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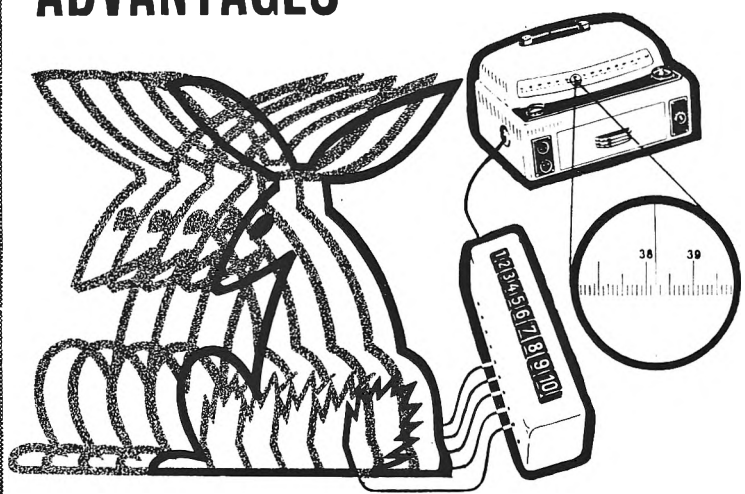

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RESEARCH PAPERS

THE METABOLISM OF 5-*p*-AMINOBENZENESULPHONAMIDO-3-METHYLISOTHIAZOLE (SULPHASOMIZOLE)

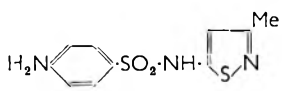
BY J. W. BRIDGES AND R. T. WILLIAMS

From the Department of Biochemistry, St. Mary's Hospital Medical School, University of London, London, W.2.

Received March 15, 1963

The fate of the sulphonamide drug, sulphasomizole, in man, dog, rabbit and rat has been studied. In man, rat and rabbit, the major urinary metabolite, is N^4-acetylsulphasomizole together with the unchanged drug. In man about 60 per cent of an oral dose of 30 mg./kg. is excreted in the urine in 24 hr., just over a third being acetylated. In the rabbit about 80 per cent of an oral dose (150 mg./kg. is excreted in 24 hr. and about two-thirds is acetylated. In the rat about 70 per cent of the dose (150 mg./kg.) is excreted in 24 hr. and just under one third is acetylated. In the dog, the main excretory product is the unchanged drug, there being no acetylation. All four species excrete small amounts (1 per cent) of the N^4-glucuronide of sulphasomizole. Other minor metabolites detected were the N^4-sulphate of sulphasomizole which was found in rat and dog urine, and an unidentified oxidation product present as a glucuronide which was detected in rabbit and dog urine.

5-*p*-AMINOBENZENESULPHONAMIDO-3-METHYLISOTHIAZOLE (I) is an antibacterial drug containing a new heterocyclic system, namely the isothiazole or 1,2-thiazole ring system. This compound and some of its derivatives were first described by Adams and Slack (1959) and its antibacterial properties were reported by Adams and others (1960).



I

The compound is marketed as a sulphonamide drug of moderate duration of action under the name of "Bidizole". The substance has been given the approved common name, sulphasomizole.

EXPERIMENTAL

Materials

Sulphasomizole, m.p. 189°, 5-*p*-aminobenzenesulphonacetamido-3-methylisothiazole (N^1-acetylsulphasomizole), m.p. 168–169°, 5-amino-3-methylisothiazole, m.p. 41–42°, 4-amino-3-hydroxybenzenesulphonic acid, m.p. 266° (decomp.) and 4-amino-2-hydroxybenzenesulphonic acid were the gifts of May & Baker, Ltd., Dagenham, Essex. 5-*p*-Acetamidobenzenesulphonamido-3-methylisothiazole (N^4-acetylsulphasomizole), m.p. 272–273° and 5-acetamido-3-methylisothiazole, m.p. 179–180°, were prepared by acetylating the corresponding amines in sodium carbonate solution with acetic anhydride (cf. Adams and Slack, 1959).

