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Research Papers

Pharmacological properties of some imidazole derivatives occurring in nature

G. BERTACCINI AND T. VITALI

Some naturally occurring histamine derivatives such as monomethylhistamine [4-(2-methylaminoethyl)imidazole], dimethylhistamine [4-(2-dimethylaminoethyl)-imidazole], spinaceamine (4,5,6,7-tetrahydroimidazo[5,4-c]pyridine) and 6-methyl-spinaceamine, and the quaternary ammonium base of histamine [4-(2-trimethyl-aminoethyl)imidazole] hitherto unknown in nature, were submitted to a pharmacological examination. The actions of monomethylhistamine resembled closely those of histamine. Dimethylhistamine was 2 to 20 times less active than histamine and showed some weak "nicotinic" effects. Trimethylhistamine had about 1% of the activity of histamine but showed a potent "nicotinic" activity on the preparations examined. Spinaceamine and 6-methylspinaceamine were virtually inactive. The importance of the *N'*-methylhistamines which behave similarly to the methyl derivatives of 5-hydroxytryptamine is discussed.

IN a systematic study of biologically active amines in the amphibian skin, skin extracts of some South-American *Leptodactylinae* were found to contain large amounts of imidazole derivatives. Using paper chromatography and biological assay of the natural compounds compared with the corresponding synthetic compounds, it was possible to identify not only histamine, monomethylhistamine and dimethylhistamine, but also two hitherto undescribed imidazole-*c*-pyridine derivatives: spinaceamine and 6-methylspinaceamine (Erspamer, Vitali, Roseghini & Cei, 1963).

Much has been written about the pharmacological properties of the *N'*-methylhistamine derivatives but many discrepancies exist in the reported data. Dale & Dudley (1921) found the monomethylhistamine to have 1/200th the activity of histamine on the cat blood pressure and 1/80th its activity on the guinea-pig uterus. In contrast to the results of Fränkel & Zeimer (1920), they also observed that the "imidazoliso-piperidine" (spinaceamine) had only 1/1500th of the activity of histamine on uterine muscle of guinea-pig and practically no action on the blood pressure of cats. Fargher & Pyman (1921) claimed that monomethyl-histamine has a negligible histamine-like action (about 1/100) and later, Garforth & Pyman (1935) found it to be approximately as active as histamine on the guinea-pig uterus. Vartiainen (1935) found the mono-methyl derivative twice as potent as histamine on the guinea-pig uterus and intestine. Huebner, Turner & Sholz (1949) observed that both mono- and di-methylhistamine exhibited 75% of the oxytocic activity of histamine. Burger (1960) claimed the monomethyl derivatives to be only one half as active as histamine in the cat, but to exert twice its action on the blood pressure of the guinea-pig. Ingle & Taylor (1963) found dimethylhistamine and other imidazolealkylamines to be 10 to 100 times

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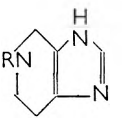
less potent than histamine. In addition, Tabor (1954) says that monomethylhistamine "has not been reported as a naturally occurring substance", but Kapeller Adler & Iggo (1957) have found both mono- and dimethyl derivatives in human urine. While the ring *N*-methylation was considered one of the main routes of histamine metabolism by many (Schayer, 1956, 1959; Schayer & Karyala, 1956; Brown, Tomchick & Axelrod, 1959), Gaddum recently (1962) pointed out the possibility that the most important mechanism by which histamine is inactivated in the body was the methylation of the side chain amino-group. To complicate matters, terminology is sometimes incorrect or incomplete since references are made to "methyl derivatives" but which kind is not stated.

Because of the discrepancies and also of the part that *N'*-methylhistamines are apparently destined to assume in the metabolism of histamine, especially after the discovery of new natural imidazole derivatives, it seemed opportune to submit the whole series of *N*-'methylated histamine derivatives (including the quaternary ammonium base of histamine hitherto unknown in nature) to a thorough pharmacological study. This we report.

Compounds. Histamine, I, R = $CH_2 \cdot CH_2 \cdot NH_2$ (2HCl); *N'*-methylhistamine[4-(2-methylaminoethyl)imidazole], I, R = $CH_2 \cdot CH_2 \cdot NH(Me)$ (HCl); *N'N'*-dimethylhistamine [4-(2-dimethylaminoethyl)imidazole], I, R = $CH_2 \cdot CH_2 \cdot NMe_2$ (HCl); *N'N'N'*-trimethylhistamine [4-(2-trimethylaminoethyl)imidazole, I, R = $CH_2 \cdot CH_2 \cdot NMe_3Cl$ (HCl): spinaceamine 4,5,6,7-tetrahydromidazo[5,4-*c*]pyridine; II, R = H; 6-methylspinaceamine 6-methyl-4,5,6,7-tetrahydroimidazo[5,4-*c*]pyridine; II, R = Me.

I



II

Dr. Vitali prepared the mono-, di- and trimethyl derivatives and both spinaceamines. Samples of histamine, nicotine and hexamethonium, were purchased from Merck and Recordati respectively. Leptodactyline and murexine were natural compounds prepared in our Institute. Weights of the compounds are quoted in terms of their free bases.

Pharmacological methods

HISTAMINE-LIKE EFFECTS

Guinea-pig ileum. Drugs were tested in a normal Krebs solution on the guinea-pig ileum prepared in the usual manner, and after treatment with atropine (10^{-7}), mepyramine (2 to 10,000 ng/ml) and hexamethonium (10 to 100 μg/ml) respectively.

Capillary permeability. This was tested on human and guinea-pig skin vessels.

Human skin vessels. Drugs were injected on the flexor surface of the forearm of 12 human volunteers as described to us by De Caro (1963). The dose of the test substance varied between 25 ng and 25 μg, the volume was always 0·1 ml. The size of the pomphus and the intensity of the erythema were examined.

Guinea-pig skin vessels. The methods was based on that of Miles & Miles (1952). After clipping away the hair, the animals were injected intravenously with 1·2 ml/kg of a 5% solution of Pontamine Sky Blue 6 BX in saline. 1 hr later the test drugs were injected intradermally in a volume of 0·1 ml. Doses of histamine varied between 50 and 500 μg. The effects of the compounds were evaluated for their ability to produce an accumulation of dye at the site of injection.

Guinea-pig bronchoconstriction. The technique used by Collier, Holgate, Schachter & Shorley (1960) was followed.

Blood pressure. Animals were anaesthetised using chloralose (90–100 mg/kg), pentobarbitone (30 mg/kg) or urethane (1–1·3 g/kg) each given intravenously. Cats and dogs received intravenous injections into the femoral vein, rabbits into the marginal vein of the ear and rats into the jugular vein after a light ether anaesthesia. Blood pressure was measured from the femoral or carotid artery by a mercury manometer.

UNSPECIFIC EFFECTS

Frog rectus abdominis and leech dorsal muscle were prepared in the usual manner. Rat diaphragm was prepared as described by Bülbring (1946). Cat sciatic nerve-gastrocnemius preparation, cat nictitating membrane and spinal cat, were according to Burn (1950).

Results

HISTAMINE-LIKE ACTIONS

The activity of derivatives as compared with histamine on various preparations is shown in Table 1.

Guinea-pig ileum. The response of the guinea-pig ileum to all the compounds was not modified after atropine (10^{-7}). After mepyramine (2 ng/ml), histamine and its monomethyl derivatives were antagonised to

TABLE 1. HISTAMINE-LIKE EFFECTS OF THE IMIDAZOLE DERIVATIVES COMPARED WITH HISTAMINE

The activity of histamine is arbitrarily taken as 100. The activities of the other drugs are expressed as percentages. ϕ = the compound is inactive or at least over 1000 times less active than histamine. Sign — = not tested. Values represent the mean of the values obtained from 3 to 6 different preparations of each test.

	H	MMH	DMH	TMH	Sp	MSp
Guinea-pig ileum	100	85	45	1	0·03	0·05
Human skin vessels	100	95	20	0·40	0·10	0·08
Guinea-pig bronchoconst.	100	90	20	—	—	—
Guinea-pig skin vessels	100	65	5	<1	ϕ	ϕ
Blood pressure						
Dog	100	60–65	45–55		ϕ	ϕ
Cat	100	75	35		0·55	0·15
Rat	100	75	35	30	—	—
Rabbit	100	85	35	(75)	<0·2	<0·2

H = Histamine; MMH the mono-, DMH the di-, and TMH the trimethyl derivatives. Sp = spinaceamine; MSp = methylspinaceamine.

the same extent. This is in accordance with the observations of Schild (1947). The dimethyl and especially the trimethyl derivative were affected to a lesser degree than histamine by the antihistamine agent. The negligible activity shown by spinaceamine is in accordance with observations by Dale & Dudley (1921) on the guinea-pig uterus and cat blood pressure.

Human skin vessels. Our results with monomethylhistamine agreed with Vartiainen's data (1935), although they were obtained with a slightly different technique. The dimethyl derivative we found to be much less active than did Vartiainen. The trimethyl derivative showed a weak but typical histamine-like effect.

Guinea-pig skin vessels. In this test the activity of monomethylhistamine came close to that exhibited on other preparations while dimethylhistamine showed little activity.

Guinea-pig bronchoconstriction. The ratio of activity between histamine and its methyl derivatives was similar to that obtained in above preparations.

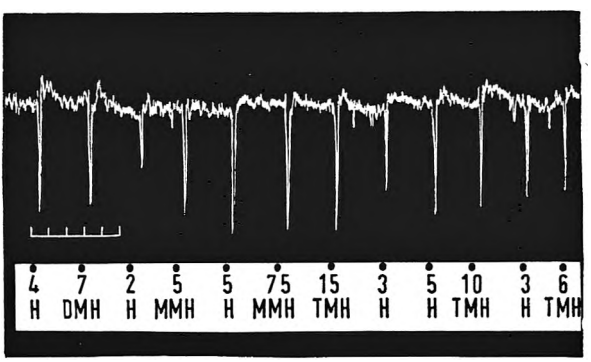


FIG. 1. Rat blood pressure. H = histamine; MMH = monomethylhistamine; DMH = dimethylhistamine; TMH = trimethylhistamine. Doses in μg. Time in min.

Blood pressure. Dog. On dog blood pressure the action of the mono- and di-methyl derivatives resembled that of histamine (60–65 and 45–55% as active respectively). Trimethylhistamine did not exhibit any histamine-like action in doses up to 25–50 μg/kg. At 100–200 μg/kg, it caused an hypertensive response similar to that produced by leptodactyline but 5 to 10 times less intense.

Cat. The cat behaves essentially like the dog in its blood pressure responses to the derivatives. The two spinaceamines were not completely inactive on this test.

Rabbit. The effects of histamine and of the methylhistamines were erratic. Sometimes they caused hypotension, sometimes hypertension and sometimes a diphasic response. The behaviour of mono- and di-methylhistamine was similar to that of histamine. Trimethylhistamine usually produced an initial fall of blood pressure followed by a more sustained hypertension. This diphasic response was not modified by mepyramine or by atropine.

Rat. In rats anaesthetised with pentobarbitone, the ratio between histamine and its methyl derivatives was the same at any dose level. In this species, trimethylhistamine mimicks histamine in its effect on blood pressure. The transient hypotension it produced was similar to that produced by histamine and seldom was it followed by a small short-lived rise of blood pressure (Fig. 1).

OTHER ACTIONS

Trimethylhistamine displayed, as expected, "nicotinic" actions on a number of biological preparations.

The "nicotinic" effects of dimethylhistamine were much less evident. Table 2 shows the activity of trimethylhistamine in comparison with that of leptodactyline, murexine, nicotine and adrenaline.

TABLE 2. NICOTINE-LIKE EFFECTS OF DIFFERENT DRUGS COMPARED WITH TRIMETHYLHISTAMINE

The figures indicate the number of moles of the different compounds required to give the same effect as 1 mole of trimethylhistamine. Values represent the mean of the values obtained from 2 to 6 different preparations of each test.

	TMH	Nic.	Lep.	Mur.	Ad.
Guinea-pig ileum	1	1			
Frog rectus	1	(0·75)	0·003	0·12	
Leech dorsal muscle	1	0·25			
Rat diaphragm	1	0·45			
Cat gastrocnemius	1		0·06	(0·20)	
Cat nictitating membrane	1	1·5	0·20		0·04
Cat, spinal	1	0·75	0·15		0·025
Dog blood pressure	1		0·16		

Nic. = nicotine; Lep. = leptodactyline; Mur. = murexine; Ad. = adrenaline.

Guinea-pig ileum. The activity ratio trimethylhistamine: nicotine which in the normal Krebs solution was 1 : 1, became 4 : 1 after mepyramine 2 ng/ml and 7 : 1 after mepyramine 100 ng/ml hence with this dose of the antihistamine, the trimethyl derivative was only 14% as active as nicotine. On the other hand, after hexamethonium, 10 μg/ml, the activity ratio trimethylhistamine: nicotine became 1 : 5 and it rose to 1 : 8 after

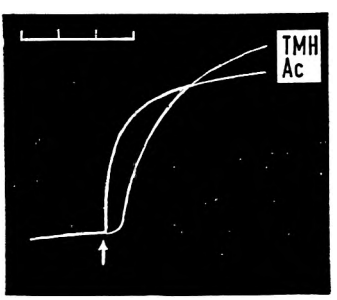


FIG. 2. Frog rectus abdominis preparation. At arrow 0·3 μg/ml acetylcholine (Ac) and after washing and relaxation of the muscle, 15 μg/ml TMH. Time in min.

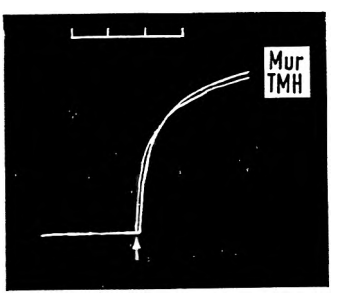


FIG. 3. Frog rectus abdominis preparation. At arrow 1·5 μg/ml murexine (Mur) and after washing and relaxation of the muscle, 10 μg/ml TMH. Time in min.

hexamethonium 100 μg/ml. With this dose, the activity ratio trimethylhistamine : histamine, which in the normal Krebs solution was 100 : 1, became 170 : 1.

Frog rectus abdominis. Trimethylhistamine caused, in this muscle, a contracture which closely resembled that produced by leptodactyline and murexine (Fig. 3) but was different from that produced by acetylcholine (Fig. 2) and nicotine. To give an approximate idea of the relative activities of these drugs, the heights of the contractions were compared after a fixed time. The minimum active dose of trimethylhistamine was 1 to 3 μg/ml and there was a satisfactory dose/response relationship. Tubocurarine showed a strong antagonistic action: after 0·5 μg/ml the contracture provoked by 25 μg/ml of the histamine derivative was reduced by 70 to 90% of the control; after tubocurarine, 1 μg/ml, the effect of the derivative was completely abolished. The dimethyl derivative showed approximately 2% of the activity of trimethylhistamine. As shown in Fig. 4 the contractures provoked by the two histamine derivatives were qualitatively identical.

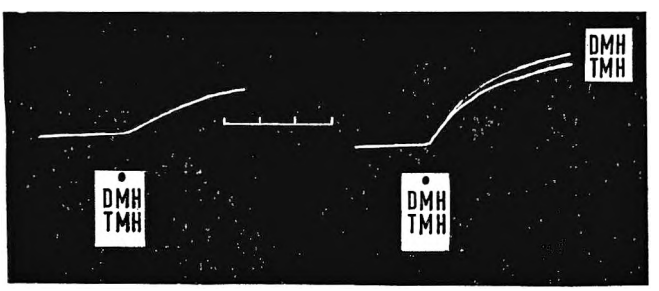


FIG. 4. Frog rectus abdominis preparation. At first dot 0·2 mg/ml DMH and 4 μg/ml TMH. At second dot 0·4 mg/ml DMH and 8·5 μg/ml TMH. Time in min.

Leech dorsal muscle. Trimethylhistamine contracture was similar to that elicited by nicotine. Eserine salicylate, 0·2 μg/ml, potentiated the response of both the derivative and nicotine by 30 to 50% of the control.

Rat diaphragm. This preparation showed little sensitivity to trimethylhistamine. The threshold blocking dose was 20 to 30μg/ml. Whereas the

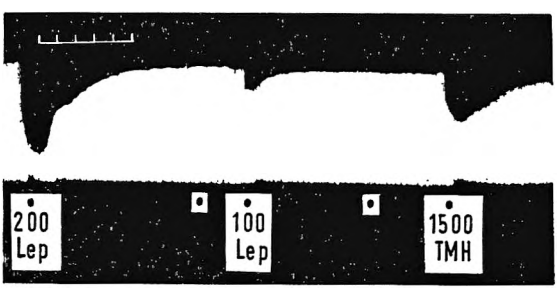


FIG. 5. Cat gastrocnemius preparation. Lep = leptodactyline; TMH = trimethylhistamine doses in μg/kg. Recording stopped for 15 min at unlabelled dots. Time in min.

derivative was about 50 times less potent than suxamethonium as a neuromuscular blocking agent, it was only 10 to 20 times weaker as a tubocurarine (0·5 μg/ml) antagonist.

Cat gastrocnemius. The action of trimethylhistamine in reducing twitches resembled that of leptodactyline (Fig. 5) though it was approximately 15 times weaker and only half as potent in antagonising tubocurarine (50 μg/kg). The minimum blocking dose was 200 to 400 μg/kg. Although the derivative was only 5 times less potent than murexine, its action was considerably more shortlived. It was approximately 1/20th as potent as suxamethonium. As with many other blocking agents, repeated administration of trimethylhistamine at 15 min intervals resulted in drug cumulation (Fig. 6). Dimethylhistamine was completely ineffective up to 1 mg/kg: higher doses could not be tested owing to its action on blood pressure.

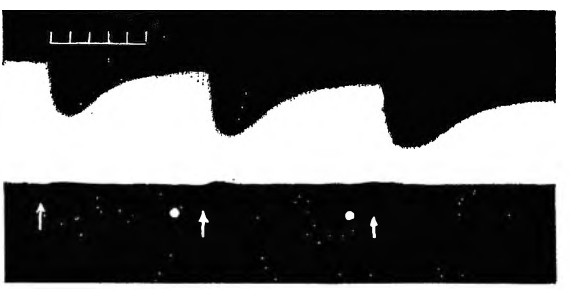


FIG. 6. Cat gastrocnemius preparation. At arrow 0·5 mg/kg TMH. Recording stopped for 15 min at dots. Time in min.

Cat nictitating membrane. The threshold dose of trimethylhistamine on this preparation was 50 to 75 μg/kg. The contraction elicited by 200 μg/kg was reduced by 50–70% after hexamethonium, 0·5 mg/kg, and completely abolished after 1 mg/kg.

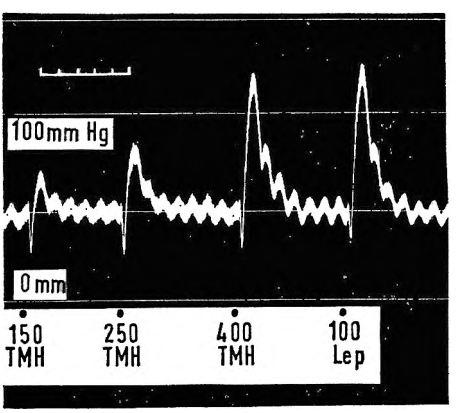


FIG. 7. Spinal cat preparation. Lep = leptodactyline; TMH = trimethylhistamine doses in μg/kg. Time in min.

Spinal cat. As with the normal cat, hypertension caused by trimethylhistamine was preceded by a small fall of the blood pressure which was not proportional to the dose administered. The rise of blood pressure was satisfactorily proportional to the dose (Fig. 7). Hypertension produced by 200 to 500 μg/kg, was reduced by about 30 to 40% after adrenalectomy. This may signify that only 30–40% of trimethylhistamine hypertension was due to release of medullary catecholamines.

Discussion

It appears from the experimental data that monomethylhistamine most closely resembled histamine in its action on blood pressure and on plain muscles, but its activity was always weaker than that of histamine.

Dimethylhistamine was 2 to 20 times less active than histamine. This tertiary amine showed a feeble nicotine-like activity on the frog rectus abdominis and on the guinea-pig ileum after mepyramine. At low doses, mepyramine inhibited the dimethyl derivative to a lesser degree than it did histamine.

Trimethylhistamine was characterised by the predominance of nicotine-like activity. Table 2 shows it to be the derivative quantitatively more closely related to nicotine, and murexine and leptodactyline to be more potent than either. As well as nicotine-like action, the trimethyl derivative had a histamine-like action, though this was less powerful than that of the two other methyl derivatives. This histaminic activity was demonstrated by the activity shown in the isolated guinea-pig ileum, in which the responses were modified after mepyramine and hexamethonium, and by the weak but specific histamine-like action exhibited on the human skin vessels.

The lack of activity of spinaceamine agrees with the findings by Dale & Dudley (1921). Its 6-methyl derivative behaved similarly.

We thus conclude that stepwise *N*-methylation in the side chain of the histamine molecule regularly reduces the histamine-like activity and this negative effect increases with the number of the methyl groups. Linkage of the side chain to the 5 position of the imidazole nucleus through a *N*-methyl group, as occurring in spinaceamine and in 6-methylspinaceamine, completely abolishes the activity. The reduction of histamine activity was accompanied by the appearance of a nicotinic activity, as with the di- and especially the trimethylhistamine.

Most of our results are similar to those obtained by others. Some discrepancies may be explained, at least partially, with the different methods of chemical preparations of the compounds. Sometimes, as with Frankel & Zeimer's (1920) findings for spinaceamine, and those of Fargher & Pyman (1921) for monomethylhistamine, the chemical synthesis probably gave contaminated products. Quantitative differences may also depend on the different animal species used for the pharmacological examination.

The relationship between chemical structure and pharmacological activity observed with histamine derivatives may bear comparison with observations

on 5-hydroxyindolealkylamines (Erspamer, 1952; Bertaccini & Zamboni, 1961) namely: progressive decay of 5-hydroxytryptamine activity with increasing introduction of *N*-methyl groups with the final appearance of nicotinic actions; loss of activity with linkage of the side-chain to the indole nucleus to form a tricyclic system—pyrrole [3,4,5-*d,e*]quinoline (Märki, Robertson & Witkop, 1961).

