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Infrared studies of hydantoin and its derivatives

T. H. ELLIOTT AND P. N. NATARAJAN

Infrared absorption spectral characteristics of hydantoin and its akyl, aryl and aralkyl derivatives are reported. The major peaks of comparative significance are discussed in detail.

THE hydantoins are a pharmaceutically important class of compounds, and the present work was undertaken primarily to provide a more certain means of characterizing those used therapeutically, and of distinguishing them from possible toxic impurities such as 5-ethyl-5-phenyl hydantoin (Nirvanol). Each of the hydantoins examined possesses a completely individual infrared spectral curve by means of which it can be readily identified. In addition, the characteristic frequencies of the imino, carbonyl, and methylene functions of hydantoin, and the influence of alkyl and aryl substituents on them, have been examined in some detail. This is because apart from the somewhat restricted original classical investigation of hydantoin and the 1-, 3- and 5-methylhydantoins by Randall, Fuson, Fowler & Dangl (1949) to assign carbonyl stretching frequencies, the similar study of hydantoin and 5-methyl- and 5-isopropyl-hydantoin by Burland & Christian (1957), and the examination of the 1,3-dihalogeno-5,5-dimethylhydantoins by Petterson, Grzeskowiak & Jules (1960), there have been no comprehensive comparative studies. Other investigations, for example by Henze & Knowles (1954), Liebermann & Kornberg (1954), Viscontini & Raschig (1959), and Fowler, Camien & Dunn (1960) have been made mainly as aids in the identification of individual compounds without the discussion of spectral details.

Experimental

Commercially available compounds were purchased. Other derivatives were prepared by a Bucherer-Bergs synthesis (Ware, 1950) or by the condensation of the corresponding amino-acid with an alkyl or aryl isocyanate and subsequent hydrolysis and cyclization (Ware, 1950). Phenylhydantoins were crystallized from an ethanol-water mixture, and methylhydantoins from ethanol. The melting points of the compounds used agreed with those recorded in the literature. Spectra were recorded on a JASCO model IR-S double-beam spectrophotometer with sodium chloride optics. The reproducibility was 0·5 cm^{-1} and the resolution 1 cm^{-1} at 1,000 cm^{-1}. All spectra were calibrated with a standard polystyrene film at 3,026, 1,603 and 1,028 cm^{-1} and the necessary corrections have been made. Peak absorptions in the 4,000 cm^{-1} to 1,250 cm^{-1} region were confirmed on a Perkin Elmer NIR 137G Infracord grating spectrophotometer, the accuracy of which in the 3,000 cm^{-1} region is better than 6 cm^{-1}.

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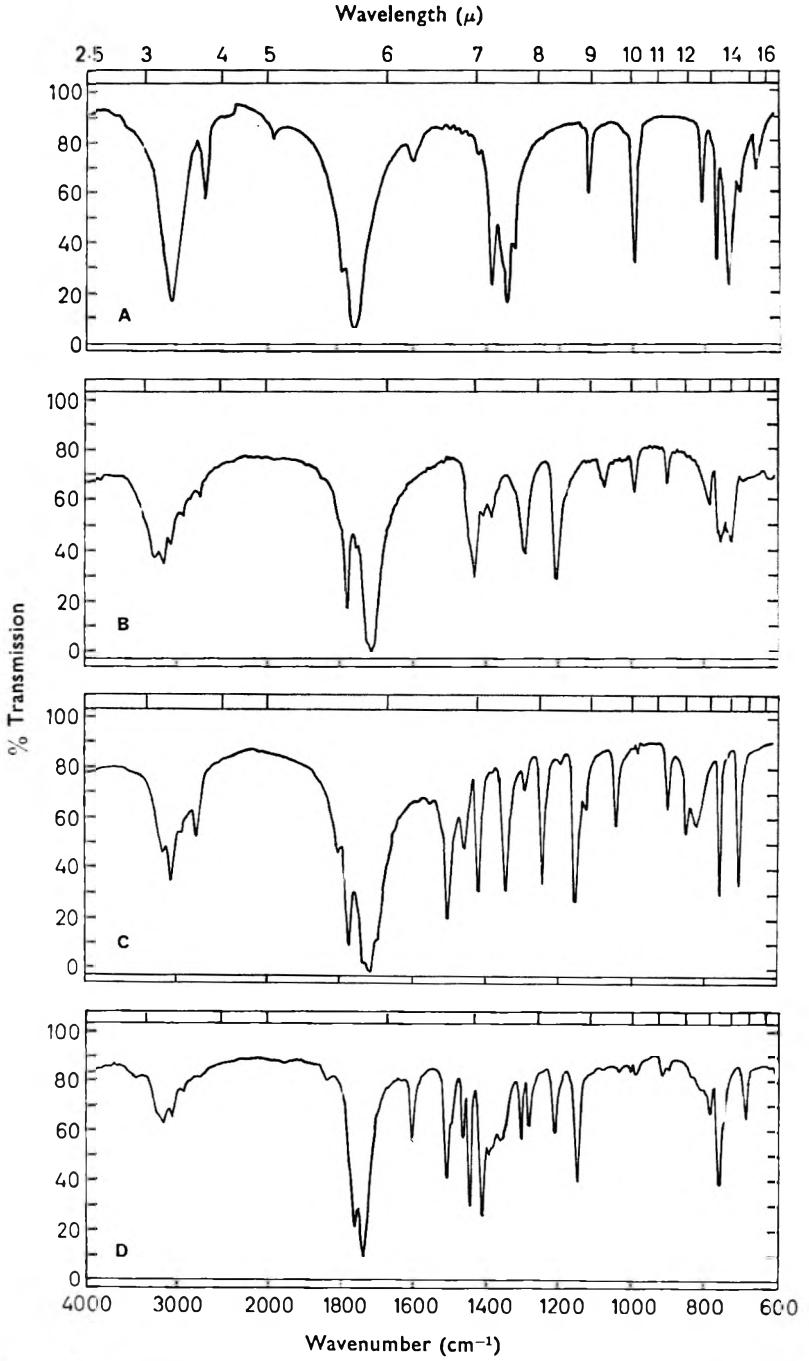
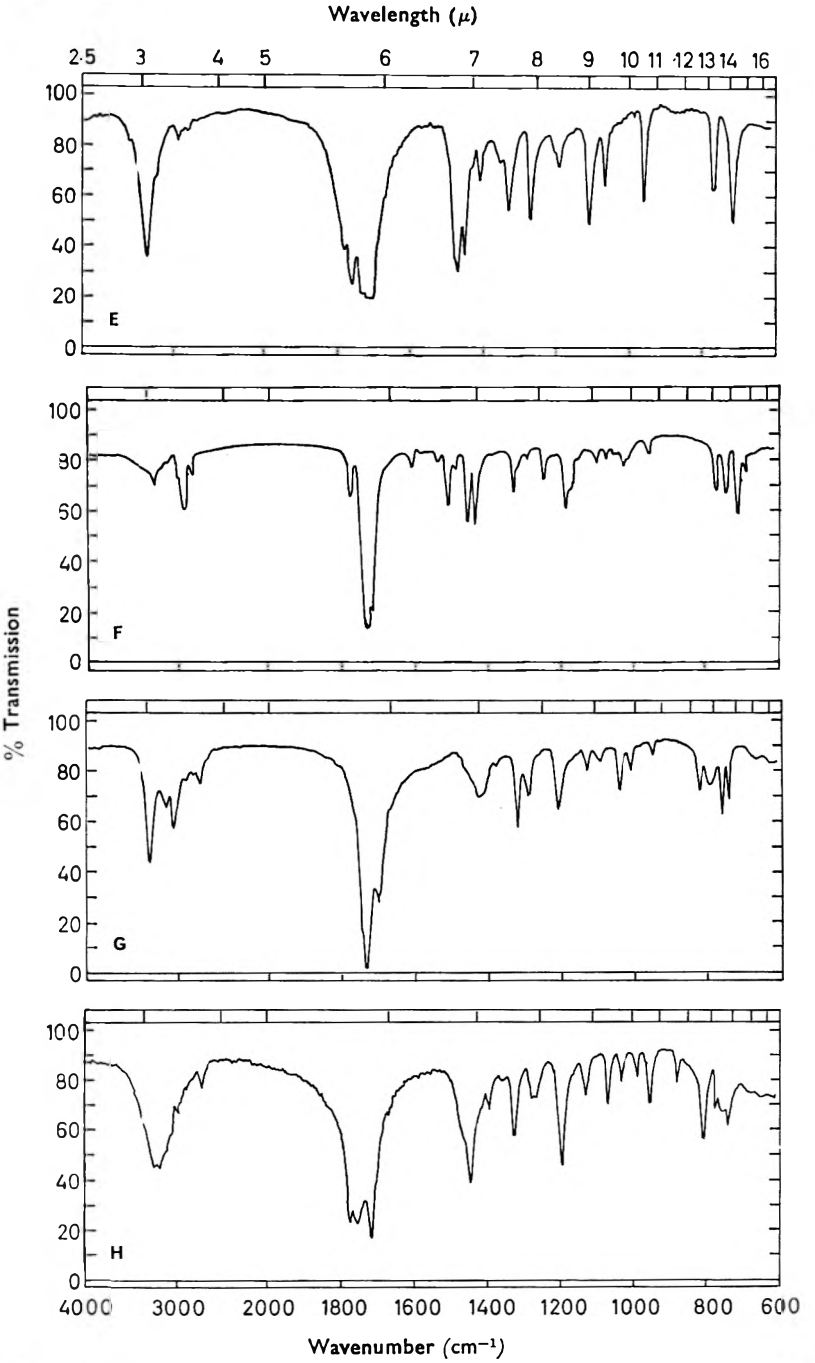


A, parabanic acid. B, hydantoin. C, 1-methylhydantoin. D, 1-phenylhydantoin.



E, 3-methylhydantoin. F, 3-phenylhydantoin. G, 5-methylhydantoin. H, 5-ethylhydantoin.

Results and discussion

C-H STRETCHING VIBRATIONS

The methylene group of hydantoin shows a significant peak of medium intensity at 2,910 cm^{-1} ascribable to in-phase stretching vibrations, and a peak of weak intensity at 2,840 cm^{-1} due to out-of-phase stretching vibrations. Alkyl substitution at position 5 results in an absorption band of varying intensity in the region 2,990 to 2,960 cm^{-1}, probably due to methyl asymmetrical stretching modes. The absorptions appearing in the 2,920 cm^{-1} region can be assigned to in-phase stretching vibrations of either the methylene group of the hydantoin ring or the alkyl side-chain, or of the methine group of the hydantoin ring. Out-of-phase methylene stretching vibrations in the region 2,880 to 2,820 cm^{-1} are seen in all derivatives with a methylene group either in the ring or in the alkyl side-chain. The absorptions in the region 2,760 to 2,700 cm^{-1} are probably overtone or combination frequencies of the deformation modes of the methyl, methylene and methine groups.

C-H DEFORMATION VIBRATIONS

Asymmetric methyl and methylene deformation frequencies in hydantoin and its derivatives occur in the region 1,476 to 1,403 cm^{-1}, and band intensities are proportional to the number of alkyl substituents. The broadest band, extending from 1,476 to 1,450 cm^{-1} is seen in 1 3,5,5-tetramethylhydantoin (P). In 3,5,5-trimethylhydantoin (O), the band extends from 1,462 to 1,452 cm^{-1}, whilst 1,5,5-trimethylhydantoin (N) shows a sharp band at 1,475 cm^{-1}. In all three compounds a further absorption of variable intensity occurs in the 1,420 cm^{-1} region.

Splitting of the infrared bands when more than one alkyl group is attached to a single carbon atom has been noted by Smith (1948), and by McMurry & Thornton (1952), and can be seen here in the spectrum of 5,5-dimethylhydantoin (J), where the multiple substitution of similar alkyl groups attached to the C-5 atom causes a split of the asymmetric absorption frequency, resulting in two bands at 1,440 and 1,430 cm^{-1}. The asymmetric C–H deformation mode of an alkyl group attached to a nitrogen atom in hydantoin derivatives is not significantly shifted. 3-Methylhydantoin (E) has two strong bands at 1,465 and 1,448 cm^{-1} and a weak band at 1,408 cm^{-1}. 1-Methylhydantoin (C) shows a medium absorption at 1,456 cm^{-1} and a strong absorption at 1,416 cm^{-1}.

The C–H symmetric deformation mode of methyl and methylene groups in hydantoin and its derivatives can be assigned to the region between 1,395 and 1,310 cm^{-1}. Hydantoin (B) itself has a medium band at 1,386 cm^{-1} and 1,3,5,5-tetramethylhydantoin (P) a strong band at 1,395 cm^{-1}. The other derivatives have usually two or sometimes more bands in this region. Multiple substitution causes, as in the asymmetric mode, a split of the symmetric deformation frequency, and alkyl substitution on a nitrogen atom is characteristic in its symmetric deformation mode; a single strong band occurs at 1,342 cm^{-1} for 1-methylhydantoin (C) and at 1,330 cm^{-1} for 3-methylhydantoin.

INFRARED STUDIES OF HYDANTOIN AND ITS DERIVATIVES

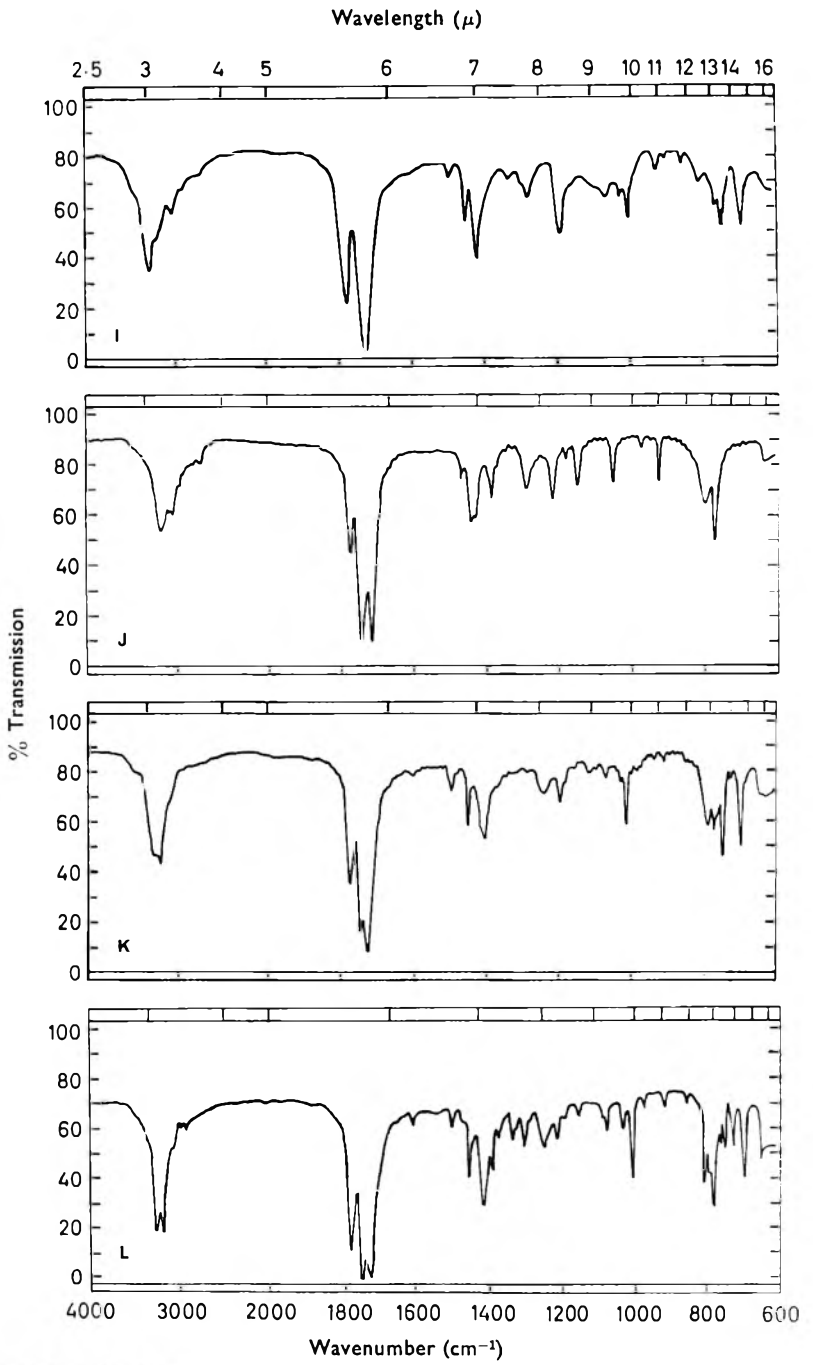
LOW-FREQUENCY VIBRATIONS

Hydantoin and all its derivatives show without exception a characteristic band varying in intensity from medium to strong in the region of 768 to 755 cm^{-1}, which is probably associated with the breathing vibration of the ring. A strong benzene ring monosubstitution band at 695 cm^{-1} is seen in all 5-phenylhydantoins and this can be regarded as diagnostic. Phenyl substitution on a nitrogen alone causes a slight shift of this band to lower frequencies; for example 3-phenylhydantoin (F) shows a weak band at 684 cm^{-1} and 1-phenylhydantoin (D) a medium absorption at 688 cm^{-1}.