Sulphasomizole N^4-*glucuronide.* A solution of sulphasomizole (9·42 g.) in dimethylformamide (20 ml.) was mixed with a solution of sodium glucuronate (6·9 g.) in ethylene glycol (30 ml.). The resulting solution was adjusted to pH 3–4 with glacial acetic acid (0·5 ml.) and then heated for 10 min. at 70°. The solution was kept in the dark for 24 hr. at room temperature. The crystals (4·3 g.), which had separated, were filtered, washed with dimethylformamide and dried with acetone followed by ether. They were purified by dissolving in 0·02N ammonium hydroxide (10 ml.), adding acetone (25 ml.) and keeping at 0° overnight (yield, 2·9 g.). The compound decomposed on heating to 150–200° and showed $[\alpha]_D^{20}$ — 65·9° (*c*, 1 in 0·02N NH_4OH) which remained constant over 24 hr. It analysed as the *sodium salt* of 5-p-*aminobenzenesulphonamido*-3-*methylisothiazole* N^4-*glucosiduronic acid monohydrate.* (Found: Na, 4·9; H_2O, 3·7 (drying *in vacuo* at 78°); $C_6H_{10}O_7$ (glucuronic acid), 37·0; $C_{10}H_{11}O_2N_3S_2$ (sulphasomizole), 55·1 per cent. $C_{16}H_{18}O_8N_3S_2Na$, H_2O requires Na, 4·7; H_2O, 3·7; $C_6H_{10}O_7$, 40·0; $C_{10}H_{11}O_2N_3S_2$, 55·5 per cent). The compound reduced Fehling's solution on warming, gave an intense naphthoresorcinol test for uronic acids, and a test for aromatic amines on diazotising in acid solution and coupling with *N*-1-naphthylethylenediamine. It was unstable in dilute acid, i.e. below pH 7, but stable in dilute alkali, i.e. above pH 7 up to 2N sodium hydroxide.

Sulphasomizole N^4-*sulphate.* Chlorosulphonic acid (1·3 ml.) was added slowly to pyridine (20 ml.) cooled in ice. To the solution, sulphasomizole (5·38 g.) was added with stirring until the drug had dissolved. After keeping the mixture at room temperature for 48 hr., it was poured into aqueous potassium hydroxide solution (5·6 g. KOH in 100 ml. water). The alkaline solution was now extracted with ether (5 × 100 ml.) to remove pyridine and concentrated under reduced pressure at 45° to 3 ml. The concentrate was then banded on Whatman seed test paper (1·6 mm. thick) and irrigated for 60 hr. by ascending chromatography with the solvent system n-butanol: ammonia solution (s.g. 0·88): water (4: 1: 5, by vol.). The portion of the paper which gave a negative *p*-dimethylaminocinnamaldehyde test, a positive diazo test (see p. 567) and a positive test for sulphate only after hydrolysis, was cut out of the chromatogram and eluted with 0·05N potassium hydroxide. The eluate was decolourised by heating with charcoal and filtered. To the filtrate, there was added slowly 1 litre of acetone, and on standing white crystals (0·86 g.) separated. The crystals were collected and purified by dissolving in water (3 ml.) and precipitating with acetone (500 ml.) (yield, 0·68 g.). The *dipotassium salt* of 5-(*p-sulphoaminobenzenesulphonamido*)-3-*methylisothiazole* was very soluble in water and crystallised as a monohydrate. (Found: K, 17·85; sulphamate-S, 6·9; sulphasomizole, $C_{10}H_{11}O_2N_3S_2$, 61·15 per cent. $C_{10}H_9O_5N_3S_3K_2$, H_2O requires K, 17·6; sulphamate-S, 7·2; sulphasomizole, 60·7 per cent).

Methods

Free and total aromatic amines in urine. These were determined by the method of Bratton and Marshall (1939). Urine containing 20–150 mg.

of sulphasomizole/100 ml. was diluted with water so as to contain between 20–40 μg./ml. Free aromatic amine (which included any N^4-glucuronide or N^4-sulphamate) in this diluted urine was determined directly by the Bratton and Marshall method. The recovery of sulphasomizole added to urine (20–100 mg./ml.) was 99–100 per cent. For total aromatic amine, the diluted urine (2 ml.) was mixed with 2N hydrochloric acid (0·5 ml.) and heated on a boiling water-bath for 1 hr. and the total aromatic amine determined by the Bratton and Marshall method. The recovery of N^4-acetylsulphasomizole added to urine (40–100 mg./100 ml.) was 99–100 per cent.

Determination of metabolites on strip chromatograms. Paper chromatography of urines of animals receiving sulphasomizole revealed the presence of up to five metabolites. Two of these were major metabolites and were isolated and identified as sulphasomizole and N^4-acetylsulphasomizole; the other three were minor products, one of which was identified as sulphasomizole N^4-glucuronide and the other two suspected to be the N^4-sulphamate and an oxidation product containing a diazotisable amino-group.

The urine (0·2–0·4 ml.) was banded across a piece of Whatman No. 31 paper (12 inches wide). The paper was chromatographed for 5 hr. with a mixture of n-propanol and ammonia solution (s.g. 0·88), in the proportion of 7:3. The appropriate strips (position determined with reference spots of the known compounds) of the paper were cut out and eluted with water (1–2 ml.) in "eluting tubes" (made by Howard Rawson Ltd.). Sulphasomizole, its N^4-glucuronide, N^4-sulphamate and suspected oxidation product were determined directly on the eluates by the Bratton and Marshall method. The eluate of the N^4-acetylsulphasomizole was hydrolysed with acid as before and the liberated amine determined. Tests on various other parts of the paper showed that no other metabolites related to sulphasomizole were present.