References

Bertaccini, G. & Zamboni, P. (1961). *Arch. int. Pharmacodyn.*, **133**, 138–156.
Bülbring, E. (1946). *Brit. J. Pharmacol.*, **1**, 38–61.
Brown, D. D., Tomchick, R. & Axelrod, J. (1959). *J. biol. Chem.*, **234**, 2948–2950.
Burger, A. (1960). *Medicinal Chemistry*, p. 516, New York: Interscience Publishers, Inc.
Burn, J. H. (1950). *Biological Standardization*, pp. 347–351, Oxford: University Press.
Collier, H. O., Holgate, J. A., Schachter, M. & Shorley, P. G. (1960). *Brit. J. Pharmacol.*, **15**, 290–297.
Dale, H. H. & Dudley, H. W. (1921). *J. Pharmacol.*, **18**, 103–110.
Erspamer, V. (1952). *Nature, Lond.*, **170**, 281.
Erspamer, V., Vitali, T., Roseghini, M. & Cei, J. M. (1963). *Experientia*, **19**, 346.
Fargher, R. G. & Pyman, F. L. (1921). *J. chem. Soc.*, **119**, 734–740.
Frankel, J. & Zeimer, L. (1920). *Biochem. Z.*, **110**, 234.
Gaddum, J. H. (1962). *Proc. XXII Inter. Con. Physiol. Scien.*, pp. 849–851, Leiden.
Garforth, B. & Pyman, F. L. (1935). *J. chem. Soc.*, 489–492.
Huebner, C. F., Turner, R. A. & Scholz, C. R. (1949). *J. Amer. chem. Soc.*, **71**, 3942–3944.
Ingle, P. H. B. & Taylor, H. (1963). *J. Pharm. Pharmacol.*, **15**, 620–623.
Kapeller-Adler, R. & Iggo, B. (1957). *Biochim. Biophys. Acta*, **25**, 394–402.
Märki, F., Robertson, A. V. & Witkop, B. (1961). *J. Amer. chem. Soc.*, **83**, 3341–3342.
Miles, A. A. & Miles, E. M. (1952). *J. Physiol.*, **118**, 228–257.
Schayer, R. W. (1956). *Brit. J. Pharmacol.*, **11**, 472–473.
Schayer, R. W. (1959). *Physiol. Rev.*, 116–126.
Schayer, R. W. & Karyala, S. A. (1956). *J. biol Chem.*, **221**, 307–312.
Schild, H. O. (1947). *Brit. J. Pharmacol.*, **2**, 251–258.
Tabor, H. (1954). *Pharmacol. Rev.*, **6**, 299–344.
Vartiainen, A. (1935). *J. Pharmacol.*, **54**, 265–282.

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Estimation of *C*-glycosides and *O*-glycosides in cascara (*Rhamnus purshiana* DC., bark) and cascara extract

J. W. FAIRBAIRN AND S. SIMIC

An assay process is described which enables the cascarosides, the aloins and the *O*-glycosides of cascara to be estimated separately. The cascarosides are separated from the aloins by partition between water and ethyl acetate; each fraction is then oxidised and hydrolysed by ferric chloride treatment to the free anthraquinones which are determined colorimetrically as aloe-emodin. The *O*-glycosides are determined colorimetrically as emodin after acid hydrolysis. Results show that commercial extracts contain only about half the theoretical amount of *C*-glycosides and about quarter of the *O*-glycosides and there is a significant breakdown of primary glycosides. These results indicate that decomposition occurs during preparation of the official extract. One commercially available extract, which passed current official standards, was shown by the method to be very deficient in activity. In view of the small proportion of *O*-glycosides in commercial extracts a shortened assay process, for cascarosides and aloins only, is described.

CASCARA bark contains the following classes of anthracene derivatives: (*a*) *cascarosides*, which are primary glycosides of barbaloin and chrysaloin and of which about four have been identified; (*b*) *aloins*, barbaloin and chrysaloin, which are *C*-glycosides of aloe-emodin anthrone and chrysophanol anthrone respectively; (*c*) O-*glycosides*, mostly based on emodin and of which about four are present and (*d*) the free *anthraquinones*, aloe-emodin, chrysophanol and emodin (Fairbairn & Simic, 1960). The cascarosides are probably the most important components. In preliminary experiments we have found them more active biologically than the aloins, which is consistent with the fact that the primary glycosides of senna are more active than the secondary ones (Fairbairn, Friedmann & Ryan, 1958). Furthermore they have a sweet taste in contrast to the aloins, and are therefore preferable pharmaceutically. A chemical method of evaluating cascara should therefore estimate the cascarosides separately from the aloins. The free anthraquinones have little purgative activity (Fairbairn, 1949) and should not be included by the assay process. Recently Auterhoff & Sachdev (1962) published a method for estimating the *C*-glycosides and *O*-glycosides of cascara but their method does not estimate the cascarosides separately from the aloins nor does it eliminate the free compounds. We have therefore attempted to devise a chemical method of assay which would estimate the three important classes of anthracene derivatives separately. Bark and extracts conforming to B.P. specifications were examined.

Experimental

EXTRACTION OF THE GLYCOSIDES FROM THE BARK AND EXTRACT

Extraction with water was unsuitable as subsequent treatment with organic solvents led to troublesome emulsions. The use of 70% methanol as extracting solvent prevented this; furthermore 5 to 30% more glycosides were extracted in a given time with this solvent than with water. Some samples of extract dissolved rapidly in the 70% methanol; others were

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much more difficult. We therefore recommend, as a general method, standing in the solvent overnight.

SEPARATION OF THE CASCAROSIDES FROM THE ALOINS

The cascarosides are much more water soluble than the aloins and we found that the partition coefficient of barbaloin, in the system ethyl acetate: water, was 1·0 whereas for cascaroside A it was almost zero. Using an artificial mixture of barbaloin and cascaroside A it was possible to effect 96 to 99% separation by distribution between ethyl acetate and water under suitable conditions. When applied to the more complex mixtures occurring in the crude drug these conditions (slightly modified) were shown to be successful by chromatographic examination of each stage.

ESTIMATION OF THE *O*-GLYCOSIDES

Acid hydrolysis followed by extraction of the liberated aglycones seemed the obvious way of estimating these glycosides. However, the hydrolysis process also converts the cascarosides to aloins, so that it must be applied *after* the separation of these two classes of substance from each other. This separation process unfortunately results in the *O*-glycosides being distributed fairly equally between the ethyl acetate and water layers. Each layer has therefore to be treated with acid and the liberated aglycones removed before the *C*-glycosides present are estimated. Details of how this is effected are given in the method of assay below.

STANDARDS

Since the four known cascarosides have a closely similar structure they are estimated as cascaroside A which we found, by preparative methods, to be present in the largest proportion. Treatment of pure cascaroside A by the ferric chloride treatment detailed below showed that 1 mg of aloe-emodin corresponded to 2·56 mg cascaroside A (an exactly similar result was obtained with cascaroside B). The aloins were estimated as barbaloin (Fairbairn & Simic, 1963) and the *O*-glycosides as emodin monoglucoside a pure sample of which was separated from the bark (Simic, 1961). Another emodin monoglucoside has also been shown to be present in cascara (Schindler, 1946).

RECOMMENDED METHOD OF ASSAY

Transfer about 1 g powdered bark or 0·5 g powdered extract, accurately weighed, to a 100 ml volumetric flask by means of 80 ml of 70% methanol. Allow to stand overnight with occasional shaking; make up to volume with 70% methanol shake well and filter. To 10 ml of the filtrate add 10 ml water and extract 2 to 3 times with 20 ml portions of carbon tetrachloride. Wash the combined carbon tetrachloride extracts with 10 ml of water, reject the carbon tetrachloride layer and return the washings to the aqueous layer. Extract the combined aqueous layers with water-saturated ethyl acetate (4 × 30 ml for the bark and 5 × 60 ml for the extract) and reserve both layers for further work.

ALOINS

Evaporate the ethyl acetate layer to small volume and transfer to a 100 ml conical flask and evaporate to dryness. Dissolve the residue in 0·3 to 0·5 ml methanol, add 10 ml N hydrochloric acid, attach a short air condenser to the flask and heat in a boiling water-bath for 15 min. Cool, transfer to a separator and rinse the flask with 2 to 3 ml N sodium hydroxide followed by 2 to 3 ml water. Shake the combined aqueous layers with 20 ml carbon tetrachloride, allow the layers to separate and draw off the lower carbon tetrachloride layer. Run the intermediate emulsified layer into a small beaker, dissolve in about 1 ml N sodium hydroxide and return to the aqueous layer. Repeat the extraction of the aqueous layer in a similar manner with two more portions of 20 ml carbon tetrachloride. Filter the aqueous layer into a 25 ml volumetric flask; wash the carbon tetrachloride layer with small quantities of water and pass through the filter and make up to volume. (Reserve the carbon tetrachloride layer for assay of the *O*-glycosides). To a suitable aliquot of the filtrate (20 ml for the bark; 5 or 10 ml for the extract but in each case made up to 20 ml with water) add 1·2 g anhydrous ferric chloride, 12 ml hydrochloric acid B.P. and heat in a boiling water-bath, under reflux, for 4 hr. Cool, extract with 3 × 20 ml portions of carbon tetrachloride and wash the combined carbon tetrachloride extracts with 2 × 10 ml water. Reject the washings. Extract the carbon tetrachloride layer with 10,5 and 5 ml N sodium hydroxide; heat the combined alkaline extracts in a boiling water-bath for 5 min (to drive off traces of carbon tetrachloride), cool and make up to 25 ml. Determine the extinction of this solution at 500 mμ, within 1 hr, and estimate the concentration of aloe-emodin from the *E* (1% 1 cm) value of 320 or from a suitable calibration curve. Calculate the percentage of aloins present as barbaloin from the fact that 1 mg aloe-emodin is equivalent to 1·61 mg $C_{21}H_{22}O_9 \cdot H_2O$.

CASCAROSIDES

To the aqueous layer obtained by the preliminary treatment, add 3 ml hydrochloric acid, and heat in a conical flask to which a short air condenser is attached in a boiling water-bath for 15 min. Cool, extract with 3 × 20 ml carbon tetrachloride and treat the intermediate emulsified layer as previously described. Filter the aqueous layer into a 50 ml volumetric flask. Wash the combined carbon tetrachloride layers with small quantities of water and pass them through the filter and make up to volume. (Reserve the carbon tetrachloride layer for assay of *O*-glycosides). To 10 ml of the filtrate add 0·6 g anhydrous ferric chloride 6 ml hydrochloric acid B.P. and heat in a boiling water-bath, under reflux, for 4 hr. Continue as for ALOINS beginning: Cool, extract with 3 × 20 ml carbon tetrachloride. . . . Calculate the percentage of cascarosides as cascaroside A. 1 mg aloe-emodin is equivalent to 2·56 mg cascaroside A.

O-GLYCOSIDES

Combine the carbon tetrachloride extracts from the "aloins" and "cascarosides" stages; extract with 10,5 and 5 ml N sodium hydroxide.

Heat the combined extracts in a boiling water-bath for 5 min. Cool and make up to 25 or 50 ml according to intensity of colour. Determine the extinction of this solution at 510 mμ, within 1 hr, and estimate the concentration of emodin from the E (1%, 1 cm) value of 330 or from a suitable calibration curve Calculate the percentage of *O*-glycosides as emodin monoglucoside. 1 mg emodin is equivalent to 1·60 mg emodin monoglucoside.

Results

Replicate assays on one sample (A) of extract were made using several separate weighings and the standard deviation of the results calculated. This indicates a reproducibility for a single assay (P = 0·95) of about ± 3·6% for the cascarosides; ± 1% for the aloins and ±6% for the *O*-glycosides. Several other commercial extracts and samples of bark were also assayed in duplicate and the results recorded in Table 1.

TABLE 1. ANALYSES OF COMMERCIAL SAMPLES OF CASCARA BARK AND EXTRACTS CONFORMING TO B.P. SPECIFICATIONS. RESULTS ARE EXPRESSED AS g/100 g AIR-DRY SAMPLE (1 g EXTRACT SHOULD BE EQUIVALENT TO 4 g BARK)
Each figure (except for extract A) is the average of two assays.

Sample	*C*-Glycosides			*O*-Glycosides (as emodin glucoside)
	Cascarosides (as cascaroside A)	Aloins (as barbaloin)	Total (as barbaloin)	
Bark				
B_1 1954	6·83	0·42	4·72	1·22
B_2 1957	5·22	0·80	4·08	0·98
B_3 1962	6·76	0·44	4·69	0·74
B_4 1962	7·67	0·69	5·51	0·91
B_5 1962	6·60	0·68	4·75	0·72
Extract				
A 1963	9·72	2·98	9·11	1·11
(Means of 6 assays)	(s. d. = 0·175)	(s. d. = 0·016)		(s. d. = 0·035)
B 1963	9·32	7·80	13·40	0·44
C 1962	6·93	5·11	9·47	0·90
D 1955	7·68	3·50	8·33	0·53
E 1955	5·40	6·77	10·17	1·0
F 1954	1·20	1·59	2·26	0·21

Discussion

OFFICIAL PREPARATIONS OF CASCARA

The results in Table 1 indicate two deficiencies in the official methods of preparing dry extract of cascara, 1 g of which should correspond approximately to 4 g of bark. For the 5 samples of bark examined the average content of *C*-glycosides (calculated as barbaloin) is 4·75%, and for *O*-glycosides is 0·90%. For the 5 samples of extract (A to E) the corresponding figures are 10·10% and 0·80%. Clearly there has been a significant loss of both classes of constituents in preparing the official extract. Secondly the proportion of cascarosides to aloins is much lower in the extract than in the bark. This indicates that decomposition has taken place during evaporation of the bulky aqueous percolate and this suggestion is confirmed by the low amount of the labile *O*-glycosides in the extract.

In spite of these deficiencies the final product contains a significant amount of the relatively thermostable aloins which would account for the

fact that cascara tablets have been accepted for a long time as an effective purgative. This is in contrast with the former official preparations of senna whose less stable *O*-glycosides largely disappear during preparation and storage of the galenicals (Fairbairn & Michaels, 1951). The results for extract F however show that poor quality extracts occasionally appear on the market. Since the proportion of individual components is similar to the other extracts the figures recorded suggest that this extract contains a small proportion of genuine extract of cascara to which has been added about 4 times the weight of water soluble diluent. The final "extract" passes all the B.P. tests for identity and purity, etc., but is obviously deficient in potency. This deficiency would be readily detected by the chemical assay process described in this paper.

SHORTENED ASSAY PROCESS

The results in Table 1 show that the *O*-glycosides only represent about 10% of the total anthracene glycosides of the extract. In view of this, and also the fact that their potency is not likely to differ markedly from the other glycosides, it is probably unnecessary to estimate them separately. If they are included in the other classes of compounds the assay process can be considerably shortened. The removal of the free compounds and separation of the glycosides into ethyl acetate soluble and water soluble fractions are carried out exactly as detailed for the assay of the extract. The *ethyl acetate fraction* is evaporated to dryness, dissolved in water to 25 or 50 ml, as described; 10 ml is heated with 0·6 g anhydrous ferric chloride and 6 ml hydrochloric acid B.P. under reflux for 4 hr. After cooling the hydrolysate is extracted with carbon tetrachloride, the latter extracted with N sodium hydroxide, made up to volume and, after colorimetric measurement, the amount of aloins calculated as already described. The *aqueous fraction* is diluted to 50 ml, and to 10 ml is added 0·6 g anhydrous ferric chloride 6 ml hydrochloric acid B.P. and the above process repeated. After colorimetric estimation the amount of cascarosides present is calculated as previously described. Besides reducing the time of assay this modified process also eliminates the troublesome middle layers which arise when the longer assay process is used. Experiments with some of the extracts analysed by the longer process showed that the normal glycosides were fairly evenly distributed between the cascarosides and the aloins when the shortened process was used.

Acknowledgements. We would like to thank Westminster Laboratories Limited, for a grant in aid to one of us (S. Simic).

References

Auterhoff, H. & Sachdev, R. (1962). *Dtsch. Apoth. Ztg.*, **30**, 921–923.
Fairbairn, J. W. (1949). *J. Pharm. Pharmacol.*, **1**, 683–694.
Fairbairn, J. W., Friedmann, C. A. & Ryan, A. H. (1958). *Ibid.*, **10**, 186*T*–192*T*.
Fairbairn, J. W. & Michaels, I. (1951). *Ibid.*, **2**, 813–830.
Fairbairn, J. W. & Simic, S. (1960). *Ibid.*, **12**, 45*T*–51*T*.
Fairbairn, J. W. & Simic, S. (1963). *Ibid.*, **15**, 325–328.
Schindler, O. (1946). *Pharm. Acta Helvet.*, **21**, 189–196.
Simic, S. (1961). Thesis, pp. 105–108, University of London.

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Absolute configuration of dextropropoxyphene at the C-3 asymmetric centre

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The configuration at the C-3 asymmetric centre of dextropropoxyphene has been related by a stereospecific route to that of the analogous centre of (−)-isomethadone and hence to (*R*)-α-methyl-β-alanine.

THE well-known stereochemical specificity of analgesics related to 3,3-diphenylpropylamines (Beckett & Casy, 1955) extends to analogues in which the two phenyl groups are placed on adjacent carbon atoms (see Table 1). Dextropropoxyphene [α-(+)-I] has achieved considerable

$$Me_2N\cdot\overset{4}{C}H_2\cdot\overset{3}{C}H(Me)\cdot\overset{2}{C}(O\cdot CO\cdot Et)(Ph)\cdot\overset{1}{C}H_2\cdot Ph$$

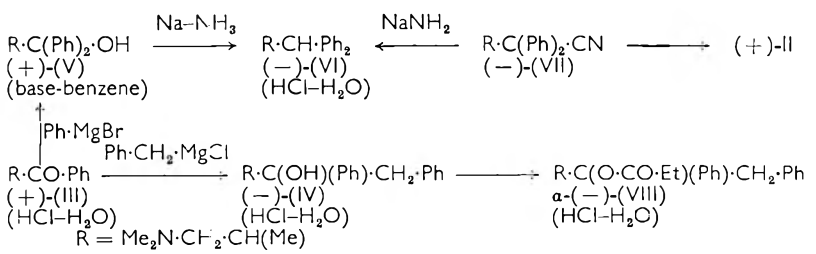
(I)

success as a clinical analgesic useful in the relief of mild to moderate pain (Beckett & Casy, 1962). This diastereoisomer possesses asymmetric centres at C–2 and C–3; the aim of the present work was to relate the configuration at C–3 of dextropropoxyphene to that of the analogous centre present at C–5 in (−)-isomethadone (II). The stereospecific

$$Me_2N\cdot\overset{6}{C}H_2\cdot\overset{5}{C}H(Me)\cdot\overset{4}{C}(Ph)_2\cdot\overset{3}{C}O\overset{2}{C}H_2\cdot\overset{1}{M}e$$

(II)

reaction sequence employed is shown below. The (+)-Mannich base (III) (obtained by resolution of racemic material by means of (−)-dibenzoyltartaric acid) was added to excess of benzylmagnesium chloride



when partially racemised α-(−)-4-dimethylamino-3-methyl-1,2-diphenylbutan-2-ol (IV) was isolated, together with the α-racemate. Reaction between the (+)-Mannich base (III) hydrochloride and the same Grignard reagent gave the optically pure α-(−)- and the partially racemised α-(−)-aminobutanol (IV). Reaction between the (+)-Mannich base (III) or the corresponding hydrochloride and phenylmagnesium bromide gave partially racemised 3-dimethylamino-2-methyl-1,1-diphenylpropan-1-ol

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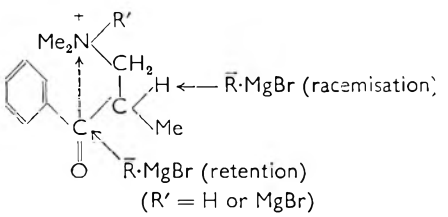
(V) (base, dextrorotatory in benzene; HCl, laevorotatory in water). Some loss of optical activity had been anticipated in the reactions of the (+)-Mannich base (III), because its asymmetric centre is adjacent to a carbonyl group and is thus prone to base-catalysed racemisation (Eliel, 1962). The greater degree of retention of optical purity that follows the use of the (+)-Mannich base (III) hydrochloride may be a result of the positively charged nitrogen atom in the molecule enhancing the reactivity of the carbonyl group towards Grignard addition and reducing the possibility of base-catalysed racemisation. The optically pure amino-



propanol (V), obtained by resolution of racemic material by means of (+)-camphor-10-sulphonic acid, was used for the stage (V) to (VI); the (+)-aminopropanol (V) gave the (−)-aminopropane (VI) hydrochloride after treatment with sodium in liquid ammonia. As the (+)-amino-cyanide (VII), the precursor of (−)-isomethadone, is converted by sodamide to the (+)-aminopropane (VI) hydrochloride (Beckett, Kirk &

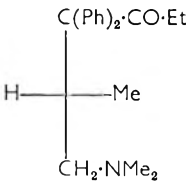
TABLE 1. ACTIVITIES OF ESTERS OF α-4-AMINO-3-METHYL-1,2-DIPHENYLBUTAN-2-OL IN THE HOT-PLATE TEST (IN MICE) (EDDY, 1959)

$X{\cdot}CH_2{\cdot}CH(Me){\cdot}C(O{\cdot}CO{\cdot}R)(Ph){\cdot}CH_2{\cdot}Ph$

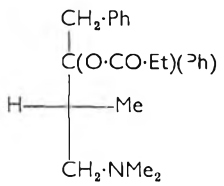
R	X	Form	ED50 mg/kg
Et	NMe_2	α-(±) (propoxyphene)	25·4
Et	NMe_2	α-(+) (dextropropoxyphene)	7·5
Me		α-(±)	4·77
Me		α-(+)	2·37

Thomas, 1962), it follows that (+)-isomethadone has the same configuration as the (−)-aminopropane (VI) hydrochloride. The last compound is related, through the (+)-Mannich base (III), to the α-(−)-butanol (IV), which gives laevopropoxyphene (VIII) on propionylation (Pohland & Sullivan, 1955). Thus (+)-isomethadone and laevopropoxyphene have identical configurations at C–5 and C–3 respectively; (−)-isomethadone and dextropropoxyphene, i.e., the more analgesically active members of the two enantiomorphic pairs, must therefore be related in the same sense. Beckett, Kirk & Thomas (1962) established the absolute configuration of (−)-isomethadone by relating this isomer to (*R*)-(−)-α-methyl-β-alanine.

