RESEARCH PAPERS

STUDIES IN THE FIELD OF DIURETIC AGENTS

PART V. A NEW ROUTE TO DISULPHAMYL DERIVATIVES OF BENZENE

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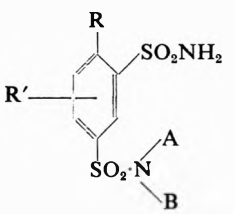
From The British Drug Houses Ltd., Graham Street, City Road, London, N.1

Received June 22, 1960

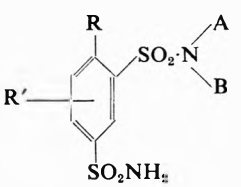
A procedure has been developed for converting sulphamyl derivatives of aniline into the corresponding sulphamyl-sulphonchlorides. The latter have been condensed with ammonia and with amines giving novel 1,2-, 1,3- and 1,4-disulphamyl derivatives of benzene, required for examination as diuretics.

WORK on disulphamyl derivatives of benzene is herein extended to some novel types containing one unsubstituted sulphamyl residue. Derivatives of 1,3-disulphamyl benzene in which only one sulphamyl group is alkylated (I; II) are not described in the literature. Their preparation presented initial difficulty. Alkylation of a disulphonamide with one equivalent proportion of alkylating agent gave a complex mixture from which a monoalkyl-derivative could not be isolated. Chlorosulphonation of a sulphonamide or *N*-substituted sulphonamide caused deamination of the sulphamyl group with formation of a 1,3-disulphonchloride (e.g., III) in place of the required 3-chlorosulphonyl sulphamylbenzene.

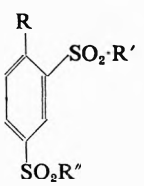
Steinkopf and others[1] have shown that sulphonfluorides are more stable than sulphonchlorides and, unlike the latter, are not readily attacked by ammonia in ether. This observation formed the starting point of our first method for the preparation of monoalkylated 1,3-disulphonamides. Toluene-4-sulphonfluoride[2] was chlorosulphonated in carbon tetrachloride solution to give toluene 2-sulphonchloride-4-sulphonfluoride (IV) in moderate yield. This reacted with ammonia in aqueous dioxan at $-10°$ to give a small yield of (V) which, with ethanolic methylamine, provided the required monomethyl disulphonamide (VI).



(I)



(II)

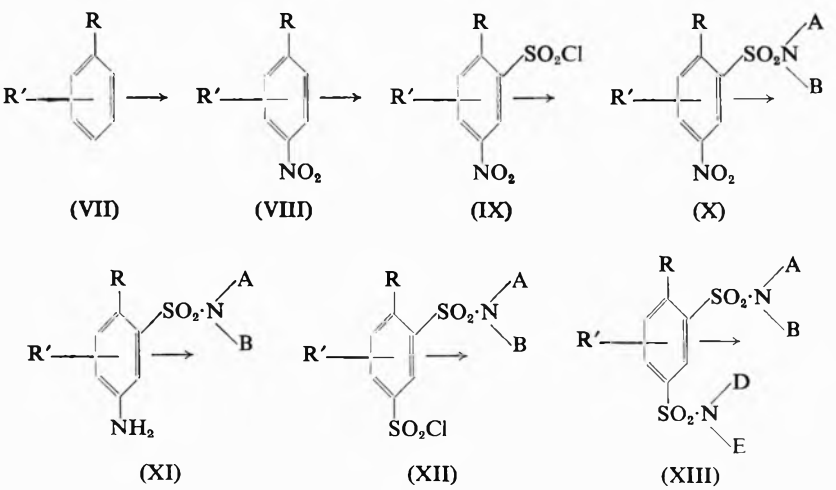


(III): $R' = R'' = Cl$
(IV): $R = Me$, $R' = Cl$, $R'' = F$
(V): $R = Me$, $R' = NH_2$, $R'' = F$
(VI): $R = Me$, $R' = NH_2$, $R'' = NH{\cdot}Me$

This method could not be extended to the preparation of 1,4-disulphamyl compounds because of the *m*-directive effect of the sulphonhalide group. A method of sufficiently wide scope was ultimately discovered by applying the reaction of Meerwein and others[3] for converting an aniline into the corresponding benzene sulphonchloride. Route (i) shows the stages in the preparation of a mixed alkylated 1,3-disulphonamide using this reaction. Conversion of (VII) to (XI) follows conventional routes. Compound (XI) is then converted into the diazonium chloride and treated with excess of a saturated solution of sulphur dioxide in glacial acetic acid, containing cupric chloride as catalyst, to give the sulphonchloride (XII). Conversion to (XIII) follows normal practice.

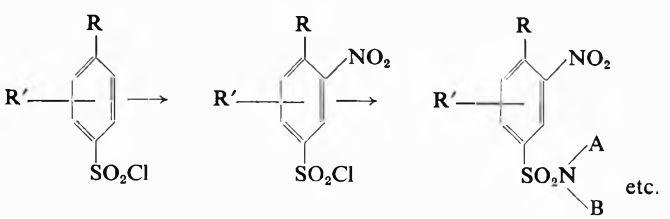
Several variations of this versatile method are immediately apparent. Thus by using a suitable benzene sulphonyl chloride (XIV) a 1,3-disulphamyl benzene derivative may be prepared according to route (ii). Numerous 1,3-disulphonamides (Table IV) were prepared in this way from benzene, toluene, ethyl, n-propyl and isopropylbenzene, 1,2- and 1,3-xylene, anisole, chlorobenzene, bromobenzene, 1,3-dichlorobenzene, 1,2- and 1,3-chlorotoluene.

Route (i)



(VII) (VIII) (IX) (X) (XI) (XII) (XIII)

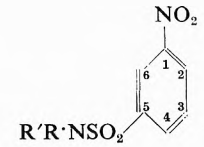
Route (ii)



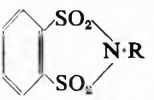
(XIV) (XV) (XVI)

TABLE I

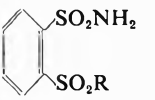
NITROSULPHONAMIDES



Substituent at position						Found				Required			
2	3	4	N·RR′	Formula	m.p. °C.	C	H	N	S	C	H	N	S
Me	—	—	NMe_2	$C_9H_{12}O_4N_2S$	92–94	44·4	5·2	11·2	12·7	44·3	5·0	11·5	13·1
Me	—	—	$N(CH_2)_5$	$C_{12}H_{16}O_4N_2S$	110–111	50·7	5·9	9·7	11·4	50·7	5·7	9·9	11·3
Et	—	—	NH_2	$C_8H_{10}O_4N_2S$	128–129	42·1	4·3	12·0	13·9	41·7	4·4	12·2	13·9
Pr^i	—	—	NH_2	$C_9H_{12}O_4N_2S$	123–124	44·0	4·6	11·8	13·2	44·3	5·0	11·5	13·1
Pr^i	—	—	NHMe	$C_{10}H_{14}O_4N_2S$	113–115	46·1	5·2	11·0	12·5	46·5	5·5	10·9	12·4
—	—	OMe	NHMe	$C_8H_{10}O_5N_2S$	178–180	39·0	4·2	11·2	13·2	39·0	4·1	11·4	13·0
—	—	OMe	NH_2	$C_7H_8O_5N_2S$	223–225	36·6	3·6	12·1	13·7	36·2	3·5	12·1	13·8
Cl	—	—	NHMe	$C_7H_7O_4N_2SCl$	70–72	33·3	3·0	11·2	12·8	33·5	2·8	11·2	12·8
Cl	—	—	NMe_2	$C_8H_9O_4N_2SCl$	103–104	36·2	3·1	10·8	12·3	36·3	3·4	10·6	12·5
—	—	Br	NH_2	$C_6H_5O_4N_2SBr$	204–205	25·8	1·8	10·0	11·6	25·6	1·8	10·0	11·4
Cl	—	Cl	NH_2	$C_6H_4O_4N_2SCl_2$	176–178	26·6	1·4	10·1	11·9	26·6	1·5	10·3	11·8
Cl	Me	—	NHMe	$C_8H_9O_4N_2SCl$	127–129	36·4	3·5	11·1	12·9	36·3	3·4	10·6	12·5
Cl	—	Me	NH_2	$C_7H_7O_4N_2SCl$	158–160	34·0	3·2	11·5	13·1	33·6	2·8	11·2	12·8
Cl	—	Me	NHMe	$C_8H_9O_4N_2SCl$	134–136	36·3	3·6	10·6	12·8	36·3	3·4	10·6	12·5
PhO	—	—	NMe_2	$C_{14}H_{14}O_4N_2S$	105	52·0	4·3	8·8	—	52·2	4·4	8·7	—



(XVII)



(XVIII)

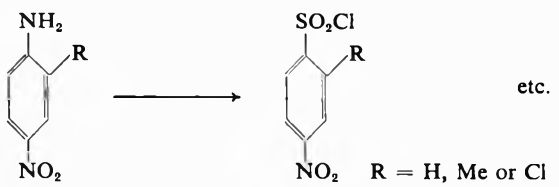
Important features of this new method of preparation of 1,3-disulphamyl benzenes include high overall yields even when using crude intermediates in the earlier stages. Thus, 2-n-propylnitrobenzene was chlorosulphonated in carbon tetrachloride solution. The crude chlorosulphonyl derivative was converted directly into the sulphonmethylamide. This, also without purification, was reduced to 3-amino-4-propylbenzene sulphonmethylamide, which was readily isolated as the crystalline hydrochloride in 60 per cent overall yield. Again, the 4-sulphonchloride of isopropyl benzene was nitrated at 40–45° with a mixture of concentrated nitric and sulphuric acids. After pouring on to ice, collecting, and washing with water, the total crude nitration product was treated directly with ammonia or with methylamine to give the appropriate sulphamyl derivatives in 63 and 65 per cent yield, respectively.

The method was applied to the synthesis of 1,2- and 1,4-disulphamyl derivatives of benzene.

The literature on the 1,2-disulphamyl derivatives of benzene is scanty. The reaction of benzene-1,2-disulphonyl chloride with ammonia or with primary amines, however, is known to lead to cyclic 1,2-disulphonimides (XVII)[4,5]. Though this ring closure occurs easily, we successfully prepared 2-chlorosulphonyl benzene sulphonamide (XVIII; R = Cl), which surprisingly proved to be a relatively stable crystalline compound. With ammonia it gave a small yield of benzene-1,2-disulphonamide (XVIII; R = NH_2), together with the cyclic imide (XVII; R = H), as the major product. The sulphonchloride (XVIII; R = Cl) condensed with dimethylamine to give 2-sulphamylbenzene sulphondimethylamide (XVIII; R = NH_2) additionally prepared from 2-nitrobenzenesulphondimethylamide by the processes already described.

Only 1,4-disulphamyl derivatives of benzene, toluene and chlorobenzene were prepared. In each case one sulphamyl group carried mono- and dimethyl substituents. Methods were essentially the same as described above. The nitrobenzene-4-sulphonchlorides (XX) required as starting materials, however, could not be obtained by nitration of sulphonchlorides or by chlorosulphonation of nitrobenzenes because of the *m*-directive effect of the $-SO_2Cl$ or $-NO_2$ substituents. They were prepared from

Route (iii)



(XIX) (XX)

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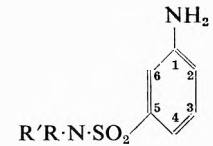
TABLE II

AMINOSULPHONAMIDES



Substituent at position			N·RR'	Formula	m.p. °C.	Found				Required			
2	3	4				C	H	N	S	C	H	N	S
Me	—	—	NH_2	$C_7H_{10}O_2N_2S$	175	44·7	5·5	15·2	17·1	45·1	5·4	15·1	17·2
Me	—	—	NHMe	$C_{10}H_{14}O_3N_2S$	163 (*a*)	49·5	5·6	11·4	12·9	49·6	5·8	11·6	13·2
Me	—	—	NMe_2	$C_9H_{14}O_2N_2S$	172–174	50·6	6·3	12·8	14·7	50·4	6·6	13·1	15·0
Me	—	—	$N(CH_2)_5$	$C_{12}H_{18}O_2N_2S$	117–118	56·7	7·3	11·3	12·7	56·7	7·1	11·0	12·6
Et	—	—	NH_2	$C_8H_{12}O_2N_2S$	130–132	47·6	5·9	14·0	16·5	48·0	6·0	14·0	16·0
Et	—	—	NH_2	$C_8H_{12}O_2N_2S$;HCl	226–228 (*d*)	40·7	5·7	12·0	13·3	40·7	5·6	11·9	13·6
Et	—	—	NHMe	$C_9H_{14}O_2N_2S$;HCl	210–212 (*d*)	43·6	5·6	11·4	12·9	43·1	6·0	11·2	12·8
Pr^n	—	—	NH_2	$C_9H_{14}O_2N_2S$;HCl	193–195	43·3	5·9	11·3	12·7	43·1	6·0	11·2	12·8
Pr^n	—	—	NHMe	$C_{10}H_{16}O_2N_2S$;HCl	208–210	45·3	6·3	10·7	12·4	45·4	6·5	10·6	12·1
Pr^i	—	—	NH_2	$C_9H_{14}O_2N_2S$;HCl	215 (*d*)	43·3	6·0	11·0	12·8	43·1	6·0	11·2	12·8
Pr^i	—	—	NHMe	$C_{10}H_{16}O_2N_2S$	103–105	52·7	7·0	12·0	14·4	52·6	7·1	12·3	14·1
Me	Me	—	NH_2	$C_8H_{12}O_2N_2S$	159–160	48·1	6·1	14·2	—	48·0	6·0	14·0	—
Me	Me	—	NHMe	$C_9H_{14}O_2N_2S$;HCl	242–244 (*d*)	43·4	5·7	11·2	13·0	43·1	6·0	11·2	12·8
Me	—	Me	NH_2	$C_8H_{12}O_2N_2S$	189–190	48·2	6·1	14·0	16·2	48·0	6·0	14·0	16·0
—	—	OMe	NH_2	$C_7H_{10}O_3N_2S$	190	41·2	5·2	13·8	15·8	41·6	5·0	13·9	15·9
—	—	OMe	NHMe	$C_8H_{12}O_3N_2S$	176–178	44·8	5·3	13·0	15·0	44·4	5·6	13·0	14·8
Cl	—	—	NH_2	$C_6H_5O_2N_2SCl$	157–159	34·9	3·5	13·6	15·1	34·9	3·4	13·6	15·5
Cl	—	—	NHMe	$C_7H_7O_2N_2SCl$	85–86	38·2	4·4	12·6	14·6	38·1	4·0	12·4	14·1
—	—	Cl	NH_2	$C_6H_5O_2N_2SCl$	168–170	35·2	3·3	13·7	15·5	34·9	3·4	13·6	15·5
Cl	—	—	NMe_2	$C_8H_{11}O_2N_2SCl$	149–151	41·3	4·9	11·7	13·7	41·0	4·7	11·9	13·7
Br	—	—	NH_2	$C_6H_5O_2N_2SBr$	160–162	28·8	2·6	11·0	13·0	28·7	2·8	11·1	12·7
—	—	Br	NH_2	$C_6H_5O_2N_2SBr$;HCl	202 (*d*)	25·0	2·8	—	39·9*	25·1	2·8	—	40·1*
Cl	—	Cl	NH_2	$C_6H_4O_2N_2SCl_2$	216–218	30·2	2·4	11·4	13·3	29·9	2·5	11·6	13·3
Cl	Me	—	NH_2	$C_7H_7O_2N_2SCl$	144–145	38·4	4·1	12·7	14·4	38·1	4·1	12·7	14·5
Cl	Me	—	NHMe	$C_8H_{11}O_2N_2SCl$;HCl	202–204	35·4	4·1	10·5	11·9	35·4	4·1	10·3	11·8
Cl	—	Me	NH_2	$C_7H_9O_2N_2SCl$	213	38·0	4·2	12·9	—	38·1	4·1	12·7	—
Cl	—	Me	NHMe	$C_8H_{11}O_2N_2SCl$	131–133	40·8	4·7	12·4	14·1	41·0	4·7	12·0	13·7
PhO	—	—	NMe_2	$C_{14}H_{16}O_3N_2S$	97–99	57·8	5·6	9·5	10·8	57·5	5·5	9·6	11·0

(*a*) Acetyl derivative.

* Total halogen.

the readily available 4-nitroanilines (XIX), which were converted into the corresponding 4-nitrosulphonchlorides (XX) by means of the diazo-reaction [Route (iii)].

Study of the above compounds as oral diuretics in the saline loaded rat (for which we are indebted to Dr. A. David and his colleagues) revealed certain correlations of structure and biological activity.

Compounds of type (I) were uniformly more potent than their isomers of type (II). As expected, the *N*-substituted disulphonamides possessed lower carbonic anhydrase inhibiting activity than the corresponding disulphamyl-derivatives, but, as with the latter group of compounds, there was no simple relation between carbonic anhydrase inhibiting activity *in vitro* and oral diuretic activity.

Many of the 1,4-disulphamyl benzene derivatives listed in Table V possessed appreciable diuretic activity, but their relatively high potency as carbonic anhydrase inhibitors led to enhanced potassium excretion, thus rendering them inferior to 5-chlorotoluene-2,4-disulphonamide (disulphamide).

Experimental

2-*Sulphamyltoluene*-4-*sulphonfluoride*. A mixture of toluene-4-sulphonfluoride[2] (97·5 g.), chlorosulphonic acid (130 g.) and carbon tetrachloride (173 g.) was heated under reflux for 3 hours on the steam bath. The mixture was cooled, poured on to ice, and the product extracted with carbon tetrachloride. The extract was washed with water and the solvent removed. Distillation of the residual oil at 0·6 mm. yielded crude 2-chlorosulphonyltoluene-4-sulphonfluoride (55 g.) b.p. 146–156° which solidified and had m.p. 41–44°. It was used without further purification.

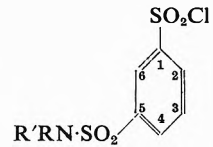
The foregoing crude product (10 g.) was added in portions with vigorous stirring and cooling to −10°, to a mixture of ammonia solution (7·4 ml., d = 0·880), water (90 ml.) and dioxan (50 ml.) and stirring was continued at −10° for 1 hour after addition was complete. The cold mixture was acidified with hydrochloric acid and the solid (5·4 g.) which separated was collected and crystallised from aqueous ethanol to yield 2-*sulphamyltoluene*-4-*sulphonfluoride* (0·95 g.), m.p. 212–214°. Found: C, 34·2; H, 3·2; N, 5·5. $C_7H_8O_4NS_2F$ requires C, 33·2; H, 3·2; N, 5·5 per cent. The mother liquors deposited toluene-2,4-disulphonamide (2·95 g.) m.p. 185°.

2-*Sulphamyltoluene*-4-*sulphonmethylamide*. The foregoing compound (0·4 g., m.p. 212–214°) was added to 25 per cent aqueous methylamine (5 ml.) and the solution allowed to stand at room temperature for 1½ hours. Excess of methylamine was distilled off and the liquid cooled and acidified. 2-*Sulphamyltoluene*-4-*sulphonmethylamide* separated and had m.p. 172–174° after crystallisation from aqueous ethanol. Found: C, 36·2; H, 4·6; N, 10·7; S, 24·4. $C_8H_{12}O_4N_2S_2$ requires C, 36·4; H, 4·5; N, 10·6; S, 24·3 per cent. The melting point was not depressed on admixture with authentic material (see below).

The following examples illustrate methods of preparation used for products listed in the Tables, which include the analyses.

TABLE III

CHLOROSULPHONYL SULPHONAMIDES



Substituent at position			N·RR'	Formula	m.p. °C.	Found				Required			
2	3	4				C	H	N	Cl	C	H	N	Cl
—	—	—	NH_2	$C_6H_6O_4NS_2Cl$	154–156	28·4	2·4	5·3	13·5	28·2	2·4	5·5	13·9
Me	—	—	NH_2	$C_7H_8O_4NS_2Cl$	162–164	31·1	2·9	5·3	13·2	31·2	3·0	5·2	13·1
—	—	Me	NH_2	$C_7H_8O_4NS_2Cl$	203–205	31·3	2·8	5·2	13·2	31·2	3·0	5·2	13·1
Me	—	—	NHMe	$C_8H_{10}O_4NS_2Cl$	126–127	34·1	3·3	4·8	—	33·9	3·6	4·9	—
Me	—	—	$N(CH_2)_5$	$C_{12}H_{16}O_4NS_2Cl$	155–156	42·5	4·5	4·3	10·9	42·7	4·8	4·2	10·5
Pr^n	—	—	NH_2	$C_9H_{12}O_4NS_2Cl$	181–183	36·4	4·0	4·5	12·3	36·3	4·1	4·7	11·9
Pr^n	—	—	NHMe	$C_{10}H_{14}O_4NS_2Cl$	93–94	38·9	4·3	4·6	11·8	38·5	4·5	4·5	11·4
Pr^i	—	—	NH_2	$C_9H_{12}O_4NS_2Cl$	205	36·5	4·1	21·4*	12·4	36·3	4·1	21·5*	11·9
Pr^i	—	—	NHMe	$C_{10}H_{14}O_4NS_2Cl$	99–101	21·0*	—	4·7	11·9	20·6*	—	4·5	11·4
—	—	OMe	NH_2	$C_7H_8O_5NS_2Cl$	183–185	29·8	3·0	5·4	12·4	29·4	2·8	4·9	12·4
Cl	—	—	NH_2	$C_6H_5O_4NS_2Cl_2$	191–192	25·1	2·1	5·1	24·3	24·8	1·7	4·8	24·4
—	—	Cl	NH_2	$C_6H_5O_4NS_2Cl_2$	186–188	25·2	2·1	4·9	24·2	24·8	1·7	4·8	24·4
Cl	—	—	NMe_2	$C_8H_9O_4NS_2Cl_2$	128–130	30·7	3·0	4·4	21·8	30·2	2·9	4·4	22·3
Br	—	—	NH_2	$C_6H_5O_4NS_2BrCl$	202–204	21·9	2·0	4·1	19·2*	21·5	1·5	4·1	19·2*
Cl	—	Cl	NH_2	$C_6H_5O_4NS_2Cl_3$	197–199	22·6	1·0	4·4	32·6	22·2	1·2	4·3	32·8
PhO	—	—	NMe_2	$C_{14}H_{14}O_5NS_2Cl$	119–121	45·0	3·7	4·2	17·5*	44·7	3·8	3·7	17·1*

* = Sulphur.