Determination in faeces. Faeces from rats and rabbits dosed with sulphasomizole were also analysed. The faeces were homogenized with water and the homogenate centrifuged. Free and total aromatic amines were determined on the supernatant. The recovery of sulphasomizole added to faeces (10–40 mg. to 20 g. wet faeces) was 72–86 per cent. Chromatography of faecal extracts showed that small amounts of sulphasomizole and its N^4-acetyl derivative only were present.

Chromatography

The R_F values of sulphasomizole and its possible metabolites are shown in Table I.

The details of the colour tests were as follows.

Diazo test. The paper was sprayed with 1 per cent sodium nitrite solution which had been freshly mixed with 0·5 vol. of 2N hydrochloric acid. After 3 min. the paper was sprayed with 0·1 per cent solution of *N*-1-naphthylethylenediamine dihydrochloride in water. With this reagent aromatic amines and the N^4-glucuronide and N^4-sulphamate show up as red or red-purple spots.

TABLE I

CHROMATOGRAPHY AND COLOUR REACTIONS OF SULPHASOMIZOLE AND ITS POSSIBLE METABOLITES

Descending chromatography on Whatman No. 4 paper was used. The solvent systems were, A, n-propanol:ammonia solution (s.g. 0·88) (7:3); B, ethanol: n-butanol:ammonia solution (s.g. 0·88):water (12:4:1:1); C, n-butanol: acetic acid:water (4:1:5); D, n-butanol:water (1:1); E, ethyl methyl ketone:water:acetic acid (200:100:1); F, ethyl methyl ketone:ammonia solution (s.g. 0·88) (200:1). The proportions of solvents are by volume. Chromatograms in solvents A, B, C, and D were run for 7 hr. and in E and F for 2 hr.

Compound	R_F values in solvent						Colour reaction‡		
	A	B	C	D	E	F	Dimethylamino-cinnamaldehyde	Diazo test	Fluorescence u.v.
4-Amino-3-hydroxybenzene-sulphonic acid	0·19	0·45	0·06	—	0·01	0·93	red	red	violet
4-Amino-2-hydroxybenzene-sulphonic acid	0·36	0·50	—	—	0·04	0·91	red	red	violet
Sulphanilic acid	0·39	0·52	0·09	0·87	0·89	0·83	red	red- purple	violet
Sulphasomizole	0·69	0·72	0·84	0·84	0·94	0·89	red	red-purple	weak violet
N^4-Acetylsulphasomizole	0·79	0·83	0·82	0·86	0·93	0·90	none	none	quench
N^1-Acetylsulphasomizole	0·86	0·74	0·80	—	0·91	0·90	red	orange-red	quench
Sulphasomizole N^4-glucuronide*	0·08	0·10	—†	0·08	—†	0·09	red slowly	red slowly	weak violet
N^4-Sulphosulphasomizole	0·36	0·32	0·22	—	0·43	0·01	red very slowly	red very slowly	none
5-Amino-3-methyl-isothiazole	0·85	0·84	0·85	0·69	0·04	0·91	red	red	weak violet
5-Acetamido-3-methyl-isothiazole	0·70	0·86	0·83	—	0·94	0·93	none	none	quench

* Gives naphthoresorcinol test.
† Hydrolysed to free amine in these solvents.
‡ See text.

p-*Dimethylaminocinnamaldehyde test.* A 1 per cent solution of *p*-dimethylaminocinnamaldehyde in ethanol was used. This solution was acidified with 0·1 vol. of 2N hydrochloric acid just before spraying the paper. Aromatic amines and urea show up immediately as red spots. The weak N^4-conjugates (glucuronide and sulphamate) show up as red spots more slowly (5–10 min. for glucuronide, 20–60 min. for sulphamate), whilst the red spot due to urea turns yellow on keeping (0·5–1 hr.). If 2N acetic acid is used instead of hydrochloric acid, the weak conjugates do not show up with the reagent.

Glucuronide test. The paper was sprayed with a freshly prepared 1 per cent aqueous solution of naphthoresorcinol in 20 per cent aqueous trichloroacetic acid and then heated in an oven at 140° for 10 min. Easily hydrolysable glucuronides show up as blue spots.

Ultra-violet light. The paper was examined with ultra-violet light of wavelength 254 mμ from a Hanovia "Chromatolite" lamp. Some compounds showed a weak violet fluorescence, others, particularly the N^4-acetylsulphasomizole, quenched the background fluorescence of the paper and showed up as dark spots.

Ultra-violet and infra-red absorption and fluorescence spectra were determined with the Unicam spectrophotometer S.P. 500, the Perkin-Elmer infracord Spectrometer and the Aminco-Bowman Spectrophoto-fluorometer, respectively.