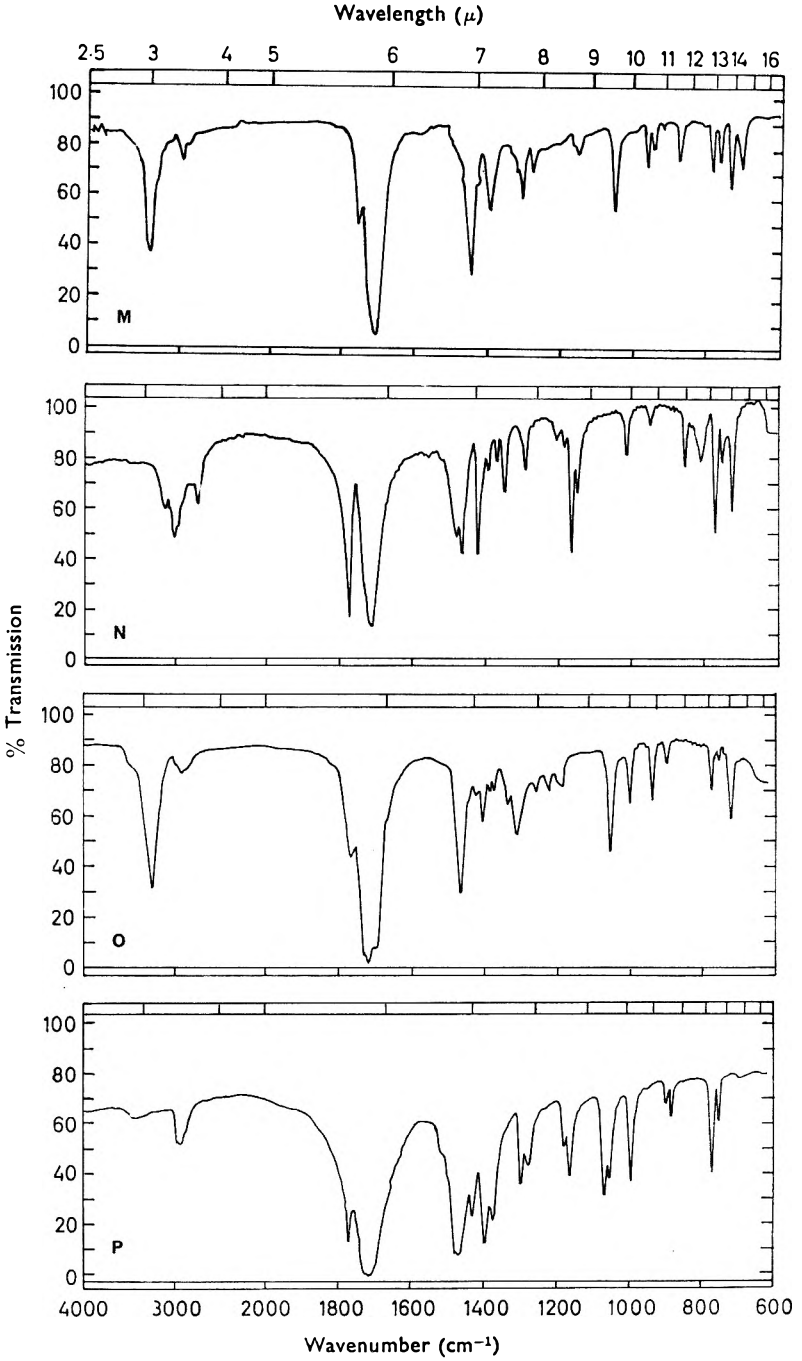
Randall & others (1949), from a consideration of the spectra of hydantoin (B), 1-methylhydantoin (C), 3-methylhydantoin (E) and 5-methylhydantoin (G), have assigned the bands occurring in the region 1,776 to 1,730 cm^{-1} to the stretching frequency of the carbonyl group in position 4 and the bands occurring in the region 1,712 to 1,695 cm^{-1} to the stretching frequency of the carbonyl group in position 2. The values recorded in this work for a more extended range of hydantoin derivatives in potassium bromide discs generally correspond with those reported by Randall & others (1949) for Nujol mull preparations. The significant difference, however, is the occurrence of a third carbonyl stretching absorption in hydantoin and all its derivatives in the region 1,756 to 1,730 cm^{-1}, which was also observed in Nujol mull preparations provided that the concentration of the hydantoin was not excessive. In relatively thick preparations, only two peaks were noted. Whether this third peak was due to polymorphism of samples in the solid state is difficult to determine, as most of the derivatives of hydantoin are insoluble in the common spectroscopic solvents. However, in those derivatives that were sufficiently soluble in chloroform for liquid spectra to be measured, viz. 1,3,5,5-tetramethyl- (P), 1,5,5- (N) and 3,5,5-trimethyl (O), 3-methyl- (E) and 3-phenylhydantoin (F) a third peak could be seen in the 1,740 cm^{-1} region and the spectra were virtually identical with those observed in potassium bromide disc preparations.

N–H STRETCHING FREQUENCY

Three absorption bands in the region of 3,390 to 3,220 cm^{-1}, 3200 to 3,120 cm^{-1}, and 3,080 to 3,040 cm^{-1} are seen in hydantoin and most of its derivatives. Substitution on both the nitrogen atoms, as in 1,3,5,5-tetramethylhydantoin (P), results in the total disappearance of these bands. A single absorption band occurs in the region of 3,280 to 3,220 cm^{-1} in the case of 3-methyl- (E), 3-phenyl- (F) and 3,5,5-trimethylhydantoin (O), all of which bear a substituent in position 3. This leads to a reasonable assignment of the absorption frequency in this region to the N–H stretching mode of the imino-group in position 1. Substitution of the imino-group in position 3 results in the disappearance of the absorption band in the 3,080 to 3,040 cm^{-1} region. Parabanic acid (A), in which both the amino-groups are situated similarly to that in position 3 in hydantoin,



I, 5-phenylhydantoin. J, 5,5-dimethylhydantoin. K, 5,5-diphenylhydantoin (Phenytoin). L, 5,5-phenylethylhydantoin (Nirvanol).



M, 5,5-phenylethyl-3-methylhydantoin (Mesantoin). N, 1,5,5-trimethylhydantoin. O, 3,5,5-trimethylhydantoin. P, 1,3,5,5-tetramethylhydantoin.

shows a single strong band at 3,040 cm^{-1}. On the basis of these observations, it seems reasonable to assume that the absorption band in the region of 3,080 to 3,040 cm^{-1} is due to the N–H stretching mode in position 3 and is possibly characteristic of the grouping CO–NH–CO in cyclic systems. The third band in the 3,200 to 3,120 cm^{-1} region could be attributed to either of the two imino-functions, though it follows more closely the pattern of the absorption band in the region of 3,080 to 3,040 cm^{-1}.

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Dual actions of some *N*-(1-phenylethyl)guanidines on the nictitating membrane of conscious cats

R. FIELDEN

After 6 hr, high doses (10 or 20 mg/kg) of some adrenergic neuron blocking drugs derived from *N*-(1-phenylethyl)guanidine produce relaxation of the nictitating membranes of conscious cats which is less than that produced by small doses (2·5 or 5 mg/kg). After 24 hr, there were marked responses to some drugs that had produced little effect after 6 hr. High doses of the drugs contract the nictitating membranes of cats treated with pempidine or (−)-*N*-(1-phenylethyl)guanidine; low doses do not. This contraction opposes the relaxation evoked by the adrenergic neuron blockade and could account for the anomalous dose-response relationships. The contraction may result from localized release of noradrenaline from the nictitating membrane.

ALTHOUGH adrenergic neuron blocking drugs generally relax the nictitating membranes of conscious cats (Exley, 1957), some may, under certain conditions, contract the nictitating membranes (Fielden, Roe & Willey, 1964; Fielden & Green, 1966a). This paper describes the two actions of some *N*-(1-phenylethyl)guanidines on the nictitating membranes of cats.

Experimental

The guanidines have been described earlier (Fielden, Green & Willey, 1965); doses refer to sulphates.

Drugs were given in sterile 0·9% saline by subcutaneous injection into the flank. From photographs of the eyes, taken at intervals, the percentage relaxation of the nictitating membranes was calculated. The eye was never fully covered by the membrane, the maximal relaxation being between 65 and 70%. Cats were used repeatedly, leaving at least a week between experiments.

In some cats the right superior cervical ganglion, together with a length of postganglionic nerve, was removed under pentobarbitone anaesthesia; sometimes the left cervical sympathetic nerve was also cut low in the neck. The animals were used 7 to 21 days after the operation.

Results

The nictitating membranes of conscious cats relaxed after administration of *N*-(1-*o*-tolylethyl)guanidine, *N*-(1-*m*-tolylethyl)guanidine, *N*-(1-*p*-tolylethyl)guanidine or *N*-(1-*p*-chlorophenylethyl)guanidine. Fig. 1 shows the degree of relaxation in cats 6 hr after injecting doses from 1·25 to 20 mg/kg. For every compound there was a dose producing a maximal effect, larger doses causing less effect or sometimes none. Such a phenomenon did not occur with xylocholine bromide at doses from 1 to 40 mg/kg. Responses were sometimes different 24 hr after drug administration. Cats which showed no overt effects 6 hr after doses of 10 or 20 mg/kg of some of the drugs had relaxed nictitating membranes

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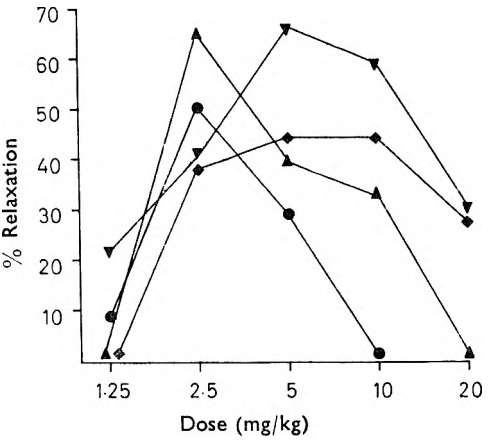

FIG. 1. Dose-6 hr-response curves for some *N*-(1-phenylethyl)guanidines. Response is the relaxation (%) of the nictitating membranes of conscious cats. Drugs were injected subcutaneously. A maximum response with these drugs is 65–70% relaxation. ▲—▲, *N*-(1-*o*-tolylethyl)guanidine sulphate. ●—●, *N*-(1-*m*-tolylethyl)guanidine sulphate. ◆—◆, *N*-(1-*p*-tolylethyl)guanidine sulphate. ▼—▼, *N*-(1-*p*-chlorophenylethyl)guanidine sulphate.

on the following day. This was particularly so after *N*-(1-*o*-tolylethyl)-guanidine. In contrast, 10 or 20 mg/kg of the *m*-tolyl compound did not relax the membranes either 6 or 24 hr after injection. These findings are summarized in Table 1.

TABLE 1. RELAXATION (%) OF THE NICTITATING MEMBRANES OF CATS 6 AND 24 HR AFTER VARIOUS DOSES OF SOME *N*-(1-PHENYLETHYL)GUANIDINES (R-CH(Me)NHC(NH)NH_2). A MAXIMUM EFFECT WITH THESE DRUGS IS 65–70% RELAXATION

R	Dose (mg/kg) (of sulphate)	Nictitating membrane relaxation %	
		6 hr	24 hr
o-Tolyl	2·5	65	25
	10	35	55
	20	0	60
m-Tolyl	2·5	50	5
	10	0	0
	20	0	0
p-Tolyl	2·5	40	15
	10	45	10
	20	30	20
p-Chlorophenyl ..	2·5	40	55
	10	60	65
	20	30	65

Cats in 2 groups of 5 were given 5 mg/kg of (−)-*N*-(1-phenylethyl)-guanidine [a potent adrenergic neuron blocking drug (Fielden & others, 1965)], alone, or together with 20 mg/kg of *N*-(1-*o*-tolylethyl)guanidine or *N*-(1-*m*-tolylethyl)guanidine or (+)- or (−)-*N*-(1-*p*-tolylethyl)guanidine. Whereas the nictitating membranes were 65% relaxed 6 hr after the

(−)-*N*-(1-phenylethyl)guanidine itself, or after the mixture with the (−)-isomer of the *p*-tolyl compound, there was little or no response to the other drug combinations. Twenty-four hr after giving the drugs the membranes of all the cats were about 30% relaxed.

In another experiment, cats in 2 groups of 4 were given 5 mg/kg of (−)-*N*-(1-phenylethyl)guanidine, alone, or with 2·5, 5 or 10 mg/kg of *N*-(1-*o*-tolylethyl)guanidine. The 10 mg/kg dose greatly reduced the response to the first mentioned compound, the 5 mg/kg dose had little effect and the 2·5 mg/kg dose none.

The experiment was repeated using pempidine tartrate (5 mg/kg) instead of (−)-*N*-(1-phenylethyl)guanidine. This time the relaxation of the membranes caused by the pempidine was completely prevented by 10 mg/kg of the *o*-tolyl compound; the other doses had little or no effect.

Eight cats were treated with (−)-*N*-(1-phenylethyl)guanidine (5 mg/kg) and 8 with pempidine tartrate (5 mg/kg). One hr later, when the nictitating membranes had relaxed, 2 cats from each group were given 2·5 or 20 mg/kg of one of the following: *N*-(1-*o*-tolylethyl)guanidine, *N*-(1-*m*-tolylethyl)guanidine or (+)- or (−)-*N*-(1-*p*-tolylethyl)guanidine. Both doses of the (−)-isomer of the latter compound had no effect. The 20 mg/kg dose of the other drugs fully retracted the membranes for at least 5 hr. The lower dose had no such actions.

In 2 cats 7 to 21 days after removal of the right superior cervical ganglion and preganglionic section of the left cervical sympathetic nerve, 20 mg/kg of *N*-(1-*o*-tolylethyl)guanidine completely retracted the left nictitating membrane for at least 5 hr, but had no effect on the right membrane.

Discussion

Except for (−)-*N*-(1-*p*-tolylethyl)guanidine, high doses of the ring-substituted *N*-(1-phenylethyl)guanidines prevent the nictitating membranes relaxing after an adrenergic neuron blocking agent or a ganglion blocking drug. Similarly high, but not low, doses contract the nictitating membranes of cats pretreated with pempidine or (−)-*N*-(1-phenylethyl)-guanidine.

The anomalous dose-response curves in Fig. 1 are therefore the result of the drugs having two opposing actions, but with different "threshold" doses. Since the compounds are potent adrenergic neuron blocking drugs, low doses relax the nictitating membranes; but, as the dose is increased, the membrane contracting action begins to predominate and so the degree of membrane relaxation decreases.

These drugs differ, therefore, from the more specific antagonists of adrenergic neuron blockade such as those described by Fielden & Green (1966b).

The time-response relationships in Table 1 suggest that the membrane contracting action of the *o*-tolyl and *p*-chlorophenyl compounds is shorter-lasting than the adrenergic neuron blocking action, so that 24 hr after a large dose only the blocking action remains. Since after *N*-(1-*m*-tolylethyl)guanidine and *N*-(1-*p*-tolylethyl)guanidine, there was no increase

in response overnight, the duration of the contracting action must be similar to that of the blocking action. With the *p*-tolyl compound, at least, the contracting action is due largely to the dextrorotatory isomer.

Although these ring-substituted *N*-(1-phenylethyl)guanidines cause slight loss of noradrenaline from tissues (Fielden & Green, 1965), the nictitating membrane contraction is unlikely to result from a general release of noradrenaline, since the *o*-tolyl compound, at least, does not retract the nictitating membrane relaxed by removal of the superior cervical ganglion, and thus made extremely sensitive to catecholamines (Lockett, 1950). Such a lack of response also precludes a direct muscle stimulant action. Ganglion stimulation is unlikely, since these drugs act in pempidine-treated cats. Since, however, removal of the superior cervical ganglion results within 48 hr in almost complete loss of noradrenaline from the nictitating membrane, but section of the preganglionic nerve does not (Kirpekar, Cervoni & Furchgott, 1962), the contraction induced by these drugs seems to be dependent on the integrity of the nerve endings and possibly on their stores of noradrenaline. The compounds may, therefore, cause contraction by a localized release of noradrenaline from the nictitating membrane.

The results described in this paper have some relevance when testing drugs for adrenergic neuron blocking activity in cats, when to induce a response it may, paradoxically, be necessary to reduce and not to increase the dose.

Acknowledgement. I wish to thank Mr R. J. Eden for his skilful assistance.

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Rhodexin A and rhodexoside in *Ornithogalum umbellatum*

J. A. SMITH AND G. R. PATERSON

Two Kedde-positive compounds, isolated in crystalline form from bulbs of *Ornithogalum umbellatum*, were shown by their infrared and ultraviolet spectra to be cardenolides. Paper chromatographic analyses of the hydrolysates of these compounds indicated that both compounds contain sarmentogenin as the aglycone moiety, and that one is a rhamnoside while the other is the corresponding rhamnoside-glucoside. The identification of the monoglycoside as rhodexin A, i.e. sarmentogenin-α-L-rhamnoside, a glycoside previously isolated from *Rhodea* (*Rohdea*) *japonica*, was confirmed by comparison with an authentic sample. The diglycoside, an apparently new compound of composition sarmentogenin-rhamnose-glucose is designated rhodexoside.

AFTER the initial pharmacological examination of bulbs of *Ornithogalum umbellatum* (Waud, 1951; Waud & Boyd, 1954; Vogelsang, 1956), two cardenolide glycosides were isolated and identified as convallatoxin and convalloside (Mrozik, Waud, Schindler & Reichstein, 1959). Of the six remaining Kedde-positive spots noted on paper chromatograms developed in toluene–butanol (1 : 1) with water as the stationary phase, that of highest Rf value was noted by these authors to be approximately the same in position as the glycoside vallarotoxin which had been isolated from *Convallaria majalis*.

We have isolated from the bulbs of *Ornithogalum umbellatum*, two Kedde-positive compounds which we identified as sarmentogenin-α-L-rhamnoside, previously isolated by Nawa (1951, 1952) from *Rhodea* (*Rohdea*) *japonica*, and sarmentogenin-L-rhamnose-D-glucose. This apparently new compound is designated rhodexoside.

Experimental and results

MATERIALS AND GENERAL PROCEDURES

Chloroform U.S.P. dried over calcium chloride and distilled before use; ethanol prepared aldehyde-free (U.S.P. XVI). Alumina, neutral activity grade 2 (Woelm), magnesium silicate, activity grade 3 (Woelm), and silica gel, 60 to 200 mesh, reagent (Fisher Scientific).

Adsorption and partition column chromatography were according to Reichstein & Shoppee (1949) and Neher (1959, 1964) respectively. For paper chromatograms, Whatman No. 1 paper and the descending technique were used. Thin-layer plates were prepared with silica gel G (Merck, Darmstadt) according to Duncan (1962), using a slurry of 1 part gel with 2 parts ethanol–water (1 : 1) shaken for 15 min before application.

Reference materials included convallatoxin (California Corporation for Biochemical Research), digoxigenin (Aldrich Chemical Co.), strophanthidin and L-rhamnose (Mann Research Lab.), and D-glucose (British Drug Houses), as well as authentic samples of sarmentogenin and rhodexin A. All other chemicals were of reagent grade.

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Enzymatic hydrolysis was with *Suc d'Helix pomatia* (Industrie Biologique Française S.A.) in McIlvaine's pH 4·8 buffer (McIlvaine, 1921), prepared from 9·86 ml of 0·2M disodium hydrogen phosphate mixed with 10·14 ml of 0·1M citric acid.

For the detection of cardenolide glycosides and aglycones, Kedde reagent (Bush & Taylor, 1952) and 2,2′,4,4′-tetranitrobiphenyl reagent (Mauli, Tamm & Reichstein, 1957) were used with paper and thin-layer chromatograms, respectively. 2,2′,4,4′-Tetranitrobiphenyl was synthesized according to Ullman & Bielecki (1901). Aniline hydrogen phthalate reagent (Partridge, 1949) was used to detect sugars on paper chromatograms.

Melting ranges (Kofler block) are uncorrected. Infrared spectra, from potassium bromide micropellets, were determined with a Beckman IR-8 spectrophotometer. Ultraviolet spectra were determined in methanol using silica cells of 1 cm path length in a Beckman DK-2 spectrophotometer fitted with a hydrogen lamp.

EXTRACTION

Bulbs of *Ornithogalum umbellatum* collected in Southern Ontario were air-dried at 40° (approximately 8 weeks), and ground to a coarse powder. The extraction procedure was similar to that of Mrozik & others (1959). The dry bulb material was extracted once with ethanol–water (1 : 1) or (2 : 1) and subsequently with ethanol until the marc was no longer bitter. Each extract was filtered through Celite 535. The combined extracts were evaporated under reduced pressure at 40° to an aqueous suspension which was subsequently diluted 1 : 4 with ethanol. The decanted supernatant extract, combined with ethanol–water (3 : 1) washings of the Kedde-negative, amber mucilaginous precipitate was evaporated to an aqueous suspension and extracted with chloroform and chloroform–ethanol (2 : 1) and (3 : 2) until one portion was negative to Kedde reagent. The chloroform–ethanol (2 : 1) extracts were washed with distilled water, sodium carbonate solution (M), and again with distilled water, before being dried over anhydrous sodium sulphate and evaporated to dryness under reduced pressure.