Hence the absolute configuration of dextropropoxyphene at C–3 is also known.*

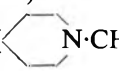


(*S*)-(−)-Isomethadone



3-(*R*)-(+)-Propoxyphene

While this work was in progress Sullivan, Beck & Pohland (1963) reported the absolute configuration of dextropropoxyphene to be 2(*S*):3(*R*). Their method for the C–3 centre involved conversion of the Mannich base (III) to the benzoyl ester of 1-dimethylamino-2-propanol by a Baeyer-Villiger oxidation. This rearrangement has been shown to proceed with retention of configuration in a number of cases, but retention has not been established in the case of amino-ketones.

With the recent report (Portoghese, 1964) that the more analgesically active enantiomer of phenampromid [ N·CH$_2$·CH(Me)·N(CO·Et)(Ph)] also has the same configuration as (−)-isomethadone, the importance of spatial configuration in analgesics possessing the structural entity >N·CH$_2$·CH(Me) is now established. This finding lends further support to the hypothesis, based originally on the results of a stereochemical study of enantiomers of methadone and related compounds (Beckett & Casy, 1954), that an overall optimum spatial configuration is one of the essential requirements for a molecule if it is to induce an analgesic response.

Experimental

Resolution of β-dimethylamino-α-methylpropiophenone (III). The Mannich base (II) (9·55 g) was added to a warm solution of (−)-dibenzoyltartaric acid (17·9 g) in acetone (200 ml). The product, after storage at 0° for 18 hr, deposited the dibenzoyltartrate (9·2 g), m.p. 115–116·5°, $[\alpha]_D^{20}$ −53 (*c* 1·0 in EtOH). The rotation of the salt was unchanged on further recrystallisation. The base, liberated from the salt with dilute aqueous ammonia solution and extracted with ether, gave a hydrochloride (4 g), m.p. 161–162°, $[\alpha]_D^{20}$ + 48 (*c* 1·0 in EtOH) [Pohland, Peters & Sullivan (1963) report m.p. 153–155°, $[\alpha]_D^{25}$ + 47 (*c* 1·0 in EtOH)].

Grignard addition to the (+)-Mannich base (III). (*a*) The (+)-Mannich base (III) (15 g) in ether (50 ml) was added to a Grignard reagent prepared from benzyl chloride (19·9 g), magnesium (4·85 g) and ether (250 ml). The mixture was heated under reflux for 4 hr, cooled and poured onto crushed ice and ammonium chloride. Hydrogen chloride was passed through the dried ethereal phase and the precipitated hydrochloride

* In applying the nomenclature of Cahn, Ingold & Prelog (1956) the sequences CH$_2$·NMe$_2$, C(Ph)$_2$·CO·Et, Me, H and C(O·CO·Et)(Ph)·CH$_2$Ph, CH$_2$·NMe$_2$, Me, H obtain in isomethadone and propoxyphene respectively.

(17 g, m.p. 226–230°) crystallised from methanol-ethyl acetate (4 : 1). After two recrystallisations α-(±)-4-dimethylamino-3-methyl-1,2-diphenylbutan-2-ol hydrochloride (10 g), m.p. and mixed m.p. 238-239° was obtained. A second crop of crystals (3·5 g) m.p. 189–192°, $[\alpha]_D^{21}$ − 19·0 (*c* 1·0 in H_2O) had m.p. 234–235°, $[\alpha]_D^{21}$ − 29 (*c* 1·0 in H_2O) after further recrystallisation. [Pohland & Sullivan (1953) report m.p. 231–232° for α-(±)-(IV)].

(*b*) A suspension of the finely powdered (+)-Mannich base (III) hydrochloride (15·0 g) in ether (150 ml) was added to a Grignard reagent prepared from benzyl chloride (38·1 g), magnesium (7·2 g) and ether (350 ml). The product was heated under reflux for 3 hr, decomposed and processed as described in (*a*) above to give the crude hydrochloride (11·2 g), m.p. 226–230°. Fractional crystallisation of the salt from methanol-ethyl acetate gave the partially racemic α-(−)-aminobutanol (IV) hydrochloride (4·8 g), m.p. 233–234·5°, $[\alpha]_D^{21}$ − 16 (*c* 1·0 in H_2O) and the optically pure α-(−)-aminobutanol (IV) hydrochloride (2·5 g), m.p. and mixed m.p. 246–247°, $[\alpha]_D^{21}$ − 53 (*c* 1·0 in H_2O) [Pohland & Sullivan (1955) report m.p. 246–247°, $[\alpha]_D^{20}$ − 53 (*c* 1·0 in H_2O)].

(*c*) The (+)-Mannich base (III) (4·75 g) in ether (25 ml) was added to a Grignard reagent prepared from bromobenzene (6·28 g), magnesium (1·26 g) and ether (50 ml). The product was heated under reflux for 2 hr, decomposed and processed as described above to give 3-dimethylamino-2-methyl-1,1-diphenylpropan-1-ol (V) hydrochloride (7·5 g), m.p. 240°. This salt melted at 244° after recrystallisation from ethanol-ether [Perrine (1953) reports m.p. 242·5° for racemic material) and had $[\alpha]_D^{21}$ − 14·0 (*c* 1·0 in H_2O or EtOH]. The free base, crystallised from light petroleum (b.p. 40–60°), had m.p. 92–92·5° [Perrine (1953) reports m.p. 92·8–93·3° for racemic material) and $[\alpha]_D^{21}$ + 13 (*c* 1·0 in benzene)].

(*d*) A suspension of the (+)-Mannich base (III) hydrochloride (5·0 g) in ether (125 ml) was added to a Grignard reagent prepared from bromobenzene (13·8 g), magnesium (2·1 g) and ether (50 ml). The product was heated under reflux for 4 hr, decomposed and processed as described above to give the aminopropanol (V) hydrochloride (5·0 g), m.p. 240°, $[\alpha]_D^{21}$ − 29·9 (*c* 1·0 in H_2O), $[\alpha]_D^{21}$ − 21·6 (*c* 1·0 in EtOH). The free base, crystallised as above, had m.p. 90–91°, $[\alpha]_D^{21}$ + 48·2 (*c* 1·2 in benzene).

Resolution of 3-*dimethylamino*-2-*methyl*-1,1-*diphenylpropan*-1-*ol*. A solution of the racemic aminopropanol (V) (26·9 g) and (+)-camphor-10-sulphonic acid (23·2 g) in acetone (100 ml) was seeded (seed material obtained by leaving the oil left after evaporating some of the above solution to stand in a desiccator until it solidified) and stored at room temperature for 48 hr. The mother liquors were decanted, the crystals dissolved in hot acetone (150 ml) and reseeded; the crystals which separated had $[\alpha]_D^{17}$ − 27·9 (*c* 1·0 in H_2O). One more recrystallisation from acetone (150 ml.) (no seed added) gave material $[\alpha]_D^{16}$ − 29·1 (*c* 1·0 in H_2O). The free base (V), liberated as usual, had m.p. 90°, $[\alpha]_D^{20}$ + 53·4 (*c* 1·2 in benzene) and gave a hydrochloride, m.p. 241–242°, $[\alpha]_D^{17}$ − 71·3 (*c* 1·0 in H_2O).

(−)-3-*Dimethylamino*-2-*methyl*-1,1-*diphenylpropane* (*VI*). Sodium (0·9 g) was added portionwise over 30 min to a vigorously stirred suspension of the (+)-aminopropanol (V) (3·5 g) in liquid ammonia (80 ml) containing ethanol (2 ml). The solvent was allowed to evaporate and the residue decomposed with ice and extracted with ether. The residue (3·1 g) from this extract gave the (−)-aminopropane (VI) hydrochloride, m.p. 198–199°, $[\alpha]_D^{17}$ − 52·5 (*c* 1·0 in H_2O). (Found: C, 74·9; H, 8·3; N, 4·6. Calc. for $C_{18}H_{24}ClN$: C, 74·6; H, 8·35; N, 4·8%). The (+)-aminopropane (V) hydrochloride derived from (+)-3-dimethylamino-2-methyl-1,1-diphenylpropyl cyanide (Beckett, Kirk & Thomas, 1962) had m.p. 199–201°, $[\alpha]_D^{20}$ + 53·8 (*c* 1·0 in H_2O). The infra-red spectra of the two samples were superimposable.

Acknowledgments. We thank Dr. A. Pohland for the gift of authentic samples of α-(+)- and α-(−)-4-dimethylamino-3-methyl-1,2-diphenylbutan-2-ol hydrochloride and for advice on the resolution of the Mannich base (III).

References

Beckett, A. H. & Casy, A. F. (1954). *J. Pharm. Pharmacol.*, **6**, 986–999.
Beckett, A. H. & Casy, A. F. (1955). *Ibid.*, **7**, 433–455.
Beckett, A. H. & Casy, A. F. (1962). *Progress in Medicinal Chemistry.* Vol. 2, p. 76. Edited by Ellis, G. P. and West, G. B. London: Butterworths.
Beckett, A. H., Kirk, G. & Thomas, R. (1962). *J. chem. Soc.*, 1386–1388.
Cahn, R. S., Ingold, C. K. & Prelog, V. (1956). *Experientia*, **12**, 81–94.
Eddy, N. B. (1959). *Chem. Ind.*, 1462–1469.
Eliel, E. L. (1962). *Stereochemistry of carbon compounds*, p. 35. New York: McGraw-Hill.
Perrine, T. D. (1953). *J. org. Chem.*, **18**, 898–903.
Pohland, A., Peters, L. R. & Sullivan, H. R. (1963). *Ibid.*, **28**, 2483.
Pohland, A. & Sullivan, H. R. (1953), *J. Amer. chem. Soc.*, **75**, 4458–4461.
Pohland, A. & Sullivan, H. R. (1955). *Ibid.*, **77**, 3400.
Portoghese, P. S. (1964). *Chem. Ind.*, 582.
Sullivan, H. R., Beck, J. R. & Pohland, A. (1963). *J. org. Chem.*, **28**, 2381–2385.

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Effect of imipramine and some analogues on the uptake of 5-hydroxytryptamine by human blood platelets *in vitro*

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The inhibitory action of imipramine and some analogues on the uptake of 5-hydroxytryptamine by human blood platelets *in vitro* was investigated. In concentrations comparable with those reached in plasma during therapy, imipramine and the isosteric compound, amitriptyline, were more effective than their metabolites. Chlorpromazine was less effective than the latter and orphenadrine ineffective at the concentrations tested.

BIOCHEMICAL studies on the mode of action of drugs used in the treatment of depression have largely been related to the effects on catecholamine and 5-hydroxytryptamine (5-HT) metabolism. The substituted hydrazine class of monoamine oxidase inhibitors, for example, causes a rise in the brain amine levels of experimental animals (Spector, Prockop, Shore & Brodie, 1958).

In man, the amine which can be most easily studied is the blood platelet 5-HT, which is taken up by platelets against a concentration gradient. Pletscher & Bernstein (1958) have shown that iproniazid raises the 5-HT content of the platelets of rabbits and of man receiving it in therapeutic dosage. Imipramine, not a monoamine oxidase inhibitor, caused a fall in the platelet 5-HT level of patients on therapy (Marshall, Stirling, Tait & Todrick, 1960). Since the uptake of 5-HT by platelets *in vitro* can also be inhibited by this compound (Marshall & others, 1960; Stacey, 1961; Long & Lessin, 1962), it has been suggested that the fall in 5-HT during therapy is due to inhibition of uptake rather than to interference with 5-HT production.

The therapeutic effectiveness of imipramine has led to the clinical trial of structurally similar compounds. We have compared the effect of these compounds and certain of their metabolites (Herrmann, Schindler & Pulver, 1959) on the uptake of 5-HT by human blood platelets *in vitro*. The effects of chlorpromazine and orphenadrine, a drug with antidepressant action probably of a different type (Robinson, 1961), have also been examined.

Experimental

Materials

The compounds investigated were: imipramine, desmethylimipramine (desipramine), 2-hydroxydesmethylimipramine, desdimethylimipramine, amitriptyline, desmethylamitriptyline (nortriptyline), chlorpromazine, orphenadrine.

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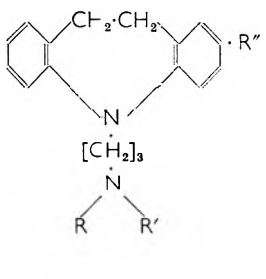
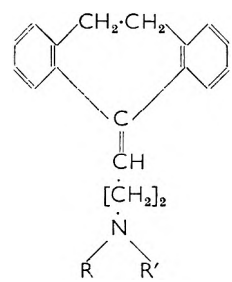
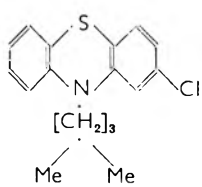
The structures of these compounds are similar, but orphenadrine may have a non-planar configuration resulting from rotation of the benzene rings.



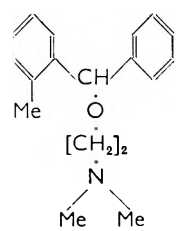
	R	R′	R″
Imipramine	Me	Me	H
Desmethylimipramine	Me	H	H
Desdimethylimipramine	H	H	H
2-Hydroxydesmethylimipramine	Me	H	OH



	R	R′
Amitriptyline	Me	Me
Nortriptyline	Me	H



Chlorpromazine



Orphenadrine

The average plasma concentration of "imipramine-like substances" in patients receiving 150 mg desipramine daily was found to be 0·85 μg/ml (Yates, Todrick & Tait, 1963). The effect of the drugs on the uptake of 5-HT by platelets was tested at concentrations of 1 and 4 μg/ml. While it might have been considered preferable to work with equimolar concentrations, the molecular weights of the drugs used are not greatly different. Those of the antidepressant drugs range from 252 to 282 while those of chlorpromazine and orphenadrine are 319 and 268 respectively; the differences in the molar concentrations are therefore comparatively small.

METHODS

20 ml of blood was drawn from normal subjects into 2 ml of a 1% solution of disodium diaminoethanetetracetate in 0·7% saline. The platelet-rich plasma was obtained by centrifugation at 200 *g* for 20 min and the platelets counted by the method of Dacie (1956). The mean of two counts, each by a different operator, on each of two separate dilutions of the platelet-rich plasma, was taken as the platelet count.

Uptake of exogenous 5-HT *by platelets.* The method previously

described (Marshall & others, 1960) was modified as follows:

(*a*) 1·25 ml platelet-rich plasma was used instead of 1·5 ml; this provided six instead of four aliquots and allowed two drugs to be tested simultaneously on the same platelets.

(*b*) Since only a small proportion of added 5-HT was removed by the platelets, additional steps were taken to prevent contamination of the platelet button by the plasma. After incubation, the tubes were chilled in ice-water to stop further uptake, diluted with 8 ml chilled saline, centrifuged at 2000 *g* for 15 min and the supernatant discarded. Any trace of diluted plasma adhering to the tube walls was then washed down with a few drops of saline and the tube recentrifuged at 400 *g* for 5 min. The saline was poured out and the residual liquid removed from the walls with filter paper. The platelet button was suspended in 5 ml saline and the 5-HT estimated in duplicate fluorimetrically as described previously (Yates & others, 1963).

The 5-HT estimates from control tubes to which saline and drug but no 5-HT had been added did not differ significantly from those to which saline alone was added; this confirms that the drugs did not interfere with the 5-HT assay, and also indicates that the drugs at the concentrations used did not release 5-HT from the platelets.

Results

The mean endogenous 5-HT level of the platelet-rich plasma was 46 ng/10^8 platelets. The platelets took up 3·5 ± 1·3 (s.d.) times their endogenous content of 5-HT.

The data for the effect of the drugs on the uptake of 5-HT by platelets are given in Table 1.

TABLE 1. INHIBITION BY ANTIDEPRESSANT DRUGS AND METABOLITES OF 5-HT UPTAKE BY PLATELETS

(5-HT concentration, 1 μg/ml free base)

Drug concentration (hydrochloride)	1 μg/ml	4 μg/ml
Drug	Percentage inhibition ± s.d. (mean of 6 estimates)	
Imipramine	54 ± 5·5	79 ± 5·8
Desipramine	30 ± 4·2	56 ± 9·5
Desdimethylimipramine	32 ± 5·0	55 ± 7·1
2-Hydroxydesmethylimipramine	30 ± 16·0	56 ± 6·1
Amitriptyline	39 ± 3·5	68 ± 6·5
Nortriptyline	24 ± 4·2	59 ± 6·8
Chlorpromazine	−6 ± 5·0	24 ± 7·8
Orphenadrine	−3 ± 12·0	−1 ± 15·4

Imipramine was the strongest inhibitor with amitriptyline next, ($P < 0·001$ and $< 0·02$ at 1 and 4 μg/ml respectively). They were each more effective than their demethylated derivatives. The three metabolites of imipramine did not differ significantly in potency. Chlorpromazine had no effect at 1 μg/ml but did have an effect at 4 μg/ml. Orphenadrine had no effect at either concentration.

Discussion

The results presented agree qualitatively with those of Stacey (1961) and Long & Lessin (1962), who found that imipramine was a more powerful inhibitor of the uptake of 5-HT by platelets than chlorpromazine. But we found the concentration of imipramine causing 50% inhibition to be about 3×10^{-6} M in contrast to $0{\cdot}5 \times 10^{-6}$ and $0{\cdot}8 \times 10^{-6}$ M obtained by the other two groups of workers. Stacey's technique, used also by Long & Lessin, differs from ours in that (i) the drug is pre-incubated with the platelets for 10 min at 37° before adding 5-HT, (ii) the mixture is gassed with 95% O_2 and 5% CO_2 and (iii) the incubation with 5-HT only lasts 20 min. We found none of these factors contributed to the difference.

Variations in degree of inhibition *in vitro* and of rate of fall of platelet 5-HT *in vivo* might perhaps be expected to correlate with observations of differing speed in clinical action; if this were so, our observations would tend to parallel the more conservative estimates of the clinical effectiveness of desipramine reported by Dick (1961) and Oltman & Friedman (1962).

Our results, together with those of Stacey (1961), indicate that, as a group, the cyclodibenzyls are the most potent inhibitors of the uptake of 5-HT by platelets by an active transport mechanism and produce effects at concentrations obtained *in vivo* during therapy. Comparison between the cyclodibenzyls suggests a small but significant superiority of the parent substances over the demethylated derivatives, paralleling the pharmacological results of Sigg, Soffer & Gyermek (1963) with 5-HT rather than those of Brodie, Bickel & Sulser (1961), Garattini, Giachetti, Jori, Pieri & Valzelli (1962) and Sulser, Watts & Brodie (1962).

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References

Brodie, B. B., Bickel, M. H. & Sulser, F. (1961). *Med. exp.*, **5**, 454–458.
Brodie, B. B., Dick, P., Kielholz, P., Poldinger, W. & Theobald, W. (1961). *Psychopharmacologia*, **2**, 467–474.
Dacie, J. N. (1956). *Practical Haematology*, 2nd ed., p. 49, London: J. and A. Churchill Ltd.
Garattini, S., Giachetti, A., Jori, A., Pieri, L. & Valzelli, L. (1962). *J. Pharm. Pharmacol.*, **14**, 509–514.
Herrmann, B., Schindler, W. & Pulver, R. (1959). *Med. exp.*, **1**, 381–385.
Long, R. F. & Lessin, A. W. (1962). *Biochem. J.*, **82**, 4P.
Marshall, E. F., Stirling, G. S., Tait, A. C. & Todrick, A. (1960) *Brit. J. Pharmacol.*, **15**, 35–41.
Oltman, J. E. & Friedman, S. (1962). *Amer. J. Psychiat.*, **119**, 370–371.
Pletscher, A. & Bernstein, A. (1958). *Nature, Lond.*, **181**, 1133–1134.
Robinson, R. A. (1961). *Act. neurol. psychiat. Belg.*, **61**, 33–38.
Sigg, E. B., Soffer, L. & Gyermek, L. (1963). *J. Pharmacol.*, **142**, 13–20.
Spector, S., Prockop, D., Shore, P. A. & Brodie, B. B. (1958). *Science*, **127**, 704.
Stacey, R. S. (1961). *Brit. J. Pharmacol.*, **16**, 284–295.
Sulser, F., Watts, J. & Brodie, B. B. (1962). *Ann. N.Y. Acad. Sci.*, **96A1**, 279–286.
Yates, C. M., Todrick, A. & Tait, A. C. (1963). *J. Pharm. Pharmacol.*, **15**, 432–439.

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Anthelmintic constituents of ferns

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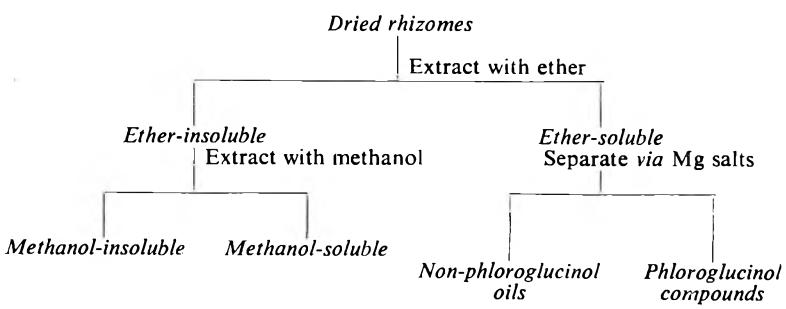
Extracts, oils and some individual phloroglucinol compounds prepared from the fern *Dryopteris dilatata* and *Dryopteris filix-mas* were tested for taeniacidal activity against the dwarf tapeworm *Hymenolepis nana*. In *D. dilatata* activity is concentrated in the phloroglucinol compounds (mainly aspidin, phloropyrone and aspidinol) of which aspidin is the most potent. In *D. filix-mas* the mixed phloroglucinol compounds (mainly flavaspidic acid and aspidinol) are active, but no more so than the total ether extract. Flavaspidic acid seems to be the most active phloroglucinol constituent of the fern. Desaspidin which occurs in a third fern, *Dryopteris austriaca*, is more potent than either aspidin or flavaspidic acid. A method of detecting phloroglucinol compounds by thin-layer chromatography is described.