Detection and Isolation of Metabolites

Isolation of sulphasomizole and its N^4*-acetyl derivative.* A chinchilla rabbit (4·3 kg.) was fed with 2 g. of sulphasomizole. The 24-hr. urine was brought to pH 10 with 2N ammonium hydroxide, filtered by suction, and evaporated to 5 ml. at 40–45° under pressure. The concentrated urine was banded on heavy Whatman No. 3 MM paper and chromatographed with solvent A (propanol:ammonia solution, see Table I), with reference spots of the drug and its N^4-acetyl derivative suitably placed on the paper. When the chromatogram had developed, strips corresponding to these two compounds were cut out and eluted with acetone. The sulphasomizole fraction on evaporation yielded 0·57 g. of the drug, m.p. 189–190°, mixed m.p. 190–191°, after recrystallisation from methanol and water (1 : 1). It was further identified by ultra-violet absorption spectra (see Table II).

TABLE II

ULTRA-VIOLET ABSORPTION SPECTRA OF SULPHASOMIZOLE AND SOME OF ITS DERIVATIVES

Compound	Absorption maxima in			
	0·1N HCl		Water or 0·1N NaOH*	
	λ max	ε max $^{10-3}$	λ max	ε max $^{10-3}$
Sulphasomizole†	245	9·3	257	14·8
	301	10·7	280	14·7
N^4-Acetylsulphasomizole†	254	16·7	255	16·8
	305	9·1	275–276	13·5
5-Amino-3-methylisothiazole† ..	230	7·0	235	5·4
	281	12·7	262	7·7

* The pK_a of the sulphonamide group of sulphasomizole is 5·03 and of N^4-acetylsulphasomizole is 4·9 (Dr. P. O. Kane of May & Baker, Ltd.). Therefore, these compounds are ionised in water.

† These compounds did not fluoresce in aqueous solution when examined in the Aminco-Bowman spectrofluorimeter. Sulphanilamide in aqueous solution is fluorescent (fluorescence maximum, 350 mμ; excitation, 275 mμ) (Bridges and Williams, 1962b).

The N^4-acetyl fraction was evaporated and the crystals were purified by dissolution in dilute sodium hydroxide and precipitation by acidification with 2N hydrochloric acid. The N^4-acetylsulphasomizole had m.p. 271–272° and mixed m.p. 271–272° and had ultra-violet and infra-red spectra identical with a synthetic sample (see Table II).

Both these compounds were isolated similarly from the urines of man and rat, after administration of the drug, but only the free drug was isolated from dog urine.

Detection of sulphasomizole N^4*-glucuronide.* The N^4-glucuronide of sulphasomizole was detected chromatographically using for solvents (A, B, D and F, Table I) in human, dog, rabbit and rat urine, as a minor metabolite. The spot corresponding to this compound gave the naphthoresorcinol reaction. The diazo and cinnamaldehyde tests were given slowly indicating a weak *N*-conjugate. On eluting the spot with water and chromatographing the eluate with the acid solvents C and E (Table I), spots with R_F corresponding to sulphasomizole were found. In solvent E a spot (R_F 0·02) corresponding to glucuronic acid was also found. Aqueous

eluates of the glucuronide spot were not hydrolysed by snail β-glucuronidase at pH 7, although the same enzyme solution was shown to be effective in hydrolysing the *O*-glucuronide, *o*-aminophenylglucuronide, at the same pH (it is to be noted that the optimum pH of β-glucuronidase is 5·2, but at this pH the N^4-glucuronide is unstable). It is believed that β-glucuronidase does not hydrolyse N^4-glucuronides (Bridges and Williams, 1962a; see also Axelrod, Inscoe and Tompkins, 1957).

N^4-*Sulphosulphasomizole.* A spot with similar R_F values and reactions to the synthetic N^4-sulphosulphasomizole was found in the urine from all rats (3) and dogs (2) tested, from 3 human urines out of 5, and one rabbit urine out of 4. These spots were eluted in appropriate experiments and estimated.

The possible oxidation product. A minor diazotisable spot with the R_F values of 0·4 in solvent A and 0·27 in solvent B was found in all the rabbit and dog urines examined and in one human urine out of five, but in none of the rat urines. The spot gave immediate tests for aromatic amine and a positive naphthoresorcinol test for glucuronic acid. This material was prepared by the elution with water of the appropriate area from a large number of preparative chromatograms of the urine of rabbits receiving sulphasomizole (total, about 8 g.) and concentration of the eluate. Attempts to isolate it were unsuccessful. However, the compound was stable to boiling for 0·5 hr. with 2N sodium hydroxide. It was hydrolysed by boiling with 0·1N hydrochloric acid for 0·5 hr. to yield a diazotisable compound of R_F 0·47 in solvent B.