ISOLATION OF GLYCOSIDE I

From 1 kg and 2 kg weights of dry bulb material were obtained respectively 2·13 g and 4·86 g dry extract which were chromatographed by adsorption on silica gel and alumina respectively. Elution was with chloroform, followed by chloroform containing increasing concentrations of methanol.

The foam-like, Kedde-positive fractions from both columns were combined. This product (1·6 g) was re-chromatographed by partition using the solvent system toluene–butanol (1 : 1) with water as stationary phase using silica gel (1·25 kg) equilibrated with water (625 g). Fractions (150 ml) were collected at 0·6 ml/min.

Fraction 7 from this column yielded crude crystals (50·5 mg) from

ether-methanol which after recrystallization four times from this solvent, gave light buff-coloured platelets (19 mg); this product is designated glycoside I.

ISOLATION OF GLYCOSIDE II

The dry extract (2·48 g) from 2 kg dry powdered bulbs was chromatographed by adsorption on silica gel with elution by chloroform followed by chloroform containing increasing concentrations of methanol.

The foam-like, Kedde-positive fractions were combined (1·49 g) and re-chromatographed on silica gel (750 g) equilibrated with water (375 g), using toluene–butanol (1 : 1)/water. Fractions (90 ml) were collected at 0·5 to 0·6 ml/min. Paper chromatography of the resulting fractions in chloroform–tetrahydrofuran–formamide (50 : 50 : 6·5)/formamide (Kaiser, 1955) indicated in fractions Nos 26 to 51, three compounds of lower Rf value than convallatoxin. These fractions were combined and the product (379 mg) was chromatographed on silica gel as above. On paper chromatography, a single Kedde-positive substance was found in fractions Nos 42 to 60, the average Rr values of which, relative to convallatoxin, were 0·77 in toluene-butanol (1 : 1)/water (Schenker, Hunger & Reichstein, 1954) and 0·17 in chloroform–tetrahydrofuran–formamide (50 : 50 : 6·5)/formamide.

Fraction Nos 48–60 were combined and the product (75 mg) was chromatographed on magnesium silicate using chloroform–methanol. Fractions from this column yielded a white crystalline product (30 mg) from ether–methanol; this product was designated glycoside II.

CHARACTERIZATION OF THE ISOLATED GLYCOSIDES

Paper chromatography of isolated glycoside I (50 μg samples) in the solvent systems toluene–butanol (1 : 1)/water, benzene–n-amyl alcohol (1 : 1)/water (Kubelka & Wichtl, 1963), and chloroform–tetrahydrofuran–formamide (50 : 50 : 6·5)/formamide, and thin-layer chromatography (20 μg samples) in ethyl acetate–methanol–water (16 : 3 : 1) indicated that glycoside I was identical in Rf values with rhodexin A.

Microhydrolyses of 0·8 mg glycoside I, 1·1 mg glycoside II, and 0·6 mg rhodexin A were made according to Mannich & Siewert (1942). Paper chromatographic analyses of 5 μl aliquots of the hydrolysates were made after 11 and 14 days using the solvent systems chloroform/formamide (Schindler & Reichstein, 1951), xylene–methyl ethyl ketone (1 : 1)/formamide (Kaiser, 1955), and benzene–chloroform (3 : 7)/formamide (Schindler & Reichstein, 1951). In addition, 2 μl aliquots of the hydrolysate of glycoside I were chromatographed on thin-layer plates developed with ethyl acetate using sarmentogenin, strophanthidin, and digoxigenin as reference compounds. In each solvent system the hydrolysates of glycoside I and authentic rhodexin A gave rise to identical spots and the major Kedde-positive spot noted in each of the glycoside hydrolysates was identical in Rf value with sarmentogenin.

Samples of each of the isolated glycosides (1·0 mg) and rhodexin A (0·6 mg) were hydrolysed according to Kiliani (1930). Paper chromatographic analyses of 5 μl aliquots were made with the solvent systems butanol/water (Kaiser, 1955) and methyl ethyl ketone–butanol (1 : 1)/borate buffer (Kraus, Jäger, Schindler & Reichstein, 1960), with D-glucose and L-rhamnose as reference sugars. A single spot, identical in Rf value with L-rhamnose, was detected in the hydrolysates of glycoside I and rhodexin A. The hydrolysate of glycoside II gave two spots of Rf values identical with those of D-glucose and L-rhamnose. These two spots appeared of equal intensity, indicating equimolar quantities of the two sugars in the glycoside.

Glycoside I had melting range 248–252° (rhodexin A, 240–246°); no significant depression was noted on admixture. The melting range of glycoside II was 180–188°. Glycosides I and II had $[\alpha]_D^{22}$ — 23·1° and $[\alpha]_D^{20}$ — 23·9° (methanol) respectively [Nawa, 1951, 1952a, gives $[\alpha]_D$ — 20° (ethanol) for rhodexin A].

The ultraviolet spectrum of glycoside I showed a single absorption maximum at 218 mμ (log ϵ^* = 4·21); for glycoside II it was at 216 mμ (log ϵ^* = 4·16). The infrared spectra of glycoside I and rhodexin A showed identical absorption bands throughout the 2·5 to 15 μ region. The series of bands noted at 5·56 μ, 5·76 μ, and 6·18 μ for glycoside I and at 5·62 μ, 5·76 μ, and 6·16 μ for glycoside II are considered to confirm their cardenolide character.

The colour changes for glycoside I and rhodexin A on addition of 84% sulphuric acid (von Euw & Reichstein, 1948) were identical, the sequence being yellow → yellow-green → green → green-blue → green over 2 hr.

Combined chloroform–ethanol extracts of a sample (0·7 mg) of glycoside II, incubated with *Suc d'Helix pomatia* (0·3 ml) in pH 4·8 buffer (4·7 ml) at 37° for 21 hr, after paper chromatography in the toluene–butanol and chloroform–tetrahydrofuran–formamide systems showed a single Kedde-positive compound identical in Rf values with rhodexin A.

Discussion

It is concluded that glycoside I is the cardenolide glycoside rhodexin A, i.e. sarmentogenin-α-L-rhamnoside. Although the rhamnoside convallatoxin (strophanthidin-α-L-rhamnoside) had been isolated previously from *Ornithogalum umbellatum*, this is the first evidence for the presence in the plant of a glycoside of sarmentogenin.

Glycoside II appeared, on the basis of the paper chromatographic analysis of the acid-catalysed hydrolysates, to contain the sugars D-glucose and L-rhamnose, together with the aglycone sarmentogenin. Since the Kedde-positive product of enzymatic hydrolysis appeared to be identical chromatographically with rhodexin A, glycoside II is concluded to be a glucoside of rhodexin A, and is designated rhodexoside. In view of the

* The calculations of ϵ for glycosides I and II were based on the molecular weight of rhodexin A, i.e. 536·6, and on the molecular weight of $C_{35}H_{52}O_{14}$, i.e. 696·8, respectively.

general observation (Klyne, 1950) that in naturally-occurring cardenolide glycosides D-sugars are usually present in β-glycosidic linkage, rhodexoside is likely to be sarmentogenin-α-L-rhamnosido-β-D-glucoside.

The presence in this plant of rhodexin A and rhodexoside is therefore analogous with the presence of convallatoxin and the glucoside of convallatoxin, convalloside. Further, it is of interest that Nawa isolated from the plant *Rhodea* (*Rohdea*) *japonica*, in addition to rhodexin A, the glycosides rhodexin B, i.e. gitoxigenin-α-L-rhamnoside (Nawa, 1952a) and rhodexin C, a glucoside of rhodexin B (Nawa, 1952b, c).

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Observations on isoprenaline-induced myocardial necroses

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Isoprenaline hydrochloride in subcutaneous doses of 2 × 10 to 2 × 680 mg/kg caused histologically detectable, characteristic heart necroses. Pathological changes were dose-dependent. The degree of necrosis was assessed by a quantitative histological method based on counting the changed morphological elements. The heart necroses due to 2 × 40 mg/kg of isoprenaline were significantly inhibited by pretreatment with different monoamine oxidase inhibitors (iproniazid, a derivative of hydrazine and pargyline and E-250 of the non-hydrazine type, all at 7 × 50 mg/kg intraperitoneally); their severity was significantly increased by reserpine (7·5 mg/kg/48 hr subcutaneously) and guanethidine (2 × 30 mg/kg subcutaneously). In these effects an important role is attributed to respective changes in catecholamine levels.

REPEATED injections of isoprenaline are claimed to produce characteristic myocardial necroses in rats (Chappel, Rona, Balázs & Gaudry, 1959a; Rona, Chappel, Balázs & Gaudry, 1959; Rona, Kahn & Chappel, 1963). The same effect has also been demonstrated in golden hamsters and cats, but not in dogs or domestic pigs (Rosenblum, Wohl & Stein, 1965a). Rats with isoprenaline-induced heart damage have been the subject of several experimental studies (Selye, Veilleux & Grasso, 1960; Wexler & Kittinger, 1963, 1965; Beznák & Hacker, 1964; Strubelt & Breining, 1964; Kako, 1965). Drugs of different kinds, like thyreostatics (Chappel, Rona & Gaudry, 1959b), monoamine oxidase inhibitors (Zbinden, 1962; Zbinden & Bagdon, 1963), β-adrenergic blocking agents (Ehringer & Gögl, 1963) and, recently, a trinitro-compound (Scriabine & Stebbins, 1966) have all been reported to mitigate the myocardial changes caused by isoprenaline.

We have made a quantitative histopathological evaluation of isoprenaline-induced cardiac necroses. We have also examined whether monoamine oxidase inhibitors not derived from hydrazine afforded protection against isoprenaline-induced heart necroses, as has been reported of monoamine oxidase inhibitors of the hydrazine type (Zbinden, 1962). Finally, reserpine and guanethidine were also used in our experiments, as they have some effects contrary to those of monoamine oxidase inhibitors.

Material and methods

Male albino rats, 150–250 g, from an inbred colony were given doses of isoprenaline subcutaneously on two occasions at intervals of 24 hr. The drug was always freshly dissolved in isotonic saline and the volume adjusted to 0·2 ml/100 g weight. The animals were killed by ether anaesthesia 24 hr after the second injection. The hearts were fixed in formalin and paraffin sections stained with haematoxylin and eosin.

The following drugs were used: (±)-isoprenaline hydrochloride,

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iproniazid hydrochloride, pargyline hydrochloride, E-250 (phenylisopropylmethylpropinylamine hydrochloride; Knoll, Ecsery, Kelemen & others, 1965), guanethidine sulphate, reserpine (Rausedyl, 2·5%; Richter).

Guanethidine was injected twice, in doses of 30 mg/kg subcutaneously, always 6 hr before the injections of isoprenaline. Reserpine, 5 and 2·5 mg/kg, was given subcutaneously 48 and 24 hr before the beginning of the isoprenaline treatment. The monoamine oxidase inhibitors, 50 mg/kg, were given daily intraperitoneally for 7 days; the administration of isoprenaline began 1 day after the last injection. The doses of all drugs except reserpine refer to the salts.

Histopathological changes were evaluated by a quantitative histological method (Gál, Leszkovszky & Lendvai, 1966) in which a numerical index is made of smaller or greater areas of necrosis, interstitial loosening or cellular infiltration; the index of severity indicating the grade of changes in the heart is obtained by converting the counted numerical values in 1 cm^2 of a section of myocardium. In each experimental group the mean value and standard error of the single animal's index numbers were calculated. Different groups were compared by means of Student's *t*-test.

Results

MYOCARDIAL LESIONS AFTER ISOPRENALINE

Gross anatomical necrosis was not seen after doses ranging from 2 × 10 to 2 × 680 mg/kg of isoprenaline in the hearts of some 200 rats.

Microscopically detectable lesions were found and were similar to those described by others in that in addition to the necrotic changes a great number of round cells and fibroblasts representing reactive-reparative processes were found in the myocardium. No pathological changes of the coronary vessels were seen. There was a well-defined dose-response myocardial pathology (Table 1).

TABLE 1. QUANTITATIVE HISTOLOGICAL EVALUATION OF HEART NECROSES INDUCED BY ISOPRENALINE

No.	Pretreatment			Isoprenaline mg/kg s.c.	Number of animals	Index number of severity	
	Drug	Dose mg/kg	Route			mean value	s.e.
1	—			2 × 10	10	2·86	0·47
2	—			2 × 40	41	16·15	1·84
3	—			2 × 160	10	21·47	3·46
4	—			2 × 680	12	31·72	4·74
5	Iproniazid	7 × 50	i.p.	2 × 40	9	9·87	1·72*
6	Pargyline	7 × 50	i.p.	2 × 40	9	6·80	1·99*
7	E-250	7 × 50	i.p.	2 × 40	9	5·94	1·28*
8	Guanethidine	2 × 30	s.c.	2 × 40	20	26·82	4·46†
9	Reserpine	5 + 2·5	s.c.	2 × 40	7	28·80	4·46†

* Differs significantly from group No. 2. $P < 0.001$.
† Differs significantly from group No. 2. $P < 0.02$.

DRUG EFFECTS ON THE NECROSES

The influences of drugs on the effect of 2 × 40 mg/kg isoprenaline were examined, since the same dose had been chosen for various experi-

ments by others (Beznák & Hacker, 1964; Kako, 1965). The results of the quantitative histological evaluation are listed in Table 1.

The index numbers of severity in the three groups pretreated with 7×50 mg/kg doses of the different monoamine oxidase inhibitors are less than in the control group and these differences are statistically significant ($P < 0{\cdot}001$).

The results obtained in the rat groups pretreated with $5 + 2{\cdot}5$ mg/kg reserpine and 2×30 mg/kg guanethidine, respectively, showed aggravation of the histopathological changes. The difference from controls for both drugs is statistically significant ($P < 0{\cdot}02$).

Discussion

Of the numerous substances known to induce myocardial necroses in experimental animals, such as digitalis (Dearing, Barnes & Essex, 1943), vasopressin (Dearing, Barnes & Essex, 1944), amphetamine (Halpern, Morard & Drudi-Baracco, 1962), acetylcholine, adrenaline, noradrenaline, methoxamine or plasmocid (Bajusz & Jasmin, 1963; Rona & others, 1963; Rosenblum, Wohl & Stein, 1965a), isoprenaline seems to have aroused the greatest interest. This can be understood, because cardiac necroses after isoprenaline can be produced quickly and simply in the rat.

This pathology has been proposed (Rona & others, 1959, 1963; Zbinden & Bagdon, 1963) as a model of myocardial infarction. On the other hand, the analogy of isoprenaline-induced necroses with human diseases and the usefulness of such experiments have been denied by Strubelt & Breining (1964). Clementi, Nidiry & Peracchia (1964) suggested that the action of isoprenaline was not mediated by any anoxic state but was exerted directly on contractile elements of the heart. Nevertheless, because isoprenaline is probably the most potent of the catecholamines in increasing the metabolism of the heart (Hornbrook & Brody, 1963; Winterscheid, Bruce, Blumberg & Merendino, 1963) and thus the demands on oxygen, the relationships of relative hypoxia and isoprenaline-induced myocardial necroses cannot be overlooked (Rona & others, 1963; Rosenblum & others, 1965b). The catecholamine-induced increase in heart metabolism is, in the view of Raab (1963), of decisive importance in the pathogenesis of human degenerative heart changes. We therefore believe that this type of experiment may be worth attention.

The necroses could be inhibited in our experiments by pretreatment with three different monoamine oxidase inhibitors. Our iproniazid experiment confirms those of Zbinden (1962) and contrasts with the results of Strubelt & Breining (1964). One question to be considered is whether the protection afforded by the monoamine oxidase inhibitors depends on the inhibition of this enzyme or whether it is due to a pharmacological action of the molecule independent from interference with monoamine oxidase (Timsit, 1965). Our results show both pargyline and E-250 to afford significant protection against the action of isoprenaline, contrary to the observation mentioned by Zbinden & Bagdon (1963), that isoprenaline-induced heart necroses could not be inhibited

by pargyline. Thus we believe that the protective action of different monoamine oxidase inhibitors may be interpreted as a consequence of changes in the monoamine metabolism due to the inhibition of the enzyme. This may lead to such an effect, e.g. by improvement of myocardial energy production, as has recently been proposed for the mechanism of the anti-anginal action of monoamine oxidase inhibitors by Pletscher (1966).

Reserpine and guanethidine increased the severity of myocardial necroses. These two drugs are known to release the catecholamines from various stores including those in myocardium (Krayer, Alper & Paasonen, 1962). According to our view the aggravation of isoprenaline-induced necroses is to be explained by the supersensitivity of the catecholamine-depleted tissues which has been demonstrated for catecholamines in general (Burn & Rand, 1958; Maxwell, Povalski & Plummer, 1959; Holtz, Osswald & Stock, 1960) and isoprenaline in particular (Schmitt & Schmitt, 1960; Lee & Yoo, 1964). Thus, after these pretreatments isoprenaline makes even larger demands on metabolism and consequently, pathological changes will become more serious. Though the heart-depressing action of reserpine (Withrington & Zaimis, 1961; Nayler, 1963) may be considered as a potential co-factor in the aggravation of the necroses, we would attribute the major role to the above changes related to catecholamines.

In a similar way, it is known that heart necroses induced by amphetamine are not aggravated but abolished by reserpine (Halpern, & others, 1962). This observation supports our view, because amphetamine, differing from isoprenaline, is considered to belong to the group of indirectly acting sympathomimetic agents (Trendelenburg, Gomez Alonso de la Sierra & Muskus, 1963). Thus, after depletion of catecholamine stores by reserpine, amphetamine's cardiovascular actions are abolished and consequently, no necroses can be produced. Isoprenaline, on the other hand, is a directly acting catecholamine, sensitivity to which is not diminished but increased by depletion of tissue catecholamine stores.

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Impurities in halothane*: their identities, concentrations and determination

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The following impurities arising during the manufacture of a proprietary halothane have been identified by gas chromatographic, mass spectrometric, nmr and infrared techniques: 2-chloro-, 2,2-dichloro-, 2-bromo-, 2,2-dibromo-, 2,2-dibromo-2-chloro- and 2-bromo-2,2-dichloro-1,1,1-trifluoroethanes, 1,1,2-trichloro-1,2,2-trifluoroethane, 1,2-dichloro-1,1-difluoroethane, 2-bromo-2-chloro-1,1-difluoroethylene, bromodichlorofluoromethane, chloroform, *trans*-2-bromo-1,1,1,4,4,4-hexafluorobut-2-ene and the *trans* and *cis* isomers of 2-chloro- and 2,3-dichloro-1,1,1,4,4,4-hexafluorobut-2-enes. Gas chromatographic methods using chlorinated biphenyl, dinonyl phthalate and polyethylene glycol as liquid phases have been developed for their determination at the ppm level.

THE inhalant anaesthetic halothane B.P. is stabilized 2-bromo-2-chloro-1,1,1-trifluoroethane containing very small amounts of impurities, the nature and biological properties of which have aroused some interest during the past year or so (Cohen, Bellville, Budzikiewicz & Williams, 1963; Corrigan, McHattie & Raventós, 1963; Linde & Butler, 1963; Sexton & Hendrickson, 1963; Albin, Horrocks & Kretchmer, 1964; Butler & Linde, 1964; Cohen & Brewer, 1964; Scherer & Weigand, 1964; Gjaldbaek & Worm, 1965).