POWDERED rhizomes of *Dryopteris filix-mas* and extracts of the plant, have been used for centuries as anthelmintics. However, the main disadvantages of such preparations are their variable composition, and toxicity. Several phloroglucinol compounds have been isolated from *D. filix-mas* and have been considered to be the active principles (Boehm, 1897; Widén, 1944) although activity has been claimed for other constituents, such as tannins and resins (Borkowski & Kowalewski, 1958). Again, aspidin, the most active phloroglucinol compound when tested *in vitro* against *Enchytraeus* is ineffective *in vivo* against *Hymenolepis fraterna* infections in mice (Büchi, 1957).

A related fern, *Dryopteris dilatata*, is reputed to be more effective than *D. filix-mas* as a taeniafuge (Rosendahl, 1911). The object of the work here reported was to compare the activities of the constituents of *D. dilatata* and *D. filix-mas* using the dwarf tapeworm *Hymenolepis nana* for both *in vitro* and *in vivo* assessments of activity.

The dried, powdered rhizomes of *D. dilatata*, for which we are indebted to Dr. G. A. Nelson of the Department of Pharmacology, University of Leeds, were fractionated according to the following scheme.



The rhizomes were collected near Golden Acre, Leeds, England, in November, 1959, dried at room temperature, and ground into a fine powder which was stored in the dark at 2° in stoppered bottles.

Ether-soluble material. Powdered rhizomes (500 g) were percolated to exhaustion at room temperature with ether and the solution evaporated down to give a dark green oil (23·2 g).

From Smith Kline and French Research Institute, Welwyn Garden City, Herts.

Treatment of ether-soluble material. The ether-insoluble material was dried in a vacuum and extracted continuously with hot methanol in a Soxhlet apparatus for 24 hr. The methanol extract was evaporated to dryness, finally in a vacuum, to give a pale brown solid (100 g). The methanol-insoluble marc (*ca* 370 g) was dried in a vacuum.

Separation of ether-soluble material into phloroglucinol and non-phloroglucinol components. The approved assay of the active principles of *D. filix-mas*, based on the amount of material extracted by aqueous barium hydroxide is not an accurate guide to the activity of the extract (Pabst & Bliss, 1932), and the isolation of the phloroglucinol constituents by means of their magnesium salts according to Ackermann & Mühlemann (1946) appears to be preferable.

The oil obtained by ether extraction of the rhizomes of *D. dilatata* was treated by the method of Büchi (1957) to give mixed phloroglucinol compounds (1·7 g). The material insoluble in magnesium hydroxide solution was dried in a vacuum and extracted to exhaustion in a Soxhlet apparatus with ether. Removal of the solvent from the extract gave non-phloroglucinol materials (7·5 g).

As a source of phloroglucinol compounds and non-phloroglucinol oils from *D. filix-mas*, a commercial sample of male fern extract, before dilution with arachis oil was used. This contained 37·8% filicin.

Isolation of non-phloroglucinol oils from male fern. Male fern extract (100 g) in ether (1,000 ml) was stirred with light magnesium oxide (300 g) suspended in water (2,500 ml) containing sodium sulphite (2·5 g). A further 500 ml of ether was added and the mixture stirred mechanically for 1½ hr. The mixture was filtered at the pump and sucked as dry as possible. The solid was dried on trays in air, then ground to a fine powder. The filtrates were extracted with ether, and the ether extract, together with 1 litre of ether, were stirred with the powder at room temperature for 3 hr. The mixture was filtered and the solid washed with ether. The filtrates were distilled down and dried in water-pump vacuum in a warm water-bath to give a greenish oil (21·3 g).

Isolation of aspidin from the phloroglucinols from D. dilatata. The crude phloroglucinol mixture (2·9 g) isolated as above was dissolved in ether, and exhaustively extracted with 2% aqueous sodium carbonate and the extract acidified with hydrochloric acid. The precipitate was centrifuged down, washed with water and dried in a vacuum to give 2 g of material. Recrystallisation of this from petroleum (b.p. 40–60°) and then from 95% ethanol gave crude aspidin (0·55 g), m.p. 110–112°. This material (0·5 g) in benzene (10 ml) was purified through a column of silica gel (Lights Silica Gel for Chromatography 100–200 mesh), 38 cm long by 2 cm diameter made up in light petroleum (b.p. 60–80°) and fitted at the top with a two-necked separating funnel (500 ml) nearly full with light petroleum (b.p. 60–80°) and equipped with a mechanical stirrer. A second separating funnel, stoppered and containing benzene, was arranged with its stem just dipping below the surface of the light petroleum in the first funnel so that, as the petroleum passed down the column it was replaced by a progressively enriched mixture of petroleum

and benzene. Fractions of 5 ml were taken and bulked according to the spots on the chromatograms, to give crude aspidin (0·325 g) which was crystallised from methanol as pale yellow needles (0·27 g), m.p. 124–125°.

Isolation of aspidinol from oils of D. dilatata. Oils (22 g) obtained by the ether extraction of dried rhizomes of *D. dilatata* were distilled in high vacuum in a short-path distillation apparatus and the fraction (1·3 g) which distilled at 110–125° (bath temp.) at 10^{-4} mm was collected. The pale-yellow solid was repeatedly recrystallised from benzene to give aspidinol (0·2 g), m.p. 140–145°.

Isolation of flavaspidic acid. Male fern extract (200 g) was treated by the method of Widén (1944) to give flavaspidic acid (5 g), m.p. 155–157° crystallised from benzene.

Thin-layer chromatography of phloroglucinol compounds from ferns. The mixtures of phloroglucinol compounds obtained by the above methods were subjected to paper chromatography on unbuffered and buffered paper impregnated with formamide and developed with benzene: chloroform using the methods of Klevstrand (1957) and Penttilä & Sundman (1961a). Although these procedures gave distinct separations of components, the Rf values could not be used as unequivocal guides to their identities.

We have found that a more convenient method of detection was by thin-layer chromatography on Kieselgel G. By this means the components could be quickly detected and the method could be applied not only to the phloroglucinol components after partial purification *via* their magnesium salts but also to the crude ether extracts of the rhizomes.

TABLE 1. CHROMATOGRAPHIC DATA

Substance	Rf value	Colour with Fast Blue Salt B Reagent
Flavaspidic acid	0	Reddish-mauve
Phloropyrone	0·17	Yellow
Desaspidin	0·25	Purple-red
Aspidin	0·35	Yellow
Aspidinol	0·6	Purple

The thin layers were made by shaking Kieselgel G (Merck) (34 g) with distilled water (60 ml) for 90 sec and then applying to the plates (10 × 20 cm) in 250 μ thickness. The plates were dried as recommended. The Shandon "Unoplan" apparatus was used. Mixtures of crude phloroglucinols (20–50 μg) or pure phloroglucinol compounds (10 μg) were applied to the plates in chloroform and developed in the tank supplied with the apparatus lined with filter paper soaked in the developing solvent. The plates were developed in chloroform:acetone (3:1 by volume) at 21·5° for approximately 50 min, by which time the solvent front had travelled 10 cm from the starting line. If greater separation of the spots was required, the plates were developed for 1½ hr. After drying, the plates were sprayed with 0·1% aqueous Fast Blue Salt B (Merck). The phloroglucinol compounds appeared as coloured spots (Table 1).

The major spots on the chromatograms were identified by comparison with pure substances isolated by the rather laborious methods from the ferns.

Biological testing

Various extracts, oils, and individual phloroglucinol compounds from the ferns were tested for activity against the dwarf tapeworm *H. nana* by an *in vitro* method based on that of Sen & Hawking (1960) and an *in vivo* method based on that of Steward (1955).

Worms for testing *in vitro* were obtained from mice killed 12–15 days after infection. The worms were freed from the small intestine by squeezing the intestine between a moistened sheet of glass and the non-cutting edge of a fine pair of angled scissors. The length of the intestine was slowly and carefully pulled between the two. The faeces were placed in a dish containing boiled and cooled tap water. The mixture was gently stirred to free the worms. Worms approximately $\frac{1}{2}$–$\frac{3}{4}$ in. in length were removed and placed in a second dish of boiled and cooled water where they were gently washed. Then they were placed in the nutrient broth medium containing penicillin and streptomycin as described by Sen & Hawking (1960). The nutrient broth containing the tapeworms was kept in an incubator at 37° until the worms were required (1–3 hr later). Special care was taken in adjusting the pH of the nutrient broth to exactly 8·5 using a direct reading pH meter. 5 mg of the substance to be tested was warmed to 37–40° in 0·5 ml of absolute ethanol. When the substance was dissolved, the volume was made up to 5 ml with nutrient broth. Various dilutions were prepared, and 3 ml of each dilution was placed in a small Kjeldahl flask. One worm was placed in each flask. The flasks were sealed with parafilm and incubated at 37°. The worms were examined at 2, 18 and 24-hr intervals. Only the 24-hr results are shown. In each experiment, controls consisting of worms in nutrient broth alone, and in nutrient broth containing ether extract of *D. filix-mas*, were set up.

The method of *in vivo* testing was based on that of Steward (1955). Mice from stock not infected with *H. nana* weighing from 14–16 g were given by stomach tube approximately 500 *H. nana* ova per mouse. Only mature ova from proglottids of adult worms were counted using a haemacytometer chamber. An appropriate dilution was made so that there were 1000 ova/ml. The diluted ova were allowed to stand at room temperature for 24–48 hr before infecting the mice.

The substances to be tested were given by mouth in a 1% sodium glycocholate solution on the 12th day of the infection. A group of infected mice received 1% sodium glycocholate only. Food was not withheld before treatment nor were the mice purged. The mice were killed on the 15th day and the tapeworms, removed from the intestine as described in the *in vitro* method, were counted. This method of removal eliminated the necessity to withhold food for 24 hr before killing. In assessing the results a form of computation similar to that of Steward (1955) was used.

Results

By the chromatographic procedure described, the major phloroglucinol compounds in *D. filix-mas* appeared as flavaspidic acid and aspidinol whereas those in *D. dilatata* were aspidin, aspidinol and an unknown substance having an Rf value of 0·17. This last substance was removed from a series of plates on which 150 mg of mixed phloroglucinol compounds had been separated, using the small scale "vacuum-cleaner" technique of Ritter & Meyer (1962). By this procedure 4·5 mg of the substance was obtained. Its infra-red spectrum showed a strong absorption peak at 1630 cm^{-1} and in the ultra-violet had maximum at 215, *ca* 311 and 355 mμ, indicating it to be phloropyrone, the structure of which has been elucidated by Penttilä & Sundman (1961b).

TABLE 2. MINIMUM CONCENTRATIONS REQUIRED TO KILL *Hymenolepis nana* AT pH 8·5 AND 37° IN 24 HR

Substance	Minimum lethal concentration
D. dilatata—	
Ether-soluble material	1 : 500,000
Mixed phloroglucinol compounds	1 : 1 million
Oils	1 : 10,000
Methanol soluble fraction of ether-insoluble material	>1 : 10,000
D. filix-mas—	
Ether extract (37·8% filicin)	1 : 500,000
Mixed phloroglucinol compounds	1 : 500,000
Oils	1 : 10,000
Individual phloroglucinol compounds—	
Aspidin	1 : 5 million
Aspidinol	1 : 100,000
Desaspidin	1 : 10,000 to 1 : 500,000
Flavaspidic acid	1 : 500,000
Phloropyrone	1 : 100,000

Various extracts of the two ferns, oils, and individual phloroglucinol compounds, were tested for anthelmintic activity. The results of *in vitro* tests are shown in Table 2. The ether extracts of the ferns are both active (1 : 500,000), as are the mixed phloroglucinol compounds (from *D. dilatata* 1 in 1 million, from *D. filix-mas* 1 : 500,000). The oils are far less active (1 : 10,000) and the methanol-soluble fraction of the ether-insoluble residue of *D. dilatata* was inactive. Of the individual phloroglucinol compounds, aspidin was most active (1 : 5 million). Changes in pH altered the minimum lethal concentration of aspidin. At pH 6 the minimum lethal concentration was 1 : 50 million, at pH 7, 1 in 20 million and at pH 9, 1 in 5 million. Next in potency were flavaspidic acid and desaspidin (1 : 500,000). However, the results with desaspidin were irregular, ranging from 1 : 10,000 to 1 : 500,000. Desaspidin did not dissolve either in 0·5 ml absolute ethanol or in the nutrient broth. Aspidinol and phloropyrone were weaker at 1 : 100,000.

All of the above extracts, oils, and individual compounds (except phloropyrone, of which there was an insufficient quantity) were tested in mice infected with *H. nana*. The results are summarised in Table 3. In general the results of these tests show greater variability than the

TABLE 3. THE ACTION OF SOME FERN CONSTITUENTS ON *Hymenolepis nana in vivo*

Treatment	Oral dose per kg	Mice surviving / Mice treated	Treated/Untreated ratios: Mice free from infection	Treated/Untreated ratios: Average computations	% Activity
D. dilatata					
Ether-soluble material	100 mg	8/8	0/8 to 0/8	141/191	26
	100 mg	7/7	0/7 to 0/7	177/147	0
Mixed phloroglucinols	100 mg	7/7	2/7 to 0/7	37/141	74
Oils	100 mg	9/10	0/9 to 1/10	190/165	0
	400 mg	19/20	2/19 to 2/19	77/53	0
	25 g	6/7	0/6 to 0/9	60/124	52
Methanol-soluble fraction of ether-insoluble materia.	100 mg	9/10	0/9 to 1/10	259/165	0
D. filix-mas					
Extract of *D. filix-mas* (containing 37·8% filicin)	50 mg	20/20	4/20 to 3/19	130/145	10
	100 mg	20/20	4/20 to 0/20	83/188	56
	250 mg	20/20	8/20 to 3/20	36/165	78
	400 mg	20/20	7/20 to 1/20	20/217	91
Mixed phloroglucinol compounds	100 mg	7/7	0/7 to 0/6	222/413	46
Oils	100 mg	7/7	1/7 to 0/7	60/67	10
	400 mg	7/7	1/7 to 0/7	51/67	24
	1000 mg	7/7	0/7 to 0/7	71/90	21
	25 g	1/8	1/1 to 0/8	2/8	100
Phloroglucinol compounds					
Aspidinol	100 mg	7/7	0/7 to 1/7	155/100	0
Flavaspidic acid	100 mg	5/6	2/5 to 1/10	66/140	53
	100 mg	6/6	1/6 to 1/10	37/140	74
	100 mg	12/12	2/12 to 4/12	85/111	23
	200 mg	3/10	0/3 to 1/10	2/140	99
	200 mg	3/10	3/3 to 1/10	0/140	100
	200 mg	6/12	3/6 to 4/12	25/111	77
Aspidin	10 mg	10/10	2/10 to 2/10	86/65	0
	50 mg	10/10	3/10 to 2/10	36/65	45
	100 mg	10/10	5/10 to 1/10	7/89	92
	100 mg	20/20	2/20 to 0/20	192/173	0
	100 mg	7/7	2/7 to 0/7	14/279	95
	100 mg	10/10	1/10 to 0/10	116/144	19
	200 mg	10/10	6/10 to 0/10	3/111	97
	200 mg	10/10	2/10 to 0/10	28/111	75
Desaspidin	10 mg	8/8	0/8 to 0/8	78/85	9
	50 mg	8/8	0/8 to 0/8	27/86	69
	100 mg	8/8	8/8 to 0/8	0/227	100
	250 mg	8/8	8/8 to 0/8	0/227	100

in vitro tests. However, of the plant extracts studied, the ether extract of *D. filix-mas*, and the mixed phloroglucinols from both *D. filix-mas* and *D. dilatata*, were all active at 100 mg/kg. The oils from the two ferns showed some activity in very high doses (25 g/kg = 0·5 ml/mouse approx.). The methanol soluble fraction of the ether insoluble material from *D. dilatata* showed no activity at 100 mg/kg, nor, surprisingly, did the ether extract of *D. dilatata*. Of the individual phloroglucinol compounds desaspidin, aspidin and flavaspidic acid were all active at 100 mg/kg, but aspidinol was inactive.

Discussion

In these experiments two methods of testing for taeniacidal activity were used. The *in vitro* method has the advantage that it requires only small quantities of test materials, but results from these tests alone could

be misleading since it is well known that drugs active *in vitro* may be inactive against the same worm *in vivo*. Moreover, these tests would indicate drugs that are toxic to all living organisms. Steward's method of *in vivo* testing compares the number and size of worms found at autopsy in the intestines of a treated group of mice, with the number and size of worms in a "control" untreated group of mice. Steward believed that large worms were more readily removed by treatment than smaller worms, and this was the basis of his computation. But another possibility is that sublethal doses of taeniacides may so damage the tapeworms that they are broken into fragments. The scolices and rostral segments of the worm remain attached to the intestinal wall and at autopsy these would be counted as small worms. Fragmentation of worms occurs in the clinical use of inadequate doses of taeniacidal drugs, hence the importance of finding the scolex in the faeces to be sure that treatment has been effective. However, larger doses of the drug cause elimination of the complete worm. Our experiments using Steward's method show that low doses of extract of *filix-mas* or of mepacrine reduce the average size (length) of the worms without reducing the number of the worms. Higher doses reduce both the number and size of the worms. Whatever the precise mechanism of action of taeniacides in this test, drugs that are effective in the clinic are also active in this test (Steward, 1957; Sen & Hawking, 1960) and these findings have been confirmed by the present authors.

In general the minimal lethal concentrations recorded in the *in vitro* experiments show less variation than the results obtained in the *in vivo* tests. An exception to this is desaspidin, which did not dissolve in the 0·5 ml of absolute ethanol from which dilutions were prepared and where the minimal lethal concentration varied in different experiments between 1 : 10,000 to 1 : 500,000. Sen & Hawking (1960) experienced similar difficulty in testing the insoluble drugs tetrachloroethylene and carbon tetrachloride. The variability of the *in vivo* tests may be illustrated by the results obtained with aspidin 100 mg/kg. In different experiments the percentage activity was 0, 19, 92 and 95 though the activity repeatedly determined *in vitro* remained constant at 1 : 5 million. In assessing the anthelmintic activity of the various fern extracts and constituents, data from both *in vitro* and *in vivo* experiments will be considered.

D. dilatata. The ether-soluble material was active *in vitro* and possibly active *in vivo* (100 mg/kg). No activity was found in the methanol extract of ether-insoluble material. Of the constituents of the ether-soluble material, the mixed phloroglucinol compounds were highly active in both tests. The oil fraction was inactive *in vitro* and inactive *in vivo* unless massive quantities were administered. Thus the main anthelmintic activity of the fern residues in the phloroglucinol compounds. The major phloroglucinol compounds were aspidin, aspidinol and phloropyrone. Of these aspidin is active in both tests, aspidinol was weakly active *in vitro* and inactive *in vivo* at 100 mg/kg, phloropyrone was weakly active *in vitro* and was not tested *in vivo*. It is concluded that the phloroglucinol

compounds are the main active constituents of *D. dilatata*, the most active single constituent probably being aspidin. Büchi (1957) found that aspidin was inactive against *H. fraterna* infections in mice but he used a low dose (10 mg/kg).

D. filix-mas. The starting material was ether extract said to contain 37·8% filicin. This was active in both tests. The oils were weakly active *in vitro* (1 : 10,000) and had possibly slight activity *in vivo*. The mixed phloroglucinol compounds were active in both tests but not appreciably more active than the original ether extract from which they were prepared. It may be that (*a*) there is an active substance present in the ether extract which is neither phloroglucinol compound nor oil, (*b*) that the various constituents enhance each others activity, (*c*) that active phloroglucinol compounds are in part destroyed by the extraction process, or (*d*) the apparent discrepancy may be due to the insensitive nature of the test procedures. Of the major phloroglucinol compounds in *D. filix-mas*, flavaspidic acid seems to be the most potent, being active in both tests, though at 200 mg/kg some of the treated mice died. However, desaspidin obtained from *D. austriaca* (Aebi, Büchi & Kapoor, 1957) proved to be the most potent phloroglucinol compound *in vivo*. In practice the potency of an anthelmintic is of less interest than the therapeutic index. Östling (1962) gives the oral LD50 values (mg/kg) in mice as desaspidin 260, phloropyrone 530, flavaspidic acid 700, and aspidin 995. So desaspidin appears to be the most potent and the most toxic of the phloroglucinol compounds. Nevertheless, both desaspidin and flavaspidic acid have been successfully used in the treatment of human tapeworm infestations (Östling, 1962; Anttonen, 1954).

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References

Aebi, A., Büchi, J. & Kapoor, A. (1957). *Helv. chim. acta*, **60**, 266–274.
Ackermann, M. & Mühlemann, H. (1946). *Pharm. acta Helvet.*, **21**, 157–177.
Anttonen, V. M. (1954). *Ann. Med. exp. Fenn.*, **32**, 15–25.
Boehm, R. (1897). *Arch. exp. Path. Pharmak.*, **38**, 35–58 and later papers.
Borkowski, J. & Kowalewski, Z. (1958). *Acta polon. pharm.*, **15**, 15–21.
Büchi, J. (1957). *J. Pharm. Belg.*, **12**, 391–417.
Klevstrand, R. (1957). *Dansk Tidsskr. Farm.*, **31**, 217–221.
Östling, G. (1962). *Nord. med.*, **67**, 15–24.
Pabst, M. L. & Bliss, A. R. (1932). *J. Amer. pharm. Ass.*, **21**, 431–441.
Penttilä, A. & Sundman, J. (1961a). *J. Pharm. Pharmacol.*, **13**, 531–535.
Penttilä, A. & Sundman, J. (1961b). *Acta chem. scand.*, **15**, 839–848.
Ritter, F. J. & Meyer, G. M. (1962). *Nature, Lond.*, **193**, 941–942.
Rosendahl, H. (1911). *Pharm. J.*, **87**, 35.
Sen, A. B. & Hawking, F. (1960). *Brit. J. Pharmacol.*, **15**, 436–439.
Steward, J. S. (1955). *Parasitology*, **45**, 255–265.
Widén, B. (1944). *Acta bot. fenn.*, **37**, 4–79.

