of reserpine such as hypothermia and sedation. It is difficult to reconcile our findings using an *in vivo* technique with those obtained by Bhagat & others (1964) using the isolated atria. It may be noteworthy to recall, however, that some organs from reserpinised animals, for example the nictitating membrane and the iris of the cat, do not show increased sensitivity to noradrenaline *in vitro*, but do so *in situ* (Burn & others, 1959; Marley, 1962).

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Biochemical and pharmacological properties of some amidopyrine metabolites

SIR,—A number of non-steroidal anti-inflammatory drugs uncouple oxidative phosphorylation, by selectively inhibiting the biogenesis of adenosine-5′-triphosphate (ATP) coupled to mitochondrial oxidative processes, without impairing cellular respiration (Adams & Cobb, 1958; Whitehouse, 1963, 1964a). Amidopyrine (4-dimethylaminoantipyrine, pyramidone) appears to be the exception to the hypothesis that this biochemical property coincides with anti-inflammatory activity, since neither amidopyrine itself nor two of its principal metabolities in man (4-aminoantipyrine and its *N*-acetyl derivative) uncouple oxidative phosphorylation at drug levels which have some resemblance to those used clinically (Whitehouse, 1963). We have now examined this point further by testing 4-aminoantipyrine (4-aminophenazone) and two other possible metabolities of amidopyrine (Jaffe, 1901; Proscher, 1902; Pechtold, 1964), namely rubazonic acid and *N*-methylrubazonic acid, for anti-inflammatory activity and ability to uncouple oxidative phosphorylation.

Drug action on phosphorylating rat liver mitochondria was studied by previously described methods (Whitehouse, 1964b) with sodium succinate as the substrate for mitochondrial respiration. 4-Aminoantipyrine and 4-nitrosoantipyrine had no effect on the phosphorylation quotient (P/O ratio) at 3 mM. Rubazonic acid, 10 μM, and *N*-methylrubazonic acid, 200 μM, inhibited phosphorylation (mitochondrial ATP biosynthesis) by approximately 50% without affecting oxygen uptake. Rubazonic acid is therefore rather more potent than either phenylbutazone or indomethacin (Whitehouse, 1964a) in uncoupling oxidative phosphorylation.

A modified ultra-violet erythema technique (Winder, Wax, Burr, Been & Rosiere, 1958) and the cotton pellet granuloma assay (Bush & Alexander, 1960) were used to assess the anti-inflammatory activity of these compounds in guinea-pigs and rats respectively. To study analgesic, antipyretic and anti-inflammatory (anti-oedema) activities in rats simultaneously, a procedure based on the method of Randall & Selitto (1957) was used. Anti-squirming ("analgesic") activity and ability to inhibit dye-release (anti-inflammatory) were also investigated in mice (Whittle, 1964). Antipyretic activity was investigated after the intravenous injection of 0·1 ml of a 1–4 dilution of T.A.B. vaccine in rabbits (Baker, Hayden, Marshall, Palmer & Whittet, 1963).

Amidopyrine and 4-aminoantipyrine were significantly active at 200 mg/kg by mouth in the Randall & Selitto test whereas rubazonic and *N*-methylrubazonic acids showed significant activity only in the lowering of the elevated paw temperature at 400 mg/kg orally. Amidopyrine, 4-aminoantipyrine and rubazonic acid did not significantly reduce the granuloma in the cotton pellet granuloma assay, when each was administered orally at 200 mg/kg/day for 7 days. (Other results are given in Table 1).

These results show that three oxidation products (metabolites) of amidopyrine are less active than amidopyrine itself as anti-erythema, anti-oedema and analgesic agents. These pharmacological assays also distinguish between amidopyrine and 4-aminoantipyrine on the one hand and the rubazonic acids on the other but there is no relation between potency in uncoupling oxidative phosphorylation and pharmacological activity.