Toluene-2,4-disulphamyl Derivatives

(i) *N^2-Substituted Compounds*

2-*Amino*-4-*sulphamyl toluene*[6]. (*a*) A solution of 2-nitro-4-sulphamyl toluene[6] (55 g.) in warm ethanol (500 ml.) containing Raney nickel catalyst (5 g.) was hydrogenated at 100° and 30 atmospheres pressure. Reaction was complete in $1\frac{1}{2}$ hours, when the mixture was boiled and filtered. The product (35 g.) separated on cooling and had m.p. 175° after crystallisation from water.

(*b*) A mixture of the nitro-compound (124·8 g.), iron powder (116 g.), acetic acid (8 ml.) and water (800 ml.) containing octanol (1 ml.), was stirred and heated under reflux for 6 hours. Ethanol (3 litres) was then added, the mixture boiled and filtered through "Hyflo". The product (93·5 g. yield) which separated had m.p. 160–162° and was sufficiently pure for the next stage of the preparation.

2-*Chlorosulphonyl toluene*-4-*sulphonamide*. A solution of 2-amino-4-sulphamyl toluene (9·3 g.) in 24 per cent hydrochloric acid was diazotised at 0–5° by the addition of a solution of sodium nitrite (3·8 g.) in water (9 ml.). The solution was added at once without cooling and with vigorous stirring to a saturated solution of sulphur dioxide in glacial acetic acid (80 ml.) containing cupric chloride dihydrate (3·5 g.). After 5 minutes the mixture was diluted with ice-water to complete precipitation of the sulphonchloride, which was collected, washed with ice-water and dried. It crystallised from 1,2-dichloroethane-light petroleum (b.p. 60–80°), m.p. 162–164° (10.4 g.).

4-*Sulphamyltoluene*-2-*sulphonpiperidide*. The foregoing sulphonchloride (13·5 g.) was added in portions with stirring, at room temperature to a mixture of piperidine (12·8 g.), water (100 ml.) and chloroform (60 ml.). After the addition was complete stirring was continued for 30 minutes when chloroform and excess piperidine were distilled off under reduced pressure. The resulting aqueous solution was acidified with hydrochloric acid when the *product* separated on cooling. It had m.p. 160–162° after crystallisation from aqueous ethanol.

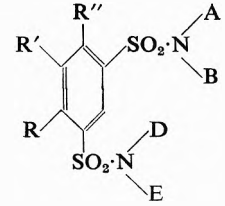
(ii) *N^4-Substituted Compounds*

4-*Nitro*-2-*sulphamyl toluene*[7]. Sodium 4-nitrotoluene-2-sulphonate dihydrate (100 g.) was added in portions with shaking to a mixture of formdimethylamide (10 ml.) and thionyl chloride (100 ml.) and the reaction completed by heating the mixture on the steam bath for 10 minutes. Residual thionyl chloride was distilled off under reduced pressure. The residue was dissolved in chloroform (400 ml.) and added with stirring to aqueous ammonia (800 ml., d = 0·880) at room temperature. After stirring for 1 hour, excess of ammonia and chloroform were boiled off, and the aqueous solution was cooled and acidified with hydrochloric acid. The product (76 per cent) had m.p. 186–187° after crystallisation from water.

4-*Amino*-2-*sulphamyltoluene*[8] was obtained in 85 per cent yield by reduction of the foregoing nitro-compound with iron powder in acidulated water. It had m.p. 164° after crystallisation from water.

TABLE IV

SUBSTITUTED-1,3-DISULPHONAMIDES



Substituent						m.p. °C.	Found				Required			
R	R′	R″	N·AB	N.DE	Formula		C	H	N	S	C	H	N	S
—	—	—	NHMe	NH_2	$C_7H_{10}O_4N_2S_2$	138–140	33·9	3·7	11·0	25·0	33·6	4·0	11·2	25·6
—	—	—	NHBu	NH_2	$C_{10}H_{16}O_4N_2S_2$	124–125	41·2	5·5	9·4	21·6	41·1	5·5	9·6	21·9
—	—	—	NH·CH_2·CH_2·OH	NH_2	$C_8H_{12}O_5N_2S_2$	132–133	34·2	4·5	10·2	22·3	34·3	4·3	10·0	22·9
—	—	—	NHPh	NH_2	$C_{12}H_{12}O_4N_2S_2$	147–149	46·2	3·9	8·7	20·1	46·1	3·9	9·0	20·5
—	—	—	NMe_2	NH_2	$C_8H_{12}O_4N_2S_2$	177–178	36·5	4·5	10·7	24·5	36·4	4·6	10·6	24·3
—	—	—	THP[1]	NH_2	$C_{11}H_{14}O_4N_2S_2$	157–159	43·6	4·7	9·2	21·0	43·7	4·7	9·3	21·2
—	—	Me	NHMe	NH_2	$C_8H_{12}O_4N_2S_2$	128–130	36·3	4·6	10·8	24·2	36·4	4·6	10·6	24·3
—	—	Me	NHEt	NH_2	$C_9H_{14}O_4N_2S_2$	143–144	39·2	5·0	9·9	22·7	38·9	5·1	10·1	23·1
—	—	Me	NHC_6H_5	NH_2	$C_{13}H_{14}O_4N_2S_2$	130–131	—	—	10·0	22·4	—	—	9·7	22·1
—	—	Me	NH·CH_2·CH_2·OH	NH_2	$C_9H_{14}O_5N_2S_2$	144–145	37·1	4·9	9·7	21·6	36·7	4·8	9·5	21·8
—	—	Me	NHPh	NH_2	$C_{13}H_{14}O_4N_2S_2$	123–125	47·5	4·3	8·9	19·8	47·8	4·3	8·6	19·7
—	—	Me	NMe_2	NH_2	$C_9H_{14}O_4N_2S_2$	136–138	39·3	5·1	10·0	22·7	38·9	5·1	10·1	23·0
—	—	Me	$N(CH_2)_5$	NH_2	$C_{13}H_{18}O_4N_2S_2$	160–162	44·7	5·7	8·9	20·3	45·3	5·7	8·8	20·1
—	—	Me	PTHP[2]	NH_2	$C_{18}H_{20}O_4N_2S_2$	176–177	55·1	5·5	7·0	16·4	55·1	5·1	7·1	16·3
—	—	Me	NH_2	NHMe	$C_8H_{12}O_4N_2S_2$	172–174	36·6	4·6	10·6	24·1	36·4	4·6	10·6	24·3
—	—	Me	NH_2	NHEt	$C_9H_{14}O_4N_2S_2$	133–135	38·9	5·1	9·9	23·0	38·9	5·1	10·1	23·0
—	—	Me	NH_2	NHBu	$C_{11}H_{18}O_4N_2S_2$	123–124	43·4	6·1	9·1	20·7	43·1	5·9	9·1	20·9
—	—	Me	NH_2	NH·CH_2·CH_2·OH	$C_9H_{14}O_5N_2S_2$	162–164	36·9	5·1	9·5	—	36·7	4·8	9·5	—
—	—	Me	NH_2	NHPh	$C_{13}H_{14}O_4N_2S_2$	150–152	48·2	4·4	8·7	19·4	47·8	4·3	8·6	19·7
—	—	Me	NH_2	NH·CH_2·CH_2·Ph	$C_{16}H_{18}O_4N_2S_2$	130–131	51·3	5·2	7·8	17·8	51·0	5·1	7·9	18·0
—	—	Me	NH_2	NMe_2	$C_9H_{14}O_4N_2S_2$	161–163	39·0	5·1	10·2	22·7	38·9	5·1	10·1	23·0
—	—	Me	NH_2	NMe·CH_2·CH_2·OH	$C_{10}H_{16}O_5N_2S_2$	142–144	39·0	5·2	9·3	20·7	38·9	5·2	9·1	20·8
—	—	Me	NH_2	$N(CH_2)_5$	$C_{13}H_{18}O_4N_2S_2$	150–152	45·3	5·8	9·1	20·1	45·3	5·7	8·8	20·1
—	—	Me	NH_2	THP[1]	$C_{12}H_{16}O_4N_2S_2$	126–128	45·2	5·1	9·0	20·2	45·5	5·1	8·9	20·3
—	—	Me	NMe_2	NHMe	$C_{10}H_{16}O_4N_2S_2$	89–90	41·1	5·4	9·7	22·1	41·1	5·5	9·6	21·9
—	—	Me	NHMe	$N(CH_2)_5$	$C_{13}H_{20}O_4N_2S_2$	118–119	47·1	6·2	8·6	19·3	47·0	6·1	8·4	19·3
—	—	Me	$N(CH_2)_4O$	$N(CH_2)_5$	$C_{16}H_{24}O_5N_2S_2$	150–151	49·5	6·2	7·4	—	49·5	6·2	7·2	—
—	—	Et	NHMe	NH_2	$C_9H_{14}O_4N_2S_2$	127–129	38·6	5·4	10·2	22·8	38·8	5·1	10·1	23·0
—	—	Et	NH_2	NHMe	$C_9H_{14}O_4N_2S_2$	157–159	38·8	5·2	10·4	23·0	38·8	5·1	10·1	23·0
—	—	Pr^n	NHMe	NH_2	$C_{10}H_{16}O_4N_2S_2$	153–155	41·1	5·7	9·6	22·0	41·1	5·5	9·6	21·9
—	—	Pr^n	NH_2	NHMe	$C_{10}H_{16}O_4N_2S_2$	145–146	41·3	5·7	9·5	22·0	41·1	5·5	9·6	21·9

4-*Chlorosulphonyl*-2-*sulphamyltoluene*. The foregoing aminosulphonamide (23 g.) was diazotised and added to a saturated solution of sulphur dioxide in acetic acid (320 ml.) at 15° as described above. The *product* precipitated by dilution with ice-water, had m.p. 203–205° after crystallisation from 1,2-dichloroethane-light petroleum (b.p. 60–80°) (yield 76 per cent).

2-*Sulphamyltoluene*-4-*sulphonethylamide*. The foregoing sulphonchloride (8·2 g.) was added in portions with stirring to a mixture of 10 per cent aqueous ethylamine (70 ml.) and chloroform (30 ml.) at 20°. After the addition was complete excess of chloroform and ethylamine were boiled off. The residual liquid was cooled and acidified with hydrochloric acid to yield the *product* which had m.p. 133–135° after crystallisation from water (yield 95 per cent).

2-*Aminotoluene*-4-*sulphonmethylamide*. A solution of 2-nitrotoluene-4-sulphonmethylamide[9] (68 g.) in ethanol (500 ml.) was hydrogenated in the presence of Raney nickel at 100° and 50 atmospheres pressure for 1 hour. The *product* (70 per cent yield), m.p. 80–83° was characterised by conversion into its *acetyl* derivative which had m.p. 163°, after crystallisation from water.

2-*Chlorosulphonyltoluene*-4-*sulphonmethylamide*. Diazotisation of the foregoing amine followed by reaction with sulphur dioxide-acetic acid yielded the *product* which had m.p. 126–127°, after crystallisation from 1,2-dichloroethane-light petroleum (b.p. 60–80°) (yield 85 per cent).

2-*Sulphamyltoluene*-4-*sulphonmethylamide* obtained by reaction of the foregoing compound with an aqueous ammonia (d = 0·880)-carbon tetrachloride two phase mixture, had m.p. 172–174°, after crystallisation from aqueous ethanol (yield 90 per cent).

2-*Nitrotoluene*-4-*sulphondimethylamide* was obtained in 80 per cent yield by reaction of the corresponding sulphonchloride[6,9] with aqueous dimethylamine-carbon tetrachloride. It had m.p. 92–94°, after crystallisation from methanol.

2-*Aminotoluene*-4-*sulphondimethylamide*, prepared in 83 per cent yield by hydrogenation of the foregoing nitro-compound in ethanol, using Raney nickel as catalyst at 100° and 40 atmospheres pressure, had m.p. 172–174°, after crystallisation from ethanol.

2-*Sulphamyl toluene*-4-*sulphondimethylamide*, prepared in 75 per cent yield from the foregoing amine by conversion into the sulphonchloride and reaction with ammonia solution (d = 0·880) (described above) had m.p. 161–163°, after crystallisation from aqueous ethanol.

2-*Nitrotoluene*-4-*sulphonpiperidide*, prepared *via* the sulphonchloride, had m.p. 110–111°, after crystallisation from aqueous ethanol.

2-*Aminotoluene*-4-*sulphonpiperidide*, obtained in 64 per cent yield by reduction of the foregoing nitro-compound with iron powder in acidulated 20 per cent ethanol, had m.p. 117–118°, after crystallisation from aqueous ethanol.

2-*Chlorosulphonyltoluene*-4-*sulphonpiperidide*, prepared from the foregoing amine, had m.p. 155–156°, after crystallisation from 1,2-dichloroethane-light petroleum (b.p. 60–80°). It was condensed with methylamine

TABLE IV—*continued*

Substituent						m.p. °C.	Found				Required			
R	R′	R″	N.AB	N.DE	Formula		C	H	N	S	C	H	N	S
—	—	Pr^i	NHMe	NH_2	$C_{10}H_{16}O_4N_2S_2$	157–159	41·2	5·5	10·0	22·2	41·1	5·5	9·6	21·9
—	—	Pr^i	NH_2	NHMe	$C_{10}H_{16}O_4N_2S_2$	172–174	41·4	5·3	9·9	21·7	41·1	5·5	9·6	21·9
—	Me	Me	NHMe	NH_2	$C_9H_{14}O_4N_2S_2$	184–186	38·9	5·2	10·2	23·1	38·9	5·1	10·1	23·0
—	Me	Me	NH_2	NHMe	$C_9H_{14}O_4N_2S_2$	157–159	38·8	5·2	10·4	23·0	38·9	5·1	10·1	23·0
Me	—	Me	NHMe	NH_2	$C_9H_{14}O_4N_2S_2$	171–173	38·7	5·1	10·0	22·6	38·9	5·1	10·1	23·0
—	—	MeO	NHMe	NH_2	$C_8H_{12}O_5N_2S_2$	208–209	34·6	4·5	9·8	—	34·3	4·3	10·0	—
—	—	MeO	NH_2	NHMe	$C_8H_{12}O_5N_2S_2$	203–204	34·2	4·7	10·0	—	34·3	4·3	10·0	—
—	—	Cl	NHMe	NH_2	$C_7H_9O_4N_2S_2Cl$	139–141	30·0	3·2	9·6	12·0*	29·5	3·2	9·8	12·5*
—	—	Cl	NH_2	NHMe	$C_7H_9O_4N_2S_2Cl$	177–179	30·0	3·2	10·1	22·2	29·5	3·2	9·8	22·5
—	—	Cl	NH_2	NHEt	$C_8H_{11}O_4N_2S_2Cl$	146–148	32·1	3·6	9·3	21·4	32·2	3·7	9·4	21·4
—	—	Cl	NH_2	$NH{\cdot}CH_2{\cdot}CH_2{\cdot}OH$	$C_8H_{11}O_5N_2S_2Cl$	162–164	30·8	3·6	9·1	20·2	30·5	3·5	8·9	20·4
—	—	Cl	NH_2	NMe_2	$C_8H_{11}O_4N_2S_2Cl$	182–184	32·6	3·8	11·9*	21·4	32·2	3·7	11·9*	21·4
—	—	Cl	NH_2	$N(CH_2)_5$	$C_{11}H_{16}O_4N_2S_2Cl$	172–174	39·1	4·4	8·4	18·9	39·0	4·5	8·3	18·9
—	—	Cl	NEt_2	NHMe	$C_{11}H_{17}O_4N_2S_2Cl$	98–100	39·0	4·8	8·2	18·8	38·7	5·0	8·2	18·8
—	—	Br	NHMe	NH_2	$C_7H_9O_4N_2S_2Br$	165–166	25·4	2·6	8·4	19·6	25·5	2·8	8·5	19·5
—	—	Br	NH_2	NHMe	$C_7H_9O_4N_2S_2Br$	175–176	25·5	2·8	8·6	19·5	25·5	2·8	8·5	19·5
Cl	—	Cl	NHMe	NH_2	$C_7H_8O_4N_2S_2Cl_2$	210–211	26·5	2·7	9·0	20·3	26·3	2·5	8·8	20·1
—	Me	Cl	NHMe	NH_2	$C_8H_{11}O_4N_2S_2Cl$	179–180	32·6	3·7	9·2	11·8*	32·2	3·7	9·4	11·9*
—	Me	Cl	NH_2	NHMe	$C_8H_{11}O_4N_2S_2Cl$	182–184	32·5	4·0	9·8	21·7	32·2	3·7	9·4	21·4
Cl	—	Me	NHMe	NH_2	$C_8H_{11}O_4N_2S_2Cl$	223–225	32·3	3·8	9·7	21·0	32·2	3·7	9·4	21·4
Cl	—	Me	NH_2	NHMe	$C_8H_{11}O_4N_2S_2Cl$	192–194	—	—	9·2	21·0	32·2	3·7	9·4	21·4
—	—	PhO	NH_2	NHMe	$C_{14}H_{16}O_5N_2S_2$	163–165	47·5	4·5	7·8	17·8	47·2	4·5	7·9	18·0

1 = 1,2,3,6-Tetrahydropyridine.
2 = 4-Phenyl-1,2,3,6-tetrahydropyridine.
* = Chlorine.

and with morpholine to yield the mixed substituted sulphonamides described in Table IV.

2-*Methoxy*-5-*nitrobenzene sulphonchloride* was prepared in 71 per cent yield by diazotisation of 2-amino-4-nitroanisole, followed by reaction of the diazonium solution with a solution of sulphur dioxide in acetic acid as described above. It had m.p. 119–120°, after crystallisation from 1,2-dichloroethane-light petroleum (b.p. 60–80°). Found: C, 33·7; H, 2·0; N, 5·8; Cl, 14·0; S, 12·8. $C_7H_6O_5NSCl$ requires C, 33·4; H, 2·4; N, 5·6; Cl, 14·1; S, 12·7 per cent.

Bromobenzene-2-*sulphonchloride*[10] was prepared in 87 per cent yield from *o*-bromoaniline by the diazo route.

2-*Bromo*-5-*nitrobenzene sulphonchloride*[11] was obtained in 93 per cent yield by nitration of the foregoing sulphonchloride with a mixture of concentrated nitric and sulphuric acids at 25–35°. It had m.p. 92°. after crystallisation from 1,2-dichloroethane-light petroleum (b.p.60–80°),

5-*Chloro*-4-*nitrotoluene*-2-*sulphonchloride*. *m*-Chlorotoluene (126·5 g.) was added with stirring to chlorosulphonic acid (300 ml.), the temperature was kept below 30° and stirring was continued for 2 hours after the addition was complete. The mixture was added slowly with stirring to crushed ice and the sulphonchloride collected, washed with ice-water and dried in air. The crude, dry sulphonchloride was added slowly with stirring to fuming nitric acid (200 ml., d = 1·50) and when the addition was complete, concentrated sulphuric acid (50 ml.) was slowly stirred into the mixture. The mixture was warmed to 40° for 1 hour when it was cooled and added with stirring to ice-water. The *product* was collected and washed with cold water. It had m.p. 108–110°, after crystallisation from light petroleum (b.p. 80–100°). Found: C, 31·4; H, 1·9; N, 5·5. $C_7H_5O_4NSCl_2$ requires C, 31·1; H, 1·9; N, 5·2 per cent.

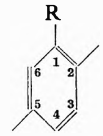
3-*Nitro*-4-*phenoxybenzene sulphondimethylamide*. A solution of 4-chloro-3-nitrobenzene sulphondimethylamide (6·6 g.) in ethanol (35 ml.) was treated with a solution of phenol (2·35 g.) in water (5 ml.) containing potassium hydroxide (1·4 g.) and the mixture heated under reflux for 4 hours. The *product* (7·4 g.) which separated on cooling and slight dilution with water had m.p. 105°, after crystallisation from ethanol. The melting point was depressed on admixture with the starting material.

2-*Chlorosulphonylbenzene sulphonamide*. A solution of 2-sulphamylaniline (17·2 g.) in 24 per cent hydrochloric acid (120 ml.) was diazotised at 0–5° by the addition of a solution of sodium nitrite (7·5 g.) in water (20 ml.), and the resultant diazonium solution added with stirring to a saturated solution of sulphur dioxide in glacial acetic acid (160 ml.) containing cupric chloride dihydrate (7 g.) at 25°. After the addition was complete, stirring was continued for a further 15 minutes when precipitation of the product was completed by the addition of ice-water. It was purified by crystallisation from 1,2-dichloroethane and had m.p. 176°. Found: C, 28·5; H, 2·5; N, 5·4; Cl, 13·6; S, 25·4. $C_6H_6O_4NS_2Cl$ requires C, 28·2; H, 2·4; N, 5·5; Cl, 13·9; S, 25·0 per cent.