Quantitative Results

Rabbits. The results for rabbits are shown in Table III. The drug is excreted in the urine mainly as sulphasomizole and its N^4-acetyl derivative. The acetyl derivative accounts for about $\frac{2}{3}$ of the drug excreted in the first 24-hr. after dosing. The two ways of estimating the acetylated amine,

TABLE III

The fate of sulphasomizole in the rabbit

Sulphasomizole administered orally; the figures quoted are per dose excreted in 24 hr. after dosing.

	Rabbit No.	1*	2*	3*	4†
	Dose, mg./kg.	150	150	150	750
	"Free" amine by direct determination	25·2	19·1	31·5	21·5
	Total amine by direct determination (24 hr. excretion)	93·8	57·0	87·8	63·7
	Acetylated amine (by diff.)	68·6	37·9	56·3	42·2
By analysis of chromatograms of 24 hr. urine	N^4-Glucuronide	1·0	·0	0·7	0·7
	N^4-Sulphamate	0·0	·0	0·0	0·0
	"Oxidation product"	1·0	2·9	1·6	1·0
	Free sulphasomizole	21·4	13·6	29·2	21·6
	"Free amine (sum of above 4 items)	23·4	18·5	31·5	23·3
	Acetylated amine	64·2	34·1	51·5	39·5
	Total urinary amine after 48 hr.	99·1	78·0	88·2	65·2
	Total urinary amine after 5 days	99·9	79·0	89·2	69·4
	Total amine in faeces after 5 days	0·0	0·0	0·0	12·4‡
	Total drug accounted for	99·9	79·0	89·2	81·8

* Female chinchilla rabbits.
† Female of mixed breed of unknown origin.
‡ This figure may be the result of contamination of faeces by urine.

i.e. by the difference between "free" and total aromatic amine and by determining the acetylated amine separated by chromatography, agreed with one another. The amounts of "free" amine obtained by two methods also agreed. It will be noted from the Table that the "free" amine is largely sulphasomizole, the rest being made up of small smounts of sulphasomizole N^4-glucuronide (about 1 per cent) and a diazotisable "oxidation product" (1–2 per cent). The presence of the N^4-sulphamate was doubtful. In the chinchilla rabbits about 80 per cent of the dose of 150 mg./kg. was excreted in the first day and most of the drug was excreted by the second day. It seems likely that there is no faecal excretion of the drug in the rabbit.

Rats. The results for female Wistar rats are shown in Table IV. The major metabolites of sulphasomizole in rats are again the free drug and the N^4-acetyl derivative. In 48-hr. about 60–90 per cent of the drug fed was excreted, but the acetylated drug accounted for only about 30 per cent of the material excreted and the main excretory product was unchanged sulphasomizole. No "oxidation product" was detected in rat urine, but the N^4-glucuronide and the "N^4-sulphamate" (each about 1 per cent) was detected in all the urines. Faecal excretion of the drug is uncertain, since the values in Table IV could be due to urinary contamination.

TABLE IV

THE FATE OF SULPHASOMIZOLE IN RATS

Sulphasomizole administered orally at a dose of 150 mg./kg. Results expressed as per cent of dose excreted in 24 hr.

	Rat No.	1	2	3
By analysis of chromatograms of 24 hr. urine	"Free" amine (direct)	—*	49·0	9·1
	Total amine in 24 hr. (direct)	—*	67·8	17·7
	Acetylated amine (by difference)	—*	18·8	8·6
	N^4-Glucuronide	1·2	1·3	0·7
	"N^4-Sulphamate"	1·0	1·6	0·4
	"Oxidation product"	0·0	0·0	0·0
	Free sulphasomizole	19·5	42·0	7·3
	"Free" amine (sum of previous 4 items)	21·7	44·9	8·4
	Acetylated amine	9·3	19·8	4·8
	Total amine excreted in 48 hr.	60·6	88·3	65·7
	(acetylated	22·3	29·0	10·6)
	Total amine excreted in 5 days	60·6	97·3	65·7
	Total amine in faeces in 5 days	19·8†	0·0	29·0†
	Total drug accounted for	80·4	97·3	94·7

* Not determined.
† Possibly due to contamination with urine.

Man. Five healthy male students (19–24 years old) were given tablets of sulphasomizole (dose, 30 mg./kg.) and the 24 hr. urine was concentrated and analysed on paper chromatograms. The results are shown in Table V. The results are fairly consistent, an average of 58 per cent (range 57–62) of the dose was excreted in 24 hr. Just over a third (36 per cent) of the excreted material occurred as the N^4-acetyl derivative and the rest was mainly the unchanged drug. Small amounts of the N^4-glucuronide (0·1–0·9 per cent) were also excreted; traces of the "N^4-sulphamate" were found in three cases, but the oxidation product was not found in most cases.