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Pharmacological actions and toxicity of dimethyl sulphoxide and other compounds which protect smooth muscle during freezing and thawing

J. FARRANT

Dimethyl sulphoxide and other non-electrolytes have been shown to protect partially, the smooth muscle of the guinea-pig uterus from damage during freezing to and thawing from −79°. The mechanism of this protection is probably physico-chemical in nature, for none of the compounds had any specific pharmacological action on smooth muscle. In the high concentrations needed for protection the compounds themselves caused some non-specific osmotic damage to the smooth muscle. All of the compounds examined had a low toxicity in mice.

IN 1949, Polge, Smith & Parkes found that glycerol protected spermatozoa from damage during freezing to and thawing from −79° and −196°. Subsequent work has shown that glycerol prevents or reduces the damage caused by freezing in a wide variety of other cells and tissues including the smooth muscle of the guinea-pig uterus (see Smith, 1961). Dimethyl sulphoxide, another water miscible non-electrolyte, was found to exert a similar action on spermatozoa and red blood cells (Lovelock & Bishop, 1959), on bone marrow cells (Ashwood-Smith, 1961 a, b) and on cells from the embryonic human lung (Porterfield & Ashwood-Smith, 1962). More recently other compounds, including pyridine *N*-oxide, methyl formamide, and methyl acetamide, have been found to protect red blood cells from haemolysis during freezing and thawing (Nash, 1961, 1962).

The ability of these compounds to protect smooth muscle during freezing and thawing has now been tested using the guinea-pig uterus. The pharmacological and toxicological properties of these compounds have also been investigated. A preliminary report of this work has been published (Farrant, 1963).

Experimental

FREEZING AND THAWING OF UTERI

In each experiment the two uterine horns from an albino guinea-pig (300–500 g) were suspended in a modified Krebs solution (NaCl 118, KCl 4·54, $CaCl_2$ 1·4, $MgCl_2$ 1·16, dextrose 11·1, $NaHCO_3$ 25, NaH_2PO_4 0·116; all in mM/litre) at 37° in two separate organ baths. A mixture of 95% O_2 and 5% CO_2 was bubbled through the solutions. Responses to standard doses of histamine (1–3 μg) were recorded using isotonic levers. The uterine horns were then transferred to separate tubes containing 10–20 ml of a solution at 37° of dimethyl sulphoxide or one of the other protective compounds. The concentration of these protective substances were, in each case, 1·4M, and the concentrations of salts and dextrose in the solutions were the same as in the original modified Krebs solution.

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The temperature was maintained at 37° and 95% O_2 and 5% CO_2 bubbled through for 30 min. The uterine horns were then transferred to tubes containing 2 ml of the same modified Krebs solution containing the test substance. The tubes were placed in the cooling chamber of the freezing apparatus (Linde, Biological Freezer 3). In this apparatus cooling is brought about by the injection of liquid nitrogen into the cooling chamber containing the samples. The controlling system was modified at the National Institute for Medical Research. The rate of cooling of the sample was measured by means of a copper constantin thermocouple and recorded potentiometrically. Unless otherwise stated, samples were cooled by approximately 3°/min to the selected temperature. When this temperature had been reached the tubes containing the uteri were transferred from the cooling chamber to beakers containing the modified Krebs solution at 37° to thaw the uteri as rapidly as possible. The solution used for thawing did not contain the protective substance. After the last particle of ice had vanished from the solution, the uterine horns were resuspended in the organ baths and attached to the isotonic lever under the same conditions of tension and magnification as before. The subsequent behaviour of the muscles was observed; standard doses of histamine were added periodically and any responses recorded. Control experiments were performed in which uteri were frozen in the modified Krebs solution without a protective substance.

PHARMACOLOGICAL ACTIONS OF THE PROTECTIVE SUBSTANCES

Three smooth muscle preparations were used for pharmacological tests *in vitro*; segments of rabbit duodenum, segments of guinea-pig ileum and guinea-pig uterine horns. The rabbit duodenal segments were suspended in Tyrode solution and the guinea-pig preparations in Krebs solution. All solutions were maintained at 37° and were bubbled with O_2 (95%) + CO_2 (5%). Isotonic responses were recorded.

WEIGHING EXPERIMENTS

Uterine horns were incubated at first in the modified Krebs solution, then in the solution containing dimethyl sulphoxide, and finally on return to the control solution. During incubation the horns were weighed every 3 min after draining off the surface fluid on the side of a beaker. Changes in weight, expressed as a percentage, were compared with the mean weight observed during the initial period in the modified Krebs solution. In some experiments, one horn of a uterus was weighed while the responses of the other horn to histamine were recorded in an isolated organ bath.

TOXICITIES OF THE PROTECTIVE SUBSTANCES

The LD50 of a 4M solution of each compound in distilled water was obtained in T.O. mice (of both sexes, weighing 15–25 g). Injections were made intravenously and intraperitoneally and there were 5 mice in each group. The long-term effects of the oral administration of some of the compounds were also studied. The maximum concentration of each

substance that the mice would drink was found by preliminary experiments. The fluid intake and weight gain of groups of 5 mice given the solutions to drink were recorded for about 6 weeks. The animals were then killed and their livers removed and fixed in Bouin's fluid. Paraffin sections were stained with haematoxylin and eosin for microscopical examination.

Results

FREEZING AND THAWING OF UTERI

When guinea-pig uteri were frozen to −30° or to −79° in the modified Krebs solution, and then rapidly thawed and resuspended in isolated organ baths, spontaneous contractions and rhythm were never recorded and responses either to the standard or to large doses of histamine could not be obtained; the muscles appeared to be dead. On the other hand, uteri frozen to and thawed from −30° or −79° in the presence of dimethyl sulphoxide (1·4M) were able to contract both spontaneously and in response to doses of histamine (Fig. 1). Protection against damage did not appear

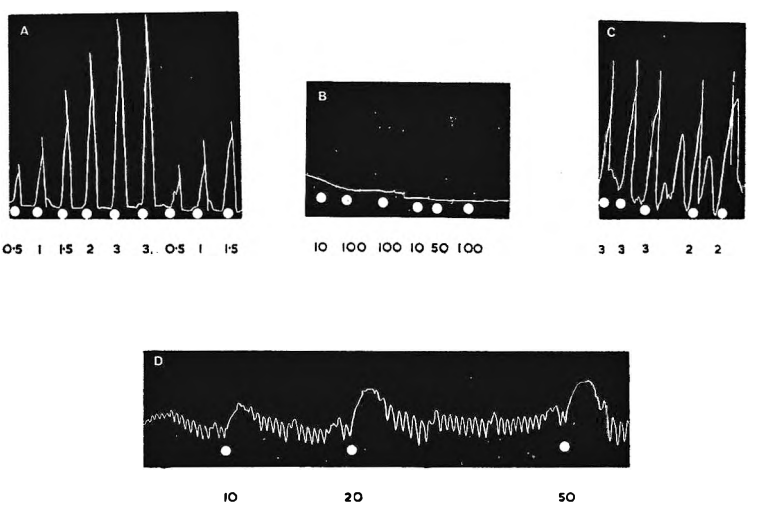

FIG. 1. Responses to histamine of guinea-pig uteri. A. Before freezing. B. After freezing to −30° in the absence of DMSO. C. After freezing to − 30° in the presence of 1·4M dimethyl sulphoxide. D. After freezing to − 79° in the presence of 1·4M dimethyl sulphoxide. At the white dots histamine was added to the 15 ml bath. Doses are in μg.

to be complete. This is shown by comparing mean dose response curves to histamine from groups of uteri before freezing and after thawing from different temperatures in the presence of dimethyl sulphoxide (1·4M) (see Fig. 2). The presence of the dimethyl sulphoxide protects the muscle only partially; the lower the temperature, the greater the damage. Under these conditions most damage seems to occur between −15° and −30°. Similar experiments were then made on uteri which had been frozen in solutions containing 1·4M of one of the other

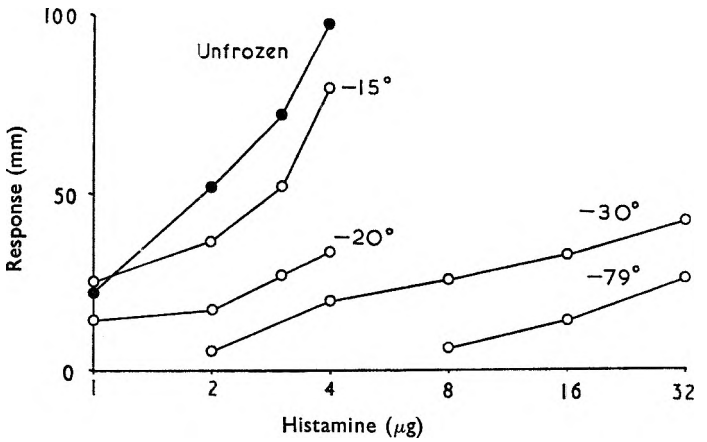

FIG. 2. Mean dose response curves to histamine from groups of uteri before freezing and after thawing from different temperatures in the presence of 1·4M dimethyl sulphoxide.

compounds. Table 1 shows that glycerol, and the four substituted amides also protected smooth muscle partially during freezing to −79°. *N*-Methylpyrrolidone and pyridine *N*-oxide did not protect the muscle.

TABLE 1. COMPOUNDS WHICH PROTECT SOME CELLS FROM FREEZING TO VERY LOW TEMPERATURES

Compound	Formulae	Protection of smooth muscle of guinea-pig uterus at −79°C
Dimethyl sulphoxide	Me_2SO	+
Glycerol	$CH_2OH(CHOH)CH_2OH$	+
Methyl formamide	HCONHMe	+
Dimethyl formamide	$HCONMe_2$	+
Methyl acetamide	MeCONHMe	+
Dimethyl acetamide	$MeCONMe_2$	+
Methylpyrrolidone	$[CH_2]_3CONMe$	0
Pyridine *N*-oxide	C_5H_5NO	0

Almost all muscles which had been partially protected from damage during freezing and thawing contracted slowly after being replaced in the Krebs solution at 37°. During the spontaneous slow contraction the addition of standard doses of histamine to the bath induced small responses which were rarely followed by a relaxation when the histamine was washed out of the bath. Furthermore, spontaneous rhythmic contractions usually occurred in the thawed preparation after the peak of the slow contraction had been reached (see Fig. 3). When spontaneous rhythm was prevented before freezing by reducing the calcium concentration from 1·4 to 0·3 mM/litre, uteri still developed spontaneous rhythm after thawing, even if the calcium was at this low level both in the freezing medium and in the thawing medium. Sensitivity to histamine increased after the spontaneous rhythm had developed, but responses to

histamine were usually less than those obtained before freezing. These points are illustrated in Fig. 3.

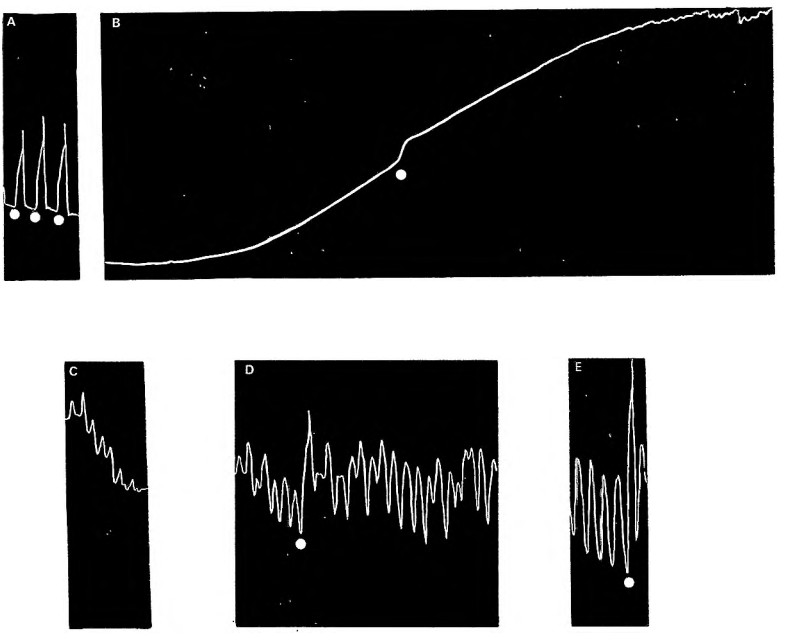

FIG. 3. Recovery of contractile ability of guinea-pig uterus after freezing to $-79°$ in the presence of 1·4M dimethyl sulphoxide. A. Before freezing. B. Immediately after thawing. C, D, E. 40, 60 and 120 min after thawing respectively. Histamine 0·5 μg was given at the white dots.

The effect of varying the rate of cooling was also examined. Fig. 4a records the cooling curves from two groups of experiments in which uteri were frozen in the presence of dimethyl sulphoxide (1·4M). Uteri in group A were cooled at 3°/min to $-30°$. Uteri in group B were cooled at 3°/min until the medium had supercooled to about $-9°$. Freezing then took place and the temperature rose to the freezing-point ($-6°$). After the freezing-point had been reached the rate of cooling was slowed because latent heat was emitted. Uteri from this group were also thawed from $-30°$. Fig. 4b shows that responses to histamine of muscles from group B were much less than those of muscles from group A. This result indicates that some damage occurs during freezing in the presence of dimethyl sulphoxide especially at temperatures close to the freezing point.

PHARMACOLOGICAL ACTIONS

The pharmacological properties of all the compounds listed in Table 1 have been examined on the smooth muscle of guinea-pig uterus and ileum, and on rabbit duodenum. In concentrations of up to 0·5% v/v (i.e. about 50 mM/litre) none of these compounds exerted any action alone

nor did they modify the responses to standard doses of acetylcholine, histamine or nicotine. However, when the dose was increased to 1·0% v/v all the compounds reduced the responses to acetylcholine, histamine or nicotine. These non-specific antagonisms were completely reversed after the protective substances had been washed out, for the control responses to the agonists were completely restored. Fig. 5 shows an example of this effect on the guinea-pig uterus. When the Krebs solution

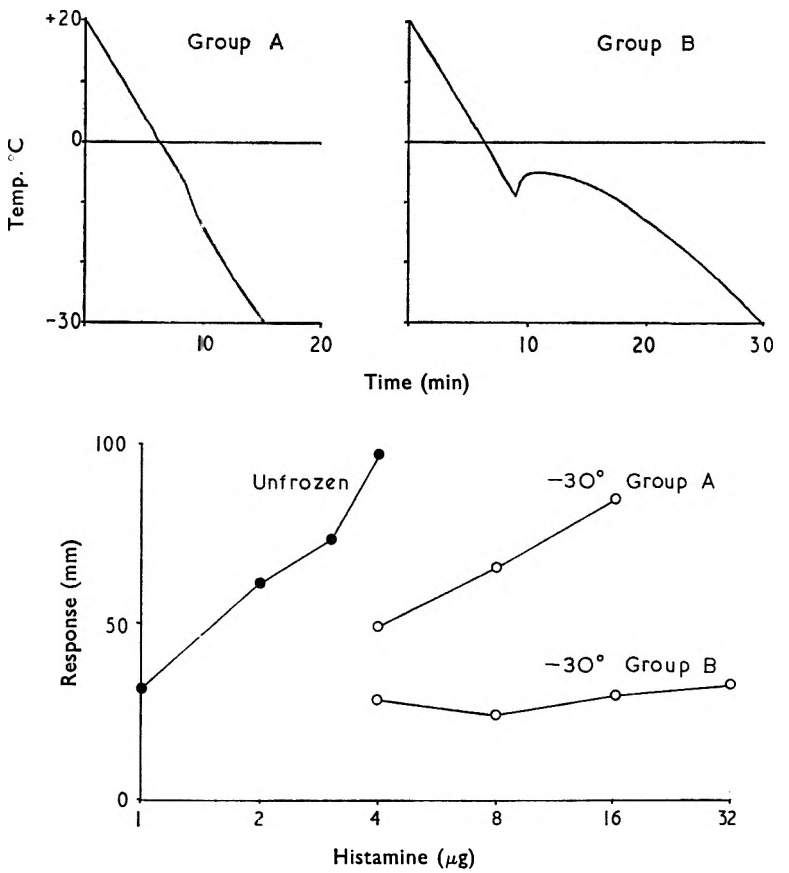

FIG. 4. a. Mean cooling curves from two groups of uteri frozen to − 30° in the presence of 1·4M dimethyl sulphoxide. b. Mean dose response curves to histamine from the two groups of uteri after thawing from − 30°.

in the bath was replaced by a Krebs solution containing 1% v/v dimethyl sulphoxide (140 mM/litre) the responses to histamine were reduced; then, when the original solution was replaced, the responses to histamine were completely restored. Fig. 5 also shows an experiment in which the uterine muscle was exposed to a solution containing 10% v/v dimethyl sulphoxide (1·4M). In this instance, the responses to histamine were completely suppressed even when large doses were given. When the dimethyl

sulphoxide was washed out of the bath there was a slow contraction of the muscle and only a partial recovery of the responses to histamine. All the protective compounds had these actions.

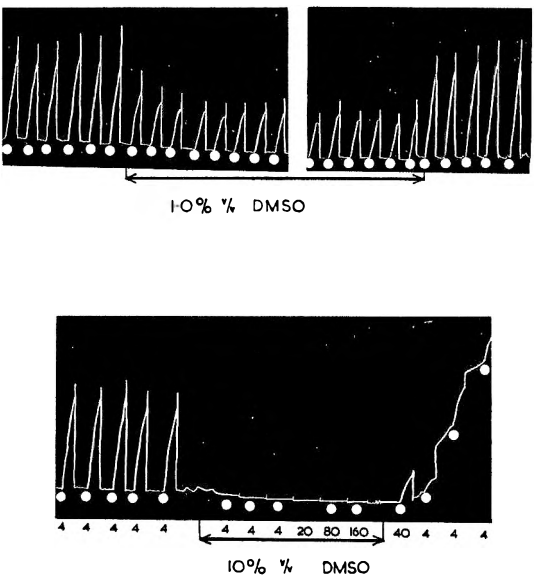

FIG. 5. Responses to histamine of guinea-pig uteri before, during and after incubation in Krebs solution containing dimethyl sulphoxide (1%, 140 mM and 10%, 1·4M). At white dots, histamine 4 μg unless otherwise marked.

WEIGHING EXPERIMENTS

Uteri incubated in Krebs solution containing dimethyl sulphoxide (10% v/v, 1·4M) lost weight but rapidly regained weight when returned to the solution without dimethyl sulphoxide. When the uteri were incubated in solutions containing lower concentrations of dimethyl sulphoxide they lost less weight (Fig. 6a). Fig. 6b records the percentage change in response to a standard dose of histamine obtained from one uterine horn after incubation in a Krebs solution containing dimethyl sulphoxide (1%, 140 mM/litre). The other horn from the same guinea-pig was treated with 1% dimethyl sulphoxide in the same way, and the changes in weight were recorded (see the top graph of Fig. 6a). When the weight of this horn had returned to normal in the presence of the dimethyl sulphoxide, the responses to histamine of the other horn were still reduced.

TOXICITIES

Acute treatment. The acute toxicity of each compound was obtained both by intravenous and by intraperitoneal injections in mice. The LD50 values in mM/kg are given in Table 2. All of the compounds examined had a very low toxicity. The least toxic substance was dimethyl sulphoxide, and the most toxic was pyridine *N*-oxide. Immediately after a

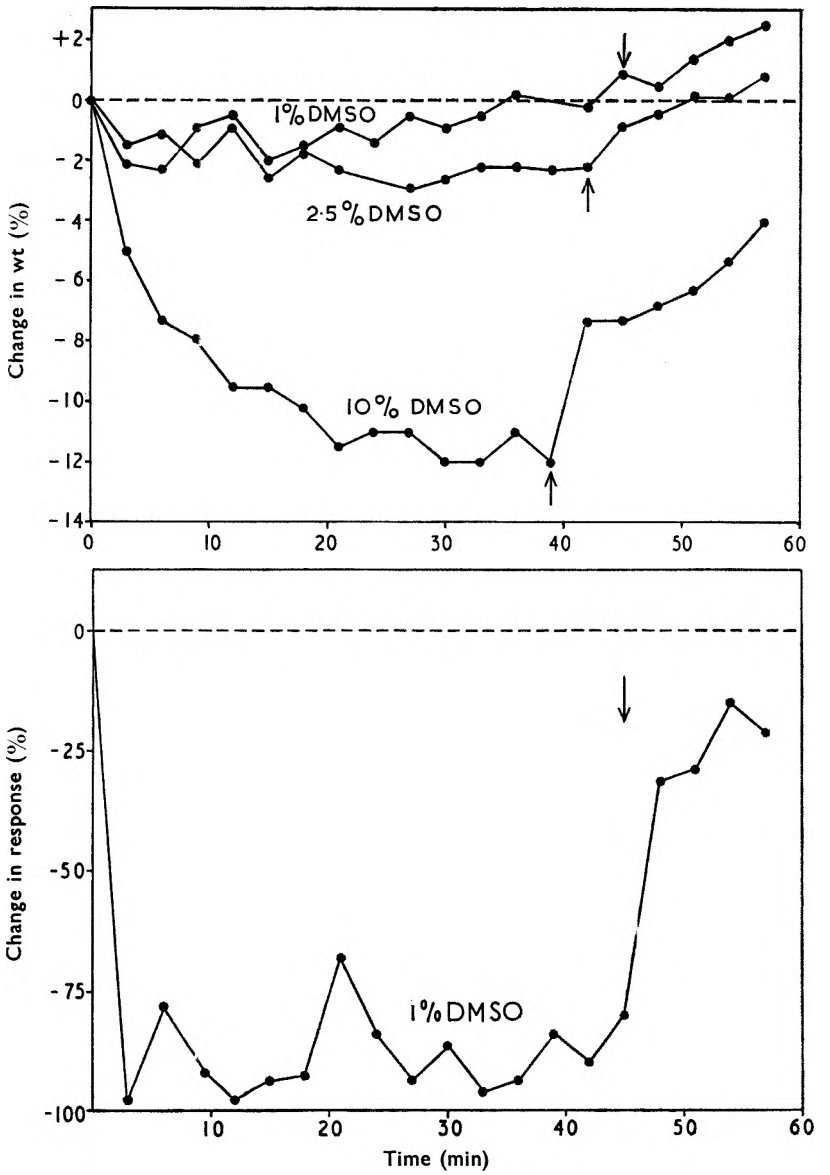

FIG. 6. a. Percentage change in weight of uteri incubated in various concentrations of dimethyl sulphoxide and after return to Krebs solution. The solutions were changed at the arrows. b. Percentage change in response to histamine of uterus incubated in 1% dimethyl sulphoxide and then returned to Krebs solution at the arrow.

lethal dose of dimethyl sulphoxide or of dimethyl acetamide the hind limbs became paralysed. A few animals died immediately after a lethal dose of a compound had been given, but most lost weight, became

hypothermic and died 2 or 3 days later. After lethal doses of glycerol intraperitoneally the animals developed ascites.