This paper deals specifically with the identification and determination of the trace impurities present in Fluothane. It does not purport to cover halothane from other sources that may be manufactured by different routes. The biological properties of the impurities are discussed elsewhere by Raventós & Lemon (1965).

The impurities found to date, many only rarely and in minute amounts, are listed in Table 1 with typical concentrations found by gas-liquid chromatography in the currently manufactured material. Also included in Table 1 are their retention times relative to 1,1,2-trichloro-1,2,2-trifluoroethane, on two stationary phases, namely chlorinated biphenyl and a mixture of dinonyl phthalate and polyethylene glycol.

The presence of most of the impurities listed in Table 1 was not unexpected in the light of the method of manufacture which involves the high-temperature bromination of 2-chloro-1,1,1-trifluoroethane (impurity No. 2). Over-bromination produces a small amount of 2,2-dibromo-2-chloro-1,1,1-trifluoroethane (impurity No. 13). The bromination probably proceeds through a free-radical mechanism, which would also be involved in the formation of the substituted butenes (impurities Nos 1, 3, 4, 7 and 8). These might be formed through a substituted butane as intermediate which, by loss of hydrogen halide, would give the butene,

e.g. $2CF_3CHCl\cdot \rightarrow CF_3\cdot CHCl\cdot CHCl\cdot CF_3 \rightarrow CF_3\cdot CH:CCl\cdot CF_3$.

Alternatively, substituted carbenes might be involved.

Of the other impurities, Nos 5, 9 and 16 occur as trace impurities in the

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* The material used in this investigation was Fluothane (I.C.I. Ltd).

TABLE 1. CONCENTRATIONS AND GAS CHROMATOGRAPHIC BEHAVIOUR OF IMPURITIES IN HALOTHANE*

Impurity	Typical concentration (ppm by weight) in the anaesthetic	Retention time relative to CF_2Cl-$CFCl_2$ on column of	
		Chlorinated biphenyl at 60°	Polyethylene glycol/ dinonyl phthalate at 60°
1	Not detected (<1)	0·12	0·38
2	1	0·22	1·00
3	Not detected (<1)	0·30	0·73
4	Not detected (<1)	0·40	1·00
5	8	0·62	2·35
6	Not detected (<1)	0·62	2·35
7	Not detected (<1)	0·62	0·73
8	Not detected (<1)	0·78	0·85
9	12	1·00	1·00
10	Not detected (<5)	3·48	Not determined
11	Not detected (<5)	4·85	Not determined
12	Not detected (<5)	6·43	10·9
13	Not detected (<5)	8·73	Not determined
14	1	Masked by halothane	1·50
15	Not detected (<5)	Masked by halothane	3·28
16	Not detected (<5)	Masked by halothane	4·22

* Fluothane (I.C.I.)

1. *trans*-2-Chloro-1,1,1,4,4,4,-hexafluorobut-2-ene.
2. 2-Chloro-1,1,1-trifluoroethane.
3. *cis*-2-Chloro-1,1,1,4,4,4-hexafluorobut-2-ene.
4. *trans*-2-Bromo-1,1,1,4,4,4-hexafluorobut-2-ene.
5. 2,2-Dichloro-1,1,1-trifluoroethane.
6. 2-Bromo-1,1,1-trifluoroethane.
7. *trans*-2,3-Dichloro-1,1,1,4,4,4-trifluorobut-2-ene.
8. *cis*-2,3-Dichloro-1,1,1,4,4,4-trifluorobut-2-ene.
9. 1,1,2-Trichloro-1,2,2-trifluoroethane.
10. Bromodichlorofluoromethane.
11. 2,2-Dibromo-1,1,1-trifluoroethane.
12. Chloroform.
13. 2,2-Dibromo-2-chloro-1,1,1-trifluoroethane.
14. 2-Bromo-2-chloro-1,1-difluoroethylene.
15. 2-Bromo-2,2-dichloro-1,1,1-trifluoroethane.
16. 1,2-Dichloro-1,1-difluoroethane.

2-chloro-1,1,1-trifluoroethane and No. 15 could be formed by bromination of No. 5. Impurities Nos 6, 11 and 15 could all arise in the course of the bromination reaction and No. 14 could be formed by elimination of hydrogen fluoride from halothane. Traces of dichloro- and trichlorofluoromethane have occurred occasionally in 2-chloro-1,1,1-trifluoroethane and one of these could be the precursor of No. 10. The occasional presence of traces of chloroform is more difficult to explain, although it is conceivable that the dichlorofluoromethane already mentioned could disproportionate to form chloroform.

It seemed possible that other compounds might be present which could be overlooked in the chromatographing because of their being masked by the peaks of known impurities or by the peak due to the anaesthetic itself. Seven such possible impurities have been prepared and their gas-chromatographic behaviour determined; this is summarized in Table 2. The columns used are capable of separating all of these compounds from known impurities and from halothane, but none of them has been detected (limit of detection 5 ppm or less) in the anaesthetic. Details of all the compounds in this paper have been published with the exception

TABLE 2. HALOGENATED ETHYLENES AND BUTENES NOT PRESENT IN HALOTHANE*

Compound	Limit of detection (ppm by weight)	Retention time relative to $CF_2Cl{\cdot}CFCl_2$ on column of:	
		Chlorinated biphenyl at 60°	Polyethylene glycol/ dinonyl phthalate at 60°
1	2	0·50	0·57
2	5	masked by halothane	1·68
3	2	0·12	0·80
4	5	1·73	2·13
5	5	2·18	2·52
6	5	5·70	6·66
7	5	6·33	8·30

* Fluothane (I.C.I.)

1. 2,2-Dichloro-1,1-difluoroethylene.
2. 2,2-Dibromo-1,1-difluoroethylene.
3. *trans*-1,1,1,4,4,4-Hexafluorobut-2-ene.
4. *trans*-2-Bromo-3-chloro-1,1,1,4,4,4-hexafluorobut-2-ene.
5. *cis*-2-Bromo-3-chloro-1,1,1,4,4,4-hexafluorobut-2-ene.
6. *trans*-2,3-Dibromo-1,1,1,4,4,4-hexafluorobut-2-ene.
7. *cis*-2,3-Dibromo-1,1,1,4,4,4-hexafluorobut-2-ene.

of 2-bromo-3-chloro-1,1,1,4,4,4-hexafluorobut-2-ene. This was prepared by bromination of 2-chloro-1,1,1,4,4,4-hexafluorobut-2-ene in ultraviolet light giving 2,3-dibromo-2-chloro-1,1,1,4,4,4-hexafluorobutane (identified by mass spectrometry) which was dehydrobrominated with potassium hydroxide to the bromochlorohexafluorobutene. Fractional distillation of the crude product gave a mixture of the *cis*- and *trans*- isomers (bp 86°/760 mm), which were then separated by preparative gas-liquid chromatography. Mass-spectrometric analysis of the isomers established their empirical formulae as C_4BrClF_6. Nuclear magnetic resonance investigation showed the coupling constants to be J_{FF} *cis* = 12·8 c/sec and J_{FF} *trans* = 1·6 c/sec, in agreement with theoretically predicted values.

IDENTIFICATION OF IMPURITIES

Mass spectrometry and gas chromatography were the main techniques used in the identification of impurities but microchemical analysis, nuclear magnetic resonance and infrared spectrometry were also used. Gas-liquid chromatographic identifications were made by correlating the retention times with those of authentic specimens of compounds expected to be present either from mass-spectrometric evidence or from the nature of the manufacturing process. Usually these identifications involved the use of liquid phases of widely differing polarity, e.g. chlorinated biphenyl and dinonyl phthalate, polyethylene glycol or a mixture of these two liquids (see Figs 1, 2, 3 and 4).

In most instances a fraction rich in a limited number of impurities, obtained either by laboratory distillation of the crude product or from a suitable point in the process stream, was used as the starting material for identification work.

Impurities of shorter retention time than halothane on a chlorinated biphenyl column. (*a*) 2-Chloro- and 2,2-dichloro-1,1,1-trifluoroethane and 1,1,2-trichloro-1,2,2-trifluoroethane. These compounds (impurities

Nos 2, 5 and 9, Table 1) were concentrated in a low-boiling fraction obtained during distillation of the anaesthetic. They were readily identified, without further separation, from their mass spectra and from their gas-liquid chromatographic behaviour on chlorinated biphenyl and dinonyl phthalate columns (Figs 1 and 2).

(*b*) *trans*- and *cis*-2,3-Dichloro-1,1,1,4,4,4-hexafluorobut-2-ene. These impurities (Nos 7 and 8, Table 1) have higher boiling points than halothane. They were concentrated by fractional distillation before isolation in millilitre quantities by preparative gas-liquid chromatography.

Fractionation was effected on a column, 6 ft long and 5/8 inch internal diameter, packed with 60–72 mesh Celite (7 parts by weight) impregnated with dinonyl phthalate (3 parts by weight). The column was run at 65° with nitrogen as carrier gas at a flow rate of 4 litre/hr. Eluted components, detected by a katharometer, were collected in traps cooled in liquid air. Sample loads of 0·5 to 1 ml were injected into a zone maintained at about 120°, at the column inlet.

Several fractionation runs were made until about 1 ml of each of the impurities had been collected. Both components gave microchemical analyses (C, 20·4; Cl, 36·0; F, 46·5%) and mass spectra consistent with isomers of dichlorohexafluorobutene. Subsequent examination by infrared spectrometry showed that they were identical with the *cis* and *trans* isomers of 2,3-dichloro-1,1,1,4,4,4-hexafluorobut-2-ene previously synthesized and characterized by Dickinson, Hill & Murray (1958).

(*c*) *trans*-2-Chloro-1,1,1,4,4,4-hexafluorobut-2-ene. This impurity (No. 1, Table 1 and present in the first peak of Fig. 1) was isolated by preparative gas-liquid chromatography, under the conditions given above, from a low-boiling distillation fraction from the anaesthetic. Elementary analyses (C, 24·0; H, 0·6; Cl, 18·0; F, 57·1%) of the isolated specimen showed that it had the empirical formula C_4HClF_6. Mass spectrometric and nuclear magnetic resonance spectrometric examinations of the compound established its structure as the required *trans*-isomer.

(*d*) *cis*-2-Chloro-1,1,1,4,4,4-hexafluorobut-2-ene. This impurity (No. 3, Table 1) is included in the third peak of Fig. 1 and is also included in the *trans*-dichlorohexafluorobutene peak obtained on a polyethylene glycol or polyethylene glycol/dinonyl phthalate column (see Figs 3 and 4). This impurity was first observed in the chromatogram, from a polyethylene glycol column, of a fraction free from the *trans*-dichlorohexafluorobutene. It was shown by mass spectrometry to have the structure $CF_3{\cdot}CH : CCl{\cdot}CF_3$, and as it appears as a separate peak from the compound already identified as *trans*-2-chloro-1,1,1,4,4,4-hexafluorobut-2-ene it follows that it must be the *cis*-isomer.

(*e*) *trans*-2-Bromo-1,1,1,4,4,4-hexafluorobut-2-ene. Although this compound (impurity No. 4, Table 1) has a slightly higher boiling point than halothane, it is concentrated in the lower-boiling fractions from the latter. It was isolated from such a fraction, in which it was the only detectable impurity, by preparative gas chromatography as described above. Mass spectrometry and microchemical analysis (C, 19·9; H, 0·4; Br, 34·2; F, 45·9%) of the isolated impurity established its molecular

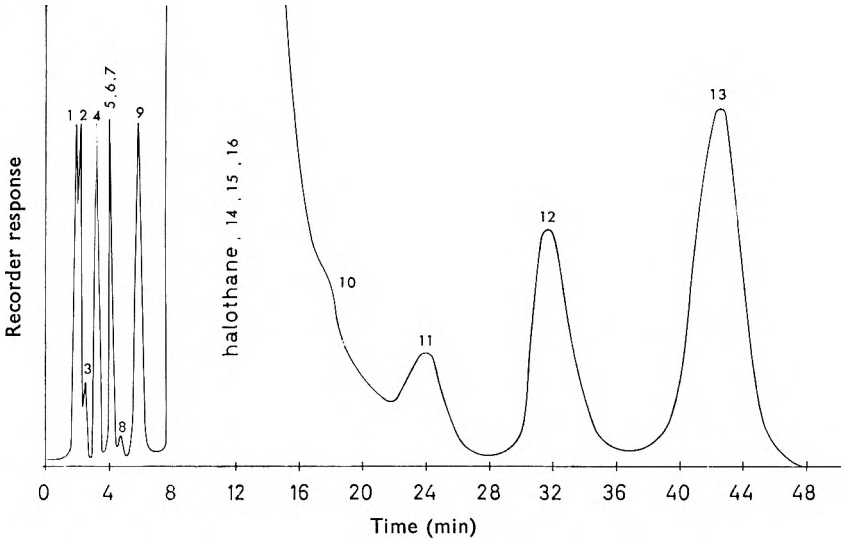

FIG. 1. Halothane impurities: chromatogram from a chlorinated biphenyl column at 60°. Impurities are numbered as in Table 1.

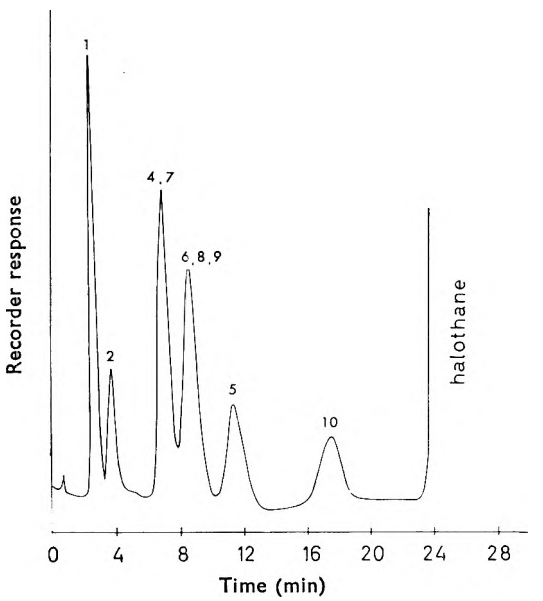

FIG. 2. Halothane impurities: chromatogram from a dinonyl phthalate column at 55°. Impurities are numbered as in Table 1.

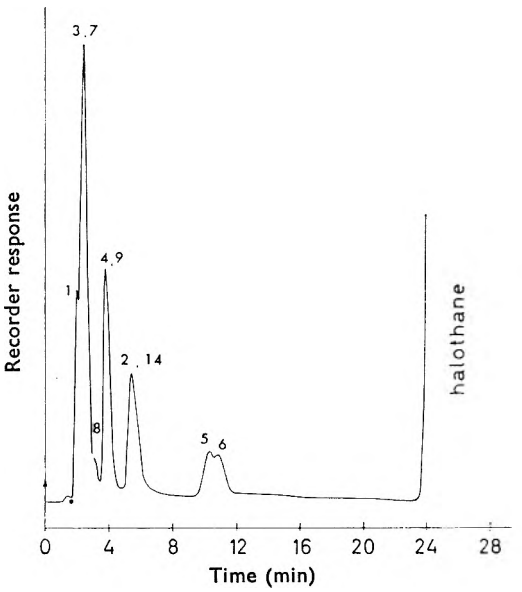

FIG. 3. Halothane impurities: chromatogram from a polyethylene glycol 400 column at 50°. Impurities are numbered as in Table 1.

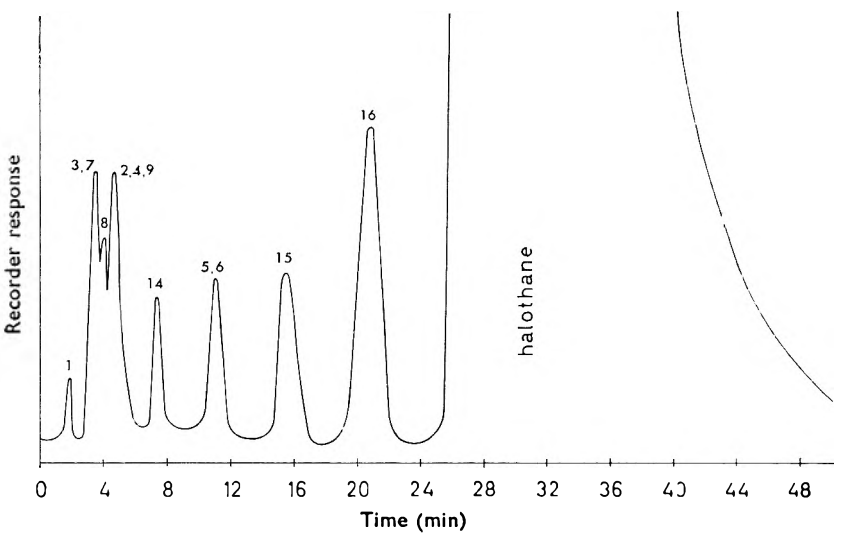

FIG. 4. Halothane impurities: chromatogram from a polyethylene glycol/dinonyl phthalate column at 60°. Impurities are numbered as in Table 1.

formula as C_4HBrF_6. It was shown to be the required *trans-* isomer by nmr spectrometry.

(*f*) 2-Bromo-1,1,1,-trifluoroethane. This impurity (No. 6, Table 1) is eluted together with *trans*-dichlorohexafluorobutene and 2,2-dichloro-1,1,1-trifluoroethane from a chlorinated biphenyl column (Fig. 1). Its presence was first noticed when a sample of the anaesthetic was chromatographed on a polyethylene glycol column which separated it completely from the former, and partially from the latter (see Fig. 3). It was tentatively identified by mass-spectrometric examination of a small fraction obtained from an analytical-scale polyethylene glycol column. Confirmation of its identity was achieved by comparing its mass spectrum and chromatographic behaviour with that of an authentic specimen of 2-bromo-1,1,1-trifluoroethane.

Impurities of longer retention time than halothane on a chlorinated biphenyl column. (a) Bromodichlorofluoromethane. A specimen of this compound (impurity No. 10, Table 1) was isolated by preparative gas-liquid chromatography from an enriched distillation fraction. The fractionation conditions were those described on p. 234, the impurity being eluted before the main component (see Fig. 2). The mass spectrum of the isolated material was consistent with the formula $CBrCl_2F$. Subsequent examination, by mass-spectrometric and gas-liquid chromatographic techniques, of an authentic specimen of bromodichlorofluoromethane confirmed this.

(b) 2,2-Dibromo-1,1,1-trifluoroethane and chloroform. The identities of these impurities (Nos 11 and 12, Table 1) were indicated by mass-spectrometric examination of the appropriate fractions obtained from an analytical-scale chlorinated biphenyl column and confirmed by correspondence of their retention times with authentic specimens.

(c) 2,2-Dibromo-2-chloro-1,1,1-trifluoroethane. This impurity (No. 13, Table 1) was isolated from crude halothane by fractional distillation and identified from its mass spectrum and elementary analysis.