(*a*) *Reaction with ammonia*. A suspension of the foregoing compound (2·0 g.) in chloroform (40 ml.) was stirred vigorously and treated at once

TABLE V

2,5-Disubstituted derivatives of benzene, toluene and chlorobenzene



R	Substituent at position 2	Substituent at position 5	Formula	m.p. °C.	Found C	Found H	Found N	Found S	Required C	Required H	Required N	Required S
H	SO_2NH_2	SO_2Cl	$C_6H_6O_4NS_2Cl$	155–157	28·6	2·5	5·5	13·7*	28·2	2·4	5·5	13·9*
H	SO_2NH_2	SO_2NHMe	$C_7H_{10}O_4N_2S_2$	160–161	33·4	4·0	11·1	—	33·6	4·0	11·2	—
H	SO_2NH_2	$SO_2NH{\cdot}CH_2{\cdot}CH_2{\cdot}OH$	$C_8H_{12}O_5N_2S_2$	150–151	34·4	4·5	10·1	23·3	34·3	4·3	10·0	22·9
H	SO_2NH_2	SO_2NMe_2	$C_8H_{12}O_4N_2S_2$	203	36·5	4·5	10·5	24·3	36·4	4·6	10·6	24·3
H	SO_2NHMe	SO_2NHMe	$C_8H_{12}O_4N_2S_2$	223–225	36·2	4·3	10·4	24·5	36·4	4·6	10·6	24·3
Me	SO_2Cl	NO_2	$C_7H_6O_4NSCl$	68–69	36·0	2·3	6·1	13·7	35·7	2·6	5·9	13·6
Me	SO_2NHMe	NO_2	$C_8H_{10}O_4N_2S$	172–174	41·8	4·2	12·5	13·8	41·7	4·4	12·2	13·9
Me	SO_2NHMe	NH_2	$C_8H_{12}O_2N_2S$	117–118	48·0	6·0	14·2	15·9	48·0	6·0	14·0	16·0
Me	SO_2NHMe	SO_2Cl	$C_8H_{10}O_4NS_2Cl$	117–119	33·8	3·4	5·1	12·7*	33·9	3·6	4·9	12·5*
Me	SO_2NHMe	SO_2NH_2	$C_8H_{12}O_4N_2S_2$	125–126	36·0	4·8	10·8	24·0	36·4	4·6	10·6	24·3
Me	SO_2NH_2	NO_2	$C_7H_8O_4N_2S$	155–156	39·3	3·6	12·9	14·5	38·9	3·7	13·0	14·8
Me	SO_2NH_2	NH_2	$C_7H_{10}O_2N_2S$	170–172	45·5	5·1	14·8	17·4	45·1	5·4	15·0	17·2
Me	SO_2NH_2	SO_2Cl	$C_7H_7O_4NS_2Cl$	134–136	31·4	2·9	5·2	23·9	31·2	3·0	5·2	23·8
Me	SO_2NH_2	SO_2NH_2	$C_7H_{10}O_4N_2S_2$	228–229	33·9	4·1	11·1	25·4	33·6	4·0	11·2	25·6
Me	SO_2NH_2	SO_2NHMe	$C_8H_{12}O_4N_2S_2$	149–151	36·3	4·9	10·6	23·9	36·4	4·6	10·6	24·3
Me	SO_2NH_2	SO_2NMe_2	$C_9H_{14}O_4N_2S_2$	173–175	39·0	5·1	10·2	22·6	38·9	5·1	10·1	23·0
Cl	SO_2Cl	NO_2	$C_6H_2O_4NSCl_2$	66–68	28·3	1·2	5·7	13·1	28·1	1·2	5·5	12·5
Cl	SO_2NHMe	NO_2	$C_7H_5O_4N_2SCl$	190–191	34·0	3·0	11·2	13·1	33·6	2·8	11·2	12·8
Cl	SO_2NHMe	NH_2	$C_7H_7O_2N_2SCl$	164–166	38·0	3·9	12·6	14·3	38·1	4·1	12·7	14·5
Cl	SO_2NHMe	SO_2Cl	$C_7H_4O_4NS_2Cl_2$	126–128	27·4	2·1	4·9	21·2	27·6	2·3	4·6	21·1
Cl	SO_2NHMe	SO_2NHMe	$C_8H_{11}O_4N_2S_2Cl$	144–145	32·0	3·8	9·4	11·6*	32·2	3·7	9·4	11·9*
Cl	SO_2NHMe	SO_2NH_2	$C_7H_8O_4N_2S_2Cl$	177–178	30·0	3·1	10·2	12·9*	29·5	3·2	9·8	12·5*
Cl	SO_2NH_2	NO_2	$C_6H_5O_4N_2SCl$	149–150	30·4	2·1	11·7	13·1	30·5	2·1	11·8	13·6
Cl	SO_2NH_2	NH_2	$C_6H_7O_2N_2SCl$	180–182	35·0	3·8	13·4	17·1*	34·9	3·4	13·6	17·2*
Cl	SO_2NH_2	SO_2Cl	$C_6H_4O_4NS_2Cl_2$	162–164	25·1	1·9	4·8	22·1	24·8	1·7	4·8	22·1
Cl	SO_2NH_2	SO_2NH_2	$C_6H_7O_4N_2S_2Cl$	229–231	26·7	2·6	10·4	24·0	26·6	2·6	10·4	23·7
Cl	SO_2NH_2	SO_2NHMe	$C_7H_9O_4N_2S_2Cl$	187–189	29·7	3·2	10·2	13·1*	29·5	3·2	9·8	12·5*
Cl	SO_2NH_2	SO_2NMe_2	$C_8H_{11}O_4N_2S_2Cl$	186–188	32·2	4·0	9·7	21·4	32·2	3·7	9·4	21·5

* = Chlorine.

with ammonia solution (3 ml., d = 0·880), when the solid dissolved and pasty material separated. The chloroform and ammonia were boiled off, the residue was acidified with hydrochloric acid and boiled with 50 per cent ethanol. The insoluble 1,2-disulphamylbenzene (0·1 g.) was collected and had m.p. 343° (decomp.). Found: C, 30·8; H, 3·2; N, 11·9; S, 27·1. *Benzene-1,2-disulphonamide*, $C_6H_8O_4N_2S_2$, requires C, 30·5; H, 3·4; N, 11·9; S, 27·1 per cent.

(*b*) *Reaction with dimethylamine.* A suspension of 2-chlorosulphonyl benzene sulphonamide (2 g.) in chloroform was stirred vigorously and treated with ethanolic dimethylamine (5 ml.; 33 per cent) when the solid dissolved immediately. The mixture was evaporated to dryness and the residue crystallised from ethanol-benzene to yield 2-sulphamylbenzene sulphondimethylamide (1·2 g.), m.p. 145–147°. The m.p. was not depressed on admixture with authentic material (see below).

2-*Nitrobenzene sulphondimethylamide* prepared by reaction of *o*-nitrobenzene sulphonchloride with 30 per cent ethanolic dimethylamine, had m.p. 80–82° after crystallisation from ethanol. Found: N, 12·0; S, 13·8. $C_8H_{10}O_4N_2S$ requires N, 12·2; S, 13·9 per cent.

2-*Aminobenzene sulphondimethylamide.* Reduction of the foregoing nitro-compound with iron powder in 1 per cent acetic acid furnished the *product* which had m.p. 85–86°, after crystallisation from aqueous ethanol. Found: C, 47·6; H, 5·9; N, 14·1. $C_8H_{12}O_2N_2S$ requires C, 48·0; H, 6·0; N, 14·0 per cent.

2-*Sulphamylbenzene sulphondimethylamide.* A solution of the foregoing compound (20 g.) in 24 per cent hydrochloric acid (240 ml.) was diazotised at 0–5° by the addition of a solution of sodium nitrite (7·6 g.) in water (18 ml.). The diazonium solution was added with stirring at 15–20° to a saturated solution of sulphur dioxide in acetic acid (160 ml.) containing cupric chloride dihydrate (7 g.). Sulphur dioxide was passed into the solution until the addition was complete, when stirring was continued for a further 20 minutes. The mixture was diluted with ice-water to complete precipitation of the sulphonchloride which was collected, washed with ice-water and dried.

The sulphonchloride (23·5 g., m.p. 96–98°) was added in portions to ammonia solution (300 ml., d = 0·880) with stirring. Stirring was continued for 1 hour after the addition was complete. The solution was boiled to remove excess of ammonia, cooled and neutralised with hydrochloric acid. The *product* (17·9 g.), had m.p. 145–147°, after crystallisation from water. Found: C, 36·2; H, 4·3; N, 10·9; S, 24·5. $C_8H_{12}O_4N_2S_2$ requires C, 36·4; H, 4·6; N, 10·6; S, 24·3 per cent.

2-*Sulphamyl*-4-*methylsulphamyl*-(*β-hydroxyethyl*)-*aniline.* A solution of 4-chloro-3-sulphamylbenzene sulphonmethylamide (28·5 g.) in 2-hydroxyethylamine (30 ml.) was heated at 150° for 1·5 hours when excess of amine was removed at 100° and 0·1 mm. pressure. The residue was dissolved in hot aqueous ethanol when the *product* crystallised on cooling. It had m.p. 162–164° after crystallisation from methanol. Found: C, 35·1; H, 4·7; N, 13·9. $C_9H_{15}O_5N_3S_2$ requires C, 34·9; H, 4·9; N, 13·6 per cent.

4-*Sulphamyl*-2-*methylsulphamyl*-(*β-hydroxyethyl*)-*aniline*, was obtained

by reaction of 2-chloro-5-sulphamylbenzene sulphonmethylamide with 2-hydroxyethylamine as described in the preceding example. It had m.p. 143–144° after crystallisation from 25 per cent methanol. Found: C, 35·3; H, 5·2; N, 13·7. $C_9H_{15}O_5N_3S_2$ requires C, 34·9; H, 4·9; N, 13·6 per cent.

5-*Methyl*-2-*sulphamyl*-4-*methylsulphamyl*-(*β-hydroxyethyl*)*aniline*, prepared by reaction of the corresponding chloro compound with 2-hydroxyethylamine at 145° for 1·5 hours, had m.p. 178–179° after crystallisation from water. Found: C, 37·3; H, 5·6; N, 12·9; S, 19·7. $C_{10}H_{17}O_5N_3S_2$ requires C, 37·1; H, 5·3; N, 13·0; S, 19·8 per cent.

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THE RELEASE OF SYMPATHETIC AMINES BY TYRAMINE FROM THE AORTIC WALLS OF CATS

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Received June 14, 1960

Horns of rat uteri in chambers, perfused with the femoral arterial blood of cats, relaxed during pressor responses to intravenous injections of tyramine, but failed to do so when half this weight of tyramine was put directly into the arterial blood bathing the uterine horn. Concentrations of tyramine, evoking only threshold inhibitions of rat uteri, have been shown to release one or more compounds from the isolated perfused aortae of cats. The substances released inhibit the spontaneous and induced contractions of rats' uteri. Finally both adrenaline and noradrenaline have been demonstrated in extracts of cats' aortae.

Chromatographic studies have shown that the concentrations of adrenaline and noradrenaline in the plasma of blood withdrawn from the lower abdominal aortae of cats rise during the pressor response to intravenous injections of tyramine[1]. The first object of this work was the direct biological confirmation of this finding, the second to discover whether these amines were released from the aortic walls.

Experimental

Anaesthesia was induced with ether and maintained by the injection of 7·5 ml. 1·0 per cent chloralose w/v in 0·9 per cent w/v aqueous NaCl per kg. through a right femoral venous cannula. A tracheal cannula was inserted.

The perfusion of a rat uterus from a cat femoral artery. One horn of a rat's dioestrus uterus was anchored in a 5 ml. bath covered with a small rubber dome through which a thread greased with petroleum jelly passed to a frontal writing lever. This horn was continuously perfused with arterial blood which entered the bath from a femoral arterial cannula through 2 inches of rubber tubing; it filled the bath to a constant volume and returned to the cat through a short length of rubber tube and a cannula set in the femoral vein.

The perfusion of the cat's aorta. Positive pressure ventilation was applied to a cat anaesthetised with chloralose, the chest was opened in the midline anteriorly, and the left lung removed after application of hilar ligatures. The thoracic aorta was prepared for perfusion by division of of the 24 intercostal branches between ligatures. The inflow cannula, bearing a side arm, was inserted just distal to the origin of the left subclavian artery after ligation of the arch. The straight outflow cannula was introduced 1 cm. above the diaphragm. The aorta was lifted out after section above the inflow and below the outflow cannulae, and was perfused with Tyrode's fluid, oxygenated and adjusted to pH 6·7, at 37° and a mean pressure of 40 mm. Hg (pulse pressure 80 mm. Hg). In

other experiments the calcium concentration in the Tyrode's fluid was reduced to one-third normal and the temperature to 28°. A diagram of the perfusion circuit, capacity 25–30 ml., is shown in Figure 1. The fluid from reservoir A was driven by a microperfusion pump B (C. F. Palmer Ltd.) through the aorta at a mean pressure registered by manometer M_1, and then traversed a resistance R consisting of thin walled rubber tubing compressed with air from pressure bottle PB at 60–70 mm. Hg pressure as recorded by manometer M_2. From this resistance the aortic effluent was either returned directly to the reservoir A through tube C_1, or was passed via tube C^2 to perfuse a horn of a rat's uterus suspended in a 5 ml. bath (UB) before returning through the overflow to reservoir A. The reservoir A, a moist chamber surrounding the aorta, the uterus bath

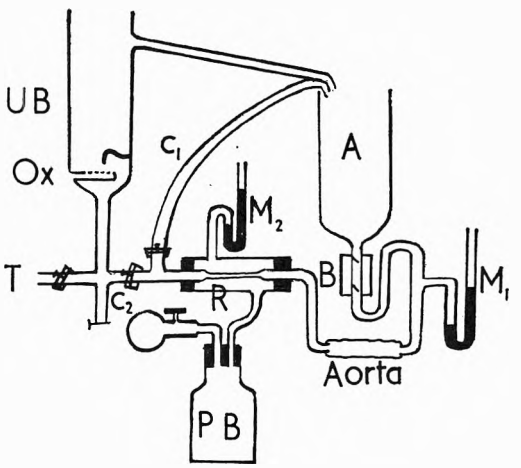

FIG. 1. Circuit diagram for perfusion of the cat aorta *in vitro*. See text for description.

UB, and coils supplying fluid through tube T to this bath whenever it was not in circuit were equipped with warming jackets perfused with water at 37° or at 28°. For the first 15 minutes of each perfusion the Ringer contained 1 mg. harmaline/25 ml.

The preparation of extracts of adrenal glands and aortae. Tissues were stored in the deep freeze for 1–4 hours before mincing with scissors, and grinding with a pinch of silver sand in 2–4 ml. of 0·1N HCl. Extracts and mortar washings were combined, immersed in a boiling water bath for 2 minutes, cooled rapidly and centrifuged. The supernatants were neutralised to litmus as external indicator immediately before bioassay. The method used for the chromatographic separation of amines and their elution has been described[1].

Bioassays. The total quantity of adrenaline plus noradrenaline in each extract was assayed as adrenaline on the rat colon. The adrenaline was assayed on the rat uterus. The relative potency of adrenaline and noradrenaline was determined for each preparation, and the absolute

quantities of the two amines in the extract were calculated by the use of simultaneous equations. Both the colon and the uterus were suspended in aerated de Jalon's Ringer and the assays were made as described by Gaddum, Peart and Vogt[2].

Drugs. Tyramine hydrochloride and harmaline (L. Light and Co. Ltd.), (−)adrenaline and (−)noradrenaline bitartrates (Burroughs Wellcome Ltd.), hexamethonium bromide (May and Baker Ltd.) and heparin (Liquemin, Roche Products Ltd.) were obtained commercially.

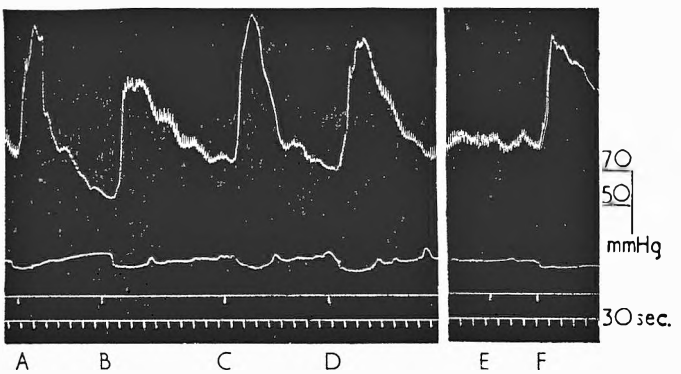

FIG. 2. The upper trace is of the mean arterial pressure of a cat under chloralose anaesthesia, in which the adrenals had been excluded from the circulation and lasting block of autonomic ganglia induced with hexamethonium; the lower traces show changes in the tone of a rat uterus perfused with the cat's femoral arterial blood. A to D intravenous injection to cat. E and F close arterial injections to uterus. A = 5 μg. (−)-adrenaline, B and F = 200 μg., E = 100 μg., and D = 300 μg. tyramine hydrochloride, C = 3 μg. (−)-noradrenaline.

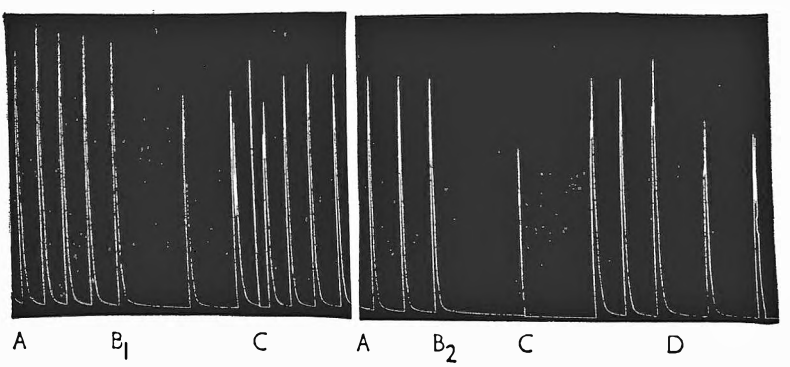

FIG. 3. The tracings show the spontaneous contractions of a rat's uterine horn suspended in oxygenated Tyrode's fluid at 37°. A to B_1 and B_2, the Tyrode's fluid had perfused a cat thoracic aorta for 15 minutes. B_1 and B_2 to C, the fluid contained 1·0 and 1·5 μg. respectively of tyramine-HCl/30 ml. which had perfused the aorta for 10 minutes. At C, the bath was emptied and filled with fresh Tyrode's fluid. At D, 0·5 μg. tyramine HCl was added to 5·4 ml. normal Tyrode's fluid in the uterus bath.

RESULTS

Horns of rats' dioestrus uteri bathed in the femoral arterial blood of chloralosed, acutely adrenalectomised, hexamethonium treated cats became quiescent after some minutes of perfusion but developed tone. They relaxed similarly whether adrenaline or noradrenaline was injected intravenously (Fig. 2) or directly into the tube leading to the chamber containing the uterus. Whereas the intravenous injection of 200 μg. tyramine hydrochloride caused relaxation of the uterus, the close arterial injection of 100 μg. was without effect (Fig. 2).

The horn of a rat's dioestrus uterus perfused with oxygenated Tyrode's fluid at 37° in series with the thoracic aorta of a cat contracted rythmically (Fig. 3) but was inhibited when 1·5 μg. tyramine hydrochloride was

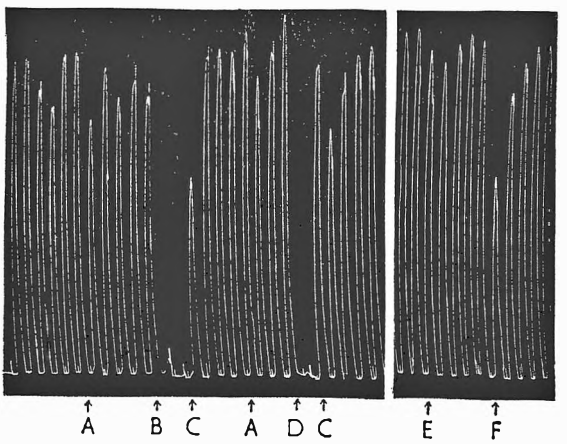

FIG. 4. The tracings show contractions of a horn of rat uterus in deJalon's fluid at 28° in response to 1 µg. acetylcholine added to the bath (5 ml.) at 3-minute intervals, then washed out. At A, the uterus is put in circuit with a perfused cat aorta. At B, 5 µg. and at D, 2·5 µg. tyramine HCl were added to the perfusion reservoir (circuit vol. 30 ml.). At C the uterus was washed and isolated from the perfusion circuit. At E 1 µg. tyramine HCl and at F 0·04 ng. adrenaline were added to the 5 ml. uterine bath (in isolation from the circuit) 1 minute before the next addition of acetylcholine.

added to the reservoir to yield a final concentration of 0·05 μg./ml. When the horn was isolated from the perfusion circuit the addition of 0·5 μg. tyramine hydrochloride to 5 ml. of bath fluid caused only slowing of spontaneous activity and some lessening of the tension developed during contractions. Similarly, when the temperature and the concentration of calcium and glucose were reduced in the perfusion fluid so that a uterus became quiescent, but could be stimulated to contract at intervals of 3 minutes by the addition of 1 μg. acetylcholine to the bath, no inhibition of these contractions resulted from the filling of the bath by fluid which had circulated through the aorta until tyramine hydrochloride was added to the circuit reservoir. Then, 2·5 μg. tyramine hydrochloride in 30 ml. produced complete inhibition of the response to acetylchloine. But, when the uterus was excluded from the circuit containing the aorta,

1 μg. tyramine hydrochloride in 5 ml. bath fluid caused only threshold reduction in the response to the fixed dose of acetylcholine (Fig. 4).