TABLE 2. TOXICITY OF COMPOUNDS IN MICE

Compound	Molecular weight	LD50 of 4M solution (mM/kg) i.v.	LD50 of 4M solution (mM/kg) i.p.	Maximum concentration taken orally (M)
Dimethyl sulphoxide	78	92	188	0·7
Glycerol	92	67	75	0·7
Methyl formamide	59	75	120	0·175
Dimethyl formamide	73	50	90	0·175
Methyl acetamide	73	55	60	0·175
Dimethyl acetamide	87	45	68	0·175
N-Methylpyrrolidone	99	20	36	0·35
Pyridine *N*-oxide	95	14	15	—

Chronic treatment. The maximum concentration that the groups of mice would drink is shown in Table 2. The fluid intake of mice which were drinking the dimethyl sulphoxide solution increased progressively (as shown in Fig. 7).

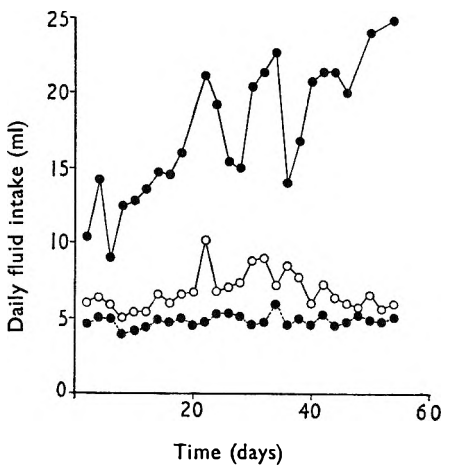

FIG. 7. Mean daily fluid intake of mice drinking dimethyl sulphoxide (0·7 M) (●—●), glycerol (0·7M) (○—○), or water (●--●).

Sections of the livers from the mice chronically treated with most of the protective agents showed mild cloudy swelling. On the other hand the livers from the mice treated with *N*-methylpyrrolidone showed severe degenerative changes. Extensive haemorrhages had taken place in some areas; in other areas fibrosis had occurred and elsewhere the parenchyma cells showed fatty degeneration.

Discussion

These results show that when the guinea-pig uterus was frozen to −30° or −79° the smooth muscle was severely damaged and no longer responded to drugs after thawing. The addition of dimethyl sulphoxide

(1·4M) to the medium partially protected the contractile mechanism of the smooth muscle during freezing so that, after thawing, responses to histamine could again be obtained. Other compounds which partially protected the muscle were glycerol, methyl formamide, dimethyl formamide, methyl acetamide and dimethyl acetamide. All these compounds are non-ionised but are sufficiently polar to form hydrogen bonds with water. Two other compounds which protected red blood cells from damage at low temperatures, pyridine *N*-oxide (Nash, 1961) and *N*-methyl pyrrolidone (Nash, private communication), did not protect smooth muscle.

Uterine smooth muscle which had been frozen in the presence of dimethyl sulphoxide was not completely protected. The lower the temperature to which the tissue was exposed the greater was the damage. Most damage occurred between $-15°$ and $-30°$. The results showed that one important factor was the length of time during which the muscle was exposed to temperatures just below the freezing point. This is indicated by the greater amount of damage observed in a group of uteri cooled slowly to $-30°$ when compared with the amount of damage in another group cooled more rapidly to the same temperature. The final temperature reached was less important. The site of damage in uteri which had been frozen in the presence of a protective substance is not clear. The fact that muscles exhibited spontaneous activity after freezing and thawing in solutions with reduced calcium suggests that the cell membrane may have been damaged.

None of the compounds tested had any specific pharmacological activity in high doses. The mechanism by which they protect the muscle from damage due to freezing and thawing must therefore be physico-chemical and not pharmacological. Lovelock (1953, 1954) suggested that glycerol and other neutral solutes entered the red blood cells and protected them during freezing and thawing by exerting their colligative properties. The same effect of lowering the concentration of salts in the solution from which ice is separating at any temperature during freezing may account for the protection of the smooth muscle. In the high concentrations required for protection they reduced or abolished responses to agonists non-specifically. These reductions in response may be due to osmotic effects, because when incubated in 1·4M dimethyl sulphoxide uterine horns rapidly lost weight. Incubation in a hypertonic solution is known to reduce the maximum tetanic tension in skeletal muscle (Howarth, 1958). Dimethyl sulphoxide may also inactivate the contractile process by some other mechanism, for, when uteri had been incubated in 140 mM/litre dimethyl sulphoxide until the weight had returned to the control value, responses to standard doses of histamine were still blocked.

All the compounds tested were remarkably non-toxic in mice. The LD50 of dimethyl sulphoxide when injected intraperitoneally as a 4M solution was 188 mM/kg. Under similar conditions the LD50 of sodium chloride is 53 mM/kg (Spector, 1956). The LD50 of dimethyl sulphoxide when injected intravenously as a 4M solution was 92 mM/kg. When

injected undiluted an LD50 of 48 mM/kg has been reported (Rosenkrautz, Hadidian, Seay & Mason, 1963). The greater toxicity of the undiluted substance may be due to the large heat of mixing. Other reported LD50 values in mice are those for dimethyl sulphoxide, 140 mM/kg intraperitoneally (Ashwood-Smith, 1961b); and for pyridine *N*-oxide, 14 mM/kg intraperitoneally (Nash, 1962). Brown, Robinson & Stevenson (1963) found that the LD50 for dimethyl sulphoxide in rats was greater than 7·5 ml/kg (about 100 mM/kg) when administered intraperitoneally.

The lack of toxicity of the compounds listed in Table 2 is confirmed by the experiments in which mice drank solutions of the compounds for a long period. Serious liver damage only occurred in those mice which had been drinking *N*-methylpyrrolidone.

At very low temperatures, cells should not deteriorate due to ageing. This is because metabolism is arrested and the physical and chemical processes involved in the degeneration of cell structures are greatly slowed. Until fairly recently these temperatures could not be used for the storage of living mammalian cells because the process of cooling to and thawing from these low temperatures caused severe damage. Although some simple organisms and particularly those which can withstand desiccation have natural systems for protection against damage during freezing and thawing, only a few types of mammalian cells could be frozen and thawed successfully without any special precautions. New possibilities were opened up by the discovery that glycerol and other compounds would protect the spermatozoa and red blood cells of several mammalian species. A wide variety of other mammalian cells and tissues have been stored at low temperatures in the presence of one or other of the protective substances (see Smith, 1961).

If a whole organ is to be stored in this way the protection of a high proportion of all the different types of cell is essential. Each cell type requires different conditions. Smooth muscle is being investigated because it is vital for the normal function of much of the digestive, excretory and reproductive systems and also it forms a major component of the vascular supply to organs. The compounds examined possess some of the ideal properties needed. They have an extremely low toxicity to the host, and therefore their removal from the stored organ before grafting may not be essential. They have no specific pharmacological effect on the tissue to be frozen.

At least three major problems remain. Firstly, in the concentration needed for protection, all the compounds so far tested themselves damaged the smooth muscle to some extent. This damage may have been due in part to osmotic changes. Secondly, protection during freezing and thawing was only partial even under the best conditions so far obtained. The damage which occurred was different from and in addition to that caused by the compound before freezing. The third problem is to determine the nature of the damage to the smooth muscle cells so that it can be reduced or prevented. Progress is already being made along these lines, but there is no immediate prospect of storing a whole organ at low temperatures.

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References

Ashwood-Smith, M. J. (1961a). *Nature, Lond.*, **190**, 1204–1205.
Ashwood-Smith, M. J. (1961b). *Int. J. Rad. Biol.*, **3**, 41–48.
Brown, V. K., Robinson, J. & Stevenson, D. E. (1963). *J. Pharm. Pharmacol.*, **15**, 688–692.
Farrant, J. (1963). *Biochem. pharmacol., Suppl.* **12**, 248.
Howarth, J. V. (1958). *J. Physiol.*, **144**, 167–175.
Lovelock, J. E. (1953). *Biochim. Biophys. Acta*, **11**, 28–36.
Lovelock, J. E. (1954). *Biochem. J.*, **56**, 265–270.
Lovelock, J. E. & Bishop, M. W. H. (1959). *Nature, Lond.*, **183**, 1394–1395.
Nash, T. (1961). *Ibid.*, **192**, 360–361.
Nash, T. (1962). *J. gen. Physiol.*, **46**, 167–175.
Polge, C., Smith, A. U. & Parkes, A. S. (1949). *Nature, Lond.*, **164**, 666.
Porterfield, J. S. & Ashwood-Smith, M. J. (1962). *Ibid.*, **193**, 548–552.
Rosenkrautz, H., Hadidian, Z., Seay, H. & Mason, M. M. (1963). *Cancer Chemotherapy Rep.*, No. 31, 7–24.
Smith, A. U. (1961). *Biological Effects of Freezing and Supercooling.* London: Arnold.
Spector, W. S. (1956). *Handbook of Toxicology.* Philadelphia: Saunders.

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Alkaloids of *Fagraea fragrans* Roxb.

ALFRED S. C. WAN AND Y. L. CHOW

The major alkaloid of the leaves and fruits of *Fagraea fragrans* Roxb. has been isolated and shown to be 4-(2-hydroxyethyl)-5-vinyl nicotinic acid lactone identical with gentiamine.

THE handsome tree *Fagraea fragrans* Roxb. (*Cyrtophyllum peregrinum* Blume), family Loganiaceae, reaches about 100 ft. high and grows abundantly in Singapore and the south of the Malayan peninsula. A decoction of the leaves and twigs is used in Malay folk medicine for the treatment of dysentery (Burkill & Haniff, 1930) and that of the bark for malaria (Holmes, 1892). The leaves and fruits have an intensely bitter taste. In their phytochemical survey of Malayan plants Douglas & Kiang (1957) found that the leaves and barks of *F. fragrans* gave a strong reaction for alkaloids.

Extraction of the leaves with ether as described in the experimental section has yielded gentianine. Its melting-point was not depressed on admixture with an authentic sample; the infra-red spectra were likewise superimposable.

Gentianine was first isolated from *Gentiana kirilowi* (Proskurnina, 1944) and subsequently from *Enicostema littorale* by Govindachari, Nagarajan & Rajapa (1957) who elucidated its structure. The alkaloid has also been isolated from other genera of the family Gentianaceae (Williaman & Schubert, 1961). This is the first report of its isolation from Loganiaceae and it is interesting to note that taxonomically the two families are closely related.

The mass spectrum of this alkaloid shows a very strong peak at m/e 175 due to the molecular ion (M) and in accord with the molecular formula $C_{10}H_9NO_2$; this is also the base peak. The intense peak at m/e 117 (M–58), relative intensity 87 %, is attributed to the loss of CH_2–O–CO; bond cleavage of this sort being common in the fragmentation of alkylated aromatic compounds (McLafferty, 1962). The peak at m/e 147 (M–28) arises from the loss of the vinyl group plus one proton from the molecular ion. Finally, loss of both CH_2=CH– and CH_2–O–CO– accounts for the peak at m/e 90 (M–85).

In the nuclear magnetic resonance spectrum, the two low field singlets (1·29 and 1·04 τ) can only be assigned to protons a and b of the pyridine ring in which both β-positions are substituted. The fact that these protons are shifted to lower fields than is usual for hydrogens in such a position is attributed to diamagnetic shielding by the vinyl and carbonyl groups (Jackman, 1959a). The multiplet between 4·6 — 2·9 τ constitutes an ABX system the pattern of which is similar to that of 4-vinyl pyridine (Varian NMR spectra catalogue No. 155). This arises from protons c, d and e. The peaks centred around 4·38 τ, equivalent to two protons, are assigned to H_c and H_d (the AB part of the system); the multiplet at about

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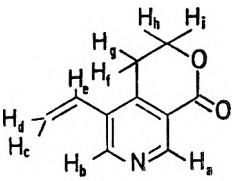
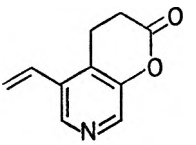
3·22 τ, equivalent to one proton, is assigned to H_e (X proton). The remaining four protons, H_f–H_i, are accounted for by the two 2-proton triplets indicating two adjacent non-equivalent methylene groups, the splitting for each being 6 c/s. The triplet at 6·92 τ is assigned to H_f and H_g; that at 5·49 τ to H_h and H_i (structure I).



I



II

The alternative structure (II) would be eliminated since a triplet due to the methylene group adjacent to a carbonyl group would then appear at around 7·9 τ instead of at 5·49 τ (Jackman, 1959b).

Experimental

PLANT MATERIAL

Leaves and fruits of *F. fragrans* were collected from plants growing in the grounds of the Medical Faculty, University of Singapore and authenticated with herbarium specimens of *F. fragrans* Roxb. at the Botanic Gardens, Singapore.

The alkaloidal content of the plant showed marked seasonal variation. The yield of alkaloid from the leaves was highest (0·32 %) at the beginning of the flowering season (May-June) and lowest (0·005%) after fruiting had occurred (October-November). The ripe fruits contained about 0·10% of alkaloids.

The ultra-violet spectra were recorded on a Bausch and Lomb Spectronic 505, the infra-red spectra on a Nihon-Bunko IRS–5 and a Perkin-Elmer 137–G, the NMR spectrum on a Varian A60. The mass spectrum was recorded by Dr. Ragnar Ryhage at the Laboratory for Mass Spectrometry, Karolinska Institutet, Stockholm.

Extraction. The air-dried, powdered leaves were moistened with ammonia and extracted by repeatedly shaking with solvent ether. The ether extract was shaken with 2% hydrochloric acid; the acid phase separated and basified with ammonia and the alkaloidal material taken up in chloroform. The chloroform solution, dried over anhydrous sodium sulphate, was evaporated to dryness under reduced pressure to yield a colourless crystalline residue, gentianine (0·32% yield).

Gentianine crystallised from hot water in colourless prisms, m.p. 82–83°, $[\alpha]_D^{20} \pm 0°$ (*c*, 1·0% in ethanol), λ_{max} (H_2O) 219 mμ (log ϵ 4·41), 285 mμ (log ϵ 3·14), inflection 240–250 mμ, ν_{max} (CCl_4) 3015, 1745, 1638 and 928 cm^{-1} [Govindachari & others (1957) cite m.p. 82–83°, $[\alpha]_D^{30} \pm 0°$ ($CHCl_3$), λ_{max} 220 mμ (log ϵ 4·38), 280 mμ (log ϵ 3·2).] Found: C, 68·6; H, 5·4; N, 8·2. Calc. for $C_{10}H_9NO_2$: C, 68·6; H, 5·2; N, 8·0%.

The NMR spectrum was recorded in deuterochloroform: carbon

tetrachloride (1 : 1) with tetramethylsilane as internal standard. The mass spectrum showed major peaks at m/e 175, 147, 117 and 90.

The *picrate*, crystallised from hot water, m.p. 126–127° [Govindachari & others (1957) cite m.p. 123–124°]. Found: C, 47·6; H, 3·1. Calc. for $C_{10}H_9NO_2.C_6H_3N_3O_7$: C, 47·5; H, 3·0%.

The alkaloid is moderately soluble in water (1·2% at 25°), completely soluble in dilute acids from which the original compound can be recovered on basifying with ammonia or aqueous sodium carbonate solution. It dissolves in 10% aqueous sodium hydroxide but is not regenerated on neutralisation. Reduction of the alkaloid with zinc and hydrochloric acid gave a secondary ring base demonstrated by the formation of copper dithiocarbamate (Feigl, 1956).

Dihydrogentianine. The alkaloid (132 mg) in ethanol (10 ml) absorbed only one equivalent of hydrogen on hydrogenation over Adam's platinum oxide under normal conditions. The product crystallised from water in colourless crystals (54 mg), m.p. 77–78°, λ_{max} (H_2O) 227mμ (log ϵ 3·9), 272 mμ (log ϵ 3·5), ν_{max} (CCl_4) 2980, 2900, 1745, 1420, 1390 cm^{-1}, ν_{max} (KBr) 1725, 1580, 1410, 1395 cm^{-1}. Found: C, 67·6; H, 6·4. Calc. for $C_{10}H_{11}NO_2$: C, 67·8; H, 6·3 %. [Govindachari & others cite m.p. 74–76°, λ_{max} 270 mμ (log ϵ 3·4)].

Lithium aluminium hydride reduction. The alkaloid (250 mg) in dry ether was refluxed (8 hr) with $LiAlH_4$ (100 mg). The cooled reaction mixture was decomposed with crushed ice; saturated ammonium chloride solution (20 ml) was added and the mixture extracted continuously with ether (12 hr). The product (150 mg) crystallised from benzene/ethanol m.p. 135–136°, λ_{max}(H_2O) 238 mμ (log ϵ 4·0), 272 mμ (log ϵ 3·5), ν_{max} (KBr) 3305, 3125, 1630, 1590, 1485 cm^{-1}. The spectrum in chloroform revealed peaks at 3640 and 3280 cm^{-1}. Found: C, 67·3; H, 7·5; N, 8·2. Calc. for $C_{10}H_{13}NO_2$: C, 67·0; H, 7·3; N, 7·8%.

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References

Burkill, I. H. & Haniff, M. (1930). *Gardens Bulletin, S. S.*, **6**, 225.
Douglas, B. & Kiang, A. K. (1957). *Malayan Pharm. J.*, **6**, 138–153.
Feigl, F. (1956). *Spot Tests in Organic Analysis*, p. 305, 5th ed. Amsterdam: Elsevier Publishing Co.
Govindachari, T. R., Nagarajan, K. & Rajapa, S. (1957). *J. chem. Soc.*, 551–556.
Holmes (1892). *Bull. Pharm.*, **6**, 116.
Jackman, L. M. (1959a). *Applications of Nuclear Magnetic Spectroscopy in Organic Chemistry*, pp. 59, 63. London: Pergamon Press.
Jackman, L. M. (1959b). *Ibid.*, pp. 55 and 57.
McLafferty, F. W. (1962). "Mass Spectrometry" in *Determination of Organic Structures by Physical Methods*, Vol. 2, Editors Nachod, F. C. & Phillips, W. D., p. 116. New York: Academic Press.
Proskurnina, N. F. (1944). *J. Gen. Chem.* (*U.S.S.R.*), **14**, 1148, in *Chem. Abstr.*, 1946, **40**, 7213.
Williaman, J. J. & Schubert, B. G. (1961). *Alkaloid-bearing Plants*, p. 87. Washington: U.S. Dept. of Agriculture.

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Stability of heparin solutions

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Heparin injection B.P., adjusted to a pH between 7·0 and 8·0, preserved with 0·15% chlorocresol, is a relatively stable biological product and may be expected to retain its potency for up to 15 years if stored at 4°, over 7 years at room temperature and 6–8 years at 37°. The stability is markedly reduced if the pH of the solution falls below 6·0, so that a rapid and marked loss in potency can be expected if heparin is sterilised in, or mixed for any length of time with, solutions of dextrose, owing to the low pH of these solutions.

TO determine the stability of solutions of heparin, now increasingly used in open heart surgery and artificial kidney treatment in which exact dosage is important, reference samples from batches manufactured over the past 15 years have been retested for potency.

As heparin is sometimes added to saline and glucose solutions before these solutions are sterilised the stability of heparin over a range of pH values at temperatures of 100° and 115° has been determined.

Experimental methods

The heparin solutions were batches of commercial origin (Pularin, Evans), a sterile solution of sodium heparin in distilled water, adjusted to a pH between 7·0 and 8·0 and preserved with 0·15% chlorocresol. The strength of the solutions tested varied from 10 to 35,000 units/ml.

Storage was at 4°, room temperature (approx. 18°) and at 37° for periods between 3 and 15 years.

Heating to 100° was in a boiling water-bath and to 115° by autoclaving in a small bench autoclave to allow a minimal time for raising and lowering the temperature

The potencies were determined by the method of Pritchard (1956) with which, with samples of commercial grade heparin, the fiducial limits of error ($P = 0.95$) of a single assay are given as $\pm 1{\cdot}7\%$ and for the mean of a duplicate assay as $\pm 1{\cdot}2\%$.

Results

Table 1 shows the change in potency of batches stored at the 3 temperatures. If changes in potency within the limits of error of the test are excluded, it was found that at 4°, 2 batches lost potency, one by 10·7%, the other by 3·8%, while two batches apparently gained in potency by 7·4% and 3·1% respectively.

At room temperature, 4 batches showed slight gains and 2 slight losses, while at 37° there was one loss and one gain in potency. Thus of 29 batches tested, 12 show a significant change, 7 showing an apparent increased potency and 5 a decrease in potency.

Fig. 1 shows the change in potency of a solution of heparin standardised to contain 660 units of heparin per ml when autoclaved for 10 min at 115° over a range of pH values from 3·0 to 10·0. Between pH 5·5 and 8·5

From The Evans Biological Institute, Runcorn.

there is no significant change but below pH 5 there is a sharp fall-off in unitage; above 8·5 there is also some loss of activity but this is less marked than at acid pH values.