Impurities masked by halothane on a chlorinated biphenyl column. There are three impurities in this category, namely 2-bromo-2,2-dichloro-1,1,1-trifluoroethane, 2-bromo-2-chloro-1,1-difluoroethylene and 1,2-dichloro-1,1-difluoroethane. All three were separated from each other and from other components on a column containing a mixture of polyethylene glycol 400 and dinonyl phthalate as the liquid phase (see Fig. 4). It was from such a column that small fractions were isolated for mass-spectrometric examination. Tentative identifications by this technique were confirmed when authentic specimens of the materials were examined by gas chromatography.

GAS-LIQUID CHROMATOGRAPHIC DETERMINATION OF IMPURITIES

By far the greatest problem in the determination of impurities in the anaesthetic was the selection of liquid phases that would not only separate them from the main component but would also give sufficient resolution of the impurities to permit quantitative estimation of each one. Of the

many possible liquid phases examined, no single one separated all the known impurities.

Before the discovery of the presence of 2-bromo-1,1,1-trifluoroethane, two columns were developed, one containing chlorinated biphenyl and the other a mixture of polyethylene glycol and dinonyl phthalate, which between them provided adequate information for the determination of the fifteen impurities then known to be present (see Figs 1 and 4). Because the bromotrifluoroethane behaves in the same way as 2,2-dichloro-1,1,1-trifluoroethane on both columns, the combined concentrations of these two impurities are obtained. This information is usually sufficient, but when individual concentrations are required an additional chromatogram must be run using polyethylene glycol as the liquid phase (see Fig. 3).

METHOD

Apparatus. Gas chromatographs fitted with flame ionization detectors, e.g. Pye Series 104, Model 4.

Reagents. 2-Bromo-2-chloro-1,1,1-trifluoroethane free from detectable impurities. Specimens of the compounds listed in Table 1; the purity of these materials need not be higher than 95%. Chromosorb P, 60–80 mesh and Celite, 72–85 mesh (Messrs. "JJ's" of King's Lynn, Norfolk). Aroclor 1254 (chlorinated biphenyl) (Monsanto). Dinonyl phthalate and polyethylene glycol 400 (May & Baker).

Gas chromatography columns and conditions. Stationary phase mixtures of Aroclor 1254 and Chromosorb P, dinonyl phthalate and Celite, and polyethylene glycol and Celite were prepared, each mixture containing 30% by weight of liquid phase. The stationary phases were then packed in copper or stainless steel tubes, 3/16 inch internal diameter to give columns of: A, 6 ft Aroclor/Chromosorb; B, 6 ft polyethylene glycol/Celite; C, 6 ft polyethylene glycol/Celite joined to 3 ft dinonyl phthalate/Celite.

The Aroclor column was operated at 60° with a carrier gas (nitrogen) flow rate of 30 ml/min; the polyethylene glycol column at 50° with a gas flow rate of 50 ml/min and the mixed polyethylene glycol/dinonyl phthalate columns at 60° with a gas flow rate of 40 ml/min, all being measured at atmospheric pressure.

All columns were conditioned at the operating temperature for 16 hr or until a stable recorder base line was obtained.

Calibration of columns. Using pure halothane as the main component, artificial mixtures of the following types were prepared: type 1 containing impurities Nos 1, 2, 4, 7–13 of Table 1, type 2 containing impurities Nos 5, 7, 14, 15 and 16 and type 3 containing impurities Nos 5 and 6. Two or three mixtures of each type were made up so as to cover the impurity concentration ranges encountered in practice (0–25 ppm).

Mixtures of types 1 and 2 were chromatographed on the Aroclor column, and those of types 2 and 3 on both the polyethylene glycol/dinonyl phthalate and polyethylene glycol columns. Five μl aliquots were used in each instance. The amplifier attenuation was set at $\times 20$ and the output signal was fed to a 1 mV potentiometric recorder. Calibration graphs

were constructed by plotting component peak heights against appropriate concentrations.

Sample analysis. Samples (5 μl) were chromatographed on each of the three columns. The concentrations of impurities Nos 1, 2, 3, 4, 8–13 of Table 1 were obtained directly from appropriate peak height measurements on the Aroclor chromatogram. (Impurity No. 3, i.e., *cis*-2-chloro-1,1,1,4,4,4-hexafluorobut-2-ene was measured as impurity No. 4 as no standard was available.) The combined concentrations of impurities Nos 5–7 were also measured. Similarly, from the polyethylene glycol-dinonyl phthalate chromatogram, the concentrations of impurities Nos 14–16, together with the combined concentration of Nos 5 and 6 measured as the former, were obtained. Individual concentrations of impurities Nos 5 and 6 were obtained from the polyethylene glycol chromatogram.

The height of the peak containing impurity No. 7 in the Aroclor chromatogram was corrected for the presence of impurities Nos 5 and 6 (measured as impurity No. 5), and, from the corrected value, the concentration of impurity No. 7 was obtained.

Discussion

In view of the exhaustive search for impurities that has been made in this proprietary brand of halothane, it seems unlikely that there is present at concentrations above about 10 ppm, any impurity other than those reported; at this level and below they would not be expected to have any significance in anaesthetic practice (see Raventós & Lemon, 1965).

Gas-liquid chromatographic methods for the determination of all the known impurities are tedious and when frequent analyses are required, as in process control, a large amount of apparatus is fully occupied. It seems probable that the use of coated capillary columns in place of packed columns would reduce the time required for an analysis and might also lead to a reduction in the number of columns required because of the higher resolving power of coated capillaries.

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The bioassay of γ-aminobutyric acid using a muscle preparation from *Ascaris lumbricoides*

A. S. F. ASH AND J. F. TUCKER

A method is described for the assay of γ-aminobutyric acid based on inhibition of the isolated dorsal muscle of *Ascaris lumbricoides*. The degree of relaxation produced by the acid was linearly related to the logarithm of the dose (0·5–2·0 μg/ml). Analyses of variance and indices of precision, calculated from the results of 6 model assays, indicated a satisfactory assay procedure. *Ascaris* was much more sensitive to γ-aminobutyric acid than to related amino-acids and was unaffected by dopa, dopamine, adrenaline, noradrenaline, 5-hydroxytryptamine or histamine (2 mg/ml) and by bradykinin (50 μg/ml).

THE distribution of γ-aminobutyric acid (GABA) and Factor I in biological tissues can be followed by measuring their inhibitory action on the impulses generated in the crayfish stretch receptor neuron (Florey, 1954; Elliot & Florey, 1956; Florey & Elliot, 1961; Levin, Lovell, Elliot & Elliot, 1961; Lovell, Elliot & Elliot, 1963). The crayfish hind gut, which is inhibited by GABA and related compounds, also provides a method of assay (Florey, 1961). Contractions of guinea-pig ileum induced by 5-hydroxytryptamine are antagonized by low concentrations of GABA (Hobbiger, 1958) but this preparation is affected by many endogenous substances.

An alternative preparation for the assay of GABA is now described using the isolated muscle of *Ascaris lumbricoides*, a round worm found in pig intestine. The method is more sensitive and specific than either of the crayfish preparations. A preliminary account of the work has been published (Ash & Tucker, 1966).

Methods

Preparation. Specimens of *Ascaris lumbricoides* were obtained fresh from the slaughterhouse and transported in a modified Tyrode solution, pH 6·5, at 37–38° (Goodwin, 1958). The composition of the medium was: NaCl 0·8, KCl 0·02, $CaCl_2$ 0·02, $MgCl_2$ 0·01, $NaHCO_3$ 0·015, Na_2HPO_4, $12H_2O$ 0·05, and glucose 0·5%. The medium was changed daily and the worms kept for not longer than 2 days at 37–38°.

The operative technique was similar to that described by Baldwin & Moyle (1947). A large active female worm was dropped from a height of about 12 inches on to the bench. This procedure stuns the worm and leads to a contraction of the musculature, which facilitates the dissection. The worm was secured, ventral surface upward, on a wax tray by passing two pins through the body, one in the region of the genital pore, the other a few mm from the head. The anterior and posterior portions of the worm were cut away and the remaining tissue was divided by a longitudinal incision in the ventral muscle. The divided edges were deflected and pinned down and the gut carefully removed with forceps. The two ventral muscle strips were then removed by cutting along the

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lateral canals. Ligatures were applied to the dorsal muscle which remained, one immediately in front of the genital pore, the other 2 to 2·5 cm further forward. Finally, tissue outside the ligatures was cut away. The isolated dorsal muscle strip was suspended in an organ bath of 10 ml capacity containing the modified Tyrode solution described above. The solution was gassed with ordinary commercial nitrogen which contained less than 0·5% oxygen. Movements of the tissue were recorded on a kymograph using a frontal writing lever with a magnification of ×20 and a load of 2 g.

Assay design. A four point design was chosen (Gaddum, 1953). To determine the precision of the assay, four known doses of GABA were used: two, A and B, were treated as "standard" and two, C and D, as "unknown". Doses were chosen, in the range 0·7 to 1·8 μg/ml, to give responses between 10 and 90% of the maximum, such that the ratio A/B = C/D = 2. The doses A, B, C and D were given in random sequence in each of 4 groups.

Results

RESPONSE OF ASCARIS MUSCLE TO GABA

GABA produced a rapid reversible inhibition of ascaris dorsal muscle in concentrations as low as 0·5 μg/ml. The inhibition, reflected as a relaxation of the preparation, was linearly related to the log dose (Fig. 1).

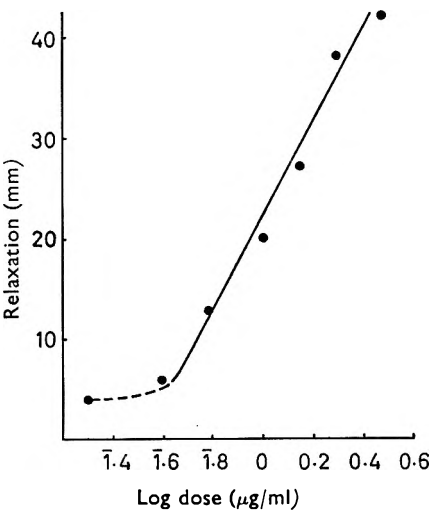

FIG. 1. Relaxation of isolated ascaris dorsal muscle in modified Tyrode solution by GABA, showing the linear relationship between dose and response which is regularly obtainable over this narrow range of doses.

Each administration of GABA was followed, after a latent period of approximately 15 sec, by a slow relaxation during a contact time of 3 min (Fig. 2). A dose cycle of 7 min allowed adequate recovery. The extent of the relaxation to any given dose tended to diminish during

the assay. This tachyphylaxis was unaffected by lengthening the interval between doses.

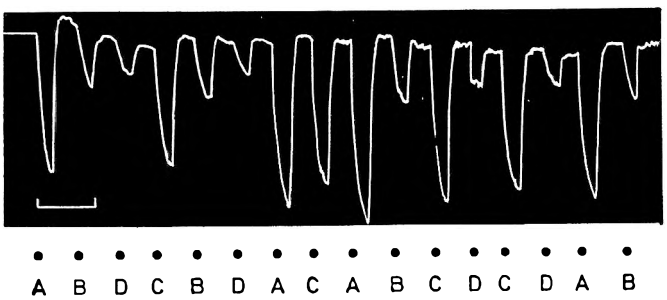

FIG. 2. Responses of isolated ascaris dorsal muscle to GABA in a model assay. Two doses of GABA, A and B (1·8 and 0·9 μg/ml), represent the "standard" and two doses, C and D (1·4 and 0·7 μg/ml), represent the "unknown". Doses were given in groups of 4, using a latin square design, allowing a contact time of 3 min and an interval of 7 min between doses. Time scale: 10 min.

ASSAY PERFORMANCE

The results of a typical assay, in which two solutions of known compositions were compared, are summarized in Table 1.

TABLE 1. INHIBITION OF ASCARIS MUSCLE BY FOUR DOSES OF GABA GIVEN IN FOUR SUCCESSIVE RANDOMIZED GROUPS

Dose (μg/ml)	Response (relaxation in mm) Groups 1	2	3	4	Sum
1·8	79	73	54	54	260
0·9	42	40	31	28	141
1·4	71	67	44	50	232
0·7	34	18	27	21	100
Sum	226	198	156	153	733

M, the logarithm of the ratio of potencies, is given by M = (Yu − Ys)/b, where Yu − Ys is the difference between the mean responses to unknown and standard, and b is the slope of the regression line (Schild, 1942). From the data in Table 1, M = 1·21. The estimated concentration of the "unknown", which had an actual concentration of 77·8 μg/ml, was then 82·7 μg/ml. The fiducial limits (95%) were 104·7 and 62·7 μg/ml.

TABLE 2. ANALYSIS OF VARIANCE OF GABA ASSAY. The probability (P) of the variations occurring by chance was calculated by means of the "F" test (Snedecor, 1956)

Source of variation	Sum of squares	Degrees of freedom	Variance estimate	F	P
Between groups	927·5	3	309·2	7·5	<0·01
Between standard and unknown	297·6	1	297·6	7·2	<0·05
Regression	3938	1	3938	949·5	<0·001
Deviation from parallelism	10·6	1	10·6	4	<0·05
Error	373·3	9	41·5		
Total	5547·0	15			

The analysis of variance (Snedecor, 1956) for this assay is summarized in Table 2. Differences between groups and between standard and unknown were significant; regressions were highly significant with negligible deviations from parallelism.

Precision of assay. The index of significance, more conveniently expressed as its reciprocal L, is a measure of assay precision. Typical values of L vary from 2 for an inaccurate assay to 30 for an accurate one (Gaddum, 1953). In 6 assays of GABA (Table 3) the mean value of L was 17.

TABLE 3. ASSAYS OF GABA ON ASCARIS MUSCLE. The precision of the assay is expressed by L, the reciprocal of the index of precision (Gaddum, 1953)

Experiment number	True value of unknown (μg/ml)	Estimated activity (μg/ml)	Fiducial limits (95%) of estimated activity (μg/ml)	L
1	77·8	73·0	54·1– 91·5	13·9
2	77·8	82·7	62·7–104·7	16·2
3	77·8	86·2	77·2– 96·4	30·0
4	77·8	78·0	50·6–106·9	12·5
5	77·8	74·7	57·5– 88·9	16·1
6	77·8	77·2	33·0–122·7	13·7

SENSITIVITY OF ASCARIS MUSCLE TO ENDOGENOUS AMINES AND ANALOGUES OF GABA

Ascaris muscle was stimulated by acetylcholine in doses as low as 0·5 μg/ml, but was unaffected by dopa, dopamine, adrenaline, noradrenaline, 5-hydroxytryptamine or histamine (2 mg/ml) and by bradykinin (50 μg/ml). In Table 4 the responses of the ascaris preparation to

TABLE 4. SENSITIVITY OF THREE PHARMACOLOGICAL PREPARATIONS TO GABA AND RELATED COMPOUNDS. Figures in parentheses are minimal effective concentrations. Data obtained in the present work* are compared with those calculated from values quoted in the literature (Bazemore, Elliot & Florey, 1956; McLennan, 1957, 1959; Robbins, 1959, Florey & Elliot, 1961†) and Florey, (1961‡).

Compound	Activity relative to GABA = 100		
	Inhibition of ascaris muscle*	Inhibition of impulse generation in the crayfish stretch receptor neuron†	Inhibition of acetylcholine induced contractions of the crayfish hind gut‡
γ-Aminobutyric acid	100 (0·5 μg/ml)	100 (1·5–10 μg/ml)	100 (2 μg/ml)
γ-Amino-β-hydroxybutyric acid ..	<0·001	50	10
Guanidinoacetic acid	43	150	200
Guanidinobutyric acid	7	33	(No action up to 100 μg/ml)
γ-Aminobutyrylcholine chloride hydrochloride	2·5	33	4
δ-Aminovaleric acid	9	5	(No data)
β-Alanine	1	5	2
ε-Aminocaproic acid	0·1	0·1	(No data)
Glutamic acid	<0·001	(Variable effects)	4

analogues of GABA are compared with values quoted in the literature for the two crayfish preparations. Five of the eight analogues of GABA are less active on the ascaris preparation than on the preparations of the crayfish.

Discussion

Studies of GABA have frequently been concerned with its possible physiological role, particularly in the central nervous system (for reviews see Elliot & Jasper, 1959; Curtis & Watkins, 1965; Elliot, 1965). There is, therefore, a need for assay preparations which are sensitive to GABA yet relatively unaffected by other common constituents in extracts of nervous tissue.

Both ascaris muscle and crayfish hind gut provide methods for the assay of GABA *in vitro* which are technically simpler than the original crayfish stretch receptor neuron preparation. The chief disadvantage of the ascaris preparation is that, unlike crayfish, it cannot readily be maintained for long periods in the laboratory. *Ascaris lumbricoides* can be kept alive for several days in suitable media at 37–38° (Baldwin & Moyle, 1947; Goodwin, 1958) but 48 hr outside the host is the limit if the worm is to be used for reliable assays of GABA. The use of commercial nitrogen to gas the bathing fluid during experiments with the preparation was introduced by Baldwin & Moyle (1947). High oxygen tensions are toxic (Laser, 1944), but it is still uncertain whether the worm is completely anaerobic (Fairbairn, 1957).

The basis of the assay is the linear relationship between the response of the muscle and the logarithm of the dose of GABA over the range 0·5–2·0 μg/ml. In the analysis of variance, the necessity for an assay design in which the effects of gradual changes in sensitivity are eliminated is illustrated by the variations between groups (Schild, 1942). The significant differences between standard and unknown, the highly significant regression and the negligible deviation from parallelism are indicative of a satisfactory assay procedure.

Ascaris lumbricoides dorsal muscle shows a greater specificity for GABA than the crayfish preparations, being less sensitive to related amino-acids. In fact, the contrasts in relative potency are such that parallel assays may be useful aids in the analysis of Factor I activity. So far there is no evidence that common endogenous amines will interfere with the use of the ascaris preparation for assay purposes.

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Diffusion from gelatin-glycerin-water gels

J. R. NIXON, P. P. GEORGAKOPOULOS* AND J. E. CARLESS

The effect of gelatin, glycerin and methylene blue concentration on the diffusion of a dye from gelatin-glycerin-water gels has been examined. The rate of diffusion depended on the pore size of the gel and on the viscosity of the interspace fluid. Except at very low concentrations of methylene blue, an aggregation factor must be added to those factors suggested by Friedman & Kraemer (1930) as controlling the rate of diffusion from gels.

THE diffusion of dyes into and from gels has been the subject of numerous investigations (Friedman & Kraemer, 1930; Calvet, 1947; Longsworth, 1954; Pontins, Kaplan & Husney, 1956; Marinin, 1958; Blyumberg & Davydkin, 1962). The direct comparison of different workers' results is not always possible because of different methods of pretreating the gels.

Although gelatin-glycerin gels have been used for many years, little systematic work has been done on their properties and the diffusion of substances from these gels has received little attention. The present work investigates the diffusion of methylene blue from gelatin-glycerin gels prepared from gelatins of varying Bloom strength.

Experimental

MATERIALS

Gelatins. The characteristics have been given in a previous paper (Nixon, Georgakopoulos & Carless, 1966). *Glycerin* was Analar grade and *methylene blue* was of B.P. quality. *Purified water* was once distilled from an all glass still (pH 5·2, specific conductivity 5 mhos cm^{-1}).

METHODS

Preparation of the gels and the *method of measuring the rigidity* were as described by Nixon & others, 1966.