Extracts of aortae taken from ten anaesthetised exsanguinated cats and assayed biologically without prior chromatographic separation of the amines were found to contain both adrenaline and noradrenaline (Table I). This was twice confirmed by chromatographic separation of these compounds. The total quantities of these amines per g. tissue and the relative proportions in which they were present varied greatly and were not affected by the induction of lasting ganglion block with hexamethomium

TABLE I

AMINES OF THE SYMPATHETIC NERVOUS SYSTEM EXTRACTED FROM THE AORTAE OF CATS

Weight of cat (kg.)	Procedure before removal of the aorta	μg./g. tissue	
		(−)Noradrenaline	(−)Adrenaline
3·8	Anaesthesia induced with ether and maintained with chloralose. Exsanguinated	0·018	0·265
3·4		0·023	0·500
4·2		0·396	0·042
3·7		0·690	0·061
2·1		0·036	2·750
1·3		0·630	2·500
3·6	As above thoracic aorta*	0·270	2·560
	abdominal aorta*	0·108	2·104
3·1	Anaesthesia induced with ether and maintained with chloralose. Adrenals excluded from the circulation. Lasting ganglion block induced with hexamethonium 30 to 40 minutes before bleeding	0·167	2·430
2·2		1·010	0·255
2·3		0·610	0·464
2·6		0·995	0·600
3·7		0·542	0·208
2·4		1·013	2·872

* Chromatographic separation of amines and elution proceded bioassay.

TABLE II

COMPARISON OF THE SYMPATHETIC AMINES PRESENT IN THE AORTIC WALLS AND THE ADRENAL GLANDS OF CATS

Weight of cat (kg.)	μg. Amine/g. tissue				Ratio, adrenaline to noradrenaline	
	Aorta		Adrenal gland			
	(−)Noradren.	(−)Adren.	(−)Noradren.	(−)Adren.	Aorta	Adrenal gland
2·1	1·57	1·62	20·2	224·0	1·03	11·2
4·3	0·96	0·50	98·0	998·0	0·54	10·2
1·1	6·15	2·26	215·0	500·0	0·36	2·3
0·9	2·55	2·91	83·3	517·0	1·14	6·3
2·1	0·43	0·85	225·0	860·0	1·98	3·8

(3 mg./kg. i.v. and 6 mg./kg. subcutaneously) and exclusion of the adrenals from the circulation 30–40 minutes before bleeding (Table I). No relation was found either between the total weight of the stores of adrenaline and noradrenaline per g. tissue or between the ratios of adrenaline to noradrenaline present in the adrenal glands on the one hand and in the aortic walls on the other (Table II).

DISCUSSION

Intravenous injections of tyramine increased the concentrations of adrenaline and noradrenaline of heparinised blood withdrawn from the

lower abdominal aortae of cats anaesthetised with chloralose which had been rested for 30–40 minutes after induction of ganglion block with hexamethomium and exclusion of the adrenals from the circulation. The identity of the adrenaline fraction, separated chromatographically has since been proved by parallel assay on rat colon, rat uterus, and the blood pressure of the guinea pig and the rat[1]. Confirmation of this observation has been obtained since horns of dioestrus uteri of rats perfused with the femoral arterial blood of cats, similarly adrenalectomised and treated with hexamethonium, relaxed during pressor responses to intravenous injections of tyramine but were unaffected when half of this intravenous dose was injected into the arterial blood approaching the uteri (Fig. 2).

It has been shown that tyramine liberates a substance from the walls of the cat aorta which inhibits both the spontaneous contractions of the rats uterus in fluid at 37° (Fig. 3), and the responses of the quiescent rat uterus in de Jalon's fluid at 28° to acetylcholine (Fig. 4) as does adrenaline[2]. Since the walls of the aorta contain adrenaline (Tables I and II) it is probable that the uterine inhibitor liberated from the aorta by tyramine was adrenaline. In the two experiments in which it was measured the final content of adrenaline per g. tissue has been lower in the tyramine treated perfused thoracic aorta than in the unperfused untreated abdominal aorta.

Intravenous tyramine was not found to cause increase in the adrenaline and noradrenaline of plasma from carotid blood[1], and did not cause relaxation of the rats uterus perfused with carotid blood[3]. Since adrenaline and noradrenaline are found in the walls both of the thoracic and the abdominal aorta (Table I) it seems probable that the high concentrations of these amines found previously in the plasma of lower aortic blood during pressor responses to tyramine are caused, at least in part, by the steady addition of these amines to the blood as it traverses the length of the aorta.

Acknowledgement. This work was undertaken while one of us (K. E.) was receiving an educational grant for training in research from the Pharmaceutical Society of Great Britain.

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RESPIRATORY TRACT FLUID AND INHALATION OF PHOSGENE

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Received June 15, 1960

Increase in the permeability of the respiratory membrane of rabbits after exposure to phosgene was followed by measuring the output and composition of respiratory tract fluid. During the latent period, levels of sodium and chloride in respiratory tract fluid were elevated but volume was normal. During the early symptomatic period, the levels of sodium and chloride were definitely elevated but volume was still normal. Just before death in the late symptomatic period, respiratory tract fluid poured out of the lungs at a rate which averaged 60 times the normal and its sodium, chloride, and lipid levels were identical to those of blood plasma. Rabbits in the last group had an intense pulmonary oedema, haemoconcentration, and little or no resistance to the lethal effect of urethane.

In a previous communication to this Journal, Boyd and Stewart[1] reported that death in frogs exposed to phosgene at 4° is due to increased permeability of the respiratory skin surface to sodium chloride. The objective of the work to be described in the present report was to determine when and to what extent inhalation of lethal doses of phosgene by rabbits increases the permeability of their respiratory membranes. This was done by measuring the composition of respiratory tract fluid and comparing this with the corresponding composition of blood plasma at intervals after exposure to phosgene. At the same intervals the degree of pulmonary oedema was estimated by histologic and chemical examination of the lungs.

Methods

The animals were healthy, adult, male rabbits. They were exposed to phosgene by the static method in a chamber of 400 l. capacity at an initial concentration of 0·27 mg./l. An exposure for 30 minutes was found in preliminary trials to kill 80 to 100 per cent of rabbits, anaesthetized as noted below. This dose is within the range of the median lethal dose of phosgene in unanaesthetized animals as reported by Spector[2].

The animals were anaesthetised to the upper level of Plane 1 of surgical anaesthesia with urethane given intraperitoneally, and arranged for the collection of respiratory tract fluid by techniques described by Boyd[3]. Thirty-one rabbits were so arranged immediately after gassing. A further 31 were gassed late in the afternoon and the survivors anaesthetised for collection of respiratory tract fluid 16 hours later. Eighteen rabbits served as controls given no phosgene, but otherwise treated as were the animals treated with phosgene.

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The output of respiratory tract fluid was measured at hourly intervals and expressed as ml./kg./24 hours. Samples collected at or near death were analysed for their content of sodium[4], chloride[5], and lipids[6]. Similar analyses were made upon samples of oxalated blood plasma collected at the same time. The haemoglobin content and per cent volume of erythrocytes (haematocrit) were determined upon these samples of oxalated blood since haemoconcentration has been reported in dogs[7–9], and guinea pigs[10] treated with phosgene. At autopsy were measured the histopathology, water, chloride, and iron[11] content of the trachea and of the hilus and periphery of the lungs, divided after the technique of Boyd and Johnson[12], and in part after the recommendations of Hemingway[13], and Poulsen[14]. Statistical analyses of data were made as described by Croxton[15] and Waugh[16].

Results

Graphical arrangement of data upon the 62 rabbits indicated that values for most measurements fell into three frequency distributions.

TABLE I

THE HISTOPATHOLOGY OF THE LUNGS AND TRACHAE*

The results are expressed as mean ± standard deviation arbitrary (1+ to 4+) units

Site	Measurement	Group I	Group II	Group III
Alveoli	Oedema	0·9 ± 0·9 A	3·5 ± 0·9 A, B	4·0 ± 0·0 A, B, C
	Congestion	0·8 ± 0·9 A	1·0 ± 1·4 A	0·0 ± 0·0 B, C
	Emphysema	0·7 ± 0·7 A	1·4 ± 1·2 A, B	0·0 ± 0·0 B, C
	Haemorrhage	0·2 ± 0·5	1·0 ± 1·5 A, B	0·0 ± 0·0 C
	Contracted arteries	1·3 ± 1·3 A	0·3 ± 0·6 B	0·0 ± 0·0 B, C
Bronchioles ..	Oedema	0·3 ± 0·3 A	0·1 ± 0·3 B	0·0 ± 0·0 B
Trachea	Oedema	1·0 ± 1·3 A	0·1 ± 0·3 B	0·3 ± 0·5 B
	Congestion	0·7 ± 1·2 A	0·3 ± 0·5	0·3 ± 0·5

* A value of $P < 0{\cdot}05$ by a t test that the mean of Groups I, II, or III equalled that of the controls is indicated by A, that the mean of Groups II or III equalled that of Group I by B, and that the mean of Group III equalled that of Group II by C.

The first frequency distribution was composed of 31 animals anaesthetised immediately after treatment with phosgene and was termed Group I. The second distribution was termed Group II; it contained 19 animals anaesthetised 16 hours after being treated with phosgene and in which the volume output of respiratory tract fluid was at no time greater than that of the controls or the rabbits in Group I. In the third group, Group III, there were 12 rabbits, anaesthetised 16 hours after phosgene treatment and in which there was a marked increase in the volume output of respiratory tract fluid just before death.

The rabbits of Group I survived a mean (± standard deviation) of 3·3 ± 1·9 hours from the time they were anaesthetized and arranged for collection of respiratory tract fluid. The corresponding survival time in Group II was 3·1 ± 2·9 hours. In Group III, the survival time was significantly less, averaging 0·7 ± 0·4 hours. The control animals were killed for measurements at the end of the day, some 6 to 8 hours after they were anaesthetised. Most normal rabbits live for 36 to 48 hours under the degree of urethane anaesthesia and surgical manipulation involved in collecting respiratory tract fluid.

A summary of histopathologic observations is presented in Table I. The measurements were made semi-quantitative by assigning to each an arbitrary value of 0 or 1+ to 4+, depending upon the visual estimate of each measurement. Values for the control rabbits were zero and have not been listed in Table I.

The immediate response to phosgene during the first 6 or 7 hours after exposure consisted of a mild oedema of the trachea, bronchioles, and alveolar tissues, associated in the latter, with some congestion, emphysema and contraction of arteries. At 16 to 20 hours in the animals of Group II, the pulmonary oedema had markedly increased, there was more emphysema and some haemorrhage, the pulmonary arteries were less contracted and there was less oedema in the bronchioles and trachea. In the animals of Group III with a premortal gush of respiratory tract fluid, pulmonary oedema was maximal, the arteries were not contracted, and there was no congestion, emphysema, or haemorrhage.

TABLE II

MEASUREMENTS UPON RESPIRATORY TRACT FLUID*

The results are expressed as mean ± standard deviation

Measurement	Units	Control rabbits	Rabbits treated with phosgene		
			Group I	Group II	Group III
Volume output ..	ml./kg./24 hr.	2·3 ± 1·7	3·0 ± 1·5	2·2 ± 2·2	136 ± 166 A, B, C
Sodium	mg./100 ml.	28·2 ± 24·1	46·3 ± 44·1	121 ± 115 A, B	329 ± 41 A, B, C
Chloride	mg./100 ml.	37·0 ± 30·1	—	206 ± 123 A	400 ± 69 A, C
Total lipid ..	mg./100 ml.	65·2 ± 24·1	—	—	345 ± 176 A
Neutral fat ..	mg./100 ml.	18·6 ± 25·3	—	—	68 ± 50 A
Total fatty acids	mg./100 ml.	42·3 ± 17·7	—	—	202 ± 70 A
Total cholesterol	mg./100 ml.	13·7 ± 22·6	—	—	87 ± 78 A
Ester cholesterol	mg./100 ml.	8·6 ± 14·1	—	—	45 ± 57
Free cholesterol	mg./100 ml.	5·1 ± 4·3	—	—	42 ± 28 A
Phospholipid ..	mg./100 ml.	28·3 ± 16·2	—	—	158 ± 111 A

* A value of $P < 0{\cdot}05$ by a t test that the mean of Groups I, II, or III equalled that of the controls is indicated by A, that the mean of Groups II or III equalled that of Group I by B, and that the mean of Group III equalled that of Group II by C.

Measurements upon respiratory tract fluid have been collected in Table II. The volume output was at no time significantly different from control values except in Group III where the mean output was increased sixtyfold. The concentration of sodium was above the normal range in a few of the animals of Group I, was significantly increased in the animals of Group II, and was further elevated in all animals of Group III. Concentrations of chloride were above normal in Group II and further increased in Group III.

These results suggested that permeability of the pulmonary capillaries and alveolar endothelium to the constituents of blood plasma was partially increased at some 6 hours after exposure to phosgene and considerably increased at 16 to 20 hours after exposure. In some 40 per cent of the latter animals (Group III), permeability to blood plasma apparently became complete, resulting in an outpouring of blood plasma into respiratory tract fluid.

To investigate the suggestion that respiratory tract fluid collected from the animals of Group III was practically pure blood plasma, samples

were measured for their lipid content. The lipid composition of blood plasma is characteristic and different from that of any other tissue or fluid of the body. Sufficient respiratory tract fluid for lipid analysis could be collected from the controls and from the animals of Group III but not from the animals of Groups I and II. As shown by data summarised in Tables II and III lipid levels in respiratory tract fluid of rabbits in Group III were some fivefold the levels of the controls and identical to lipid levels in blood plasma of these animals.

Measurements upon blood have been summarised in Table III. The haematocrit and chloride levels were augmented over control values in Group II. The haematocrit and levels of chloride and all lipids except neutral fat were above control levels in Group III, some values being significantly higher than in Groups I and II.

TABLE III

MEASUREMENTS UPON BLOOD PLASMA*

The results are expressed as mean ± standard deviation units per 100 ml. of blood plasma

Measurement	Units	Control rabbits	Rabbits treated with phosgene		
			Group I	Group II	Group III
Sodium	mg.	261 ± 20	260 ± 13	264 ± 17	269 ± 16
Chloride	mg.	327 ± 13	331 ± 32	341 ± 25 A	362 ± 26 A, B, C
Total lipid ..	mg.	297 ± 80	312 ± 121	333 ± 139	356 ± 111
Neutral fat ..	mg.	118 ± 58	126 ± 87	140 ± 103	86 ± 85
Total fatty acids	mg.	201 ± 74	205 ± 97	221 ± 116	205 ± 75
Total cholesterol	mg.	58 ± 17	62 ± 25	67 ± 22	104 ± 51 A, B, C
Ester cholesterol	mg.	26 ± 13	31 ± 19	31 ± 16	54 ± 30 A, B, C
Free cholesterol	mg.	32 ± 97	31 ± 14	36 ± 13	50 ± 27 A, B
Phospholipid ..	mg.	96 ± 12	101 ± 36	101 ± 43	129 ± 49 A
Haemoglobin ..	g.†	11·4 ± 1·6	10·8 ± 0·7	12·4 ± 2·3 B	12·6 ± 2·1 B
Haematocrit ..	ml.†	35·0 ± 4·2	33·8 ± 7·0	41·3 ± 10·1 A, B	39·6 ± 6·2 A, B

* A value of $P < 0{\cdot}05$ by a t test that the mean of Groups I, II, or III equalled that of the controls is indicated by A, that the mean of Groups II or III equalled that of Group I by B, and the mean of Group III equalled that of Group II by C.

† Measured as units per 100 ml. of whole blood.

The water, chloride, and iron content of the lung periphery, hilus, and trachea were measured at autopsy to obtain a further estimate of the degree of oedema and congestion. The results are summarised in Table IV. Water and chloride levels in the periphery were elevated above control values in Groups I and II and increased further in Group III. The results agree with the histological data on alveolar oedema (Table I) but suggest that there was a greater diffusion of water and salt into the lung parenchyma and stroma at 6 to 7 hours than appeared as oedema in microscopic examination.

Data in Table IV indicate that the blood vessels of alveolar tissues contained more blood at 6 to 7 hours than controls, which corresponds with the congestion noted in Table I. The amount of blood (iron) was significantly lower in Group II than in Group I and this did not correspond with the microscopic interpretations noted in Table I. The findings of less iron in the periphery of rabbits in Group III than in all other groups corresponds to the absence of congestion noted microscopically (Table I).

The water content of the hilus was increased in the rabbits subjected to phosgene, but not to the same extent as in the periphery except in

Group II. This suggests that oedema of the bronchi occurred in Group II but not in Group I and III. Shifts in the iron content of the hilus were similar to those of the periphery, indicating that there was probably no significant change in the iron content of the bronchi.

The water content of the trachea was lower in the rabbits of Group III than of Group I which agreed with the histological findings. The iron content of the trachea was increased above the controls in Group I which also agreed with the microscopic findings.

TABLE IV

MEASUREMENTS UPON THE LUNGS AND TRACHEA*

The results are expressed as mean ± standard deviation

Measurement	Units	Control rabbits	Rabbits treated with phosgene		
			Group I	Group II	Group III
Periphery, water	g./100 g. wet wt.	78·8 ± 2·4	82·4 ± 2·8 A	81·4 ± 2·1 A	84·6 ± 1·4 A, B, C
Periphery, chloride	mg./g. dry wt.	8·7 ± 0·9	13·7 ± 2·8 A	13·4 ± 3·0 A	16·6 ± 4·6 A, B, C
Periphery, iron	µg./g. dry wt.	420 ± 61	502 ± 180 A	391 ± 98 B	280 ± 86 A, B, C
Hilus, water	g./100 g. wet wt.	78·6 ± 1·9	79·8 ± 3·5	80·7 ± 3·1 A	82·0 ± 3·5 A
Hilus, iron	µg./g. dry wt.	404 ± 69	495 ± 186 A	352 ± 65 A, B	330 ± 82 A, B
Trachea, water	g./100 g. wet wt.	69·6 ± 5·9	71·4 ± 4·7	70·0 ± 7·5	67·6 ± 3·9 B
Trachea, iron	µg./g. dry wt.	191 ± 77	401 ± 308 A	187 ± 84 B	151 ± 56 B

* A value of $P < 0.05$ by a t test that the mean of Groups I, II, or III equalled that of the controls is indicated by A, that the mean of Group II or III equalled that of Group I by B, and that the mean of Group III equalled that of Group II by C.

DISCUSSION

The clinical course of phosgene poisoning is characterised first by a latent period of several hours following exposure. The rabbits of Group I were examined during this interval. Much pathological change had occurred during the latent period. Inhalation of the gas produced a mild irritation of the trachea which was temporary and not seen the next day. Irritation of the alveolar sacs was evidenced by mild oedema, congestion, and, probably compensatory, emphysema. Contraction of the pulmonary arteries was pronounced in many rabbits. Pulmonary vasoconstriction may have been due to increased susceptibility of the pulmonary arteries to, or to increased output of, noradrenaline which can also produce pulmonary congestion[17]. The condition made the animals susceptible to the lethal effect of urethane anesthesia.

The symptomatic phase of phosgene poisoning follows the latent period. Rabbits examined in this stage could be divided into two groups, one with advanced pulmonary oedema and a normal volume output of respiratory tract fluid (termed Group II), and a second with extreme pulmonary oedema and a tremendous premortal gush of respiratory tract fluid (termed Group III). The symptomatic phase is divided clinically into an early stage, a blue stage, and a grey stage[18]. The rabbits of Group II were in what corresponded approximately to the

early stage or first part of the blue stage in human poisoning. The animals of Group III were approximately in the late blue stage or grey stage.

The rabbits of Group II had the same resistance to urethane anaesthesia as the animals of Group I. Microscopically pulmonary oedema had progressed markedly but the water and chloride content of the lungs were not greater than in Group I. Respiratory tract fluid was normal in volume but contained markedly increased levels of sodium and chloride. There was more compensatory alveolar emphysema. The arteries were appreciably less contracted and there was a significant amount of pulmonary haemorrhage. The bronchioles and trachea had returned approximately to normal in microscopic appearance and water and iron content. The haematocrit and blood haemoglobin were appreciably elevated. In this group, therefore, pulmonary oedema had progressed considerably, but compensatory mechanisms made the rabbits no less resistant to urethane anaesthesia than the rabbits of Group I.