TABLE V

THE FATE OF SULPHASOMIZOLE IN MAN

Five healthy male subjects took 27–32 mg./kg. of sulphasomizole. The urine was collected for 24 hr., concentrated and chromatographed. The results are expressed as per cent of dose.

	Metabolite	Subjects				
		J.W.B.	J.B.	W.J.	R.W.	P.S.
Fractions obtained chromatographically	N^4-Glucuronide	0·7	0·1	0·1	0·9	0·2
	"N^4-Sulphamate"	0·1	0·0	0·2	0·0	0·1
	"Oxidation product"	0·0	0·0	0·0	0·0	0·1
	Sulphasomizole	40·9	43·2	35·1	30·1	35·1
	N^4-Acetylsulphasomizole	16·6	18·2	24·1	31·8	21·1
	Total in 24 hr.	58·3	61·5	59·5	62·8	56·6
	Total in 48 hr.	76·4	—	—	—	—
		19·5*				

* Acetylated

Dogs. Two male Corgi dogs each received sulphasomizole, 5 g. daily, during chronic toxicity studies. A 24 hr. sample of urine collected on the 1st and 5th day was sent to us by May & Baker, Ltd.

These urines were concentrated as before and chromatographed. The results are shown in Table VI. No acetylation product was found and most of the drug was excreted unchanged, about 36 per cent being eliminated in 24 hr. All the minor metabolites were also found and they amounted to 2–4 per cent of the dose or nearly 10 per cent of the material excreted.

TABLE VI

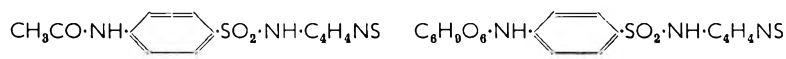
THE FATE OF SULPHASOMIZOLE IN THE DOG (CORGI)

	Metabolite	Dog 1*	Dog 2†
Fractions obtained chromatographically	N^4-Glucuronide	0·3	0·7
	"N^4-Sulphamate"	1·1	1·7
	"Oxidation product"	1·1	2·0
	Sulphasomizole	33·8	31·0
	N^4-Acetylsulphasomizole	0·0	0·0
	Total for 24 hr.	36·3	35·4

* This dog (10 kg.) received 5 g. of the drug, 24 hr. previously.
† This dog (10 kg.) had received 5 g. of the drug daily for 5 days, the urine analysed was for the fifth day.

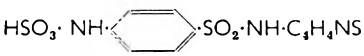
DISCUSSION

In Table VII the data on each species of animal has been averaged and the amount of each metabolite found in the urine has been calculated as a percentage of the total drug excreted. The major transformation product of sulphasomizole is N^4-acetylsulphasomizole (II) and it is clear that the extent of acetylation varies with species. About two-thirds of the excreted drug is acetylated in the rabbit, just over one-third in man, just under one-third in the rat and none at all in the dog. The N^4-glucuronide of sulphasomizole (III) is a minor constituent of the urine and we are of the opinion that it is an artifact and not a true metabolite. Other work carried out in this department (Bridges and Williams, 1962a) on the formation of N^4-glucuronides of aromatic amines strongly suggests that these compounds

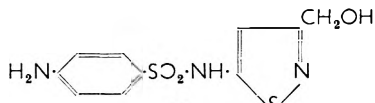


II III

are formed in the urine non-enzymically. The occurrence of an aryl sulphamate (N^4-sulphosulphasomizole, IV) was suspected in rat and dog urine and these compounds (aryl sulphamates) are known metabolites of some aromatic amines *in vivo* (Boyland, Manson and Orr, 1957; Parke, 1960) and *in vitro* (Roy, 1960; 1961). There are also reports in the Japanese literature of the occurrence in urine of the sulphamates of sulphathiazole (Uno and Veda, 1960), sulphisoxazole (Uno and Kono, 1960), and sulfamethylthiadiazole (Uno and Okazaki, 1960). A minor metabolite, which we suspect is the glucuronide of an oxidation product



IV



V

of sulphasomizole, was found by paper chromatography in rabbit and dog urine. This compound did not appear to be a phenolic glucuronide and we suggest that it may be derived from sulphasomizole oxidized at the 3-methyl group (V).

TABLE VII

URINARY METABOLITES OF SULPHASOMIZOLE IN VARIOUS SPECIES

Average values have been calculated from the other Tables

	Chinchilla rabbits	Wistar* rats	Corgi dogs	Man
Number of Animals	3 ♀	3 ♀	2 ♂	5 ♂
Oral dose of drug, mg./kg.	150	150	500	30
Total drug excreted in 24 hr. in per cent of dose	79·5	71·5	35·9	59·7
Metabolites found in urine	Per cent of total drug excreted			
Free drug	26·9	68·1	90·2	61·8
N^4-Acetylated drug	68·3	29·0	0·0	36·8
N^4-Glucuronide	1·1	1·5	1·4	0·7
"N^4-Sulphamate"	0·0	1·4	3·9	0·1
"Oxidation product"	2·2	0·0	4·2	0·0

* The figures for rats are for 48 hr. after dosing.