TABLE 1. CHANGES IN POTENCY OF BATCHES STORED AT THREE TEMPERATURES

Batch No.	Manufactured	Original potency	Storage period		Change in potency %
			Years	months	
Temperature 4° C					
24	July 1947	1,000 u/ml	15	2	+ 0·6
34	June 1948	5,000 ,,	14	3	− 0·2
40	July 1948	4,400 ,,	14	2	− 0·8
41	,,	1,300 ,,	14	2	− 10·7
42	,,	5,500 ,,	14	2	+ 3·1
44	,,	5,000 ,,	14	2	− 0·7
221	Sept. 1952	5,000 ,,	10	2	+ 7·4
289/500	Nov. 1954	10 ,,	8	3	− 0·7
76	Feb. 1950	5,000 ,,	5	10	− 3·8
72	Dec. 1949	25,000 ,,	4	5	− 0·8
Room temperature (18° C)					
54	Oct. 1948	5,000 u/ml	7	6	+ 3·4
343	May 1956	5,000 ,,	6	4	+ 0·1
69	Sept. 1949	5,000 ,,	6	3	− 3·2
345	June 1956	35,000 ,,	6	3	0·0
360	Nov. 1956	5,000 ,,	5	10	+ 2·3
80	May 1950	5,000 ,,	5	7	− 3·2
376	June 1957	5,000 ,,	5	3	− 0·7
390	Sept. 1957	25,000 ,,	5	−	+ 0·2
393	Oct. 1957	5,000 ,,	4	11	+ 4·9
177	Oct. 1951	5,000 ,,	4	2	− 0·5
429	Oct. 1958	1,000 ,,	3	11	− 0·9
220	Sept. 1952	25,000 ,,	3	3	+ 3·2
Temperature 37° C					
221	Sept. 1952	5,000 u/ml	9	9	− 15·9
,,	,,	5,000 ,,	3	4	+ 3·6
282	June 1954	5,000 ,,	8	4	− 0·6
335	Mar. 1956	5,000 ,,	6	8	− 0·2
,,	,,	5,000 ,,	6	3	− 1·5
337	,,	5,000 ,,	6	8	− 0·3
,,	,,	5,000 ,,	6	3	+ 0·9

Fig. 2 shows the effect of heating solutions buffered at pH 2·99, 4·96, 7·5 and 9·96 for a period of 8 hr at 100°. The control material was unheated. This again shows the marked destruction of heparin when heated at an acid pH.

Discussion

The results show that correctly prepared solutions of heparin are stable when stored at 4° for 15 years, at room temperature for over 7 years and at 37° for 6–8 years. Two batches showed a loss in potency, one of 10·7% after 14 years at 4° and the other of 15·9% after 9¾ years at 37°. The remaining batches, with the exception of one showing an apparent 7·4% increase, do not vary widely from expected limits and, since it is unlikely that an increase could occur, it is probable that the original assay was at fault. If we accept these three observations, the percentage change between observations within groups does not differ significantly from the difference between groups at the 3 temperatures tested, suggesting that any change in potency is due to a factor other than storage temperature.

One of the most probable causes of a loss of potency is the incorrect

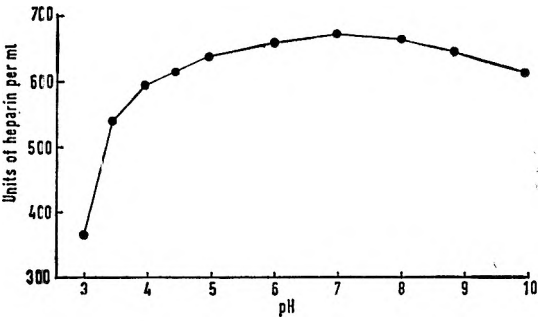

FIG. 1. Potency of heparin solutions after autoclaving at 10 lb./inch² for 10 min at pH values between 3·0 and 10·0.

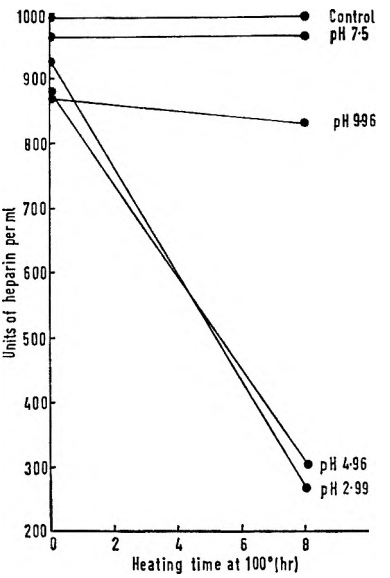

FIG. 2. Potency of heparin solutions, after heating at 100° C for 8 hr at different pH values.

adjustment of the pH of the product. This effect is shown clearly in Figs 1 and 2. The pH is important not only to the stability of the stored product, but also to the stability of mixtures of heparin and dextrose when these are intended as an anticoagulant for blood for heart-lung machines maintaining extra-corporeal circulation in open heart surgery, As injection dextrose B.P. has a pH range of 3·5–6, autoclaving heparin in this solution could destroy 50% of its original potency, and bring about coagulation of the blood.

If heparin is required in a dextrose solution it is best prepared by adding the sterile heparin solution to the bottle containing the sterilised dextrose solution as shortly as possible before use.

Reference

Pritchard, J. (1956). *J. Pharm. Pharmacol.*, **8**, 523–529.

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Colorimetric method for the estimation of norethynodrel in tablets containing mestranol

J. F. CHISSELL

The colour method proposed for the estimation of norethynodrel in tablets with mestranol utilises a reaction similar to the well known Zimmerman reaction, the reagents employed being *m*-dinitrobenzene and benzyltrimethylammonium hydroxide solution and a solvent mixture consisting of ethyl acetate and ethanol. Maximum extinction was at a wavelength of 510 mμ and Beer's law is obeyed for a concentration range 5 to 20μg norethynodrel per ml final solution.

AN attempt to use a conventional method for estimating norethynodrel utilising light absorption was unsatisfactory since the material exhibited little characteristic absorption, whereas mestranol had a significant absorption at a wavelength at about 280 mμ.

The first procedure adopted in this Laboratory was an extension of the British Pharmacopoeia method for methyltestosterone tablets involving the precipitation of the 3-ketosteroid with an acid solution of 2,4-dinitrophenylhydrazine. The precipitate was collected by filtration and ultimately for light extinction of a chloroform solution of the derivative which was compared against that obtained by similarly treating a known standard solution of norethynodrel. Maximum extinction was at 390 mμ and Beer's law was obeyed over the range 2·5 to 10 μg norethynodrel per ml expressed as final concentration of solution.

Although satisfactory results were obtained by this method it involved several lengthy manipulations and suffered from the disadvantage that complete precipitation of the 2,4-dinitrophenylhydrazone occurred only on prolonged standing. A quicker method was therefore sought.

Experimental

REAGENTS

Analar *m*-dinitrobenzene, used to prepare a 0·5% w/v solution in ethanol: benzyltrimethylammonium hydroxide (40% w/w aqueous solution) and used at this concentration (Corker, Norymberski & Throw, 1962): Analar ethyl acetate. Norethynodrel (Roussel-UCLAF).

PREPARATION OF CALIBRATION CURVE

The optimum intensity of colour consistent with simplicity of manipulation and reproducible results was achieved as follows:

A chloroform solution of norethynodrel (0·005%) was prepared and quantities equivalent to 50, 100, 150 and 200 μg transferred to separate 10 ml graduated flasks and the contents evaporated to dryness in a boiling water-bath. A fifth flask was used to prepare a reagent blank.

The solid residue in each flask was dissolved by the addition of 0·3 ml of a solution of *m*-dinitrobenzene, followed with mixing by 0·15 ml of benzyltrimethylammonium hydroxide solution.

From Roussel Laboratories Ltd., Columbus House, Wembley, Middlesex.

After 30 min at 20°±0·5° the contents of the flasks were diluted to volume with 10% v/v ethanol in ethyl acetate.

The extinctions of the red-violet colour were read immediately on a spectrophotometer (Uvispec) in 1 cm cells at 510 mμ and the calibration curve plotted from the results. This was a straight line which passed through the origin and Beer's law was obeyed over the range of concentrations employed.

ESTIMATION OF NORETHYNODREL IN TABLETS

Several tablets were reduced to a fine powder and an amount of powder equivalent to about 2·5 mg norethynodrel accurately weighed and transferred to a 100 ml graduated flask. Approximately 70 ml of chloroform was added, the mixture shaken for 15 min and the volume made up to 100 ml at 20° with chloroform.

At the same time a standard chloroform solution of norethynodrel (0·0025%) was prepared. Quantities of norethynodrel equivalent to 125 μg (= 5 ml) were transferred to separate 10 ml graduated flasks from the standard and test solutions. A third 10 ml graduated flask containing 5 ml of chloroform was used to prepare a reagent blank. The content of each flask was evaporated to dryness in a boiling water-bath and the assay completed as described for the calibration.

The extinction of the test and standard colours was compared against the reagent blank.

Discussion

The reaction between norethynodrel and *m*-dinitrobenzene was complete after 30 min and the colour intensity remained constant for a further 20 min but thereafter it decreased.

The reaction is not light sensitive and reagent blank values are low.

The quantities of the reagents used were not critical although the procedure described above is standard in our laboratory. Other solvent systems may be used successfully for diluting the reaction mixture, but 10% v/v ethanol in ethyl acetate provided maximum intensity of colour without precipitation of the reactants.

Unknown quantities of norethynodrel were assayed in an identical manner to that described above for the calibration curve. For the assay of tablets containing norethynodrel the colour was preferably compared against that produced by a similar quantity of pure norethynodrel acting as a standard. Rigorous temperature control is unnecessary in this case and reagent grade solvents and reagents may be used. The tablet excipients do not interfere with the colour development, nor does the associated mestranol, indeed no significant colouration was produced even when this substance was present in ten-fold excess over the quantity demanded by the tablet formulation.

The results obtained from 10 determinations on tablets each containing a theoretical 2·5 mg norethynodrel and 0·1 mg mestranol as active components showed a mean recovery of 2·47±0·057 (s.d.). The percentage recovery from theoretical was 98·8±2·3 (s.d.).

Investigations using other steroids have shown that this colour reaction is not restricted to norethynodrel, and preliminary results for methylandrostanolone in tablets have shown that a similar colour reaction is obtained with maximum extinction at about 560 mμ.

Results with methyltestosterone have been disappointing.

Acknowledgements. The author wishes to acknowledge the assistance of A. J. Newbery who was responsible for the analyses contained in this report.

References

Corker, C. S., Norymberski, J. K., & Throw, R. (1962). *Biochem. J.*, **83**, 583–588.

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Kinetics of degradation of 2-hydroxyiminomethyl-1-methylpyridinium methanesulphonate

MOUNG CHOUNG FAN, J. GRAHAM NAIRN AND GEORGE C. WALKER

The decomposition of 2-hydroxyiminomethyl-1-methylpyridinium methanesulphonate (P_2S) was studied at different temperatures and pH values. The initial rate of decomposition was found to be first order with respect to P_2S concentration. The pH for maximum stability appears to be about 5.

THE methanesulphonate of 2-hydroxyiminomethyl-*N*-methylpyridinium, P_2S, is one of the salts effective in reactivating cholinesterases which have been poisoned by anticholinesterase compounds, notably the organic phosphates (Ladell, 1958). P_2S has been reported to be unstable to heat, changing from pale yellow to orange-brown in aqueous solution, and producing cyanide as one of the end products in alkali medium. The rate of this change has also been found to depend on the temperature and pH of the solution (Creasy & Green, 1959; Kondritzer, Ellin & Edberg, 1961). More detailed information about these dependencies would be of interest. We have therefore examined the rate of degradation of P_2S in relation to temperature and pH. The results of studies on pralidoxime iodide (2-PAM, pyridine-2-aldoxime methiodide) were reported while this study was in progress (Ellin, Carlese & Kondritzer, 1962).

TABLE 1. BUFFERS FOR P_2S SOLUTIONS

pH	Composition	Dilution
1	0·1N hydrochloric acid	None
2	50·0 ml 0·2M potassium chloride and 7·8 ml 0·2M hydrochloric acid	to 200 ml
3	6 ml glacial acetic acid	to 1000 ml
4	1·8 ml 0·2M sodium acetate and 8·2 ml 0·2M acetic acid	None
5	7·0 ml 0·2M sodium acetate and 3·0 ml 0·2M acetic acid	None
6	12·3 ml 0·2M disodium phosphate and 87·7 ml 0·2M monosodium phosphate	to 200 ml
7	61·0 ml 0·2M disodium phosphate and 39·0 ml 0·2M monosodium phosphate	to 200 ml
9	50·0 ml 0·025M borax and 4·6 ml 0·1M hydrochloric acid	to 100 ml
11	50·0 ml 0·05M disodium phosphate and 4·1 ml 0·01M sodium hydroxide	to 100 ml
13	01·N sodium hydroxide	None

Methods

The pH of the test solutions was maintained by using buffer solutions or excess of acid or alkali. The buffers are listed in Table 1 and were found to be "transparent" throughout the ultra-violet range of the

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spectrophotometer (Beckman DK 2) and did not interfere with the absorption of the P_2S solutions. The spectra of freshly made solutions of P_2S (Aldrich Chemical Company, recrystallised from ethanol, m.p. 156·5–157·5°) show absorption peaks of 292 mμ in acid medium and 336 mμ in alkaline medium (Creasy & Green, 1959). P_2S was found to follow Beer's Law, but in view of the absorption of *N*-methyl-α-pyridone, a decomposition product, at 293 mμ in 0·1N HCl and 0·1N NaOH, all assays were made by adding 1·0 ml of 1N NaOH to a 5 ml sample where necessary and the absorption measured at 336 mμ (Greasy & Green 1959).

Solutions of P_2S in various media, at a concentration of about 23μg/ml were introduced into colourless 10 ml ampoules which were immersed in thermostatically-controlled baths at 40°, 50° and 60°. At appropiate intervals, depending upon the rate of degradation, two ampoules of each medium from each bath were removed and immediately assayed for P_2S and cyanide.

The cyanide content was estimated according to Aldridge's method (Aldridge, 1945) whereby the cyanide is converted into cyanogen bromide

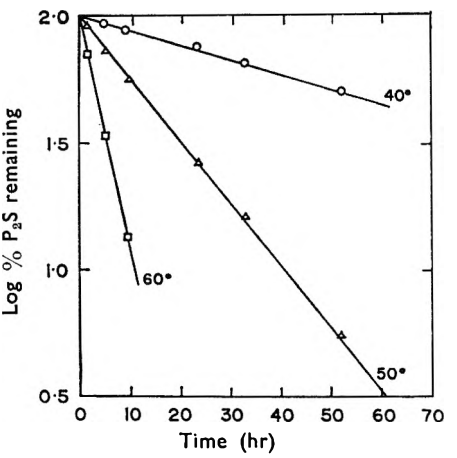

FIG. 1. Typical first-order rate plots for the decomposition of P_2S at pH 10·9 at different temperatures.

and then allowed to react with benzidine and pyridine. The magenta-coloured complex of cyanide was found to absorb in the visible range with a peak at 526 mμ. As this complex is formed only in neutral or acid medium, 2 ml samples were made acid by adding 0·2 ml of 50% sulphuric acid where necessary and the concentration of cyanide estimated spectrophotometrically.

Results

P_2S DEGRADATION

The observed rate of degradation of P_2S follows the classical first order equation $\ln c = kt + \text{constant}$, where k is the first order rate constant at a constant pH. First order plots for the decomposition of P_2S at pH 10·9 are shown in Fig. 1. Using the Arrhenius equation $k =$

$Ae^{-E/RT}$ the temperature dependence of the rate constant is shown in Fig. 2. The pH dependence of the rate constant is shown in Fig. 3 which shows a pattern similar to that for pralidoxime iodide (Ellin & others, 1962) and it is probable that P_2S and pralidoxime iodide degrade in the same manner.

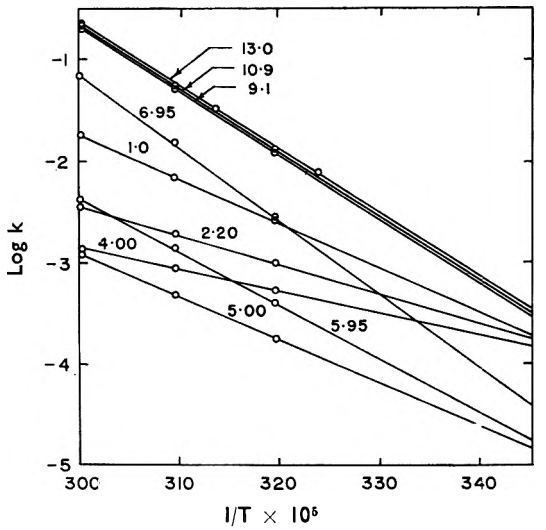

FIG. 2. Temperature dependency of the rate constant for the decomposition of P_2S at different pH values.

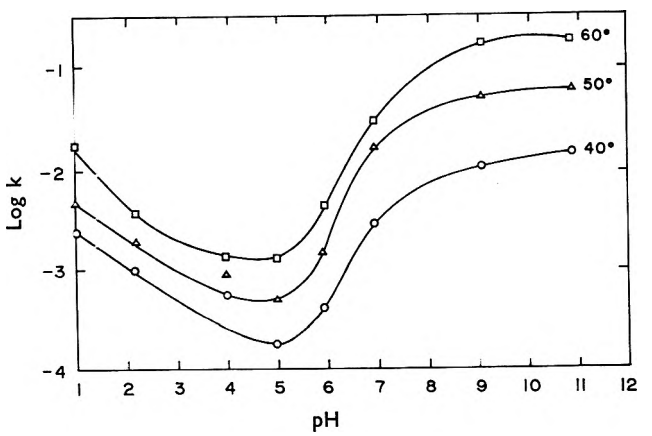

FIG. 3. pH profile of the logarithm of the first-order rate constants for the decomposition of P_2S.

First order rate constants for the degradation of P_2S at lower temperatures may be obtained from Fig. 2. The predicted half-lives of P_2S at various pH values and temperatures are shown in Table 2. It should be borne in mind. however, that the constants were obtained from the initial overall first order reactions only. Fig. 3 and Table 2 suggest

that the maximum stability of P_2S occurs at about pH 5 in aqueous solutions.

TABLE 2. PREDICTED HALF-LIFE (DAYS) OF P_2S IN AQUEOUS SOLUTIONS AT VARIOUS TEMPERATURES AND PH VALUES

Temp. °C	pH 1·0	2·20	4·00	5·00	5·95	6·95	9·10	10·90	13·0
60	1·65	7·776	20·06	24·10	4·18	1·03	0·14	0·15	0·13
50	4·04	14·04	32·74	59·71	20·22	1·82	0·59	0·51	0·49
40	11·4	28·59	51·44	160·4	69·66	10·02	3·30	2·17	2·09
30	31·7	56·30	69·23	432·0	251·3	57·61	10·48	10·10	9·56
20	97·83	123·1	155·1	1320·0	977·3	371·9	53·76	51·35	49·03
10	339·2	272·6	288·7	4271·0	4174·0	2821·0	313·0	302·4	288·4

CYANIDE FORMATION

The amount of cyanide ion found in the solutions at various temperatures and pH is shown in Table 3. Below pH 4, no cyanide was found even after prolonged periods of heating. Curves plotted from the various cyanide determinations did not appear to follow any general

TABLE 3. CYANIDE ION FORMED IN AQUEOUS SOLUTIONS OF P_2S AT VARIOUS TEMPERATURES AND PH VALUES

pH	μg/ml CN^- 40°	50°	60°
4	0 (210)*	0 (209)*	0 (80)*
5	0 (229)	0·013 (120)	0·035 (60)
5·95	0·01 (70)	0·043 (70)	0·35 (60)
6·95	0·29 (70)	0·65 (50)	1·31 (50)
9·10	0·83 (40)	1·44 (50)	

* Figures in brackets represent the number of hr.

pattern, although the amount of cyanide formed was higher when more P_2S was degraded. Assuming that P_2S degrades in a similar manner to pralidoxime iodide, then one mole of cyanide should be formed from one mole of P_2S (Ellin, 1958). However, the relation between cyanide formed and P_2S degraded is not constant. This would suggest that part of the degraded P_2S remained as intermediates and further studies of these intermediates and their rate constants would be necessary before a logical prediction of the amount of cyanide formed is possible.

Acknowledgements. The research for this paper was supported (in part) by the Defence Research Board of Canada, Grant number 9370-06.

References

Aldridge, W. N. (1945). *Analyst*, **70**, 474–475.
Askew, B. M. (1956). *Brit. J. Pharmacol.*, **11**, 417–423.
Creasey, N. H. & Green, A. L. (1959). *J. Pharm. Pharmacol.*, **11**, 485–490.
Davies, D. R., Green, A. L. & Willey, G. L. (1959). *Brit. J. Pharmacol.*, **14**, 5–8.
Ellin, R. I. (1958). *J. Amer. chem. Soc.*, **80**, 6588–6590.
Ellin, R. I., Carlese, J. S. & Kondritzer, A. A. (1962). *J. pharm. Sci.*, **51**, 141–148.
Kondritzer, A. A., Ellin, R. I. & Edberg, L. J. (1961). *Ibid.*, **50**, 109–112.
Ladell, W. S. S. (1958). *Brit. med. J.*, **2**, 141–142.

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Measurement of body temperature in conscious small laboratory animals by means of an oesophageal thermocouple

R. T. BRITTAIN AND P. S. J. SPENCER

A device is described for making rapid repeated measurements of body temperature in conscious small laboratory animals. A thermocouple, sealed inside an 18 gauge hypodermic needle is inserted into the oesophagus and the temperature read directly on a calibrated meter.

THE effects of a drug on body temperature, or the modification by a drug of a temperature change induced by a second drug or by other means, affords a suitable way of indicating certain types of pharmacological activity in laboratory animals. Sympathomimetic drugs are capable of invoking a hyperthermic response by an action on central adrenergic receptors (von Euler, 1961). Lessin & Parkes (1957) have shown that in mice the hypothermic effects of chlorpromazine and reserpine are related to their sedative properties. Morphine induces a biphasic temperature response in rats comprising an initial fall followed by a rise of temperature (Gunne, 1960) and Chodera (1963) has used this effect to investigate the interaction of morphine with monoamine oxidase inhibitors. In most experiments of this nature the body temperature of small laboratory animals is measured using a thermometer, thermocouple or a thermistor inserted in the rectum; in a few experiments skin thermocouples have been used. Measurement of body temperature using a skin thermocouple suffers from the disadvantage that local changes in blood flow inevitably affect local skin temperature without affecting the true body temperature. The methods using the rectal route necessitate substantial immobilisation of the animal and thus impose much stress which may in turn exert a decisive effect on the temperature recorded. In addition, it has been our experience that the use of rectal thermocouples in mice, rats and guinea-pigs by a single operator is a relatively slow process and frequent repeated measurements of body temperature are difficult with large numbers of animals.