Resistance to urethane anaesthesia was significantly less in the rabbits of Group III. Respiratory tract fluid poured out of the lungs at a volume which averaged 60 times the normal rate. Respiratory tract fluid appeared to have become defibrinated plasma since its concentrations of sodium, chloride, and lipids were the same as those of blood plasma. Histologically there was a 4+ pulmonary oedema in all of these rabbits with no congestion, no emphysema, no haemorrhage, and no contracted arteries. The water and chloride levels of the lungs were greater than in the rabbits of Group II. The iron content of the lungs was even lower than in the controls, indicating that there was less blood than normal in the lungs. Levels of chloride and most lipids of blood plasma were elevated above normal and above the levels in Groups I and II. In this terminal group, therefore, the pulmonary capillaries had lost their semipermeability, blood plasma appeared in respiratory tract fluid, the pulmonary stroma was filled with oedema fluid, the blood supply of the lungs was reduced, haemoconcentration occurred, and resistance to urethane anaesthesia was reduced almost to zero.

Acknowledgement. The authors wish to acknowledge the assistance of Drs. G. H. Ettinger and W. C. Stewart and of J. Gastle, M. L. MacLachlan, P. E. Sheppard and M. E. T. Stevens.

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ASSAY OF DIGITALIS

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Received June 1, 1960

The uses of the outer walls of the right ventricles of guinea pigs and of auricles of rabbits for the assay of digitalis have been reinvestigated. The results obtained are compared with those obtained by slow intravenous infusion in guinea pigs (B.P. 1958) and the pigeon method (U.S.P. XV). The results obtained by the use of isolated auricles indicate that this method is reasonably accurate, cheap and less time-consuming than other methods and has the further advantage that the test sample is compared with the standard on the same tissue.

The chemical methods for the assay of the potency of digitalis preparations do not give satisfactory results, as digitalis contains several active principles with different chemical and pharmacodynamic properties. Unless it becomes possible to cultivate leaves with a fixed or nearly fixed content of glycosides, it is necessary to depend on biological methods.

Knaffl-Lenz[1] introduced the guinea pig method which is now official in B.P. 1958[2]. Hanzlik[3] proposed the use of pigeons for the assay of digitalis, as simple, economic and reasonably accurate. Hagg and Woodley[4] described a new intravenous pigeon method for the standardisation of digitalis and showed that results obtained by their method agree reasonably well with those of the cat method of Hatcher and Brody[5]. This method, as modified by Braun and Lusky[6], has been recognised as an official method in U.S.P. XV[7].

Trevan and Boock[8] suggested the use of isolated rabbit auricles for the estimation of digitalis, as they show a reversible increase of amplitude when digitalis is added to the bath and the increase is proportional to the dose added. Stewart[9] has studied the influence of digitalis preparation and of cardiac glycosides on an electrically stimulated isolated outer wall of the right ventricle of a guinea pig. He has shown that a digitalis preparation can be standardised by computing $\log_{10}$ time to zero amplitude for a standard and a test preparation, as the index of precision is highest when time for zero amplitude is taken as the metameter, in preference to first increase in amplitude, beginning of plateau, maximum amplitude, and duration of plateau. In the present study the uses of the outer walls of right ventricles of guinea pigs and of rabbit auricles for the assay of digitalis have been reinvestigated and compared.

The results obtained by these two methods are compared with the results obtained by B.P. 1958[2] method and U.S.P. XV[7] method.

Methods and Materials

Isolated Ventricle Method

Throughout the investigation, male albino guinea pigs weighing between 350 g. and 450 g. were used.

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A suitable wide glass tube closed at one end was used as the isolated organ bath (Fig. 1), which was suspended in a thermostatically controlled water bath to give a temperatue of 35° ± 1°.

Pure oxygen at the rate of 200 ml. per minute was bubbled through the Ringer solution[9]. A spring lever was balanced by a definite load, to keep the tension constant for all the preparations of ventricles. The ventricles were stimulated by a square wave stimulator—duration 5 msec., volts 50, rate one per 20 sec. Platinum electrodes (20 S.W.G.) were used; one was kept free near the ventricle preparation in the organ bath and the other was in contact with the ventricle preparation (Fig. 1). Extracts from two samples of *Digitalis purpurea* leaves were prepared by

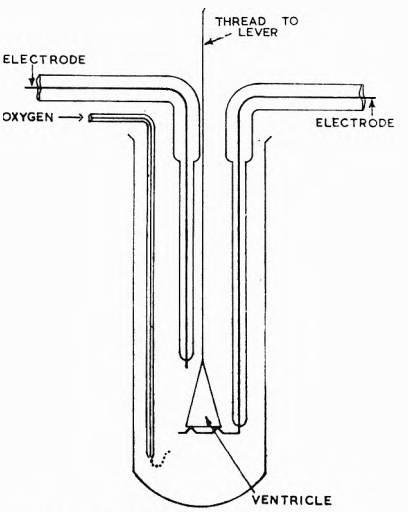

Fig.1. Isolated organ bath.

the B.P. 1958[2] procedure. To ensure the uniformity of the alcohol content in each preparation, ethanol was completely removed under vacuum from a definite volume of each extract and the residues obtained were taken up in a fresh quantity of a similar volume of ethanol (80 per cent v/v). The samples of *Digitalis purpurea* used had been standardised by B.P. 1958[2] and U.S.P. XV[7] procedures.

A guinea pig is killed by a blow on the head and the chest is opened immediately. The pulmonary artery is tied by means of a cotton thread after exposing the heart. The heart is then removed and put in warm well-oxygenated Ringer solution. The outer wall of the right ventricle is removed as quickly as possible and attached to the platinum electrode and suspended from the pulmonary artery side in oxygenated Ringer solution maintained at a temperature of 35° ± 1°. Before the addition of the drug, the ventricle preparation was allowed to settle for at least 2½ hours, as it was observed that during this period the amplitude of contraction often varies.

Three doses of each preparation were tried in a definite dose ratio. Each dose was tried on six ventricular strips. The preparation of the ventricle could not be used again, as it does not recover its response to stimulation even after repeated washings.

A typical kymographic record of an experiment is shown in Figure 2.

The time in minutes required for the first increase of amplitude (the first point at which the increase in amplitude is 1 mm. over the initial amplitude), beginning of plateau of maximum contraction (taken as the time required to reach 95 per cent of the maximum amplitude), duration

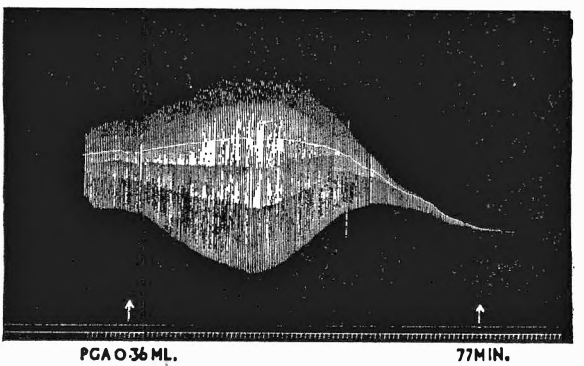

FIG. 2. Effect of digitalis on recorded contractions. Guinea pig, abino male, 370g.

of plateau (time embraced by the 95 per cent limits on maximum contraction), maximum amplitude, half of maximum amplitude and zero amplitude is recorded.

RESULTS

From the results obtained, it was seen that $\log_{10}$ time to maximum amplitude may be taken as a suitable metameter for the assay of digitalis. Analysis of variance[10] worked out for the two samples in comparisons with the standard, with respect to $\log_{10}$ time to maximum amplitude is tabulated in Tables I and II.

From the Tables it is seen that the component "Preparation" shows that there is an overall difference in response between each of the two

TABLE I

VALIDITY TESTS FOR PARALLELISM, CURVATURE AND DIFFERENCE OF CURVATURES WHEN $\log_{10}$ TIME TO MAXIMUM AMPLITUDE FOR STANDARD AND THE TEST A IS COMPUTED

Nature of variance	Degrees of freedom	Sum of squares	Mean square
Preparations	1	1,906·8	1,906·8
Regression	1	1,568·2	1,568·2
Parallelism	1	20·2	20·2
Curvature	1	2·7	2·7
Difference of curvatures ..	1	12·5	12·5
Between doses	5	3,510·4	
Error	30	1,287·0	42·9

preparations and the standard and the component "Regression" indicates that there is an increase in effect with an increase in dose.

Mean square for "Parallelism" is not significantly large for both the samples, indicating that the responses to the two preparations and the standard are similar, and shows that there is no significant deviation from parallelism.

The non-significance of "curvature" indicates that the regression for each of the preparations is satisfactorily linear. This is further enhanced by the non-significance of "difference in curvatures".

The Isolated Auricles Method

An adult rabbit is killed by a blow on the head and the heart removed immediately, following usual procedure, without damaging the auricles. Taking normal precautions, the auricles were separated and suspended

TABLE II

VALIDITY TESTS FOR PARALLELISM, CURVATURE AND DIFFERENCE OF CURVATURES WHEN LOG_{10} TIME TO MAXIMUM AMPLITUDE FOR STANDARD AND THE TEST B IS COMPUTED

Nature of variance	Degrees of freedom	Sum of squares	Mean square
Preparations	1	529·0	529·0
Regression	1	2,185·0	2,185·0
Parallelism	1	7·0	7·0
Curvature	1	48·4	48·4
Difference of curvatures ..	1	36·1	36·1
Between doses	5	2,805·5	
Error	30	2,043	68·10

in an organ bath containing a well oxygenated Ringer solution, as described by Trevan[8], maintained at a temperature of 37° ± 1°. A light lever, consisting of a spindle mounted on centres and having a spring collar for gripping straws, was used. The auricles were allowed to beat until they gave a uniform amplitude.

A measured volume of test extract and standard extract prepared as per B.P. 1958[2] procedure was taken and evaporated to dryness on a water bath, taking care not to over heat. The residues obtained were taken up in similar volumes of normal saline and filtered through cotton wool plugs.

Different doses of the extracts prepared as above were then added to the organ bath containing the auricles to find out a suitable dose which would give a measurable increase in amplitude. Two doses of test extract and two doses of the standard extract in the dose ratio of 1 : 2 were added to the organ bath and the increases in amplitude recorded. The speed of the drum is adjusted in such a way that each beat is recorded separately and clearly. Each dose was duplicated, triplicated or added four times, depending upon the working of the auricles. Normally the doses were duplicated, as it was observed that at a later stage the auricles lose sensitivity and also that the first few doses are considered as trial doses.

The kymograph record (Fig. 3) is taken, for each dose, first before the addition of the drug, then 1 minute after the addition of the drug and, finally, 5 minutes after the addition. The actual increase in the amplitude during the last 4 minutes is measured for comparison purposes.

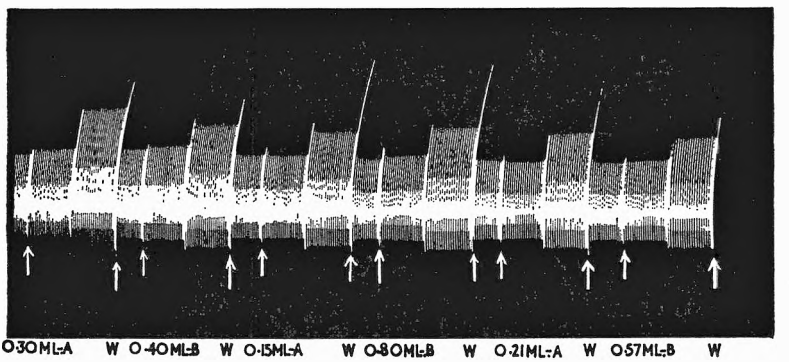

FIG. 3. Tracing showing the increase in auricular beat of isolated rabbit auricles after addition of digitalis in saline. A = Extract from leaf sample A. B = Extract from sample B. W = wash.

The auricles are washed in four changes of Ringer and allowed to beat for 20 minutes before another dose of the drug is added. Used in this way, the same auricles can respond to give satisfactory records for up to sixteen doses of the drug.

TABLE III

RESULTS OF ASSAY OBTAINED BY B.P. 1958, U.S.P. XV, ISOLATED OUTER WALL OF RIGHT VENTRICLE AND ISOLATED AURICLES METHODS

Digitalis purpurea	B.P. I.U./g.	U.S.P. XV I.U./g.	Isolated right ventricle I.U./g.	Isolated rabbit auricles I.U./g.
A	10·78	9·91	9·34	9·10
B	4·60	4·68	5·50	3·20
*C	—	11·12	—	9·77

* A third sample of *Digitalis purpurea* leaf

DISCUSSION

One important advantage of the auricle method over the right ventricle method is that only one animal is required for the assay and that the same auricles can be used for several doses of test and the standard; whereas in the case of ventricles, only one dose of one drug could be tried on one preparation.

In the case of the isolated ventricles, the study of the analysis of variance for digitalis leaf A shows that there is a linear relationship between dose and $\log_{10}$ time to the maximum contraction of ventricle and $\log_{10}$ time to beginning of plateau. When digitalis leaf B was studied, it was found that a linear relationship existed between dose and $\log_{10}$ time to maximum amplitude and $\log_{10}$ time to zero amplitude.

Log_{10} time to first increase for both the samples hardly alters with the increase in dose, therefore this could not be considered as a possible metameter.

Difference in time required for different metameters with different doses of the drug shows that there is a direct relationship of concentration of digitalis and the time required for its action.

In the case of isolated auricles, the initial amplitude increases with the addition of the drug, showing probably that some drug is fixed on or in the tissue, even after repeated washings. However, this does not interfere with the rise in amplitude, which remains proportional to the quantity of the drug added.

Acknowledgements. We wish to thank Mr. J. N. Darroch of the Statistical Laboratory for his advice in statistical analysis and Mr. E. D. Davison for valuable help during the experimental work. One of the authors, J. G. Bhatt, is greatly indebted to the Pharmaceutical Society of Great Britain for the grant without which this work would not have been possible.

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PHARMACOGNOSTIC STUDY OF *VALERIANA PYROLAEFOLIA* DECAISNE

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Received August 15, 1960

The macroscopical and microscopical characters of the roots, rhizomes and stolons of *Valeriana pyrolaefolia* Decaisne have been described.

Valeriana officinalis has been used in medicine in Western countries as an antispasmodic in the treatment of hysteria[1]. Wallis and Sanyal[2] described the macroscopical and histological characters of *V. wallichii*, an Indian substitute for valerian. In India a number of other species[3] of valeriana grow. The pharmacognosy of *V. hardwickii* and *Nardostachys jatamansi* has been described by Mehra and Garg[4]. In the present investigation the pharmacognostic characters of *V. pyrolaefolia* have been studied. This species is taxonomically closely related to *V. wallichii*. It differs[3,4] (Fig. 1) from the latter in having smaller broadly ovate radical leaves with obtuse apex and sessile cauline leaves. Both the species occur interspersed in more or less the same areas and there is a chance of their being admixed by unskilled collectors. Preliminary chemical investigation of *V. pyrolaefolia* also indicated that it may form a valuable substitute for the official valerian. An investigation of this drug was, therefore, undertaken.

Material and Methods

The material was collected from Kashmir, between Gulmarg and Khillanmarg. The authenticity was established by comparison with the description given in standard texts[3,5]. Roots, rhizomes and stolons were fixed in 50 per cent ethanol. Both free hand sections and paraffin embedded sections were cut for histological studies. Usual methods[6] of staining with safranin and fast green were followed.

Macroscopy

The dried drug (Fig. 2A) consists of vertical rhizomes, stolons and sub-horizontal rhizomes with numerous long wiry roots. The vertical rhizomes are knotty, undifferentiated into nodes and internodes and show roots and root scars. They are 0·4–0·8 cm. in diameter. The stolons vary in length from 5 to 8 cm. and in diameter from 0·25 to 0·5 cm., show distinct nodes and internodes and longitudinal wrinkles. The roots are seen arising from the undersurface of the nodes. The sub-horizontal rhizomes are more or less similar to the vertical rhizomes but can be differentiated by the presence of roots and root scars on the lower surface only. The roots are slender, 1–2 mm. in diameter and up to 10 cm. in length. The drug shows a short fracture, a distinct valerianaceous odour, a bitter camphoraceous taste and dark brown colour.

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Microscopy

RHIZOME. The old thick rhizome in diagrammatic transverse section (Fig. 2B) is circular in outline and shows from without inward, cork, cortex, endodermis, pericycle, a ring of open collateral vascular bundles and a large central pith. The cork (Fig. 2D) consists of 3 to 6 layers of radially arranged suberised, lignified, tangentially elongated rectangular cells. They are brownish-black in colour and occasionally contain oil globules. They measure R, 12–**24**–52 μ; T, 15–**31**–56 μ; L, 14–**33**–59 μ. A single layer of thin walled tangentially elongated cells measuring R, 11–**14**–19 μ; T, 35–**49**–57 μ, form the cork cambium. The cortex is

FIG. 1. The flowering plant of *Valeriana pyrolaefolia* × $\frac{1}{4}$.

composed of 16 to 20 layers of cells. The outer 2 to 4 layers of cells are polygonal in outline and are collenchymatous. They measure R, 23–**30**–46 μ; T, 28–**41**–59 μ; L, 35–**51**–81 μ. The inner layers of the cortex are thin walled, parenchymatous and are rounded to polygonal in outline. They show well-marked intercellular air spaces and measure R, 38–**48**–58 μ; T, 42–**57**–98 μ; L, 21–**40**–63 μ. Almost all the cells contain oil globules. The endodermis consists of rectangular, suberised and lignified cells. They frequently show passage cells and measure R, 13–**17**–20 μ; T, 31–**46**–70 μ; L, 24–**39**–63 μ. The pericycle is composed of thin walled parenchyma cells which measure R, 9–**15**–23 μ; T, 17–**32**–42 μ; L, 33–**45**–56 μ. The vascular bundles (Fig. 2C) are open collateral and are from 13 to 16 in number. The phloem is collenchymatous and shows sieve tubes and companion cells. The xylem

consists of xylem vessels, fibres and xylem parenchyma. The fibres are few, thick walled, pitted and measure up to 180 μ in length. The pith is large, showing lacunae and consists of parenchymatous cells. These cells contain starch grains and oil globules and measure R, 49–**51**–81 μ; T, 39–**60**–73 μ; L, 37–**53**–59 μ.

STOLON. The stolon in diagrammatic transverse section (Fig. 3B) is similar to the rhizome. It differs from the rhizome in having 12 to 13 vascular bundles and by the absence of lacunae in the pith. The cork is

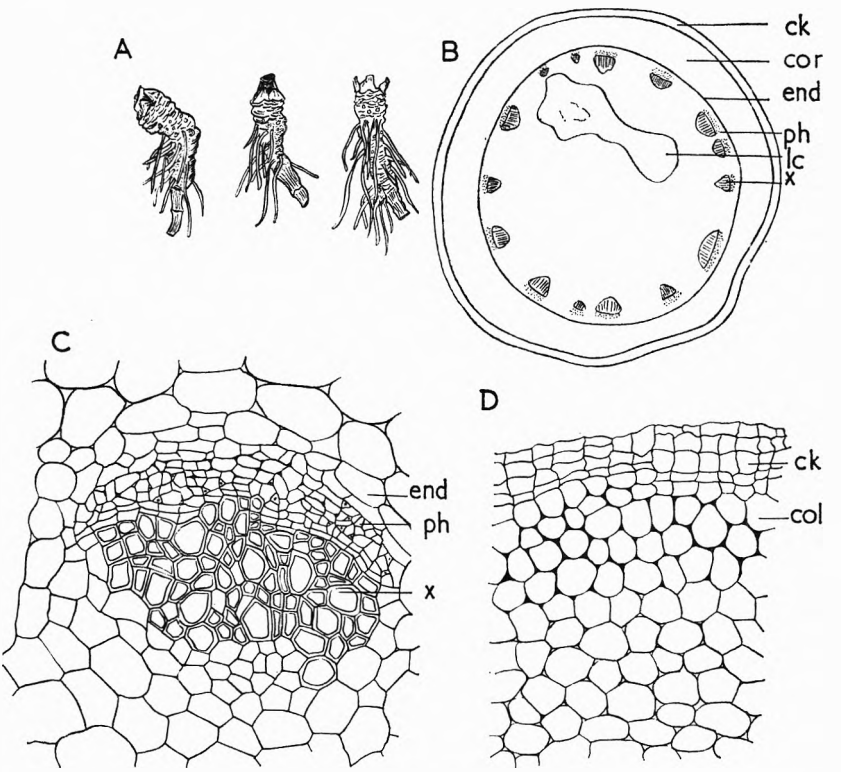

FIG. 2. A, the dried drug of *V. pyrolaefolia* × 2/3. B, T.S. rhizome of *V. pyrolaefolia* (diagrammatic) × 6; ck, cork; cor, cortex; end, endodermis; lc, lacunae; ph, phloem; x, xylem. C. T.S. rhizome of *V. pyrolaefolia* showing vascular bundle × 175; end, endodermis; ph, phloem; x, xylem. D, T.S. rhizome of *V. pyrolaefolia*, showing cork and cortex × 125; ck, cork; col, collenchyma.

1 to 2 layers thick and is brownish-black in colour. The cells measure R, 11–**26**–47 μ; T, 8–**21**–36 μ; L, 31–**56**–77 μ. The cortex consists of about 13 layers of cells. The outer 3 to 4 layers are collenchymatous as in case of the rhizome. They measure R, 14–**27**–41 μ; T, 21–**31**–49 μ; L, 45–**67**–97 μ. The next 9 to 10 layers of cells are parenchymatous, show intercellular air spaces and are filled with starch grains and oil globules. They measure R, 28–**47**–61 μ; T, 27–**51**–73 μ; L, 31–**54**–87 μ. The endodermis is single layered and consists of broadly rectangular suberised and lignified cells. The passage cells are also

present. The endodermis cells measure R, 11–**21**–28 μ; T, 17–**32**–45 μ; L, 35–**46**–56 μ. The pericycle is formed of 1 to 3 layers of parenchyma cells measuring R, 7–**12**–18 μ; T, 17–**21**–28 μ; L, 49–**59**–89 μ. The vascular bundles have 4 or 5 layers of collenchyma associated with the phloem. The xylem consists of xylem vessels, tracheids and xylem parenchyma. The pith cells are also similar to those of rhizome and measure R, 24–**39**–52 μ; T, 21–**35**–59 μ.