Measurement of diffusion coefficient. Diffusion was measured by a method based on that of Friedman & Kraemer (1930). The dye was uniformly distributed in the molten gel (200 ml) and the container allowed to equilibrate at 25° ± 0·1° for 16 hr. A similar volume of water at the same temperature was added and the whole placed in a shaking thermostat bath (108 strokes/min; 39 mm throw). Absorptiometer readings on samples of the aqueous phase were made at intervals up to 50 hr. The samples were always returned after use to the master solution, and at the end of the experiment a check was made with a depth gauge, to be certain that no significant change in volume had occurred.

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* This work forms part of an M.Pharm. thesis by P.P.G. (present address Pharmacy Department, University of Thessaloniki, Greece).

The diffusion coefficient can be derived from the equation of March & Weaver (1928) which in its expanded form becomes

$$V = \tfrac{1}{2} - (0{\cdot}327e^{-4{\cdot}117T} + 0{\cdot}0766e^{-24{\cdot}14T} + 0{\cdot}0306e^{-73{\cdot}68T} + 0{\cdot}0160e^{-123T} + 0{\cdot}0100e^{-200T} + 0{\cdot}0067e^{-299T} \ldots) \quad \ldots \quad (1)$$

where V = the fraction of material which has diffused from the gel at a given time.

A theoretical curve was constructed from which values of T could be read using experimentally determined values of V.

The diffusion coefficient was calculated from T by means of the equation

$$T = \frac{Da^2}{t} \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad (2)$$

where D = diffusion coefficient of a solute moving into the solution above the gel; a = the thickness of the gel and also the depth of solution above the gel; t = time in sec. All values of D recorded are the mean of six readings.

Results and discussion

Gels containing 20% w/w glycerin and 10 mg% methylene blue were used to examine the effect of gelatin concentration and Bloom number on the rate of diffusion. There was a linear decrease in diffusion coefficient between 10 and 15% w/w gelatin (Fig. 1). The slope of the line depended

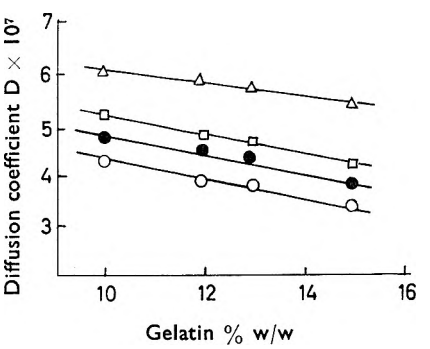

FIG. 1. Diffusion of methylene blue from gelatin-glycerin gels. Glycerin 20% w/w, methylene blue 10 mg %. Temperature 25 ±0·1°. Gelatin Bloom numbers: △ 99; □ 154; ● 200; ○ 250.

on the Bloom number of the gelatin used, the percentage fall in diffusion rate between the two concentrations of gelatin being: Bloom No. 99, 11·4; Bloom No. 154, 20; Bloom No. 200, 20·8 and Bloom No. 250, 21·1. The increase in Bloom number also resulted in a slower rate of diffusion.

Measurements were confined to this narrow range of gelatin concentrations because of experimental difficulties. At gelatin concentrations below 10% w/w, slight mechanical rupture of the gel surface after prolonged shaking of the system prevented the calculation of a true diffusion coefficient. Simple extrapolation of the curves towards low gelatin

concentrations would be incorrect, as at some point they would bend upwards towards the theoretical diffusion coefficient of methylene blue from glycerin-water mixtures into water. At high gelatin concentrations the experimental difficulty was microbial decomposition of the surface layers of the gel due to the extended experimental time necessary to calculate a diffusion coefficient. Extrapolation to zero diffusion rate gave a gelatin concentration of approximately 25% for all the gelatins except the 99 Bloom strength where the value was 40%. It is doubtful whether diffusion would cease at these concentrations as although an increase in gelatin concentration decreases the available area for diffusion due to the smaller pore size of the gel (Friedman & Kraemer, 1930) the capillaries are of different size and would allow passage of some dye molecules.

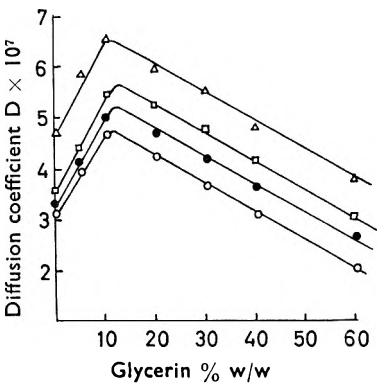

FIG. 2. Effect of glycerin concentration on the diffusion of methylene blue. Gelatin 10% w/w; methylene blue 10 mg %. Temperature 25 ±0·1°. Gelatin Bloom number: △ 99; □ 154; ● 200; ○ 250.

The effect of glycerin on the diffusion coefficient depended on its concentration (Fig. 2). The curves, which were parallel for all gelatins studied, exhibited a maximum at approximately 10% glycerin. The reduction in diffusion rate at higher glycerin concentrations was paralleled by a sharp increase in the viscosity of the glycerin-water interspace fluid. This increased viscosity was the same irrespective of the Bloom number of the gelatin and, as the concentration of the latter remained constant, the pore size of the gel network produced by a given gelatin would not vary. However, as the chain length increased the smaller pore size caused by the possibility of a greater number of linkage points along the chain led to a reduction in diffusion rate as shown in Fig. 2. The rise in diffusion rate at glycerin concentrations below 10%, where the interspace fluid viscosity remains constant, is probably due to a polarity effect of the solvent causing a decrease in the intermolecular forces of attraction between methylene blue molecules thus producing a less aggregated molecule which would more easily pass through the gel network.

The rigidity of the gels containing a given percentage of gelatin was increased by the addition of glycerin (Nixon & others, 1966) but this

increase as such played no part in determining the diffusion coefficient. Only when the pore size was decreased by an increased concentration of gelatin, resulting in an increased rigidity, did this factor affect the diffusion rate. Although there appears to be no simple relationship between rigidity and diffusion rate, the data from a large number of experiments using both different Bloom number gelatins and gelatin-glycerin ratios allow a single line to be drawn through the points (Fig. 3).

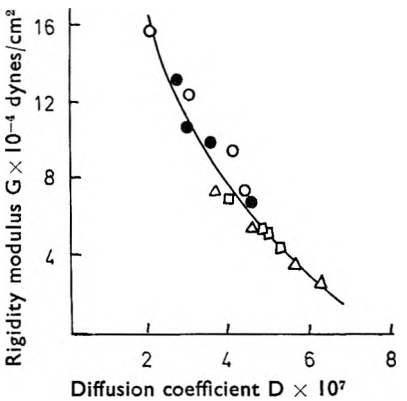

FIG. 3. Rigidity against diffusion coefficient at different % gelatin-glycerin ratios (10/10; 10/20; 10/40; 10/60). Methylene blue 10 mg %; temperature 25 ±0·1°. Gelatin Bloom numbers: △ 99; □ 154; ● 200; ○ 250.

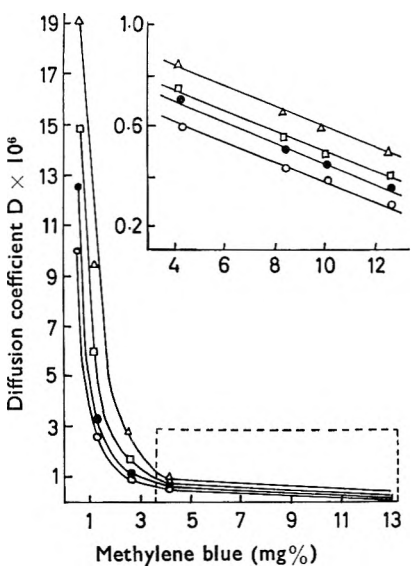

FIG. 4. Diffusion coefficient against methylene blue concentration. Gelatin concentration 12 % w/w; glycerin concentration 20% w/w; temperature 25 ±0·1°. Gelatin Bloom numbers: △ 99; □ 154; ● 200; ○ 250. Inset portion is enlargement of area within dotted lines.

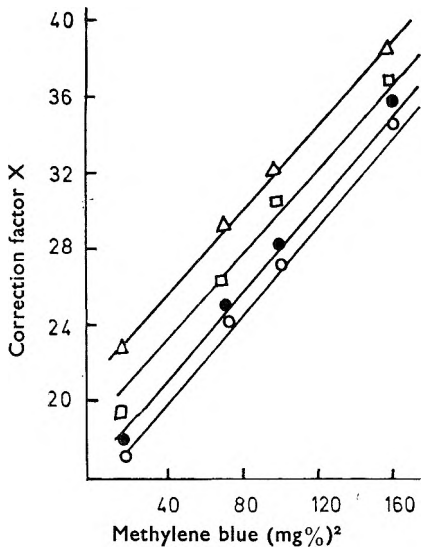

FIG. 5. Effect of concentration of methylene blue on correction factor X. Gelatin Bloom numbers: △ 99; □ 154; ● 200; ○ 250.

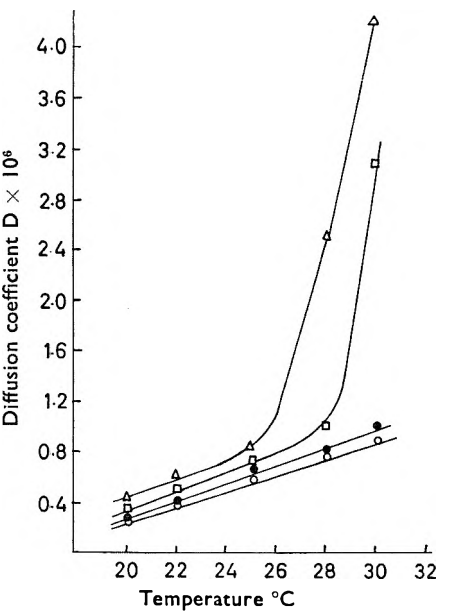

FIG. 6. Effect of temperature on the diffusion coefficient. Concentrations: gelatin 12% w/w; glycerin 20% w/w; methylene blue 4·2 mg %. Gelatin Bloom numbers: △ 99; □ 154; ● 200; ○ 250.

In these experiments, because the gelatin concentration remained constant, the increased rigidity was due to an increase in inter-chain linkage with the longer chain length gelatins.

The effect of methylene blue concentration was complicated by dissociation of the molecular aggregates at low concentrations of the dye. At concentrations above 4 mg% the diffusion coefficient was found to be a slowly decreasing linear function of the methylene blue concentration (Fig. 4 inset). The size of the molecular aggregate obviously approaches an optimum close to the pore size of the gel. When this is reached only those molecular aggregates smaller than the pores of the gel will pass through, resulting in a low diffusion rate.

At dye concentrations lower than approximately 2 mg% free movement within the gel network becomes possible as the molecular aggregates of methylene blue become small (Fig. 4) and at very low concentrations the diffusion coefficient becomes very large.

When considering the diffusion coefficient of the dye from the gels it is necessary to consider a fourth factor which affects the rate of diffusion. An aggregation factor, X, has to be included in Friedman & Kraemer's equation. The equation (3) relates the diffusion coefficient of the unaggregated molecule in water to its diffusion in the gel.

$$D_{water} = D_{gel}\,(1 + 2{\cdot}4\ r/R)(1 + a)(1 + \pi)(1 + X) \quad .. \quad (3)$$

where D = diffusion coefficient; r = radius of diffusing molecule; R = radius of gel pore; a = viscosity correction factor; π = mechanical blocking correction factor; X = aggregation factor.

At low methylene blue concentrations, aggregation has no significance and Friedman & Kraemer's equation holds and can be written

$$D_{water} = D_{gel}^{(1+X)\to 1}\,(1 + 2{\cdot}4\ r/R)(1 + a)(1 + \pi) \quad .. \quad .. \quad (4)$$

The X factor can be calculated from

$$1 + X = D_{gel}^{(1+X)\to 1}/D_{gel} \quad .. \quad .. \quad .. \quad .. \quad .. \quad (5)$$

where $D_{gel}^{(1+X)\to 1}$ is the diffusion coefficient at very low concentrations of dye and D_{gel} is the diffusion coefficient at the dye concentration for which X is required.

It is necessary to know values of X in order to calculate the true diffusion coefficient of the non-aggregated dye molecules. Over the region where the factor has great significance it is proportional to the square of the dye concentration (Fig. 5), and as the curves for the different gelatins are parallel, X will depend on the pore size of the gel. Although important in calculating the diffusion coefficient of the unaggregated molecule the X factor cannot be taken as a direct measure of either pore size or degree of aggregation.

Increases in temperature caused significant increases in the rate of diffusion of methylene blue from the gel, due to both the decrease in rigidity and the increased thermal agitation of both the gelatin and methylene blue. These result in a progressively larger pore size. Up to a temperature of 25° this increased diffusion was linear and the curves for

the different gelatins were parallel (Fig. 6) suggesting that only the different pore size of the gels was causing differences between the gelatins. Above this temperature diffusion coefficient was non-linear and the temperature at which this non-linearity occurred was lower with the shorter chain length gelatins. The long chain 250 Bloom number gelatin retained its linearity up to a temperature of 30°.

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Desipramine and amphetamine metabolism

S. CONSOLO, E. DOLFINI, S. GARATTINI AND L. VALZELLI

Desipramine (2·5–5 mg/kg) increases the urinary excretion of amphetamine given intraperitoneally at doses of 7·5–15 mg/kg and it decreases the excretion of *p*-hydroxyamphetamine. The rate of removal of brain amphetamine is decreased by desipramine (5 mg/kg, i.p.) when amphetamine is injected intraperitoneally but not when it is injected intracerebrally. It is suggested that desipramine impairs the hydroxylation of amphetamine in the liver thereby increasing the level of circulating amphetamine and eventually of brain amphetamine.

IMIPRAMINE and other tricyclic antidepressant agents are known to increase and prolong the pharmacological effects of (+)-amphetamine (Carlton, 1961; Lapin & Shchelkunov, 1963; Stein, 1964, 1966; Morpurgo & Theobald, 1965). Recently Valzelli, Consolo & Morpurgo (1966) showed that imipramine and desipramine prolong the hyperthermia induced by amphetamine in rats and that they also increase the amphetamine levels in brain and liver.

These findings prompted an investigation to establish whether desipramine was able to affect the formation of *p*-hydroxyamphetamine, the major metabolite of amphetamine in rats (Axelrod, 1954a, b; Alleva, 1963; Dring, Smith & Williams, 1966).

Materials and methods

Male Sprague-Dawley rats, 165 ± 5 g, were kept in Makrolon cages at constant room temperature (22°) and relative humidity (60%). (+)-Amphetamine sulphate and desipramine were injected intraperitoneally and the urines were collected 24 hr after dosing.

Amphetamine and *p*-hydroxyamphetamine were determined in urine and amphetamine in brain by the method of Axelrod (1954 a, b). Free *p*-hydroxyamphetamine was determined in urines before acid hydrolysis. Suitable controls indicated that desipramine did not interfere with the determination of urinary amphetamine or *p*-hydroxyamphetamine. The brains of animals pretreated with desipramine (5 mg/kg i.p.) did not affect the recovery of (+)-amphetamine added *in vitro*.

In some experiments (+)-amphetamine was introduced into the brain by the method of Valzelli (1964).

Results

Table 1 gives the results obtained on measuring amphetamine and *p*-hydroxyamphetamine in urines of rats treated with different doses of amphetamine alone or with desipramine. It is evident that desipramine treatment changed the pattern of amphetamine metabolism by increasing the urinary excretion of amphetamine and decreasing that of *p*-hydroxyamphetamine.

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* This work has been partially financed by a grant from J. R. Geigy, S.A., Basel.

TABLE 1. AMPHETAMINE AND *p*-HYDROXYAMPHETAMINE CONTENT IN RAT URINE 24 HR AFTER DIFFERENT DOSES OF (+)-AMPHETAMINE ALONE OR WITH DESIPRAMINE. Desipramine was always given 1 hr before (+)-amphetamine. Each figure is the average of at least 8 determinations.

Treatment	mg/kg i.p.	ml of urine/ rat in 24 hr	μg/ml of urine (± s.e.)	
			Amphetamine	Total *p*-hydroxyamphetamine
Amphetamine	7·5	4	72·5 ± 1·7	67·6 ± 13·6
Desipramine .. .. Amphetamine	2·5 + 7·5	4	178·5 ± 1·4	3·2 ± 1·1
Amphetamine	10·0	4	80·0 ± 0·9	80·8 ± 13·9
Desipramine .. .. Amphetamine	2·5 + 10·0	4	158 · 5 ± 2·0	n.m.*
Amphetamine	15	3	126·8 ± 3·7	135·2 ± 8·6
Desipramine .. .. Amphetamine	5 + 15	3	200·0 ± 0·5	n.m.*

* n.m. = not measurable

The pH of urines was in all instances about 6·5. This measurement was made since according to Asatoor, Galman, Johnson & Milne (1965) and Beckett, Rowland & Turner (1965) the amount of amphetamine excreted unchanged by the rats increases with acidity of the urine.

TABLE 2. TOTAL EXCRETION IN URINE AND RECOVERY OF AMPHETAMINE AND *p*-HYDROXYAMPHETAMINE AFTER ADMINISTRATION OF AMPHETAMINE ALONE OR IN COMBINATION WITH DESIPRAMINE TO RATS

Desipramine μg/rat i.p.	Amphetamine base μg/rat i.p.	Amphetamine μg/24 hr urine	Free *p*-OH-amphetamine μg/24 hr urine	Total *p*-OH-amphetamine μg/24 hr urine	Amphetamine + *p*-OH-amphetamine μg/24 hr urine	% hydroxylation relative to excretion	Recovery % amphetamine + *p*-OH-amphetamine
—	730	290	42	271	561	48	76
416	730	714	16	16	730	2	100
—	960	320	52	324	644	50	67
416	960	632	15	15	647	2	67
—	1460	380	79	405	785	52	53
832	1460	600	15	15	615	2	42

The total excretion of amphetamine, and both free and total *p*-hydroxyamphetamine, is showed in Table 2 which also includes the percentage hydroxylation relative to excretion and the percentage recovery of drug and metabolite. From these data it is even more evident that desipramine almost completely blocks the hydroxylation of amphetamine. The percentage of the dose of amphetamine excreted decreases with increase in dose while the percentage hydroxylation remains constant. Both the percentage recovery and hydroxylation are in good agreement with data recently published using [^{14}C]amphetamine (Dring & others, 1966).

To establish whether desipramine was able to modify the level of brain amphetamine because of its inhibition of the hydroxylation process or by a direct effect on the disposition of brain amphetamine, the rate of decrease of brain amphetamine was determined. This was done both in the presence and absence of desipramine, when (+)-amphetamine was

injected intraperitoneally or intracerebrally and desipramine intraperitoneally 1 hr previously. Fig. 1 summarizes the results. The rate of removal of brain amphetamine was decreased by desipramine when amphetamine was injected intraperitoneally but not intracerebrally.