Acknowledgement. The work was supported by a grant from May & Baker, Ltd. We are grateful to Dr. H. J. Barber, Dr. J. P. Johnson and Dr. R. Slack of May & Baker Ltd., for materials and assistance.

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HISTAMINE AS AN IMPURITY IN SAMPLES OF HISTIDINE

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Received March 22, 1963

Commercial samples of histidine are shown to contain histamine, generally in small quantities, but occasionally between 100 and 260 μg./g. When this fact is taken into account in incubation experiments of tissue extracts no evidence is found for the presence of histidine decarboxylase in extracts of cat liver or kidney. This is in contrast to the original claim by Holtz, Heise and Spreyer.

The first and often quoted evidence for the presence of histidine decarboxylase in cat tissue is the finding by Holtz and Heise (1937) and by Holtz, Heise and Spreyer (1937-1938) of the formation of histamine by cat liver brei incubated with histidine. This finding has not been confirmed (Waton, 1956) and the present experiments provide a possible explanation for this discrepancy since it was found that commercial samples of histidine may contain high amounts of histamine, the presence of which can easily simulate formation of histamine in incubation experiments with animal tissue if this factor is not excluded by control experiments. Hitherto the presence of histamine as impurity in commercial samples of histidine has been mentioned only by MacKay and Shepherd (1960) and by White (1960).

Methods

Nineteen samples of histidine were assayed for histamine. Of fifteen samples, commercially obtained, twelve were the L-monohydrochloride, one the DL-dihydrochloride, one the free DL-amino-acid and one the free D-amino-acid. In addition, four samples of the free DL-amino-acid prepared several years ago in this laboratory, were examined.

The samples were made 1/200 either in water if the salt, or in dilute hydrochloric acid (0·02N) if the free amino-acid was used, and assayed on the atropinised guinea-pig ileum preparation suspended in 5 ml. oxygenated Tyrode's solution. The contractions obtained were abolished by doses of mepyramine maleate and recovered in parallel with histamine, which according to Reuse (1948) is good evidence for the identification of histamine.

Preparation and incubation of tissue extracts was carried out according to the method of Holtz, Heise and Spreyer (1937–1938). Fresh cat liver or kidney tissue was minced and ground with silver sand and 0·05M disodium phosphate buffer (1 g. tissue/5 ml. buffer), centrifuged and the supernatant shaken with kaolin (1 g./20 ml.) for 15 min. to remove histaminase from the extracts; after further centrifugation, the supernatants were removed and used for incubation.

Samples of 5 ml. of liver extracts or of 3 ml. of kidney extracts were mixed with an equal volume of histidine (4 mg./ml.) with or without 1 ml. or 0·6 ml. of toluene respectively. Immediately after mixing nitrogen was

blown into each flask for 5 min. and the flasks were stoppered and incubated for 18 hr. at 37°. Their histamine content was assayed on the arterial blood pressure of a cat anaesthetised with pentobarbitone sodium.

RESULTS

Histamine Content in Samples of Histidine

In Table I are shown the histamine contents obtained for 19 samples of histidine. Three samples contained either no histamine or less than 2 μg./g., six samples contained 4–8 μg./g., seven samples 10–20 μg./g. but three samples contained as much as 106, 160 and 240 μg./g.

TABLE I

HISTAMINE CONTENT (μg./g.) OF 19 SAMPLES OF HISTIDINE

	Histidine samples	Histamine μg./g.
1.	Commercial L- monohydrochloride ..	6
2.	" " " ..	6
3.	" " " ..	8
4.	" " " ..	8
5.	" " " ..	10
6.	" " " ..	14
7.	" " " ..	16
8.	" " " ..	16
9.	" " " ..	20
10.	" " " ..	106
11.	" " " ..	160
12.	" " " ..	240
13.	" DL- dihydrochloride	<2
14.	" free DL- amino-acid	<2
15.	" free D- amino-acid	<2
16.	Old laboratory free DL- amino-acid ..	4
17.	" " " " " " ..	6
18.	" " " " " " ..	14
19.	" " " " " " ..	14

Incubation of Extracts with histidine

Two samples of histidine, No. 9 and No. 10 of Table I containing 20 and 106 μg./g. histamine respectively were chosen for the incubation experiments.

Liver Extracts

Samples of liver extracts incubated for 18 hr. with histidine contained detectable amounts of histamine only if the histidine sample contained a high amount of histamine. The histamine assayed was fully accounted for by the histamine impurity of the histidine sample.