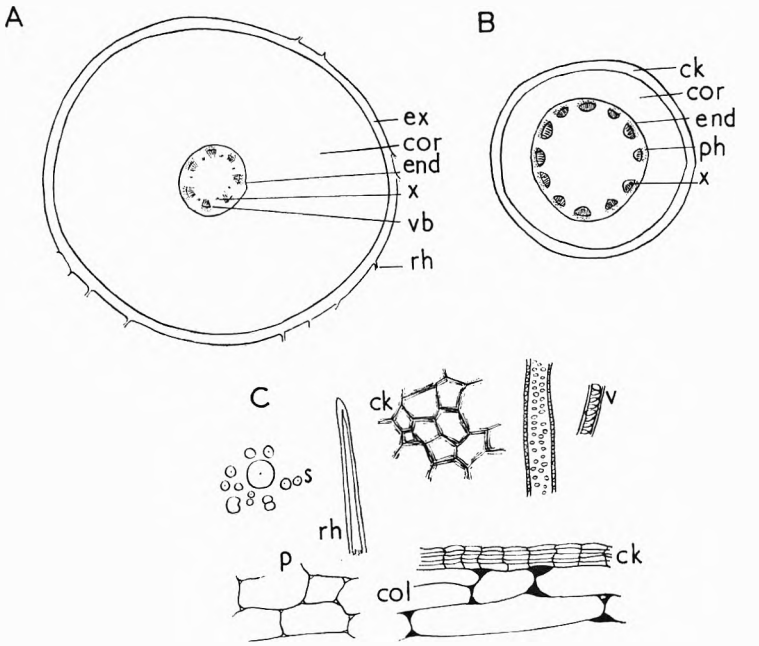

FIG. 3. A, T.S. root of *V. pyrolaefolia* (diagrammatic) × 30; cor, cortex; end, endodermis; ex, exodermis and piliferous layer; rh, root hair; vb, vascular bundle; x, primary xylem. B, T.S. stolon of *V. pyrolaefolia* (diagrammatic) × 6; ck, cork; cor, cortex; end, endodermis; ph, phloem; x, xylem. C, microscopic view of *V. pyrolaefolia* powder × 80; ck, cork; col, collenchyma; rh, root hair; p, parenchyma; s, starch; v, vessels.

ROOT (Fig. 3A). The outermost layer of the root, the piliferous layer, consists of collapsed rectangular cells which are dark-brown in colour and measure R, 7–**11**–17 μ; T, 14–**22**–27 μ; L, 34–**67**–95 μ. It is suberised and lignified. Frequently these cells give rise to root hairs which are also suberised and lignified. The exodermis consists of rectangular cells, the anticlinal walls of which are wavy. These cells are also suberised and lignified and measure R, 12–**26**–35 μ; T, 37–**51**–63 μ; L, 37–**47**–70 μ. The cortex consists of about 21 layers of cells. The outermost 1 to 2 layers are collenchymatous and are polygonal to rectangular in outline. They measure R, 13–**28**–42 μ; T, 21–**27**–49 μ; L, 56–**81**–129 μ. The rest of the cells of the cortex are parenchymatous, circular to ovoid in outline and measure R, 18–**37**–56 μ; T, 24–**39**–53 μ; L, 77–**106**–167 μ. They contain starch grains and oil globules. The endodermis is formed of

suberised and slightly lignified rectangular cells. Passage cells are occasionally present. The cells of the endodermis measure R, 4–**31**–21 μ; T, 14–**23**–35 μ; L, 32–**43**–77 μ. The pericycle consists of 1 to 2 layers of collenchyma cells, polygonal in outline, measuring R, 9–**15**–21 μ; T, 14–**23**–31 μ; L, 28–**47**–81 μ. The xylem after secondary growth consists of xylem vessels and tracheids. The pith cells are polygonal, show slight thickening at the angles and measure R and T, 11–**13**–18 μ. The primary structure of the root (Fig. 3A) varies from tetrarch to heptarch.

Powdered Drug

Macroscopy

The powdered drug is dark yellowish-brown in colour. It gives a bluish-black colour with ferric chloride reagent and a yellowish-brown colour with 5 per cent potassium hydroxide solution. It has a strongly valerianaceous odour and a slightly bitter aromatic taste.

Microscopy

The powdered drug under the microscope (Fig. 3C) shows rounded to irregular starch grains with central dot-like hilum. The starch grains are usually simple but may be 2-compound and measure up to 24 μ in diameter, the average diameter being 7 μ. Fragments of pitted or spiral vessels are also discernible. The powder also shows fragments of collenchymatous tissue and cork cells as well as thin walled parenchyma cells. Occasionally thick walled root hairs may be seen. When the powder is mounted in chloral hydrate, no crystals appear as reported in the case of *V. wallichii*[2].

Discussion

It will be clear from the foregoing description that the characters of *V. pyrolaefolia* are quite distinct from the characters reported[3] for *V. wallichii*. The former can be differentiated from the latter by the smaller size of the rhizome and stolon and by distinct differences in the cell dimensions of the various tissues. It can be further differentiated by the absence of secondary xylem in the interfascicular region and by the absence of lacunae in the pith of the stolon. In *V. pyrolaefolia* the pericycle is collenchymatous in roots while in *V. wallichii* it is not so. The powder of *V. pyrolaefolia* can also be differentiated from that of *V. wallichii*[2] by the shape and size of starch grains as well as by the fact that it does not give any crystals on treatment with chloral hydrate solution.

Acknowledgement. Our thanks are due to Dr. K. N. Gaind, Head of the Pharmacy Department, Panjab University for encouragement during the course of this investigation.

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INFLUENCE OF HALOPERIDOL (R 1625) AND OF HALOPERIDIDE (R 3201) ON AVOIDANCE AND ESCAPE BEHAVIOUR OF TRAINED DOGS IN A "JUMPING BOX"

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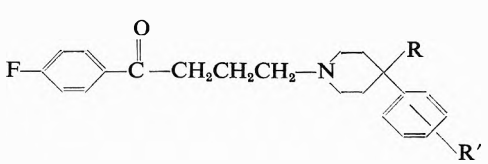
Received August 3, 1960

The experimental details of a technique for establishing conditioned responses on the basis of escape and avoidance behaviour of dogs in an electrically charged "jumping box" are described. The method is similar to the well-known Warner technique for rats. The influence of two new neuroleptic agents, haloperidol (R 1625) and haloperidide (R 3201), on the behaviour of trained dogs is reported. One and five hours after injection the effects of both compounds are qualitatively and quantitatively indistinguishable. Haloperidol has a longer duration of action. These results are of particular interest in view of the fact that haloperidide is about ten times more active than haloperidol as an antagonist of apomorphine-induced emesis in dogs.

The literature contains many descriptions of techniques for establishing conditioned responses on the basis of an escape response of the animal as a whole from an electrically charged grill. The first apparatus of this sort—referred to as a "jumping box" in this paper—was described for rats in 1932 by Warner [1,2]. It consists of a box with two compartments separated by a partition. The floor of each compartment is a grill which can be charged electrically. The rat can escape the aversive stimulus (shock) by making the only appropriate "escape response", that is to jump into the other compartment. The rat can also avoid the aversive stimulus by making a conditioned "avoidance response" when presented with a warning signal, for example the sound of a buzzer or bell, a short time before presentation of the shock. Many modifications of this "jumping box" technique are described for rats[1–13].

The purpose of this paper is to describe the experimental details of a "jumping box" technique for dogs which we have tried to standardise. To illustrate the technique, the effects of two drugs on the behaviour of trained dogs will be described. Both compounds are neuroleptics of the butyrophenone-series and were originally synthesised in this laboratory.



Haloperidol (R 1625): R = OH; R′ = *p*-Cl

Haloperidide (R 3201): R = CON⟨ ⟩ ; R′ = *m*-Cl

Pharmacological and clinical properties of haloperidol have been described by many authors[14–55]. The pharmacology of haloperidide will be described in detail in subsequent papers from this laboratory. One point of particular interest in connection with this study is the extremely high activity of haloperidol and of haloperidide as antagonists of apomorphine-induced emesis in dogs. One to eight hours after subcutaneous injection we find haloperidide (ED50 = 0·0025 mg./kg. s.c.) about 10 times more active than haloperidol (ED50 = 0·025 mg./kg. s.c.) in this test[35,37,47].

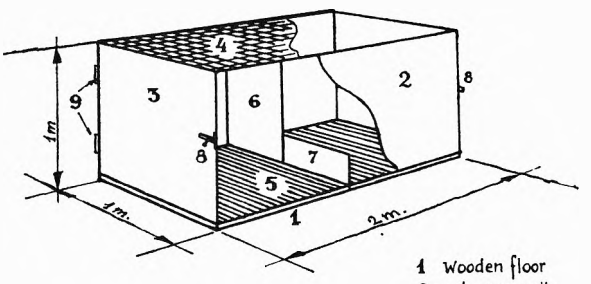
METHOD

Subjects. Eight adult male mongrel dogs of unknown age were the subjects. They had initial weights in kg. (dog 1: 12·5; 2: 13·2; 3: 10·8; 4: 8·5; 5: 10·9; 6: 8·2; 7: 9·7; 8: 11·2).

Administration of the drugs. Aqueous solutions of haloperidol and of haloperidide were prepared for subcutaneous injection of 0·5 ml./kg. weight. Each dog received at random, using an 8 × 8 latin square design, all eight of the following doses at weekly intervals:

haloperidol (R 1625):	0·005 mg./kg.	= x_1
	0·02 mg./kg.	= x_2
	0·08 mg./kg.	= x_3
	0·31 mg./kg.	= x_4
haloperidide (R 3201):	0·005 mg./kg.	= y_1
	0·02 mg./kg.	= y_2
	0·08 mg./kg.	= y_3
	0·31 mg./kg.	= y_4

Apparatus (*jumping box*). The cage, Figure 1, consists of a steel frame, 2 m. long, 1 m. wide and 1 m. high, made of L-shaped steel. The bottom is made of $\frac{3}{4}$ in. thick wood. The top is covered with 2 cm. square galvanised wire gauze, the walls are made of $\frac{1}{8}$ in. thick asbestos cement



1 Wooden floor
2 asb.-cem. wall
3 asb.-cem. door
4 wire gauze top
5 grate
6 perspex wall
7 hurdle
8 bolts
9 hinges

FIG. 1.

sheets. Both ends of the cage can be opened and are used as doors. They are covered with asbestos cement and provided with hinges and bolts. The floor consists of a grate made of 8 mm. iron bars, electrically isolated in two groups dividing the cage in two compartments (A and B), alternatively being connected to the shock source.

In the middle of the cage, separating the two compartments a perspex wall of 50 cm. wide and $\frac{5}{8}$ in. thick, bars half of the transit from one compartment into the other. The free half shows a smaller wall or hurdle of about 20 cm. high of the same material, which the animal must jump over to reach the other compartment.

By means of a variac (1 k. VA.) a voltage of $\pm$30–50 V., a.c. at 50 c.p.s. is given to the floor grid of one compartment. This voltage may be

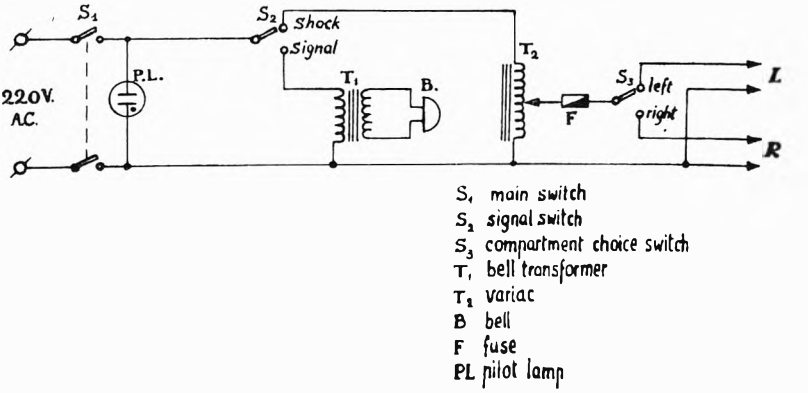


FIG. 2.

switched off by the two-way signal switch to connect the signal bell (5 V., with step-down transformer). During the signal period a second two-way switch makes it possible to electrify the second compartment. By switching back the signal switch to shock-position, the shock-voltage will be connected to the first compartment (Fig. 2).

Description of one cycle. Avoidance conditioning in dogs was progressively achieved by subjecting the animals twice a day, except Saturday and Sunday, at 5-hour intervals to a series of 10 cycles of conditions. Each cycle, Figure 3, has a duration of 1 minute (T_3) and consists of periods T_1 (1 to 15 seconds) and T_2 (59 to 45 seconds).

The dog is placed on the unelectrified grid floor of compartment A of the box.

(*a*) Warning stimulus: a bell (S_1) is rung for a maximum of 15 seconds (T_1) or until the animal jumps from compartment A into B. If jumping occurs during the 15 second period (T_1) it stops the bell and is defined as an "avoidance response" (R_1). During the T_1 period both compartments of the box are current-free.

(*b*) Aversive stimulus: if the animal does not leave its compartment during the signal period S_1, a continuous electric shock (S_2) is delivered during a period T_2 with a maximal duration of 45 seconds. The T_2 period can be terminated by an "escape response".

(*c*) "Silence stimulus" (S_3), that is, absence of S_1 or S_2. A new response occuring during the same cycle under S_3 is stimulated by shock, necessitating another escape response, and defined as a "paradoxal response" (R_3). R_3 may obviously occur after R_2 during the same cycle.

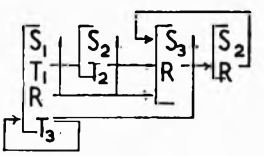
A series of 10 cycles of 10 minutes' total duration is defined as a session.

Training period. During the training period the eight dogs were subjected daily to a morning and afternoon session, with 5 hours in between, except on Saturdays and Sundays. Except for the first day of the training period, the observer did not actively interfere with the behaviour of the dogs. Training was continued until 20 out of 20 avoidance responses (R_1) were observed on a given day. Such an animal was considered to be "adequately" trained.

Design of the experiment. The eight "adequately" trained dogs were subjected to two daily sessions 5 days a week for 8 weeks.

	Sessions		Hours before (−) or after (+) injection	
	a.m.	p.m.	a.m.	p.m.
Monday	−4	−3	−48	−43
Tuesday	−2	−1	−24	−19
Wednesday	1	2	+1	+5
Thursday	3	4	+25	+30
Friday	5	6	+49	+54

On Wednesday morning each animal was given a subcutaneous dose of haloperidol (x_1 to x_4) or of haloperidide (y_1 to y_4) as described above. At the end of the 8 weeks each dog had received all doses of both compounds in a random order.



S_1 = warning stimulus (bell).
T_1 = duration of S_1 (1 to 15 seconds).
S_2 = aversive stimulus (shocks).
T_2 = duration of S_2 (1 to 45 seconds).
S_3 = "silence" stimulus with a duration of 0 to 59 seconds.
T_3 = 60 seconds, i.e., the period with which these conditions recycle.
R = "response", i.e., jumping from one compartment to the other.
R_1 = R occurring under S_1 (avoidance response).
R_2 = R occurring under S_2 (escape response).
R_3 = R occurring under S_3 (paradoxal response).

FIG. 3. Symbolisation of one cycle (ref. 56).

RESULTS AND DISCUSSION

1. *Training Period*

The relevant data concerning the behaviour of the eight dogs during the training period are summarised in Tables I, II and III.

All dogs were trained for 8 or more days. A minimum of 5 and a maximum of 11 days was required for adequate training.

Table I shows the significance of the criteria $\overline{T}_1$, $\overline{T}_2$, fR_2 and fR_3 to decrease as a function of time. $\overline{T}_1$ reaches a lower limit of roughly 3 seconds after about 2 weeks of training, whereas $\overline{T}_2$, fR_2 and fR_3 approaches zero.

It is obviously easier for a dog to learn how to avoid aversive stimulation in situation S_1 (bell) than in situation S_3 (silence). Escape or avoidance responses were observed in all trials throughout the training period.

TABLE I

TRAINING PERIOD (8 DAYS): DAILY VALUES FOR THE 8 DOGS (20 CYCLES PER DAY)

d	$\overline{T}_1$*	$\overline{T}_2$	fR_2	fR_3†	fR
1	8·6	1·5	5·8	4·0	160
2	6·5	0·41	3·1	5·8	160
3	5·8	0·44	2·4	3·9	160
4	5·2	0·15	1·1	4·0	160
5	4·6	0·06	0·38	0·88	160
6	4·3	0·09	0·50	1·4	160
7	3·8	0·02	0·25	0·63	160
8	3·4	0·07	0·25	0·63	160

* $\overline{T}_1$: mean duration of T_1 per cycle (max. 15 seconds).
$\overline{T}_2$: mean duration of T_2 per cycle (max. 45 seconds).
fR_2: mean frequency of R_2 per 20 cycles (max. 20).
fR_3: mean frequency of R_3 per 20 cycles (max. 20).

† Frequency computed on the basis of a simple alternative criterion: response or no response in a given cycle. It should be noted, however, that several responses R_3 per cycle were sometimes observed.

Table II summarises relevant data on the individual characteristics of the behaviour of each dog during the 8 days.

On the basis of these data the following alternative criteria are proposed for the purpose of classifying the eight dogs in four categories A, B, C and D:

(1) "slow dog": $\overline{T}_1 > 5$ seconds.

(2) "quick dog": $\overline{T}_1 < 5$ seconds.

(3) $fR_3/fR_2 < 1$.

(4) $fR_3/fR_2 > 1$.

Hence

		fR_3/fR_2	
		<1	>1
$\overline{T}_1$	slow	Dog 7 (A)	Dogs 1, 2, 3, (B)
	quick	Dog 5 (C)	Dogs 4, 6, 8, (D)

For the six dogs of categories B and D we found a highly significant positive correlation between $\overline{T}_1$ and $\overline{T}_2$ ($\overline{T}_2$ = 0·19, 0·19 and 0·45 for category B and T_2 = 0·01, 0·06 and 0·06 for category D). Dogs No. 5 and 7, however, making relatively less paradoxal responses (R_3), are characterised by high values of $\overline{T}_2$ (0·81 and 0·82).

Table III shows that there is no striking correlation between the frequency of R_2 and of the *n*th cycle, the probability of occurrence varying from 6 to 17/128 per cycle. Paradoxal responses, however, occur most frequently ($P < 0{\cdot}05$) during the first cycles of a given session. No significant correlation was found between body weight and any of these training period data.

Pre-injection period. Throughout the eight experimental weeks all eight dogs were subjected to two daily sessions of 10 cycles each on both

TABLE II

TRAINING PERIOD (8 DAYS): MEAN VALUES FOR EACH DOG (160 CYCLES PER DOG)

	Dog No.							
	1	2	3	4	5	6	7	8
$\bar{T}_1$*	6·5	6·2	5·7	4·1	4·5	3·4	7·4	3·9
	(2)	(3)	(4)	(6)	(5)	(8)	(1)	(7)
$\bar{T}_2$	0·45	0·19	0·19	0·01	0·81	0·06	0·82	0·06
	(3)	(4½)	(4½)	(8)	(2)	(6½)	(1)	(6½)
fR_2	17	19	14	2	11	7	34	6
	(3)	(2)	(4)	(8)	(5)	(6)	(1)	(7)
fR_3	35	32	26	16	8	17	14	23
	(1)	(2)	(3)	(6)	(8)	(5)	(7)	(4)
$fR_2 + fR_3$	52	51	40	18	19	24	48	29
	(1)	(2)	(4)	(8)	(7)	(6)	(3)	(5)
fR_3/fR_2	2·1	1·7	1·9	8·0	0·73	2·4	0·41	3·83
	(4)	(6)	(5)	(1)	(7)	(3)	(8)	(2)

* $\bar{T}_1$: mean duration of T_1 per cycle (max. 15 seconds).
$\bar{T}_2$: mean duration of T_2 per cycle (max. 45 seconds).
fR_2: total frequency of R_2 (max. 160).
fR_3: total frequency of R_3 (max. 160).

TABLE III

TRAINING PERIOD (8 DAYS) TOTAL FREQUENCY OF R_2 (fR_2) AND OF R_3 (fR_3) FOR THE 8 DOGS (MAXIMUM FREQUENCY: 8 DOGS × 8 DAYS × 2 SESSIONS = 128)

			Ranking of	
*n**	fR_2	fR_3	fR_2	fR_3
1	7	26	9	1
2	12	23	5	2
3	17 (57)	21 (109)	1	3
4	13	20	3½	4
5	8	19	8	5
6	6	14	10	8
7	13	12	3½	9
8	14 (53)	16 (62)	2	6
9	9	15	7	7
10	11	5	6	10
	110/1280	171/1280		

* n = the n th cycle of a session.

pre-injection days, i.e., on Monday and on Tuesday. Only two "errors" (of type R_3) occurred in these 2,560 (8 dogs × 8 weeks × 2 days × 20 cycles) cycles, showing that all subjects were "adequately" trained.