It was decided that an alternative method to those in current use was necessary to measure body temperature in small laboratory animals. Accordingly a small thermocouple was sealed inside a bent 18 gauge hypodermic needle, 4·5 cm long (Fig. 1). The bend makes it easier to insert the thermocouple into the oesophagus of mice, rats or guinea-pigs, which are held as for oral administration of drugs. The oesophageal thermocouple was specifically designed for use with a commercially available electric thermometer*. The thermocouple is connected to the electric thermometer which contains a thermocouple amplifier and its power supply. The amplifier is fully transistorised and is of the chopper

From Research Division, Allen & Hanburys Limited, Ware, Hertfordshire.

* Electric thermometer Model BA 9000, Allen & Hanburys (Surgical Engineering Division) Limited, Bethnal Green, London, E.2.

type, the chopper being run at mains frequency. There is feedback over the amplifier which linearises it, stabilises its gain and gives a high imput impedance, so reducing the effect of thermocouple resistance on the calibration. The output is displayed on a calibrated 0·1 mA meter, the recorded temperature being read directly in degrees centigrade. Over a wide range (5 to 40°) of laboratory temperatures and recorded body temperatures the accuracy of the instrument is within $\pm 0{\cdot}2°$. In particular, for a laboratory temperature of 20° and a recorded body temperature of 37° the accuracy is within $\pm 0{\cdot}1°$.

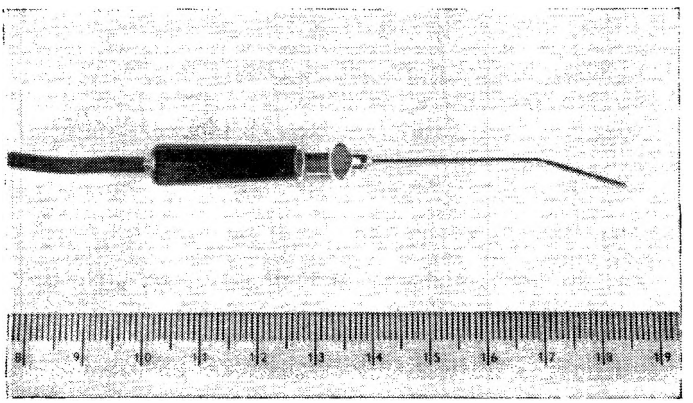


FIG. 1. The oesophageal thermocouple. (Each small division of the scale is 1 mm).

The measurement of body temperature in small laboratory animals by this method is a rapid, simple and acccurate procedure. Following insertion of the thermocouple in the oesophagus a constant temperature reading is obtained in 3 to 4 sec. In a total time of approximately 8 sec, a single operator can remove a mouse from its cage, measure and record its oesophageal temperature, and replace the animal in the cage. The body temperature of 10 mice can be individually measured and recorded in less than 90 sec.

The use of this oesophageal thermocouple is illustrated in the following experiment. Reserpine induces marked sedation in mice and rats characterised by ptosis, locomotor inactivity and hypothermia. The severity of the depression may be assessed by visual methods and the antagonism of reserpine-induced depression has been used to detect possible antidepressant activity in test compounds (Sulser, Watts & Brodie, 1962; Wilson & Tislow, 1962). However, visual assessment of the degree of sedation is liable to subjective error and quantitative comparison of active compounds is difficult. In our experience antagonism of the depressant effects of reserpine, for example with imipramine, amphetamine, monoamine oxidase inhibitors and other drugs, is always accompanied by either a rise in temperature (if established depression is being reversed) or prevention of a fall in temperature (if depression is being prevented). Since temperatures could now be

measured quickly and accurately, a new quantitative method was available for comparing the activities of compounds found to have anti-reserpine activity. As an example, some results are given for the effects of amphetamine in established reserpine depression. Ten male white (Tuck) mice, weighing 18 to 22 g, each received an intravenous injection of reserpine, 1 mg/kg; 4 hr later, 5 animals were given amphetamine, 15 mg/kg, orally. The remaining 5 animals received distilled water orally and served as reserpine controls. The anti-reserpine effect of amphetamine is clearly demonstrated by its action on reserpine-induced hyopthermia (Fig. 2).

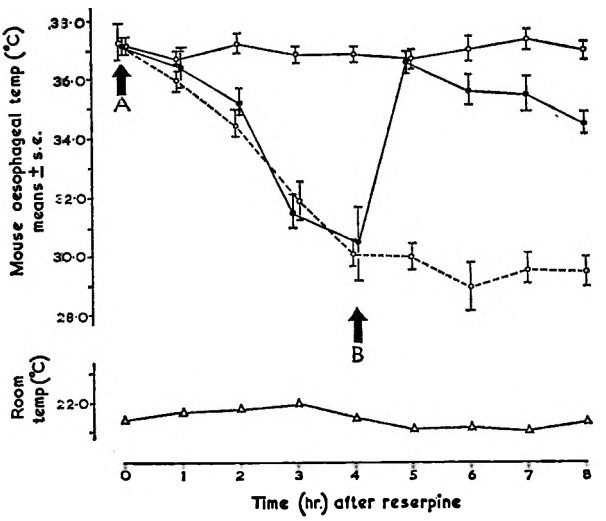


FIG. 2. Effect of amphetamine on the body temperature of reserpine-treated mice. ○—○ Untreated mice. ○----○ At A, reserpine 1 mg/kg. i.v. ●—● At A, reserpine 1 mg./kg. i.v., at B, amphetamine 15 mg/kg orally. △—△ Laboratory temperature.

It is obvious that other quantitative applications will be found for this apparatus because of the possibility of a single operator measuring the body temperatures of large numbers of small laboratory animals accurately, quickly and if desired at frequent time intervals without unduly disturbing the animals.

Acknowledgement. The authors are indebted to Mr. V. M. Johnston for the photographic illustration.

References

Chodera, A. (1963). *Arch. int. Pharmacodyn.*, **144**, 362–369.
Euler, C., von (1961). *Pharmacol. Rev.*, **13**, 361–398.
Gunne, L. M. (1960). *Arch. int. Pharmacodyn.*, **129**, 416–428.
Lessin, A. W. & Parkes, M. W. (1957). *Brit. J. Pharmacol.*, **12**, 245–250.
Sulser, F., Watts, J. & Brodie, B. B. (1962). *Ann. N. Y. Acad. Sci.*, **96**, 279–288.
Wilson, S. P. & Tislow, R. (1962). *Proc. Soc. exp. Biol. N. Y.*, **109**, 847–848.

J. Pharm. Pharmacol., 1964, **16**, 500

Letters to the Editor

Spectroscopic studies of some promazine metabolites and 3-hydroxypromazine

SIR,—Recently (Beckett & Curry, 1963), it was shown that the absorption spectra of the semiquinones formed by dissolving phenothiazine compounds in 50% sulphuric acid, could be used to identify the parent compounds. Particular consideration was given to hydroxylated promazine derivatives, because of their importance as possible biotransformation products of promazine. At that time, only three of the four possible monohydroxylated derivatives were available as reference compounds, and it was necessary to predict the spectroscopic properties of 3-hydroxypromazine. We now report the verification of these predictions.

The 3-hydroxypromazine sample (m.p. 143–143°) ran as a single compound in five solvent systems (chloroform : ethanol, 90 : 10; chloroform : diethylamine, 98 : 2; and chloroform alone, all on alumina plates, and n-butanol : methanol : formic acid : water, 40 : 40 : 2 : 18; and ethanol : acetic acid : water, 50 : 30 : 20; both on silica gel plates). The infra-red spectrum of the compound was consistent with its stated structure. The ultra-violet absorption spectrum of the unoxidised material was similar to those of 1-, 2-, and 4-hydroxypromazines previously reported. In solution in 50% sulphuric acid the absorption spectrum had peaks at 278, 343, 372 and 568 mμ, differing from the corresponding spectrum of 2-hydroxypromazine in the presence of the peak at 372 mμ. (See Fig. 1).

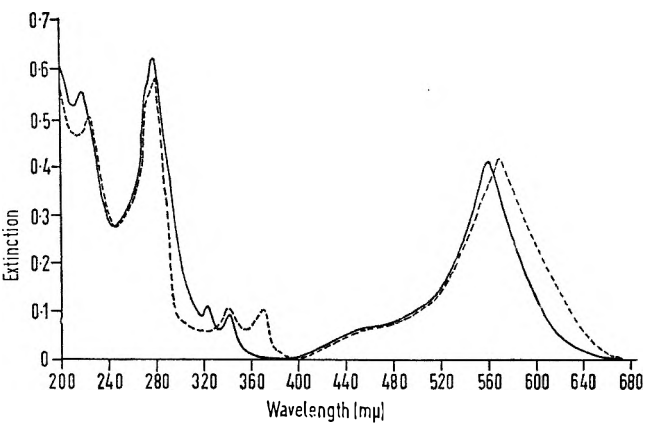


FIG. 1. Absorption spectra of 2- and 3-hydroxypromazines in solution in 50% sulphuric acid, recorded on a Unicam SP800 spectrophotometer. Dilution of the solution for the ultra-violet spectral record to one-tenth of the concentration for the visible spectrum, was effected at approx. 320 mμ. The minor peak at 325 mμ in the spectrum of 2-hydroxypromazine is additional to those previously reported (Beckett & Curry, 1963).

Key, —— 2-hydroxypromazine, - - - - 3-hydroxypromazine.

From urine of psychiatric patients receiving promazine, four glucuronides (C and D, as major and A and B as minor components) were isolated from the main glucuronide fraction, by continuous electrophoresis, paper chromatography, column chromatography and preparative thin layer chromatography.

They were found to be hydroxy primary amine sulphides by spot tests, e.g., ninhydrin +ve, sodium nitroprusside −ve, ferric chloride +ve and sodium periodate +ve. The Rf values of A, B, C and D, with the solvent system n-butanol : methanol : formic acid 98% : water, 40 : 40 : 2 : 18; on thin layer silica gel plates were respectively 77, 75, 67 and 57 (3-hydroxypromazine, Rf = 60).

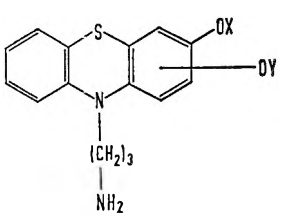
TABLE 1. ABSCRPTION MAXIMA OF CERTAIN PROMAZINE DERIVATIVES IN 50% H_2SO_4 SOLUTION

Compound	λmax (mμ)					
2-hydroxypromazine	278	325	343		(440)	558
3-hydroxypromazine	278		343	372	(450)	568
3-methoxypromazine	279		342	372	(450)	565
3,7-dimethoxyphenothiazine ..	297		351	383		592
A	277		?	?	?	547–557
B	277		343	(368)	(450–470)	554
C	277		343	370	(450–480)	552
D	277		343	370	(450–480)	552
aglycone of B	277		340	374	?	566
aglycone of C	277		338	(372)	?	566
aglycone of D	277		340	372	(450)	567

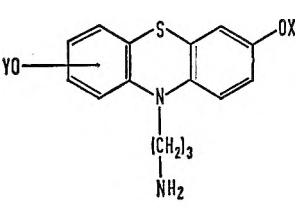
Figures in brackets indicate shoulders on the main peaks.

The electronic spectra of B, C and D in 50% sulphuric acid were similar to that of 3-hydroxypromazine except for a shift of the main peak from 566–7 mμ to 552–4 mμ (see Table 1). Not enough of A was isolated to obtain a satisfactory spectrum, but a peak in the 550 mμ region was observed. After hydrolysis of B, C and D with β-glucuronidase and subsequent extraction, the spectra observed in 50% H_2SO_4 were similar to that of 3-hydroxypromazine except for less intense minor peaks in the 340–370 mμ region. Although this evidence suggests that each of the aglycones of B, C and D are 3-hydroxydesdimethylpromazine, their multiplicity precludes this possibility. Moreover, the glucuronides A, B, C and D change very rapidly to sulphoxides on spectrophotofluorometric examination, whereas 3-methoxypromazine changes slowly to the sulphoxide; more than monohydroxylation of the aromatic nucleus of promazine is thus indicated in these glucuronides.

The structural formulae of B, C and D are then I or II,



I



II

where X = $C_6H_9O_6$ and Y = H,
or X = H and Y = $C_6H_9O_6$

3,7-Dihydroxylation of the promazine nucleus is unlikely because the spectroscopic properties of these glucuronides are completely different from that of 3,7-dimethoxyphenothiazine (see Table 1), which may be considered a suitable reference compound, i.e. the effects exerted in the sulphuric acid test by methoxy and hydroxy groups in phenothiazines are very similar and the addition of an aliphatic side chain on the nuclear nitrogen atom makes only minor differences (Beckett & Curry, 1963).

Acknowledgements. We thank Dr. Albert A. Manian of N.I.H. Bethesda, U.S.A., for supplying a sample of 3-hydroxypromazine prepared by the Research Institute of Temple U. Philadelphia, and Dr. J. C. Craig of the School of Pharmacy, University of California, U.S.A. for supplying a sample of 3,7-dimethoxyphenothiazine.

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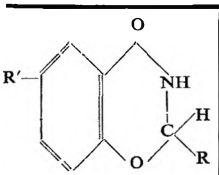
Reference

Beckett, A. H. and Curry, S. H. (1963). *J. Pharm. Pharmacol.*, **15**,246 *T*–253*T*.

Structural requirements for the antiphlogistic activity in some novel derivatives of chlorthenoxazin

SIR,—Kadatz (1957) described the synthesis and the anti-inflammatory properties of chlorthenoxazin, a benzoxazine derivative [2-(2-chloroethyl)-2,3-dihydro-4-oxobenz-1,3-oxazine]. Some years later Baroli, Bottazzi, Ferrari, Garzia, Trabucchi & Vargiu (1963), Ferrari & Garzia (1963), Arrigoni-Martelli (1964) and Arrigoni-Martelli & Conti (1964) described the synthesis and the pharmacological properties of some new derivatives of this compound with various substituents on the nucleus, particularly the 6-amino-derivative (A 350). For the purpose of investigating more deeply the structure-action relationships of this class of compounds, we synthesised a number of new derivatives of chlorthenoxazin (AP 67). The anti-inflammatory activity has been studied on three experimental models of phlogosis of the hind paw of the rats: carrageenin-, dextran-, formalin-oedema (for methods see Arrigoni-Martelli, 1964; Arrigoni-Martelli

TABLE 1. ANTI-INFLAMMATORY ACTIVITY



	Dose mg/kg oral	% Inhibition (± s.d.) of oedema induced by: Carrageenin	Dextran	Formalin	Oral LD 50 mg/kg (with confidence limits), rat
R = CH_2–CH_2–Cl R′ = H (AP 67) Chlorthenoxazin	200	37·0 (±3·2)	22·9 (±4·8)	12·1 (±3·6)	> 2000
R = CH_2–CH_2–Cl R′ = NH_2 (A 350)	195	54·9 (±1·9)	38·8 (±4·2)	27·5 (±4·2)	1958 (1847–2024)
R = Et R′ = H (A 301)	131	33·0 (±2·8)	24·2 (±4·7)	15·6 (±3·9)	1310 (1156–1573)
R = Et R′ = NH_2 (A 302)	189	41·5 (±2·2)	35·3 (±5·1)	18·1 (±3·4)	1890 (1756–1981)
R = Me R′ = H (A 309)	102	25·8 (±2·4)	22·3 (±4·8)	7·5 (±3·1)	1025 (851–1270)
R = Me R′ = NH_2 (A 310)	141	35·8 (±3·1)	32·6 (±5·9)	12·3 (±3·9)	1415 (1282–1593)
R = $CHMe_2$ R′ = H (A 319)	185	20·5 (±2·9)	12·7 (±6·4)	6·4 (±2·1)	1850 (1781–2021)
R = $CHMe_2$ R′ = NH_2 (A 321)	200	26·4 (±2·5)	20·9 (±5·8)	16·6 (±3·9)	> 2000
Phenylbutazone	128	48·2 (±3·1)	29·9 (±4·7)	28·6 (±5·4)	1280 (1156–1325)

& Conti, 1964). The results obtained with some selected compounds—reported in Table 1—show the following:

(1) The presence of the NH_2-group on the C-6 of the nucleus increases the antiphlogistic activity independently from the length and the shape of the side-chain. The acute toxicity is unchanged or diminished.

(2) The replacement of the methyl group on the side-chain with an ethyl-group led to an increase of the antiphlogistic activity.

(3) The branching of the side-chain led to a reduction of the antiphlogistic activity.

(4) The presence of the chloroethyl-group on the side-chain confers a more pronounced anti-inflammatory activity, particularly when its presence is accompanied by the introduction of NH_2-group on the C-6 of the nucleus.

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May 18, 1964

References

Kadatz, R. (1957). *Arzneimitt.-Forsch.*, **7**, 651–653.
Baroli, F., Bottazzi, A., Ferrari, W., Garzia, A., Trabucchi, I. & Vargiu, L. (1963). *Ibid.*, **13**, 884–887.
Ferrari, W. & Garzia A. (1964). *Ibid.*, **14**, 55–56.
Arrigoni-Martelli, E. (1964). *Ibid.*, **14**, 56–58.
Arrigoni-Martelli, E. & Conti I. (1964). *Med. exp.*, **10**, 164–168.

Role of the polymethylene chain in derivatives of demecarium bromide on the inhibition of monoamine oxidase

SIR,—Inhibition of monoamine oxidase has been shown by several structurally unrelated compounds. We have recently reported (Pant, Parmar & Bhargava, 1964) that demecarium bromide [decamethylene-bis(3-dimethylaminophenyl *N*-methylcarbamate)dimethobromide], a potent inhibitor of brain acetylcholinesterase inhibits monoamine oxidase present in isolated rat liver mitochondria. In the present study the effect of the tetramethylene, hexamethylene, octamethylene and dodecamethylene derivatives as well as the decamethylene derivative has been investigated on monoamine oxidase in rat liver mitochondria to show the effect of the number of methylene groups connecting the two neostigmine molecules present in these compounds.

Monoamine oxidase activity was determined by the conventional Warburg manometric method (Creasey, 1956). The oxygen uptake has been shown by Parmar & Nickerson (1961) to reflect the true enzyme activity during oxidative deamination of tyramine. The effect of these compounds was also investigated on the oxidation of tryptamine. The inhibition of monoamine oxidase produced by these compounds at the final concentration of 8×10^{-5}M using tyramine and tryptamine as substrates is shown in Table 1. Inhibition of the enzyme increased with increase in the number of methylene groups in the compounds during oxidative deamination of tyramine or tryptamine. The compound with 4 methylene groups had no inhibitory effect on the enzyme activity at this concentration. Further increase in its concentration to $3{\cdot}2 \times 10^{-3}$M also produced no

inhibition of enzyme. In the absence of substrate no oxygen uptake could be observed with these agents even at 3×10^{-3}M. Furthermore, the degree of inhibition was unaltered by pre-incubation of the enzyme preparation with these compounds for varying length of time before the addition of either tyramine or tryptamine. The parent substance, neostigmine, has been shown to have no inhibitory effect on monoamine oxidase under similar experimental conditions (Pant, Parmar & Bhargava, 1964).

TABLE 1. INHIBITION OF RAT LIVER MITOCHONDRIAL MONOAMINE OXIDASE

Substrate	Inhibition %				
	$[CH_2]_4$	$[CH_2]_6$	$[CH_2]_8$	$[CH_2]_{10}$	$[CH_2]_{12}$
Tyramine	0·0	15·6	17·9	37·0	55·4
	0·0	11·0 (13·5)	16·1 (17·0)	41·0 (39·0)	52·2 (52·9)
	0·0	14·0	17·1	39·2	51·2
Tryptamine	0·0	9·8	18·0	41·0	88·4
	0·0	6·0 (8·1)	11·3 (15·3)	42·2 (41·3)	71·0 (78·7)
	0·0	8·6	16·5	40·8	76·8

Per cent inhibition was calculated from the decrease in the oxygen uptake. Each reaction vessel contained mitochondria equivalent to 250 mg of fresh rat liver tissue; 0·01M tyramine or tryptamine in a total volume of 3·0 ml with 0·066M phosphate buffer pH 7·4. The inhibitors present in the side arm at the final concentration of 8×10^{-6}M were incubated with the enzyme preparation for 10 min before adding either tyramine or tryptamine. The enzyme system was then incubated at 37° for 1 hr in an atmosphere of oxygen. Figures in parentheses are averages.

On the basis of these observations it can be assumed that the polymethylene chain seems to be involved in the binding of these compounds to the active site(s) to produce enzyme inhibition. A similar explanation on the role of an alkyl chain in monoamine oxidase inhibition was put forward by Barsky, Pacha, Sarkar & Zeller (1959) who found monosubstitution of the second nitrogen atom of isonicotinic acid hydrazide produced optimal inhibition with the butyl derivative. At present it is difficult to provide an explanation for increase in the inhibition of the enzyme along with increase in the number of methylene groups present in the chain and also why the compounds should inhibit two different enzymes, monoamine oxidase and brain acetylcholinesterase, the former being an oxidative enzyme which chances to be a flavoprotein (Belleau & Moran, 1963) and the latter a hydrolase without any cofactor requirements. Further studies with these compounds may prove useful in the elucidation of the nature of the active site(s) of the enzyme monoamine oxidase and its role in physiological functions.

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References

Barsky, J., Pacha, W. L., Sarkar, S. & Zeller, E. A. (1959). *J. biol. Chem.*, **234**, 389–391.
Belleau, B. & Moran, J. (1963). *Ann. N.Y. Acad. Sci.*, **107**, 822–839.
Creasey, N. H. (1956). *Biochem. J.*, **64**, 178–183.
Pant, M. C., Parmar, S. S. & Bhargava, K. P. (1964). *Canad. J. Biochem.* in the press.
Parmar, S. S. & Nickerson, M. (1961). *Biochem. Pharmacol.*, **10**, 8.

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