These results suggest that *either* (1) the anti-inflammatory activity of amidopyrine is not due to its conversion *in vivo* to the rubazonic acids, even

TABLE 1. PHARMACOLOGICAL ACTIVITIES OF AMIDOPYRINE AND SOME OF ITS OXIDATION PRODUCTS (METABOLITES)

Compound	Ultra-violet erythema	Squirming test Relative activities		Antipyretic test
	Oral ED 50 mg/kg (confidence limits)	Reduction of squirms	Reduction of vascular permeability (dye-release)	Antipyretic index
Amidopyrine	16 (10 to 25)	1·00	1·00	27·0
4-Aminoantipyrine	24 (15 to 38)	0·62	0·77	13·0
Rubazonic acid	42 (24 to 79)	0·14	0·38	—
N-Methyl rubazonic acid ..	120 (55 to 260)	0·15	0·26	12

though the latter compounds (unlike amidopyrine) behave like many non-steroidal anti-inflammatory drugs in uncoupling oxidative phosphorylation, *or* (2) insufficient of the two rubazonic acids, when administered orally, reaches those sites where amidopyrine exerts its anti-oedema and anti-erythema properties. In view of the antihistaminic activity of amidopyrine (Domenjoz, 1960) and evidence that histamine is implicated in the inflammatory response (Bhatt & Sanyal, 1964), the first conclusion does not necessarily preclude the hypothesis that ability to uncouple oxidative phosphorylation determines anti-inflammatory activity in other drugs which, unlike amidopyrine, have little or no antihistaminic activity.

We thank Dr. G. J. Durant for synthesising the rubazonic acids.

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October 15, 1964

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Alkaloids of *Voacanga schweinfurthii* Stapf: voacorine and voacangine

SIR,—In a previous communication (Fish, Newcombe & Poisson, 1960), the isolation of voacamine and vobtusine from the stem bark of *V. schweinfurthii* was described. Modification of the extraction procedure has yielded several other alkaloids of which one, voacorine, has been isolated and characterised and a second, voacangine, has been identified by its chromatographic properties and ultra-violet spectrum.

The total alkaloidal fraction of the bark was obtained by cold percolation with 70% ethanol, concentration of the percolate, adjustment to pH 9·0 and extraction with ethyl acetate. The extract was purified by shaking with 5% acetic acid, basifying the acid extract with solution of ammonia and then re-extracting with ethyl acetate. This solution was evaporated to dryness and the residue, dissolved in benzene, chromatographed on alumina. Elution with ether separated several alkaloidal fractions which were analysed by comparison with known alkaloids on circular chromatograms using Whatman No. 1 paper discs, 24 cm diameter, buffered to pH 4·7 with potassium hydrogen phthalate solution; development was with ether saturated with buffer solution. Ether eluted from the alumina column a mixture of voacangine and an unidentified alkaloid, then voacamine and, finally, a mixture of vobtusine and voacorine.

Voacangine and the unknown alkaloid were separated by thin layer chromatography using a modification of the method of Demole (1958), employing silicic acid bound with tragacanth and developing with a mixture of ether and chloroform (2:1). Neither alkaloid was obtained in crystalline form but their ultra-violet spectra were determined on solutions in ethanol. Voacangine showed absorption maxima at 225 and 287 mμ and although no values for log ϵ could be obtained, the maxima were of the same relative proportions as published (Janot & Goutarel, 1955). The other alkaloid showed a single sharp peak at 220 mμ possibly indicative of a ψ indoxyl chromophore (Scott, 1964) which thus distinguishes it from all other alkaloids so far separated from *Voacanga* species.

Concentration of the vobtusine–voacorine fraction precipitated the vobtusine and further concentration of the mother liquor yielded voacorine as rosettes of white, feathery crystals which were recrystallised from methanol and then from acetone.

Voacorine. Uncorrected m.p. 271° (decomp.), λ_{max}(EtOH) 225 mμ (log ϵ 4 66), 286 (4·20), and 295 (4·21) agreed with published figures (Goutarel & Janot, 1956; La Barre & Gillo, 1956). Infra-red spectrum coincided with that described by Goutarel & Janot (1956) and with that obtained using authentic voacorine. Found: C, 70·25; H, 7·4; N, 8·1, $C_{43}H_{52}N_4O_6$ requires C, 71·6; H, 7·3; N, 7·7%. This most recent formula was given by Budzikiewicz & others (1963) on interpretation of the mass spectrum of voacorine.

Our investigations have revealed a marked similarity between the alkaloidal constituents of the stem barks of *V. schweinfurthii* and *V. africana* Stapf, species which are very closely related taxonomically (Pichon, 1947).

We wish to thank Dr. J. Poisson, Faculty of Pharmacy, University of Paris, for samples of known alkaloids.