Table IV summarises the most important data pertaining to the behaviour of the dogs during the pre-injection period. Obviously $\bar{T}_1$ is not correlated with a particular week, showing the absence of significant after-effects of previous doses. Neither are the $\bar{T}_1$-values correlated with the sessions, the reaction time remaining about the same throughout.

There are no significant differences between morning and afternoon experiments as far as $\overline{T}_1$ is concerned.

Surprisingly, we found no correlation of the average reaction time per dog for the training period and for the pre-injection days as shown by the following rankings of $\overline{T}_1$:

Dog	Training period	Pre-injection days
1	6·5 (2)	1·9 (6)
2	6·2 (3)	3·6 (3)
3	5·7 (4)	1·8 (7)
4	4·1 (6)	4·1 (1)
5	4·5 (5)	3·7 (2)
6	3·4 (8)	1·6 (8)
7	7·4 (1)	3·2 (4)
8	3·9 (7)	3·0 (5)

The effects of haloperidol and of haloperidide. All eight dogs were treated at random and at weekly intervals with eight different doses, as described. The effects of each dose were measured at six time intervals

TABLE IV

PRE-INJECTION DAYS

Week	$\overline{T}_1$	Dog	$\overline{T}_1$	Cycle	$\overline{T}_1$ a.m.	$\overline{T}_1$ p.m.
1	2·7	1	1·9 (6)	1	2·8	2·8
2	3·1	2	3·6 (3)	2	3·3	3·1
3	2·7	3	1·8 (7)	3	3·2	3·3
4	2·9	4	4·1 (1)	4	3·2	3·4
5	2·8	5	3·7 (2)	5	3·3	3·4
6	3·1	6	1·6 (8)	6	3·3	3·4
7	3·0	7	3·2 (4)	7	3·3	3·3
8	2·9	8	3·0 (5)	8	3·1	3·3
average	2·9	average	2·9	9	3·3	3·2
				10	3·2	3·1
				average	2·9	2·9

after injection. A total of 1920 post-injection cycles (8 dogs × 4 doses × 6 sessions × 10 cycles) are therefore available for each drug. The frequency of the various types of responses was computed as follows:

	fR	fR_1	fR_2	fR_3	Maximum
Haloperidol ..	1726	1557	69	14	1920
Haloperidide ..	1786	1719	67	9	1920
Total	3512	3276	136	23	3840

The animals failed to respond 194 times after haloperidol and 134 times after haloperidide. Avoidance responses were observed more frequently after haloperidide ($fR_1 = 1719$) than after haloperidol (1557). The frequency of escape responses (fR_2) and paradoxal responses (fR_3) after haloperidol was also slightly greater. On the basis of these figures, haloperidol seems to be somewhat more active than haloperidide.

This difference in activity, however, is mainly due to the longer duration of action of haloperidol (Table V). One and 5 hours after injection both substances are about equi-active. The second day, however, haloperidol

is still strikingly active and haloperidide much less so. The two lower dose levels of both compounds (0·005 and 0·02 mg./kg.) have no influence on escape behaviour. They do produce, however, a slight but significant

TABLE V

EFFECTS OF HALOPERIDOL AND OF HALOPERIDIDE AT VARIOUS INTERVALS AFTER INJECTION (SEC.)

		1st day		2nd day		3rd day		
	Dose*	a.m.	p.m.	a.m.	p.m.	a.m.	p.m.	Maximum
$\overline{T_1}$	x_1	3·2	3·4	3·0	2·8	3·0	2·9	15
	x_2	3·7	3·3	3·2	3·2	3·2	2·9	15
	x_3	11·6	12·0	3·1	3·6	2·3	3·2	15
	x_4	13·1	14·4	8·8	10·3	4·4	4·3	15
	Σx	8·1		4·8		3·4		15
	y_1	3·4	3·7	2·7	2·7	2·6	2·5	15
	y_2	3·6	4·7	3·5	3·2	3·1	2·8	15
	y_3	9·4	13·2	3·4	3·2	3·1	2·9	15
	y_4	12·5	14·1	5·6	4·4	3·6	3·4	15
	Σy	8·1		3·6		3·0		15
$\overline{T_2}$	x_1	—	—	—	—	—	—	45
	x_2	0·04	—	—	—	—	—	45
	x_3	17·2	15·6	—	—	—	—	45
	x_4	27·9	32·1	10·3	11·8	—	—	45
	Σx	11·6		2·8		0		45
	y_1	—	0·16	—	—	—	—	45
	y_2	—	0·14	—	—	—	—	45
	y_3	12·0	16·4	—	—	—	—	45
	y_4	25·3	27·8	—	—	—	—	45
	Σy	10·2		0		0		45
fR	x_1	80	80	80	80	80	80	80
	x_2	80	80	80	80	80	80	80
	x_3	50	52	80	80	80	80	80
	x_4	33	29	62	60	80	80	80
	Σx	484		602		640		640
	y_1	80	80	80	80	80	80	80
	y_2	80	80	80	80	80	80	80
	y_3	60	53	80	80	80	80	80
	y_4	38	35	80	80	80	80	80
	Σy	506		604		640		640
fR_1	Σx	432		485		640		640
	Σy	439		640		640		640
fR_2	x_1	—	—	—	—	—	—	80
	x_2	1	—	—	—	—	—	80
	x_3	13	12	—	—	—	—	80
	x_4	10	16	4	13	—	—	80
	Σx	52		17		0		640
	y_1	—	1	—	—	—	—	80
	y_2	—	3	—	—	—	—	80
	y_3	11	20	—	—	—	—	80
	y_4	11	21	—	—	—	—	80
	Σy	67		0		0		640
fR_3	x_1	—	—	—	—	—	—	80
	x_2	3	1	2	—	—	—	80
	x_3	—	3	3	1	—	—	80
	x_4	—	—	1	—	—	—	80
	Σx	7		7		0		640
	y_1	—	2	—	—	—	—	80
	y_2	—	1	—	1	—	—	80
	y_3	—	2	2	—	—	—	80
	x_4	—	—	—	1	—	—	80
	Σy	5		4		0		640

* See text for details.

increase of the reaction time of the avoidance response as well as a few paradoxal responses. After injection of 0·08 mg./kg. of both drugs these same effects were much more pronounced and significant inhibition of escape behaviour was observed with both drugs up to 5 hours after dosage.

The highest dose (0·31 mg./kg.) produced essentially similar effects. The duration of action of this dose is clearly more prolonged. The frequency of the paradoxal responses (fR_3), however, was significantly lower (Table V). As shown in Table VI we find considerable variation among dogs during the post-injection period. On the basis of different individual total frequencies of avoidance (fR_2) and escape-loss (480-fR) the eight dogs may be classified in four categories (A to D).

	fR_2	480-fR	Dogs
A	normal	high	No. 7
B	normal	normal	No. 2, 5, 6, 8
C	normal	low	No. 1, 3
D	high	low	No. 4

There is no significant relation between the behaviour of the animals during the training period and their sensitivity to haloperidol and haloperidide. We found furthermore no correlation between the frequency

TABLE VI

INDIVIDUAL FREQUENCIES OF THE VARIOUS TYPES OF RESPONSE (R, R_1, R_2 AND R_3) AFTER INJECTION (COMBINED DATA PER DOG FOR ALL EIGHT DOSES AND ALL SIX SESSIONS AFTER INJECTION)

Dog No.	fR	fR_1	fR_2	fR_3
1	463 (7)	459 (7)	4 (8)	3 (4)
2	438 (5)	421 (6)	17 (4)	2 (5½)
3	479 (8)	466 (8)	13 (5)	1 (7)
4	457 (6)	411 (3½)	46 (1)	2 (5½)
5	428 (3)	409 (2)	19 (2)	6 (1)
6	429 (4)	411 (3½)	18 (3)	0 (8)
7	389 (1)	381 (1)	8 (7)	4 (3)
8	424 (2)	412 (5)	12 (6)	5 (2)
Max.*	480	480	480	480
Total	3507	3370	137	23

* 1 dog × 8 doses × 6 sessions × 10 cycles = 480.

of paradoxal responses (fR_3) observed during the training- and the post-injection periods (Tables II and VI) or between the individual sensitivity of the animals to the drugs and their average reaction times (T_1) during the pre-injection days (Table IV).

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A NEW COLORIMETRIC METHOD FOR THE DETERMINATION OF CHLORAL HYDRATE

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Received September 13, 1960

A colorimetric method is described for the determination of chloral hydrate, particularly in the presence of its decomposition products. Quinaldine ethiodide reacts with chloral hydrate in alkaline solution to produce a stable, blue cyanine dye. Chloroform, trichloracetic acid and formic acid do not react under the conditions described. The colour produced conforms to the Beer-Lambert law up to 100 μg./10 ml. of reaction mixture.

In the course of work on the stability of chloral hydrate preparations it became necessary to determine chloral hydrate in the presence of its decomposition products, viz. trichloracetic acid, formic acid, hydrochloric acid[1] and possibly chloroform. Conventional macro methods for estimating chloral hydrate, based on alkaline hydrolysis or total chlorine estimation, are subject to interference from the decomposition products mentioned above and in an attempt to find a more specific method, attention was turned to colorimetric methods.

With one exception, colorimetric methods for the estimation of chloral hydrate[2–7] are based on the reaction of chloral hydrate and pyridine in alkaline solution to produce a red colour, Fujiwara's reaction[8] (also attributed to Ross[9]). Although sensitive, this is not specific for chloral hydrate and a positive reaction is given by many poly-halogenated aliphatic compounds, including chloroform and trichloracetic acid. As a result, chloral hydrate in the presence of trichloracetic acid must be determined by difference. Meyer and Lee-Motter[5] determine both compounds together by means of the Fujiwara reaction and trichloracetic acid after hydrolysis of the chloral hydrate and removal of the chloroform produced; Friedman and Cooper[7] use a similar procedure but make use of the maximum at 370 mμ instead of that at 540 mμ. Fujiwara's reaction has been used for the colorimetric estimation or detection of many polyhalogen compounds; trichloroethylene[10,11], trichlorethanol[5,7,10] carbon tetrachloride[12], urochloralic acid[5], chloroform[13,14], chloralose[15] trichloracetic acid[5,7,10,16], tertiary acetylenic halogenated alcohols[17] and chloral urethane[18].

Stehwien and Kühmstedt[19] describe a colour reaction of chloral hydrate, which they adapted for quantitative purposes, based on the reaction of chloral hydrate, hydroxylamine hydrochloride and 2,6-diaminopyridine in acid solution to produce a red pyrisatin dye. Although apparently specific, the reaction conditions are critical and the reaction is relatively insensitive. In addition 2-6-diaminopyridine is not readily available.

Other colour reactions of chloral hydrate described in the literature[20–23] also lack selectivity in that similar colours are produced by other aldehydes or halogen compounds.

The colour reaction of Feigl[24], although specific, has the disadvantage of using concentrated sulphuric acid, which produces colours or charring with many organic compounds.

In 1934 Ogata and Suzuki[25] reported the preparation of cyanine dyes from chloral hydrate by condensation in alkaline solution with quinaldine

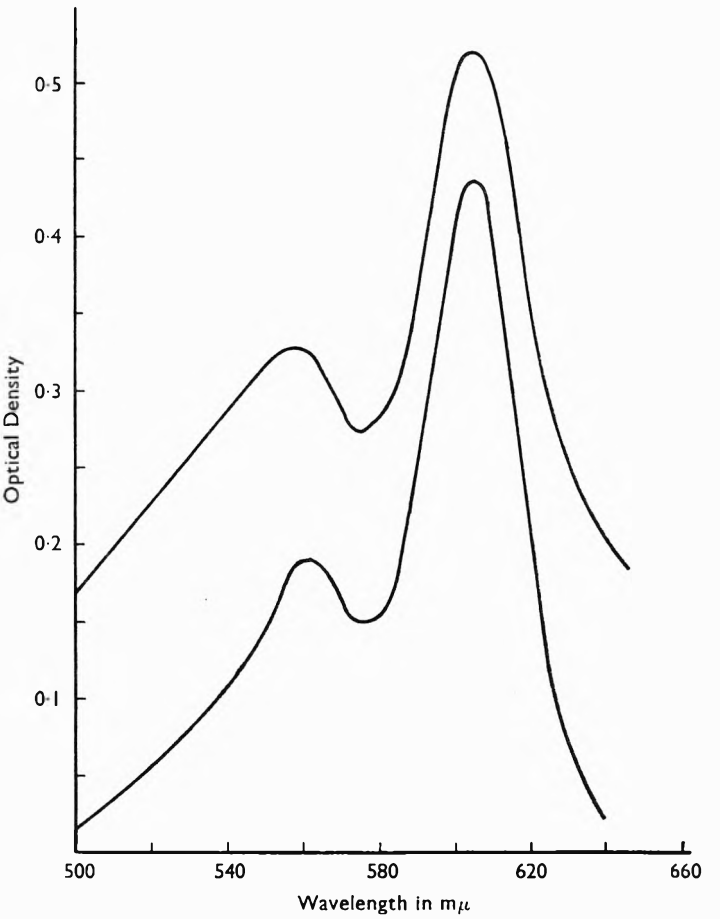


FIG. 1. Comparison of absorption spectra.
Upper curve: colour obtained from chloral hydrate.
Lower curve: 1,1′-Diethyl-2,2′-carbocyanine iodide.

ethiodide, lepidine ethiodide and α-picoline ethiodide. This reaction was examined with a view to adapting it to the quantitative colorimetric estimation of chloral hydrate.

EXPERIMENTAL

Preliminary experiments showed that quinaldine ethiodide was the most sensitive reagent and was studied in detail; chloroform and trichloracetic

acid were found not to react and the reaction was therefore investigated further.

Ammonia and (mono)ethanolamine were found to be the most effective alkalies and the presence of a water soluble alcohol increased the sensitivity of the reaction. The colour produced has a sharp maximum at 605 mμ and a wider maximum at 555–560 mμ (Fig. 1). The maximum at 605 mμ was used in subsequent quantitative measurements. The following variables were studied in more detail to determine the optimum conditions (all experiments were carried out in test-tubes graduated at 10 ml. with a final volume of reaction mixture of 10 ml.).

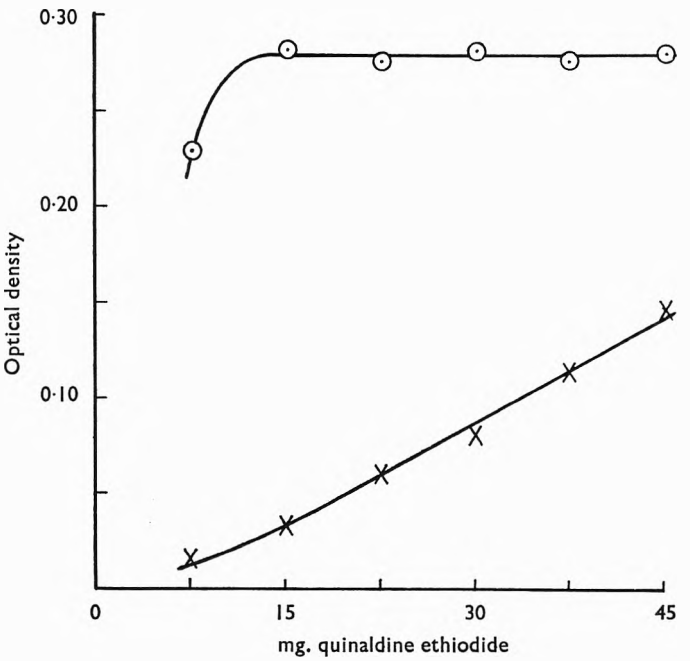


FIG. 2. Effect of varying quantities of quinaldine ethiodide.

⊙—⊙ Optical density at 605 mμ; 50 μg. chloral hydrate measured against appropriate blank.

×—× Optical density at 560 mμ; blank measured against water.

Alkali. Ammonia and ethanolamine were found to be the most effective alkalies and the less volatile ethanolamine was studied in detail. The colour produced is constant for 0·2 to 0·8 ml. of 0·1N ethanolamine in 10 ml. of reaction mixture.

Quinaldine ethiodide. The effect of varying amounts of quinaldine ethiodide on the colour produced from 50 μg. of chloral hydrate is shown in Figure 2. The colour produced is constant with 15 mg. or more in 10 ml. of reaction mixture but with increasing amounts the faint pink colour of the blank (maximum at 560 mμ) increases as shown in Figure 2 and the blue colour due to the chloral hydrate assumes a reddish tint.

Alcohol. Isopropanol and n-propanol were found to be effective in improving sensitivity; isopropanol was used throughout. Maximum colour is produced with 6 or more ml. of isopropanol in the reaction mixture.

Time and temperature. The reaction is slow at temperatures of 50° or below, but the rate of reaction increases at higher temperatures. 60° was chosen as a convenient working temperature. The colour reaches a maximum after 50 minutes at 60° and is constant for a further 40 minutes heating.

Chloral hydrate. Using the conditions described in the method the colour produced was proportional to the amount of chloral hydrate present up to 100 μg. in 10 ml. of reaction mixture. The colour produced is stable for at least 24 hours.

Method

Reagents. Quinaldine ethiodide solution 1·5 per cent w/v: dissolve 1·5 g. quinaldine ethiodide in water and dilute to 100 ml. and filter if necessary. 0·1N Ethanolamine: dissolve 6·1 g. ethanolamine B.P. in water and dilute to 1 litre. Isopropanol: analytical reagent grade. Standard chloral hydrate solution (50 μg./ml.): Dissolve 0·2500 g. chloral hydrate B.P. in water and dilute to 500 ml.; dilute 10 ml. of this solution to 100 ml. with water.

Apparatus. Test-tubes graduated at 10 ml. Unicam SP.500 spectrophotometer.

Procedure. Prepare an aqueous solution or extract of the sample to contain about 50 μg. chloral hydrate in 1 ml. Pipette 1 ml. of this solution into a graduated test-tube, pipette 1 ml. of standard chloral hydrate solution into a second graduated test-tube and pipette 1 ml. of water into a third graduated test-tube to serve as a blank. Add to each test-tube 1 ml. of quinaldine ethiodide solution and 6 ml. of isopropanol, mix and add 0·5 ml. of 0·1N ethanolamine; dilute to 10 ml. with water, mix, and place in a water bath at 60° for 1 hour. Remove from the water bath, cool, and measure the optical densities of the sample and standard against the blank at 605 mμ.

Calculate the amount of chloral hydrate present from the ratio:

$$\frac{\text{Optical density of sample}}{\text{Optical density of standard}}$$

If necessary prepare a solution or extract of the sample with isopropanol and take a suitable aliquot. Adjust the amount of isopropanol added later accordingly.

Results

Some results obtained by the method are shown in Table I together with the results obtained by the B.P. or B.P.C. method, where applicable. The B.P.C. preparations were freshly prepared from accurately weighed quantities of chloral hydrate and diluted to volume in 100 ml. volumetric flasks.

The accuracy of the method is comparable with that of the appropriate B.P. or B.P.C. method.

Specificity of the reaction. 5 mg. quantities of the following substances (i.e., 100 times the quantity of chloral hydrate normally taken) were tested under the conditions described: trichloracetic acid, acetic acid, formic acid, oxalic acid, citric acid (added as equivalent amounts of sodium salts) chloroform, bromoform, hexachlorethane, pentachlorethane, chlorbutol, formaldehyde, acetaldehyde, benzaldehyde and glucose. In each case no blue colour was produced.

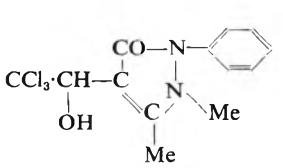
TABLE I

COMPARISON OF RESULTS FROM THE PROPOSED METHOD OF ESTIMATING CHLORAL HYDRATE WITH THOSE OBTAINED USING THE OFFICAL METHOD

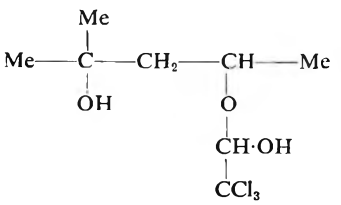
	Chloral hydrate content per cent w/v		
	Calculated	Found	
		B.P. or B.P.C. method	Proposed colorimetric method
Syrup of Chloral B.P.C.	20·0	19·8	20·4
Mixture of Potassium Bromide and Chloral B.P.C.	2·29	2·30	2·32
Mixture of Chloral B.P.C.	9·14	9·11	9·10
Mixture of Chloral and Potassium Bromide for Infants B.P.C.	3·33	—	3·30
2-Methyl-2-hydroxy-4-(βββ-trichloro-α-hydroxyethoxy) pentane $CCl_3{\cdot}CH(OH){\cdot}CH(Me){\cdot}CH_2{\cdot}C(Me)_2{\cdot}OH$	62·3*	62·0*	63·0*
Dichloral phenazone $C_{11}H_{12}N_2O{\cdot}2\,CCl_3CH(OH)_2$	63·7*	63·6*	63·5*
Dichloral phenazone tablets	413**	409**	414**
Proprietary Product A containing chloral glycerolate	5·85	—	5·85
Proprietary Product B containing chloral hydrate, valerian and strontium bromide	2·11	—	2·18
Proprietary Product C containing chloral hydrate, sodium bromide and hyoscine hydrobromide	18·25	—	18·27

* per cent w/w. ** mg./tablet.