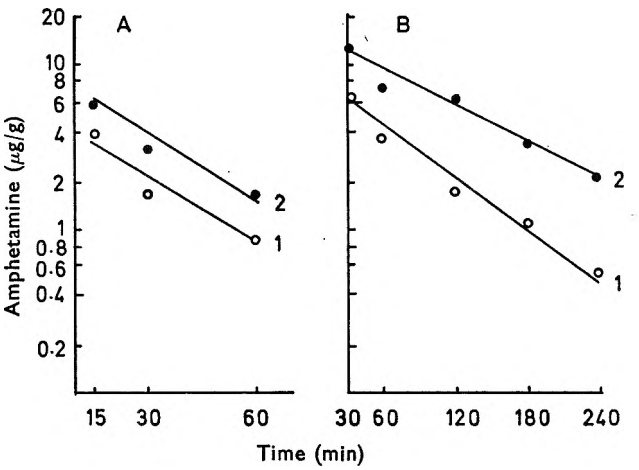

FIG. 1. A. Level of brain amphetamine after intracerebral injection of 50 μg of (+)-amphetamine (0·1 ml) alone (1) or following a pretreatment with desipramine (5 mg/kg i.p. 1 hr before) (2). B. Level of brain amphetamine after i.p. injection of 7·5 mg/kg of (+)-amphetamine alone (1) or following a pretreatment with desipramine alone (1) or following a pretreatment with desipramine (5 mg/kg i.p. 1 hr before) (2). The percentages removed (K) were:

$$K_{1A} = 3{\cdot}2;\ K_{2A} = 2{\cdot}7;\ K_{1B} = 1{\cdot}2;\ K_{2B} = 0{\cdot}76$$

The variance analysis shows that the lines 1A and 2A are not significantly different from parallelism. The lines 1B and 2B are significantly different from parallelism ($P < 0{\cdot}01$).

Discussion

The increase and prolongation of the pharmacological effects of amphetamine in the presence of tricyclic antidepressant agents, may be explained, at least in rats, by a difference in the disposition of amphetamine. Previous work established that the levels of amphetamine in brain and liver were increased by imipramine and similar agents (Valzelli & others, 1966). This suggested that impairment of the hepatic metabolism of amphetamine would increase circulating amphetamine and therefore give rise to higher levels of brain amphetamine. Since it is well known that the major metabolite of amphetamine in rats is *p*-hydroxyamphetamine, it should be at the hydroxylation stage that the tricyclic antidepressant agents exert their inhibiting effect. Our findings corroborate this by showing that the urinary excretion of *p*-hydroxyamphetamine is markedly decreased by desipramine. Indeed amphetamine derivatives substituted in the *para* position by chlorine or an hydroxyl group do not have their hyperthermic activity potentiated by tricyclic antidepressant agents (Valzelli & others, 1966).

The block of this hydroxylation probably occurs at the level of the hepatic microsomes. Desipramine does not show any major effect on the rate of removal of brain amphetamine when this amine is injected directly into the brain. It remains to be established whether desipramine inhibits the microsomal enzymes or prevents the uptake of amphetamine by preventing a contact between the enzyme and the substrate.

Imipramine and desipramine are hydroxylated in the 2 and 10 positions (Herrmann & Pulver, 1960; Bickel, 1966; Crammer & Scott, 1966). Since desipramine is rapidly formed from imipramine but is slowly metabolized in rats (Dingell, Sulser & Gillette, 1964) it might be expected that a competitive inhibition with the hydroxylation of amphetamine would be effective *in vivo* for several hours. Other investigations have recently shown that tricyclic antidepressants inhibit the metabolism of tremorine, presumably by a similar mechanism (Sjoqvist & Hammer, 1966). The hydroxylation of tyramine to dopamine is also inhibited by desipramine in rabbits (Lemberger, Kuntzman, Conney & Burns, 1965). A possible inhibition of the uptake of amphetamine at the microsomal level is supported by the finding that tricyclic antidepressant agents prevent the uptake of several drugs (Costa, Boullin, Hammer & others, 1966).

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Quantitative determination of physostigmine in solution

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A chemical assay is described for the indirect determination of physostigmine (eserine) in solution. The method involves a chemical reaction between methylamine, a hydrolysis product of physostigmine, and carbon disulphide to form a dithiocarbamic acid which is subsequently titrated with a standard aqueous sodium hydroxide solution. The alkaline hydrolysis of the physostigmine sample is carried out at room temperature using a closed system. The difference in the amount of methylamine found in a physostigmine hydrochloride solution before hydrolysis (blank) and after hydrolysis (sample) is indicative of the amount of intact alkaloid present. This indirect determination of methylamine makes it possible for the first time, to determine conveniently the amount of the alkaloid in solution in the presence of its degradation products.

AQUEOUS solutions of physostigmine (eserine) salts are known to decompose, gradually turning red on standing at room temperature. It has been well established that the first decomposition products are colourless eseroline, methylamine, and carbon dioxide. This decomposition is followed by rapid oxidation of eseroline to the red-coloured rubreserine, and more slowly to eserine blue, eserine brown and possibly other products (Ellis, 1943). Since pharmacological activity is lost during the first stage of decomposition to eseroline (Ellis, Krayer & Plachte, 1943), one should be able to determine the pharmacological activity of a physostigmine solution at any given time, if the amount of alkaloid present can be accurately determined.

Previous attempts to determine the alkaloid include spectrophotometric measurements of rubreserine (Ellis, 1943; Hellberg, 1947, 1949) or methylamine (Hellberg, 1947, 1949), titrimetric determinations of the alkaloid (Hellberg, 1947; Chatten, 1955; United States Pharmacopeia, 17th Revision) or methylamine (Hellberg, 1947), polarography (Parrak, Mohelska & Machovicova, 1961; Parrak & Radejova, 1962), and the determination of pharmacological activity (Ellis & others, 1943). These methods have not proved entirely satisfactory because of the lack of specificity either for physostigmine or one of the decomposition products, the inability to account for some decomposition products before analysis, or too laborious or inconvenient a procedure.

We have attempted to develop a method for the quantitative measurement of physostigmine in solution, directly or indirectly, and preferably in the presence of its degradation products.

The method presented determines the alkaloid by indirectly measuring the methylamine present before and after alkaline hydrolysis of equal aliquots of a solution of physostigmine hydrochloride. The methylamine is allowed to react with carbon disulphide to form a dithiocarbamic acid which is titrated with standard aqueous sodium hydroxide.

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Experimental

Materials. Carbon disulphide C.P., hydrochloric acid C.P., methylamine hydrochloride, physostigmine (Mann Research Laboratories, N.Y.), potassium hydrogen phthalate, sodium hydroxide, acetonitrile, 2-propanol, hydrochloric acid 4N, sodium hydroxide 4N and 0·2N*, methylamine hydrochloride solution (1·0 g of methylamine hydrochloride dissolved in water and diluted to 250 ml). All chemicals are of analytical reagent grade unless otherwise indicated.

Apparatus. Metrohm Potentiograph E336 (automatic recording titrator), micro combination glass-saturated calomel electrode, 5 ml piston burette, 50 ml round bottom flasks and 60 ml dropping funnels both with ground glass joints (24/40), and 5 ml microburettes.

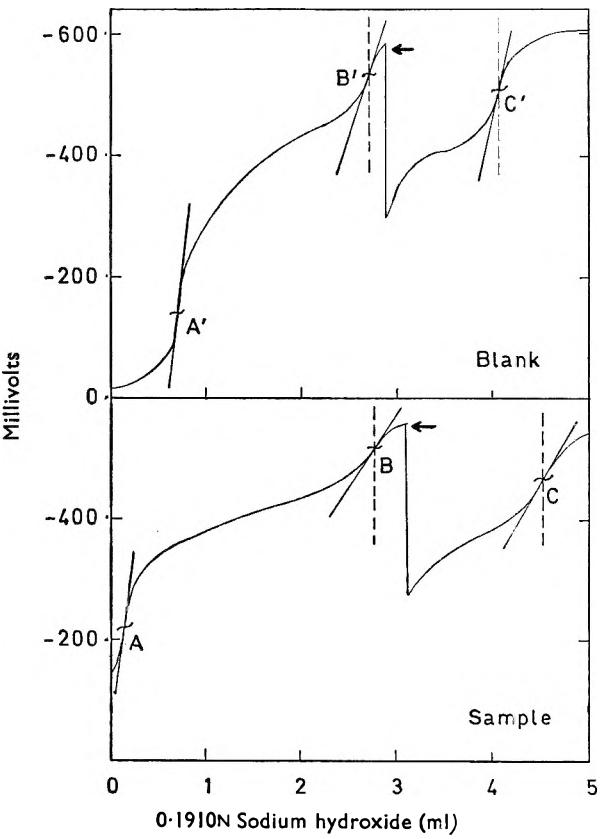

FIG. 1. Titration curves of non-hydrolysed (blank) and hydrolysed (sample) physostigmine hydrochloride solutions with sodium hydroxide. End points A′ and A, B′ and B indicate neutralization points of excess hydrochloric acid and hydrochloric acid salts of organic bases, respectively. Just after end points B′ and B at the arrows, carbon disulphide is added to react with methylamine present to form a dithiocarbamic acid which is neutralized at points C′ and C.

* Standardized against potassium hydrogen phthalate dried at 120° for 1 hr.

PROCEDURE

Dissolve an accurately weighed sample of physostigmine (about 250 mg) in about 0·5 ml of 4N hydrochloric acid and dilute to 50 ml with water. This provides nine 5·0 ml aliquots each equivalent to 24·7 mg of the alkaloid base. Place 5·0 ml aliquot of this solution in a 50 ml round bottom flask and add 4N sodium hydroxide (1·0 ml). Close the flask immediately with a stoppered dropping funnel containing 2-propanol (5 ml) and 4N hydrochloric acid (1 ml) and stir vigorously for 2 hr to allow for complete hydrolysis of the alkaloid. Add slowly the slight excess of acid in the alcohol in the funnel, stirring to ensure complete trapping of the methylamine vapour released on hydrolysis. Add a further 10 ml of 2-propanol and 5·0 ml of the methylamine hydrochloride solution via the funnel, followed by a further 10 ml of 2-propanol. Then remove the funnel and add 15 ml of acetonitrile to the contents of the flask. Adjust the pH, if necessary, to ensure that the titration curve falls within the confines of the recorder chart. Titrate the solution with sodium hydroxide (0·2N) to a point just past the second potentiometric end point (B) at which add carbon disulphide (2 ml) (Fig. 1). Allow 10 min for the reaction then titrate to the third potentiometric end point (C).

The amount of standard sodium hydroxide consumed between the second (B) and third (C) end points is a direct measure of the dithiocarbamic acid produced, and an indirect measure of the methylamine released as a result of the hydrolysis of the physostigmine and the added solution of methylamine hydrochloride.

Make a blank titration using a 5·0 ml aliquot of the same physostigmine hydrochloride solution and treating it exactly as the sample omitting the hydrolysis with 4N sodium hydroxide.

Results and discussion

The procedure described is a modification of that developed by Critchfield & Johnson (1956) for the determination of primary and secondary aliphatic amines in the presence of tertiary amines which do not react with carbon disulphide to form a dithiocarbamic acid.

A few modifications to the original method were necessary. To obtain an adequate separation between the end points B and C, B′ and C′ (Fig. 1), a 5·0 ml aliquot of the methylamine hydrochloride solution was added. To sharpen the end points, the less polar solvent system of 2-propanol (50%) and acetonitrile (30%) was used. To insure complete hydrolysis of the physostigmine (*ca.* 0·1 m-equiv.) in the 5·0 ml aliquots, 4N sodium hydroxide (1 ml *ca.* 4 m-equiv.) and a reaction period of 2 hr were required. The closed system used was necessary to prevent loss of methylamine.

Fig. 1 shows typical titration curves which indicate the three potentiometric end points obtained, and the time of carbon disulphide addition.

The results of five sample and three blank determinations made on the 50·0 ml of physostigmine hydrochloride solution are shown in Table 1.

CALCULATIONS

From the stoichiometric relationships one mole of sodium hydroxide is equivalent to one mole of physostigmine. On a weight basis each mole of sodium hydroxide is equivalent to 275·34 g of the alkaloid. Therefore, each ml of 0·2N sodium hydroxide is equivalent to 55·068 mg of alkaloid.

The 0·480 ml difference between the mean values of the sodium hydroxide (0·1910N) required for the sample and blank determinations (Table 1) is equivalent to 25·24 mg of physostigmine. The amount of alkaloid represents 102·2% of the amount originally dissolved in the solution.

Another aqueous solution of physostigmine hydrochloride (5·0 ml equivalent to 25·9 mg of base) was analysed similarly, except that in the blank determinations 5·0 ml of deionized water was used instead of a non-hydrolysed aliquot of the same physostigmine hydrochloride solution. Calculations showed that 100·2% of the original physostigmine in the solution was found (Table 1).

TABLE 1. INDIRECT DETERMINATION OF METHYLAMINE IN ALKALINE-HYDROLYSED PHYSOSTIGMINE SAMPLES AND CONTROL SOLUTIONS OF NON-HYDROLYSED PHYSOSTIGMINE AND WATER

Volume (ml) of sodium hydroxide (0·1910N) consumed by dithiocarbamic acid[1]			
Determinations of		Determinations of	
Sample 1	Blanks[2]	Sample 2	Blanks[3]
1·845	1·380	1·915	1·370
1·910	1·371	1·820	1·385
1·875	1·390	1·905	1·380
1·855		1·890	1·385
1·850		1·870	1·390
		1·905	
		1·860	
		1·860	
		1·875	
Mean = 1·867	1·387	1·878	1·382
s.d. = 0·0245	0·0061	0·0297	0·0076

[1] Dithiocarbamic acid resulting from the methylamine and carbon disulphide interactions.
[2] Containing physostigmine (see text).
[3] Containing water instead of physostigmine.

The close agreement between the two sets of determinations demonstrates the lack of reactivity of the nitrogen in the urethane side-chain of physostigmine with carbon disulphide, and thereby underlines the specificity of the entire method for the analysis of the alkaloid under the conditions outlined.

Work is in progress to ascertain whether this method, or some modification of it, can be used for the determination of physostigmine salicylate or other salts in aqueous solutions alone or in the presence of certain additives such as preservatives and antioxidants.

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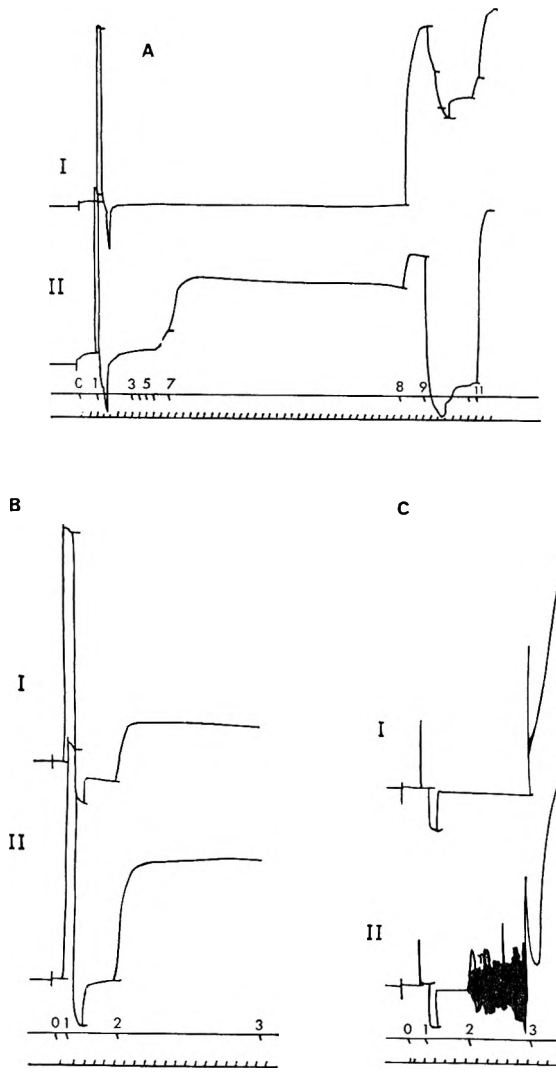
Calcium-dependent stimulating action of triiodothyronine on the isolated muscle of the frog

SIR,—The precise mechanism of action of the iodinated thyroid hormone or its substitutes is far from being clearly understood. Their generally delayed effects suggest some intricate, enzymatically induced actions. Very few immediate effects on isolated organs, tissues, or part of tissues, have so far been reported (Smith & Whalen, 1960; Lee, Lee & Yoo, 1965; Haldar, Freeman & Work, 1966).

We have found, however, that the rectus muscle of the frog, isolated in Ringer solution, is sensitive to relatively low concentrations of triiodothyronine, the threshold for inducing contracture usually being between 10 and 50 μM. In



addition, there are two procedures which enhance the effects of triiodothyronine on this muscle. These are (1) to keep the isolated recti in Ringer solution lacking calcium ions (Fig. 1A), or (2) to keep them in Ringer solution lacking potassium ions (Fig. 1B). Both procedures have in common the property of increasing the sensitivity of the muscles to triiodothyronine as well as to calcium chelating agents such as sodium edetate.

Under these sensitizing conditions, the threshold of the stimulating effect of triiodothyronine is lowered by at least five- to tenfold, and the amplitude of the induced contracture is increased two to four times.

In addition to the rectus, other skeletal frog muscles, like the pectoralis or sartorius, are sensitized by the same experimental procedures and actually the enhanced reactions follow individual response patterns, i.e. contracture, contracture with superimposed contractions, or bursts of repetitive contractions (Fig. 1C).

We may assume, theoretically at least, that ultimately the excitability of a given tissue is a direct or indirect function of the lability of its membrane-bound calcium ions. In our experiments, this concept is given support by the known chelating properties of the iodinated thyronine compounds (Lardy, 1955). It can be further tested by adding half of the stoichiometric amount of calcium ions, corresponding to the triiodothyronine concentration, to the solution of the muscle caused to contract in Ca-free medium. This addition reverses the contracture induced by the hormone, thereby suggesting that its action is calcium dependent.

If before addition to the Ringer solution, the triiodothyronine solution is treated with an adequate calcium chloride concentration and its effect on paired muscles compared with that of the untreated solution, we find that it has lost the greater part of its stimulating effect.

These muscle-stimulating actions of triiodothyronine are enhanced by cholinesterase inhibitors and by concentrations of barium chloride or caffeine which just fail to produce contraction. Conversely, sub-minimal concentrations of triiodothyronine potentiate the stimulation produced by caffeine or barium.

FIG. 1A. *Rana rid.* ♀, 110 g. Both recti muscles. Isotonic, vertically writing pens, amplifying 2 x under a pull of 2 g, in normal Ringer solution. Time marks = 30 min. 1. Acetylcholine 0·3 mM. After 15 min, at 2, muscle I was washed three times with Ringer solution, muscle II with Ca-free Ringer, both under a pull of 9 g lasting 30 min. At 3 to 7, both muscles received triiodothyronine 3 nM, 30 nM, 0·3, 3 and 10 μM. The first contracture occurred after 3 μM for the treated muscle in Ca-free solution, a concentration of triiodothyronine which produced no effect on the control. At 8, after 22 hr, a final reactivity test was made with 3 mM caffeine, followed by 3 washes as at 2. At 10, again 3 mM and at 11, 10 mM caffeine.

B. *Rana rid.* ♀, 88 g. Both recti muscles with the conditions described in A. 30 min after the initial acetylcholine test, the two muscles were washed 3 times: I with Ringer solution, II with K-free Ringer. At 2, both received a 30 μM concentration of triiodothyronine. At 3, 10 hr after beginning, the experiment was terminated.