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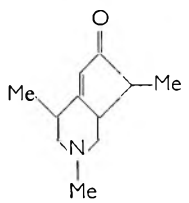
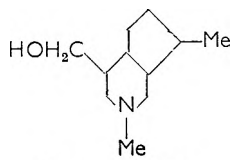
Hypoglycaemic properties of tecomine and tecostanine

SIR,—Tecomine (I) and tecostanine (II) are two alkaloids isolated by Hammouda & Motawi (1959) and Hammouda, Plat & Le Men (1963a) from the leaves of *Tecoma stans* (Juss.). The leaves of the various species of *Tecoma*



Tecomine (I)



Tecostanine (II)

have long been used orally by the natives of Mexico as antidiabetic remedies (Colin, 1926; 1927). The structures of the two alkaloids have also been elucidated (Hammouda, Plat & Le Men, 1963b; Jones, Fales & Wildman, 1963).

The present communication describes the biological assay for hypoglycaemic properties of the two alkaloids compared with tolbutamide. Normal healthy albino rabbits weighing 1·5–2 kg fasted for 12 hr were injected with tecomine and tecostanine salt solutions in isotonic saline. Their hypoglycaemic potency was calculated and related to that of tolbutamide given orally and measured by the procedure outlined by Marks (1926) for the biological assay of insulin. Blood sugar was determined by the method of Nelson (1944).

The results (Table 1) show tecomine and tecostanine to be potent hypoglycaemic agents when given intravenously. The average lethal dose was found to be 300 mg/kg in mice.

TABLE 1. HYPOGLYCAEMIC ACTION OF THE ALKALOIDS

Substance administered	Dose mg/kg	Route	Blood sugar response mg/100 ml		Time of maximal response hour : min	Mean reduction %	Hypoglycaemic potency of tolbutamide %
			Initial†	Maximal†			
Tolbutamide	250	Oral	100·1	73·7	3 : 50	26 ± 2	—
Tecomine citrate ..	20*	i.v.	98·3	52·3	3 : 14	47 ± 3·5	179
Tecostanine hydrochloride	20*	i.v.	104·9	48·6	3 : 23	49 ± 3	186

† Average of four rabbits.
* Calculated as the free base.

The two alkaloids represent a new nucleus not investigated before for hypoglycaemic effect. Further work on their detailed action on blood sugar, their beneficial effects in diabetes and their structure activity relationships is now in progress.

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Beta sympathetic inhibitory receptors in the small intestine of the guinea-pig

SIR,—The sympathetic inhibitory receptors of the gut of the cat, rat, rabbit, and dog were defined by Ahlquist (1948) as alpha receptors, but it has since been demonstrated that beta receptors also are present in the small intestine of the dog (Ahlquist & Levy, 1959) and rabbit (Furchgott, 1960). Activation of either type of receptor causes an inhibition of the gut.

In the guinea-pig, McDougal & West (1954) showed that the sympathetic inhibitory receptors on the intramural cholinergic neurones have the properties of alpha receptors, and more recently, Harry (1964) in this department has distinguished alpha inhibitory receptors in the circular smooth muscle layer. The results described below (Wilson, 1960) formed part of a communication to the British Pharmacological Society in January, 1960: they give evidence for the presence of beta inhibitory receptors in the longitudinal smooth muscle layer of the guinea-pig gut.

Isolated preparations of guinea-pig proximal small intestine were suspended at 36°, in Krebs solution containing 1 in 10,000 sodium metabisulphite as an antioxidant and bubbled with a mixture of 95% oxygen and 5% carbon dioxide. Longitudinal contractions were produced at 4 min intervals by direct stimulation of the smooth muscle with histamine or methacholine. Graded doses of noradrenaline, adrenaline, isoprenaline or phenylephrine were added to the organ bath 2 min before the next addition of agonist drug and caused an inhibition of the longitudinal contractions. The four sympathomimetic amines produced parallel log dose-inhibitory response curves, but the slope of the curves for the inhibition of histamine was steeper than the slope of the curves for the inhibition of methacholine. Estimates of the pA_2 values of the sympathomimetic amines (Table 1) showed a more pronounced inhibition of histamine than of methacholine, with a ten- to twenty-two-fold difference in potency for the inhibition of the two agonists. The sequence of inhibitory potency of the sympathomimetic amines was isoprenaline the most active, followed by noradrenaline, then

adrenaline and finally phenylephrine which had a very low potency. This order was the same whether the agonist was histamine or methacholine. The potency of isoprenaline was statistically significantly greater than that of noradrenaline