The related compounds, chloral formamide and butyl chloral hydrate, produced a green and reddish-violet colour respectively. Chloralose and the condensation product of chloral hydrate and phenazone, 4(βββ-trichloro-α-hydroxyethyl)-2,3-dimethyl-1-phenyl-pyrazol-5-one (I) produced no colour: the hemi-acetal with hexylene glycol, 2-methyl-2-hydroxy-4-(βββ-trichloro-α-hydroxy-ethoxy)-pentane (II) and the addition compound, dichloral phenazone, produced a blue colour in proportion to their chloral hydrate content.



I

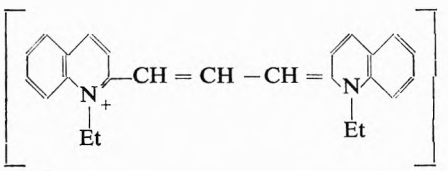


II

Discussion

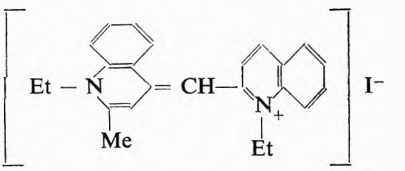
Paper chromatography of the blue colour extracted from the reaction mixture showed it to be identical in chromatographic properties, in neutral, acid and alkaline solvents, with a sample of 1,1′-diethyl-2,2′-carbocyanine iodide, III, prepared by the method of Hamer[26]. The absorption spectrum of (III) is shown in Figure 1.

The faint pink colour of the blank, which increased with increasing quantities of quinaldine ethiodide, as shown in Figure 2, is believed to be due to the formation of a red isocyanine dye (IV), produced by the condensation of two molecules of quinaldine ethiodide.



III

1,1′-diethyl-2,2′-carbocyanine iodide

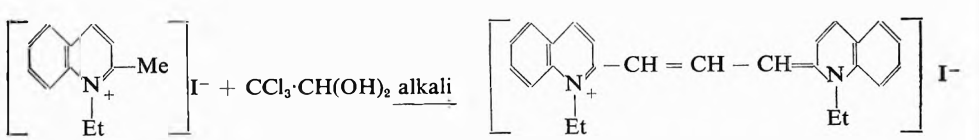


IV

1,1′-diethyl-2′-methyl-2,4′-isocyanine iodide

(III) has been synthesised from quinaldine ethiodide by condensation with formaldehyde[27], chloroform[28] or ethyl orthoformate[26] as a source of the central carbon atom. Under the conditions described under the method, these compounds do not react.

Ogata and Suzuki formulated the reaction as involving the CCl_3 group of chloral hydrate, but the failure of compounds such as trichloracetic acid, chloral formamide and chloroform to produce a blue cyanine dye under the above conditions suggests that the $CH(OH)_2$ group is involved, with the elimination of one molecule of chloroform, thus:



$$+ CHCl_3 + 2H_2O + HI$$

Acknowledgements. The authors wish to thank Mr. B. W. Mitchell for help in the preparation of this paper.

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A NOTE ON THE OXIDATIVE DEAMINATION OF ISOMERS OF 5-HYDROXYTRYPTAMINE AND OTHER INDOLEALKYLAMINES

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Monoamine oxidase from guinea pig tissues oxidises 4-hydroxytryptamine, 6-hydroxytryptamine and 7-hydroxytryptamine at about 50–70 per cent of the rate of 5-hydroxytryptamine. For the rat liver the relative rate of oxidation of 6-hydroxytryptamine is less. 5-Methoxytryptamine and tryptamine are substrates for amine oxidase as good as, or better than 5-hydroxytryptamine, whereas iso-5-hydroxytryptamine and iso-tryptamine are oxidised less readily than 5-hydroxytryptamine and tryptamine. 5-Hydroxy-β-hydroxy-*NN*-dimethyltryptamine(β-hydroxybufotenine) does not seem to be a substrate for amine oxidase from guinea pig kidney.

Monoamine oxidase (MAO) is probably the enzyme of major importance for the inactivation of 5-hydroxytryptamine (5–HT) and tryptamine in the mammalian organism. This note is intended to give a concise account of the action of MAO on the 4-, 6-, and 7-isomers of 5–HT, as well as on some other natural and synthetic indolealkylamines.

Experimental and Results

The MAO preparations employed in this study were obtained by homogenation, in a Waring blender, of guinea pig liver and kidney, and rat liver with 9 volumes of 0·067 M phosphate buffer at pH 7·4. Manometric determination of enzymic activity was made in a conventional Warburg apparatus at 37° and pH 7·3. The total volume of the reaction mixture in the flasks was 2·6 ml.; 2 ml. were represented by the tissue

TABLE I

Enzymic oxidation of various substrates by a guinea pig liver homogenate. The oxidation rate is given as a percentage of that of 5-hydroxytryptamine

Substrate	Per cent oxidation rate	Substrate	Per cent oxidation rate
5-HT	100	5-Methoxytryptamine	90
4-HT	49	Iso-5-hydroxytryptamine	10
6-HT	49	Tryptamine	118
7-HT	71	Psilocybine	0

homogenate, 0·2 ml. by a 0·01 M solution of the substrate, and 0·4 ml. by distilled water. This was eventually replaced by solutions of KCN, semicarbazide or MAO inhibitors (iproniazid, pheniprazine 2-phenylcyclopropylamine or SKF–385).

The main results are listed in the accompanying Tables I and II and in Figure 1.

Tables I and II show the enzymic oxidation of various substrates by guinea pig liver and kidney homogenates, respectively. The oxidation rate in the initial 20-minute period of observation is given as a percentage of that of 5-HT. The average oxygen consumption for 5-HT was 39 μl. using guinea pig liver homogenates, and 37 μl. using guinea pig kidney homogenates.

Three MAO inhibitors were tested on the guinea pig liver preparation using 7-HT as substrate. At final concentrations of 10^{-6} M and 10^{-5} M, β-phenylisopropylhydrazine produced, after 30 minutes, a 10 and 98 per cent inhibition of oxygen uptake, respectively; iproniazid was in-

TABLE II

ENZYMIC OXIDATION OF VARIOUS SUBSTRATES BY A GUINEA PIG KIDNEY HOMOGENATE. THE OXIDATION RATE IS GIVEN AS A PERCENTAGE OF THAT OF 5-HYDROXYTRYPTAMINE

Substrate	Per cent oxidation rate	Substrate	Per cent oxidation rate
5-HT	100	Iso-5-hydroxytryptamine	26
4-HT	48	Tryptamine	130
6-HT	54	Iso-tryptamine	22
7-HT	74	Psilocybine	5
5-Methoxytryptamine ..	94	β-Hydroxybufotenine	0

effective at a concentration of 2×10^{-5} M, but produced a 95 per cent inhibition of enzymic activity at a concentration of 2×10^{-4} M; finally, 2-phenylcyclopropylamine produced, at a concentration of 10^{-5} M a 95 per cent reduction in the oxygen consumption.

Using 5-HT and 6-HT as substrates, and homogenates of guinea pig liver and kidney as enzyme preparations, cyanide 3×10^{-3} M produced, after 10 minutes, a 20 to 40 per cent inhibition of oxygen consumption, and semicarbazide 10^{-2} M a 40 to 45 per cent reduction. It is known that these substances, while ineffective on the intracellular amine oxidase of mammalian tissue, reduce the oxygen uptake by inhibiting further oxidation of the aldehyde formed in the primary oxidation reaction. In fact, addition of cyanide and semicarbazide hindered the appearance of any coloration of the reaction mixture in the vessels (brown with 5-HT, 6-HT and 7-HT, and bluish with 4-HT).

DISCUSSION

There is no doubt that the enzyme responsible for the oxidation of the examined amines is monoamine oxidase.

It appears from the results that all isomers of 5-HT are good substrates for amine oxidase, although none of them is as good as 5-HT. On the whole, the most easily attacked, among the isomers, is 7-HT, the most resistant 6-HT. In fact, rat liver MAO oxidises 6-HT at only 15 per cent of the rate of 5-HT. 5-Methoxytryptamine also and, as known for a long time, tryptamine are excellent substrates for MAO. It is remarkable that transposition of the lateral chain to the 2-position of the indole nucleus (iso-5-hydroxytryptamine and iso-tryptamine) produces

a sharp reduction of the MAO attack. Similarly, hydroxylation of the lateral chain, at the β-position, renders bufotenine completely resistant to MAO.

Results obtained in this investigation may be of some interest because 4–HT and 6–HT are likely to be biogenic indolealkylamines, the first deriving from the decarboxylation of 4–hydroxytryptophan[1,2], the second from the hydroxylation, at the 6-position of the indole ring, of

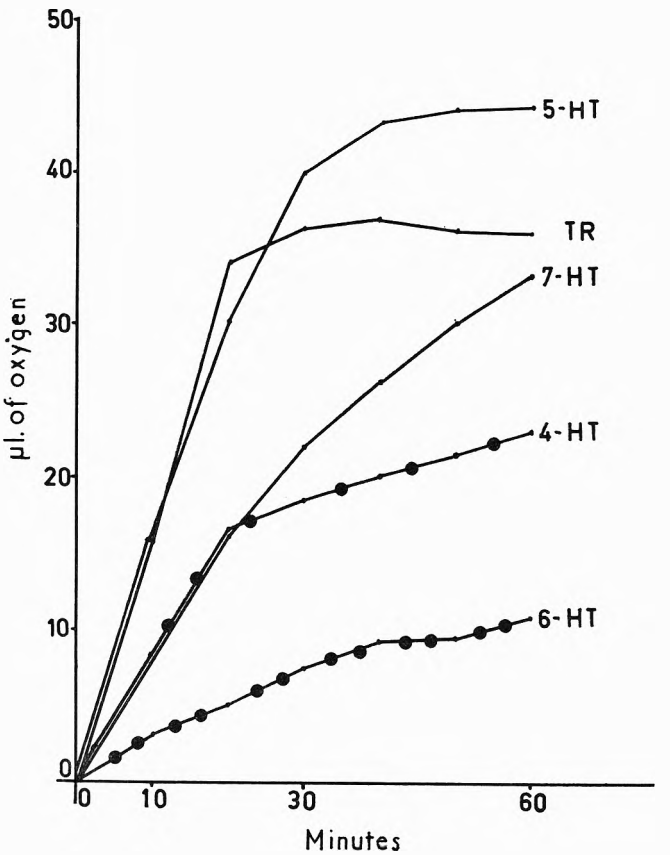

FIG. 1. Oxidation of 5-hydroxytryptamine (5–HT), 4-hydroxytryptamine (4–HT), 6-hydroxytryptamine (6–HT), 7-hydroxytryptamine (7–HT) and tryptamine (TR) by a homogenate of rat liver. Abscissa: time in minutes. Ordinate: µl. of oxygen used.

tryptamine[3], and because 5-methoxyindoleacetic acid, the deamination product of 5-methoxytryptamine, has been isolated from the bovine pineal gland[4]. Moreover, they can give a satisfactory explanation of the observation that the pharmacological actions produced *in vivo* by 4–HT and 4-hydroxytryptophan are more sustained than those produced by 5–HT and 5-hydroxytryptophan[5].

Acknowledgements. We are indebted to Dr. A. Hofmann, Sandoz Ltd., Basle, for a sample of psilocybin, and to Dr. F. Häfliger, J. R. Geigy Ltd., Basle, for samples of iso-5-hydroxytryptamine and iso-tryptamine. All the other amines used in this study were synthetised in the Farmitalia Research Laboratories, Milan, by Dr. C. Pasini, Dr. V. Colò and Dr. D. Chillemi.

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BOOK REVIEW

METHODEN DER ORGANISCHEN CHEMIE (Houben-Weyl). Fourth Edition. Edited by Eugen Müller. Volume V, Part 4. Halogenverbindungen. Herstellung von Brom- und Iodverbindungen. Reactivität und Umwandlung von Chlor- Brom- und Iodverbindungen. Pp. xlviii + 894 (including Index). Georg Thieme Verlag, Stuttgart, 1960. Moleskin, DM.180.00.

The pace at which organic chemistry continues to develop is marked by the fact that the chemistry of hydrocarbons and halogen compounds allotted to a single volume in the original plan for the new Houben-Weyl is now to be treated in no less than four separate volumes. The need for this large expansion arises from the rapid growth of fluorocarbon chemistry, and from the perhaps rather better known development of methods for the production of organic chemicals from petroleum sources. The somewhat unusual properties of fluorocarbons, which set them apart from the other organo-halogen compounds based on chlorine, bromine and iodine, both in synthesis and properties, has led to a separate treatment of fluorocarbons in Volume 5, Part 3. The second volume on halogen compounds, Volume 5, Part 4, at present under review, is concerned with the preparation of bromo and iodo compounds (the preparations of chloro-compounds is dealt with in Part 3) and the properties of chloro-, bromo- and iodo-compounds. The opening chapter provides a comprehensive review of brominating and iodinating reagents, including the elementary halogens, halogen acids, alkali- and organo-hypohalites, inter-halogen compounds, phosphorus halides, thionyl halides, acid halides, and *N*-halogen compounds, describing general properties. methods of preparation, purification and drying. Succeeding chapters are devoted to preparative methods with these reagents, and include the addition of halogen and halogen acids to unsaturated systems; the replacement of hydrogen, hydroxyl, amino, carboxyl and other groups by halogen; special addition reactions of organo-halogen compounds with olefines, epoxides, carbonyl compounds and aliphatic diazo compounds; bromomethylation and the haloform reaction. Only two chapters are devoted to the properties of halogen compounds. They provide a comprehensive survey of elimination and replacement reactions of halogen compounds, and include a classification based on detailed reaction mechanism. The influence of neighbouring substituents on mechanism and reaction rate is discussed in detail, and this section greatly enhances the value of the book. In keeping with the rest of this series, Volume V, Part 4, is excellently referenced, providing a ready access to the literature for those requiring more detailed information.

J. B. STENLAKE.

LETTERS TO THE EDITOR

Indole Compounds and Growth

SIR,—The possible evolutionary relationship of 5-hydroxytryptamine (5–HT) in animals to auxin (3-indoleacetic acid, IAA) in plants has recently been discussed by Woolley[1]. The fact that one is a base which has been hydroxylated whereas the other is the corresponding acid suggests that in the course of evolution the usefulness of the indolic nucleus for specific purposes has long been recognized by Nature. In animals where the tissue fluids are usually alkaline, the indolic base has been evolved, whereas in plants where the cell sap is usually acidic the corresponding acid has proved to be more useful. In each situation, the compound probably exists predominantly in the unionized form.

Auxin causes plant cells to grow, probably by changing the permeability of the cell wall and so allowing an increased uptake of water and other metallic ions. If 5–HT is the counterpart in the animal kingdom of IAA in the plant world, then it is reasonable to expect some similarity in basic biochemical roles. This is in fact true since both 5–HT and IAA increase the permeability of plant cells[2,3] and animal cells[3]. We have also found that one precursor of 5–HT, namely 5-hydroxytryptophan, increases the permeability of plant cells whereas tryptamine does not, and the same is true for animal cells. This latter finding may explain why higher vertebrates utilise 5–HT and not tryptamine, and why only traces of tryptamine have been detected in mammalian tissues. The insertion of the 5-hydroxy group in the molecule of tryptamine may also be a device which has been evolved to retard the passage of 5–HT from the blood to the brain, since injected 5–HT does not pass in measurable amount into the brain. What 5–HT the brain needs it makes for itself from 5-hydroxytryptophan, a substance which readily penetrates the blood-brain barrier.

During a systematic examination by paper chromatography of the presence of indole compounds in animals and plants, the following results were obtained with pure compounds and these may be linked with some of the above considerations. Hydroxylated indolic compounds always migrated up the paper at a slower rate than did the corresponding unsubstituted ones; indolic amino-acids always migrated up the paper at a slower rate than did the corresponding amines; and 5–HT migrated at a faster rate than did IAA when an alkaline solvent was used, and the reverse was true in acid systems. For this study, the compounds used were 5-hydroxytryptamine creatinine sulphate, tryptamine hydrochloride, 5-hydroxytryptophan, tryptophan, 5-hydroxyindoleacetic acid and indoleacetic acid. They were detected as coloured spots after treatment with Ehrlich's reagent. The solvent systems were butanol : acetic acid : water (4 : 1 : 5), butanol : acetic acid (100 : 1), butanol saturated with N HCl, isopropanol : acetic acid : water (20 : 1 : 2 and 53 : 1 : 46), sodium chloride solution (8 per cent w/v) : acetic acid (100 : 1), n-propanol : water (3 : 1), isopropanol : water (3 : 1), sodium chloride solution (8 per cent w/v), n-propanol : ammonia : water (20 : 1 : 2), and isopropanol : ammonia : water (20 : 1 : 2 and 53 : 1 : 46). Further work is in progress to determine whether 5–HT plays a role in the growth of cells in higher vertebrates.

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The Excretion of Scillaren A by Rats

SIR,—As part of a survey of the metabolism and excretion of cardiac glycosides being carried out in this department[1–4] we have examined the bile and urine of rats after doses of scillaren A. The excretory products have been separated by paper chromatography and estimated colorimetrically. The squill glycosides (bufadienolides) do not give colours with the reagents normally used to detect the unsaturated lactone ring in the cardenolides and we have therefore used the pink colour given by these glycosides with 80 per cent sulphuric acid to detect them on paper and to estimate them colorimetrically after elution of the glycoside areas from paper chromatograms.

The bile of male albino rats (150–300 g.) was collected for 6 hours after intravenous injection of doses of 1 μg./g. of body weight of scillaren A.[3] After dilution to about 15 ml. with water the bile from each rat was extracted with chloroform in a liquid-liquid extractor for 4 hours and the extracts streaked across strips of Whatman Paper No. 1 ($1\frac{1}{4}$ in. × 18 in.) and the chromatograms developed with the solvent mixture chloroform : methanol : water (10 : 4 : 5) by the descending method. After 5 hours the papers were dried at 100° for 10 minutes and a longitudinal strip $\frac{1}{4}$ in. wide cut from the chromatogram and treated with 65 per cent v/v sulphuric acid. Under these conditions scillaren A gave a pink colour on the paper strip. The corresponding area on the remainder of the strip was cut and eluted with methanol. The methanol extracts were evaporated to dryness, 1·75 ml. of 65 per cent v/v sulphuric acid added and the optical density of the pink colour produced was measured in an EEL Colorimeter (green filter 624). The colour obtained reached a maximum intensity in 10 minutes and was stable for at least 25 minutes (5 μg. of scillaren A could be readily detected and with the quantities assayed an error of ± 2 per cent was possible).

Using this procedure, extraction of known quantities of scillaren A from bile gave mean recoveries of 82 per cent (79–85 per cent, P = 0·95) and this figure was used as a correction factor to obtain a close approximation of the scillaren A content of the bile collected in the excretion experiments.

Only one band was detected in the chromatograms of the bile extracts obtained after doses of 1 μg./g. This was eluted and identified as scillaren A by rechromatography on paper with the original glycoside using four different solvent systems for development: chloroform : methanol : water (10 : 8 : 5), toluene : butanol (8 : 2) saturated with water, ethyl acetate : butanol : chloroform (16 : 16 : 68) saturated with formamide, and chloroform : benzene : butanol (70 : 10 : 10) saturated with formamide.

Quantitative determination of the amount of glycoside present in bile showed that 84 per cent (79–90 per cent, P = 0·95) of the dose was excreted in 5 hours.

Chloroform extracts of urine collected for 12 hours after intraperitoneal doses of 1 μg. and 2 μg./g. of scillaren A were chromatographed but no glycosides or metabolites could be detected on the papers indicating urinary excretion was very low during this period.

It appears from these results that scillaren A is similar to the polar digitalis glycosides lanatoside A and lanatoside C[3] and to ouabain[4] in being excreted mainly in the bile and without chemical modification.

Acknowledgement. This work is supported by a grant from the National Health and Medical Research Council of Australia.

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Carcinoid Tumours and Pineapples

SIR,—The presence of 5-hydroxytryptamine (5–HT) and its precursors in fruits has been reported in recent years[1,2]. The ingestion of large quantities of banana and tomato may lead to erroneous chemical diagnoses of carcinoid tumours by producing an increased urinary excretion of 5–HT and its metabolites. These fruits should be eliminated therefore from the diets of patients whose urinary indoles are being measured. To the list of forbidden fruits, Bruce[3] in Australia has recently added the pineapple. Firstly he showed that fresh and canned pineapple juice contain much 5–HT (12–25 μg./ml.), and secondly he found that the rate of excretion of 5-hydroxyindoleacetic acid (5–HIAA) was increased 10-fold after the ingestion of 500 ml. commercial canned pineapple juice.

During a systemmatic examination over 2 years ago of the presence of indole compounds in plants, we had detected only traces of indole derivatives in fresh pineapples, and Foy and Parratt[4] this year obtained a similar result using the fruit gathered from the trees in Nigeria. A re-investigation of the problem was thus needed.

Fresh pineapple, three brands of canned pineapple juices, and a sample of bottled juice were extracted with acetone, and after removal of the acetone the extracts were subjected to paper chromatographic analysis and to bioassay using the rat uterus preparation. The concentrations of 5–HT in no case exceeded 1·5 μg./ml. juice and there were only traces of tryptophan and indoleacetic acid. It seems unlikely therefore that the ingestion of much pineapple juice would increase the excretion of 5–HIAA above the range (2–10 mg./day) found in patients who do not have carcinoid tumours. It should be pointed out that none of the preparations of pineapple used in the present work was of Australian origin.

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