C. *Rana rid.* ♀, 85 g. Both sartori muscles with the conditions as described in A. 30 min after the initial test to acetylcholine 0·3 mM, both muscles were washed 3 times, I with Ringer solution, II with K-free Ringer, both under a pull of 9 g lasting 30 min. At 2, both muscles received a 56 μM concentration of triiodothyronine. This produced a pronounced and rapid twitching in muscle II which persisted until the muscle was washed. 3 hr later, at 3, the final reactivity was tested with 5·6 mM caffeine.

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Antagonism of morphine analgesia by reserpine and α-methyltyrosine and the role played by catecholamines in morphine analgesic action

SIR,—Since Vogt (1954) showed that morphine can deplete hypothalamic noradrenaline the participation of catecholamines in the analgesic action of morphine has been proposed by many authors (Radouco-Thomas, Radouco-Thomas & Le Breton, 1957; Schaumann, 1958; Paeile & Muñoz, 1966, and others).

The effect of reserpine on the analgesic action of morphine has now been examined by means of two different tests; also, the effect of pretreatment with α-methyltyrosine on morphine analgesia was investigated. According to current views (Spector, 1966) α-methyltyrosine is a potent inhibitor of tyrosine hydroxylase which leads to a selective depletion of brain noradrenaline and dopamine without affecting 5-hydroxytryptamine.

The increase induced by morphine (5 mg/kg, i.v.) of the pain threshold when the tooth pulp of the rabbit is electrically stimulated was abolished 24 hr after the administration of reserpine (2 mg/kg, i.v.). In the same way, chronic treatment with reserpine (0·2 mg/kg, s.c., once a day, during 14 days) much reduced the

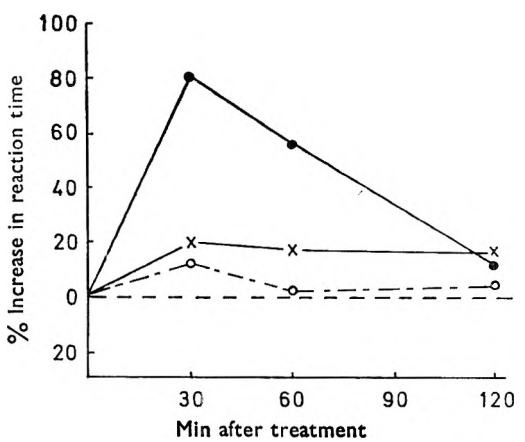

FIG. 1. Antagonism of morphine analgesic action by α-methyltyrosine in the mouse. Two doses of 100 mg/kg, i.p. of α-methyltyrosine were injected 8 and 4 hr respectively before morphine (10 mg/kg, i.p.). Analgesia was measured by the hot plate test (58° ± 0·5), according to Garcia Leme & Rocha e Silva (1961). The points on the curves represent the means of 40, 35 and 33 animals in the groups treated with morphine alone (●), morphine after α-methyltyrosine (×) or α-methyltyrosine alone (○), respectively.

analgesic response to morphine injected before the 15th dose of reserpine and abolished the analgesic response when the narcotic was tested 4 hr after that dose of the tranquillizer.

The increase produced by morphine (10 mg/kg, i.p.) in the reaction time to heat stimulation of the mouse paw (hot plate test, 58·0° ± 0·5°) was abolished 4 hr after reserpine (1 mg/kg, i.p.). The analgesic effect of morphine partially returned over a 144 hr period following reserpine treatment. On the other hand, the pretreatment with two sucessive doses of α-methyltyrosine (each of 100 mg/kg, i.p.) given 8 and 4 hr respectively before the injection of morphine nearly abolished the analgesic effect of the narcotic, measured by the hot plate test in the mouse (Fig. 1).

These results support the view that the analgesic action of morphine is mediated though liberation of brain catecholamines.

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A new nerve muscle preparation: the obturator nerve-anterior gracilis preparation of the rat

SIR,—It is well known that the rat phrenic nerve-diaphragm preparation is insensitive to certain muscle relaxant drugs (Paton & Zaimis, 1952). An alternative and possibly more sensitive preparation appears to be the obturator nerve-anterior gracilis preparation of the rat. This muscle is 1·0 mm thick (Quilliam, 1955), or about twice the thickness of the diaphragm. A good diagram of the rat anterior gracilis and obturator nerve is given by Quilliam (1955). The anterior gracilis is a strap-like muscle which arises from the pubis and is inserted into the upper end of the tibia. It is exposed by removing the skin from the thigh and removing the connective tissue. The muscle can be identified easily because the obturator nerve enters its upper border and then divides into two parallel branches which run longitudinally down the muscle towards its insertion. The obturator nerve is exposed by cutting through the pectineus muscle and is followed up to the obturator foramen. At this point the nerve can be easily sectioned and carefully separated from the underlying muscle. The origin of the anterior gracilis is exposed by removal of the pectineus muscle. The bony origin is then cut free from the pelvis with bone cutting forceps and the muscle separated from adjacent muscles. In a similar manner the insertion is separated from the rest of the tibia. The muscle and nerve are then free and can be set up in a similar manner to the phrenic nerve-diaphragm in Krebs solution, oxygenated with oxygen 95% and carbon dioxide 5% at 37°.

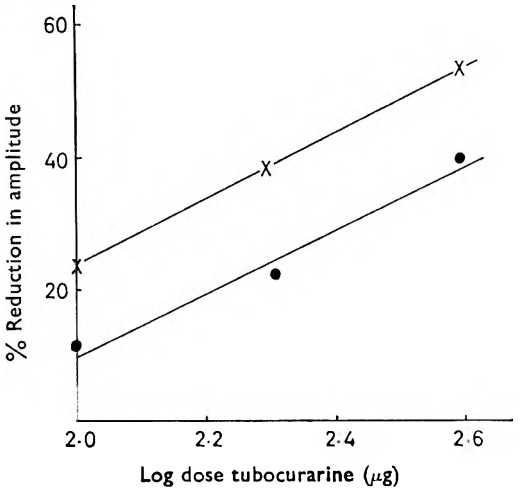

FIG. 1. Semi-log dose response curves showing difference in sensitivities to tubocurarine on the rat obturator nerve-anterior gracilis, ×——×, and the rat phrenic nerve-diaphragm, ●——●, preparations. Each point on the curves represents the mean of two values. In this experiment the range was too small to be shown. Both preparations were set up in the same 100 ml bath containing Krebs solution, gassed with oxygen (95%) and carbon dioxide (5%) at 37°. Each nerve was stimulated by supramaximal square wave pulses at a frequency of 12 min. Contractions were recorded using spring loaded levers writing on a smoked drum. The gracilis contractions were recorded above those of the diaphragm. In this experiment normal contractions were recorded for 90 sec, after which tubocurarine was added to the bath and recordings taken for a further 90 sec. Between responses the bath was washed out at 3 min intervals for 30 min.

The obturator nerve-anterior gracilis and phrenic nerve-diaphragm preparations were compared side-by-side in the same 100 ml bath. Each nerve was stimulated by supra-maximal square wave stimuli, having a pulse width of 0·5 msec, at a rate of 12/min from two different stimulators. Recordings were made on the same smoked drum using spring-loaded levers, the contractions of the gracilis being recorded above those of the diaphragm. Contractions were recorded for 60 to 90 sec, being constant within a single experiment, both before and after adding a neuromuscular blocking agent. After each response, the bath was washed out at 3 min intervals for 30 min.

In each of nine experiments, the obturator nerve-anterior gracilis preparation was consistantly more sensitive than the phrenic nerve-diaphragm preparation to tubocurarine (See Fig. 1). Similar results have been found with gallamine and with suxamethonium.

From these results, it would appear that a nonrespiratory muscle in the rat is more sensitive than a respiratory muscle to both kinds of neuromuscular blocking agent. This differs from the view of Paton & Zaimis (1952).

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A simple flow recorder

SIR,—We have designed a simple flow recorder based on the emptying of known volumes of fluid by a syphon to measure flow of fluids, such as in the rabbit ear perfusion, the rat hind limb perfusion or urinary flow. This apparatus, which does not appear to have been described hitherto, has the advantage of simplicity over the conventional electronic or mechanical types of flow recorders at present in use (see references).

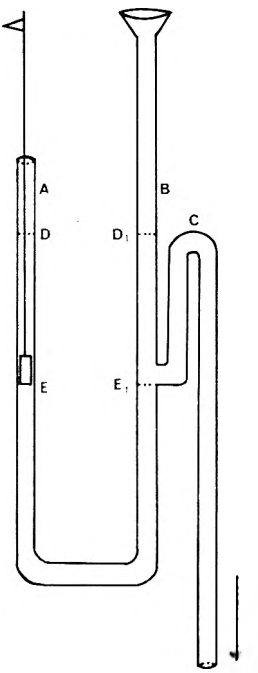

FIG 1. Diagrammatic sketch of the simple flow recorder.

In Fig. 1 the limb "B" of the U-tube is the receiver of the perfusion fluid. The apparatus is first filled with water to the level $E - E_1$. As the outflow collects in the limb "B", the level of the liquid also rises in the limb "A", carrying with it the float and writing point. When the level of the liquid reaches $D - D_1$, the syphon comes into action, bringing back the level of the liquid to $E - E_1$, and simultaneously the float drops to the original point. In this way a record of the emptying of each selected volume may be marked on a drum. The duration is conveniently marked with a time marker and, in this fashion, rate of flow may be measured.

By selecting the diameter of the tubes and the length of the syphon system, the instrument can be used to record flows ranging from a few drops to large volumes. The tubes are calibrated before use.

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The effect of hemicholinium on the depletion of catecholamine induced by reserpine

SIR,—Reserpine-induced depletion of catecholamines from the adrenal medulla has been attributed both to a direct effect on the organ and to an effect secondary to stimulation of centres in the central nervous system (CNS). Callingham & Mann (1958) and Stjärne & Schapiro (1959) concluded that the depletion was due to a direct action on the organ, while Brodie, Olin, Kuntzman & Shore (1957), Holzbauer & Vogt (1956) and Mirkin (1961) showed that depletion occurred only indirectly. In the experiments demonstrating a direct effect, rats were used, whereas in those indicating the involvement of a neural component rabbits were most often used. It is possible that the differing results are explainable by a species difference.

Feldberg, Minz & Tsudzimura (1934) have shown that acetylcholine is the neurotransmitter liberated during splanchnic stimulation of the adrenal gland. Recent work in our laboratory (Stitzel, Campos & Shideman, 1965) has indicated that hemicholinium (HC-3), an inhibitor of acetylcholine synthesis, mimics the effect of splanchnic nerve section and impairs the depleting action of reserpine in rabbits. A similar experimental design is now used to examine the extent of CNS stimulation in the depletion caused by reserpine of catecholamines from rat adrenal medulla.

TABLE 1. EFFECT OF HEMICHOLINIUM (HC-3) ON RESERPINE-INDUCED CHANGES IN CATECHOLAMINE CONCENTRATIONS IN TISSUES OF THE RAT AND RABBIT. The results are based on 4–7 measurements and are expressed as means ± s.e.m. The heart and brain levels are expressed as μg/g of tissue, while those of the adrenal gland are expressed as μg/gland. For experimental details see text.

Treatment	Heart	Brain	Adrenal
Rats			
Control	0·99 ± 0·05	0·51 ± 0·07	22·3 ± 3·02
Reserpine	0·27 ± 0·04	0·33 ± 0·05	14·3 ± 3·03
HC-3 + Reserpine	0·20 ± 0·08	0·28 ± 0·07	12·0 ± 3·05
Rabbits*			
Control	2·13 ± 0·10	0·64 ± 0·10	83·8 ± 3·9
Reserpine	0·40 ± 0·05	0·20 ± 0·02	26·0 ± 7·0
HC-3 + Reserpine	0·41 ± 0·09	0·20 ± 0·03	67·1 ± 7·6

* Taken from the data of Stitzel & others (1965).

Male Sprague-Dawley rats weighing 200–300 g were given HC-3 (50 μg/kg) in three doses at intervals of 2 hr, beginning 1 hr before the injection of reserpine (5 mg/kg, i.p.). The first dose was given intraperitoneally and the second and third subcutaneously. Animals were killed by a blow on the head 2 hr after the last dose of HC-3 (i.e. 5 hr after reserpine). The adrenals, heart and brain stem were removed, weighed and homogenized in 0·01N hydrochloric acid. The catecholamine content in these tissues was measured spectrophotofluorometrically by the trihydroxyindole method of Shore & Olin (1958).

Reserpine greatly decreased the amine content of the heart and brain stem and pretreatment with HC-3 failed to affect this depletion (Table 1). These results are in complete agreement with our previous experiments with rabbits. Table 1 shows that reserpine also reduced the total amine content of the rat adrenal gland and that this effect could not be antagonized by HC-3. It is apparent, therefore, that not even this maximally tolerated dose of HC-3 could impair the amine-depleting action of reserpine in the rat, whereas in the rabbit, adrenal amine loss was almost completely prevented. HC-3 alone had no effect on amine levels in any of the tissues examined of either of the species.

It appears that the discrepancies noted in the literature can be accounted for by a species difference. Our experiments support the hypothesis (Kroneberg & Schümann, 1957) that reserpine-induced depletion in the adrenal gland of the rat is due to a direct action of the drug on the gland, while in the rabbit reserpine appears to cause amine depletion only indirectly.

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The anti-nialamide effect of pantothenic acid and pyridoxine

SIR,—It has been suggested that monoamine oxidase inhibitors may produce a relative deficiency of pyridoxine (Gillespie, Terry & Sjoerdsma, 1959; Jones, 1961; Joseph & Berkman, 1965; Anon., 1965) and, bearing in mind the central role of coenzyme A in cerebral metabolism, we have examined the possibility of influencing the action of a monoamine oxidase inhibitor drug, nialamide, by the administration of pantothenic acid and pyridoxine.

Nialamide (20 mg/kg i.p.) reduces the sleeping time of mice if administered 24 hr before the intraperitoneal injection of 75 mg/kg of hexobarbitone sodium (Holtz, Balzer, Westermann & Wezler, 1957). Groups of mice were examined using this finding as a basis for the evaluation of the effect of pantothenic acid and pyridoxine. The sleeping time to hexobarbitone alone was established in 20 mice and then 20 mice were treated according to Holtz & others (1957), the sleeping times being measured as described by Holtz, Balzer & Westermann (1958). Another 10 mice, also injected with nialamide 24 hr previously, were given pantothenic acid (62·5 mg/kg) and pyridoxine (12·5 mg/kg) intramuscularly, 30 min before the hexobarbitone sodium. Two more groups of 17 mice were given nialamide and 24 hr later one group had pantothenic acid (125 mg/kg) and the other pyridoxine (25 mg/kg) 30 min before the barbiturate. Other groups were given pantothenic acid (125 mg/kg, 10 animals), or pyridoxine (25 mg/kg, 17 animals) alone or together (10 animals) at half these doses. A *t*-test was made on the groups.

Nialamide reduced the hexobarbitone-induced sleeping time by an average of 65% ($P < 0{\cdot}0001$). Pantothenic acid and pyridoxine antagonized the effect of the nialamide, the sleeping time being now reduced only by 30%. The protective effect against nialamide was also statistically significant

Pantothenic acid alone with nialamide decreased sleeping time by 41%. This protective effect was significant too ($P < 0{\cdot}01$); the protection given by pantothenic acid and pyridoxine together was higher than that given by pantothenic acid ($P < 0{\cdot}03$).

Pyridoxine in the presence of nialamide decreased sleeping time by 51%, an effect that was not significant.

Pantothenic acid or pyridoxine, separately or together, in the absence of nialamide, had no statistically significant effect on hexobarbitone-induced sleeping time.

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Tissue histamine and catecholamines in the reparative process

SIR,—Histamine is recognized as one of the chemical mediators in the process of inflammation (Bhatt & Sanyal, 1964; Schayer, 1964).

It has recently been suggested (Spector & Willoughby, 1964) that the inflammatory changes following injury may be due to local inactivation of endogenous anti-inflammatory substances, like catecholamines. In the rat there is a reduction in the level of catecholamines in the injured skin (Moller, 1962) with a rise in the monoamine oxidase activity (Raekallio, 1963). When monoamine oxidase inhibitors are used, there is a striking diminution in the increased permeability caused by an injurious stimulus. This has lent further support to the above view (Spector & Willoughby, 1964).

The relevant studies, so far, have been made during the exudative phase of inflammation. The present experiments were undertaken to assess the relative roles played by histamine and by catecholamines in the reparative phase in the rat.

The skin histamine was depleted by repeated injections of polymyxin B (Parratt & West, 1957). The catecholamine metabolism was blocked by monoamine oxidase inhibitors, like nialamide or iproniazid; and catechol-*O*-methyl transferase inhibitors, like pyrogallol or quercitin. The quantity of the granulation tissue developed under various experimental circumstances was measured by the cotton pellet method (Finney & Somers, 1958). The strength of the fibres was assessed by measuring the tensile strength of the scar tissue (Fenton & West, 1963). The animals were killed for various estimations 10 days after producing injury.

In the polymyxin B treated animals there was a marked reduction in the tensile strength with only a slight reduction in the granulation tissue. The tensile strength of the scar tissue was also reduced in animals treated with monoamine oxidase inhibitors though the total amount of the granulation tissue was little affected.

In animals treated with pyrogallol, the amount of the granulation tissue as well as the tensile strength were reduced. A large reduction in the tensile strength but not in the granulation tissue, was seen in the rats treated with quercitin.

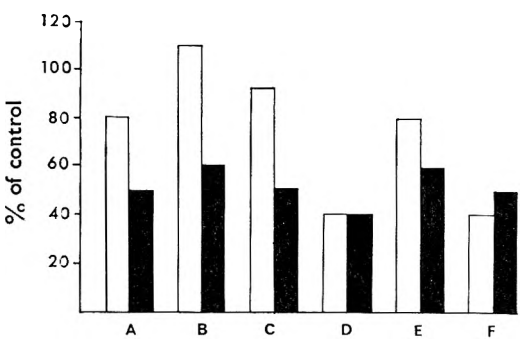

FIG. 1. The effect of various drugs on the granulation tissue formation (open columns) and tensile strength (solid columns) of scar tissue in the rat. A. Polymyxin B, 5 mg/kg. B. Nialamide, 80 mg/kg. C. Iproniazid, 40 mg/kg. D. Pyrogallol, 200 mg/kg. E. Quercetin, 50 mg/kg. F. Betamethasone, 2·5 mg/kg. Drugs were injected intraperitoneally daily for 10 days.

When the animals were treated with a potent anti-inflammatory corticosteroid such as betamethasone, the total amount of granulation tissue as well as the tensile strength were much reduced. The results are illustrated in Fig. 1.

It was thus seen that the processes which either lead to a reduction in the tissue histamine content or which block the pathway of catecholamine disposal, affect the reparative process in such a way as to produce a poor quality of granulation tissue without markedly affecting the total quantity.

Since both histamine and catecholamines take part in the early exudative phase as well as in the late reparative process, it is possible that a dynamic balance between these two biogenic amines may be one of the factors determining the ultimate inflammatory response of the tissues.

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Letters to the Editor

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