TABLE 1. ESTIMATES OF MEAN pA_2 VALUES ± STANDARD ERRORS FOR THE INHIBITION OF METHACHOLINE AND HISTAMINE CONTRACTIONS, AND APPROXIMATE DOSE EQUIVALENTS RELATIVE TO (±) -ISOPRENALINE

Sympathomimetic amine	Inhibition of methacholine		Inhibition of histamine	
	pA_2 value	Isoprenaline dose equivalents	pA_2 value	Isoprenaline dose equivalents
(±) -Isoprenaline	6·78 ± 0·10	1	7·79 ± 0·08	1
(−) -Noradrenaline	5·94 ± 0·05	7	7·20 ± 0·08	4
(−) -Adrenaline	5·69 ± 0·12	12	6·90 ± 0·17	8
(−) -Phenylephrine	2·75 ± 0·20	10,700	4·09 ± 0·21	5,000

(four and seven times; P = 0·001), adrenaline (eight and twelve times; P = 0·001) or phenylephrine (five thousand and almost eleven thousand times; P = 0·001), but the approximately two-fold greater potency of noradrenaline compared with that of adrenaline was not statistically significant.

Whatever the cause of the observed difference in the inhibition of histamine and methacholine contractions by the sympathomimetic amines, their parallel log dose-inhibitory response curves and their similar relative potencies for the inhibition of either agonist drug are suggestive evidence for an action on a single type of sympathetic receptor. The relative potencies of isoprenaline, noradrenaline, adrenaline and phenylephrine reported above are commensurate with the relative potencies of the same four amines on receptors in the rabbit duodenum which had a high sensitivity to isoprenaline, were blocked by dichloroisoprenaline, but were resistant to blockade by *N*-(2-chloroethyl)-dibenzylamine (dibenamine) or phentolamine (Furchgott, 1960). These receptors were classified as beta receptors. Similarly, in the present experiments with the guinea-pig, the relative inhibitory activities of the four sympathomimetic amines and the high potency of isoprenaline are consistent with an action on receptors of the beta type. The recording of inhibition as a reduction in the contractions caused by direct stimulation of the longitudinal smooth muscle with histamine or methacholine, excludes the inhibitory effects of the sympathomimetic amines on the cholinergic neurones and enables the beta receptors to be sited in the longitudinal smooth muscle.

The results of these experiments together with the findings of McDougal & West (1954) and of Harry (1964), give evidence for the presence of both alpha and beta receptors in the small intestine of the guinea-pig, and moreover, give evidence that the two types of receptor have different locations in the gut.

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October 22, 1964

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Book Review

PHARMACOGNOSY OF AYURVEDIC DRUGS. (Kerala). By K. Narayana Aiyer and M. Kolammal. Pp. 116. Department of Pharmacognosy, University of Kerala, Trivandrum, India. Series 1, No. 6, 1963. Rs. 7.50.

Number six of this series describes sixteen ayurvedic drugs from twenty-six plants. Of the plants described, one of them, *Cynodon dactylon*, Bermuda grass or Dog's Tooth grass, p. 18, is well-known in this country because its rhizome is used as a substitute for couch gass, *Agropyron repens*. Another plant, p. 31, *Mimosa pudica*, is the well-known sensitive plant commonly studied in botanical physiology. A third plant, *Gloriosa superba*, is well known as a hot-house climber. The remaining plants are native Indian plants not usually cultivated in this country. Each plant is illustrated by a full-page drawing; two of them, namely *Gloriosa superba* and *Asteracantha longifolia*, are represented in colour, the remainder being carefully executed line-drawings. The illustrations were prepared by two artists, Sri T. K. P. Iyer and Sri K. K. Warier.

The description of each drug begins with quotations in Sanskrit characters from the ancient writings followed by a transliteration into arabic alphabet. These notes have been prepared by two physicians acting under the guidance of the Director of Indigenous Systems of Medicine and of the Principal, Ayurveda College, Trivandrum. The information about each drug is given under two headings; first under the Ayurvedic name with quotations and transliteration from the Sanskrit; then under the systematic botanical name or names with a full description in modern form. Details are given in a regular sequence as follows. Synonyms and names in four native languages; Distribution and Habitat; Habit and General Features; External Morphology; and, when a particular plant member is used as the officinal part, that is described in detail. For seven of the drugs, the histology of the underground plant member is described and illustrated by carefully executed drawings.

The book is a valuable contribution to the study of the vegetable materia medica of Indian indigenous drugs.

T. E. WALLIS

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