A typical experiment is illustrated in Fig. 1 which gives an assay, on the arterial blood pressure of the cat, of samples of 1 ml. of liver extract incubated with histidine either samples 10 or 9. If no histamine had been formed or destroyed, the 1 ml. liver extract would have contained 212 ng. of histamine, if histidine sample 10, and 40 ng., if histidine sample 9 had been used for incubation. The 1 ml. of the liver extract incubated with histidine sample 10 either with or without toluene, gave a depressor action (at E and B) smaller or equal to that of 200 ng. histamine (at A and L) and slightly less than that of the liver extract which had not been

incubated but to which the same amount of histidine sample 10 had been added (at H). The effect of 2 mg. histidine sample 10 which is equivalent to the amount of the added histidine in 1 ml. of liver extract is shown for comparison (at K).

The 40 ng. histamine present in 2 mg. histidine sample 9 was insufficient to lower the blood pressure (at J) and the 1 ml. of liver extract to which 2 mg. of this sample of histidine had been added remained ineffective whether the sample had been incubated with (at F) or without toluene (at C). The figure shows further that the liver extract produced no fall in arterial blood pressure either when incubated without histidine in the absence or presence of toluene (at D and G) or when not incubated but containing the histidine (at I).

From these results it is not only evident that there is no formation of histamine by liver brei on incubation with histidine but also that by the use of a sample of histidine with a high histamine impurity, an apparent formation of histamine is simulated if this impurity is not taken into consideration.

FIG. 1

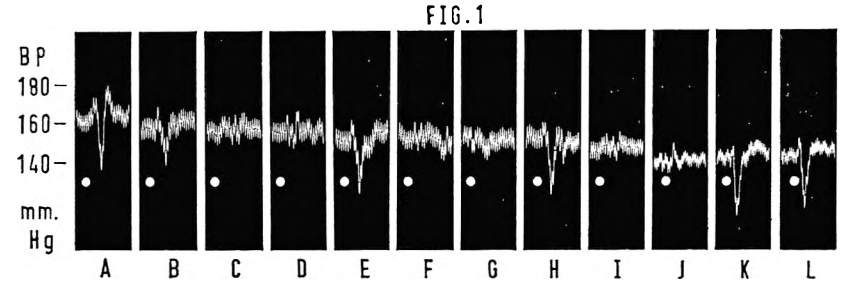

FIG. 2

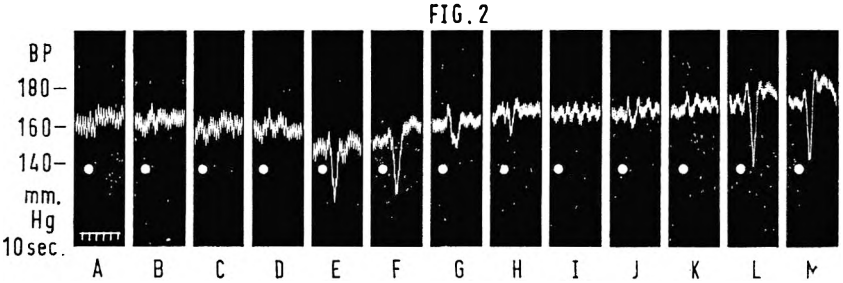

FIG. 1. Arterial blood pressure of cat anaesthetised with pentobarbitone sodium. Assay of liver extract incubated for 18 hr. Effects of 200 μg. histamine (A and L) of 2 mg. histidine sample 9 (J) and of 2 mg. histidine sample 10 (K). Effects of 1 ml. liver extract incubated with histidine sample 10 (B and E), with 2 mg. histidine sample 9 (C and F) and without histidine (D and G). B-D incubated without, and E-G with toluene. Effects of 1 ml. unincubated liver extract with 2 mg. histidine sample 10 (H) and sample 9 (I).

FIG. 2. Preparation as in Fig. 1. Assay of kidney extract incubated for 18 hr. Effects of 200 μg. of histamine (E and M), of 2 mg. histidine sample 9 (K) and of 2 mg. histidine sample 10 (L). Effects of 1 ml. of kidney extract incubated with histidine sample 9 (A and C) and with histidine sample 10 (B and D). A and B incubated without, and C and D incubated with toluene. Effect of 1 ml. unincubated kidney extract (J). Effect of 1 ml. kidney extract with 2 mg. histidine sample 0 tested after standing at 18° for 25 sec., 5, 10 and 20 min. (F-I).

Kidney Extracts

No histamine was detected in any of the 1 ml. samples of kidney extract tested after incubation for 18 hr. with histidine, with or without toluene. This result which is illustrated in Fig. 2,B was also obtained when histidine No. 10 with the high histamine impurity had been used.

The incubated kidney extract injected into the same cat, on which the liver extract had been assayed, produced no depression whether incubation was with histidine sample 9 or 10 and whether incubation was without or with toluene (Fig. 2, A–D) yet the injection of 200 ng. histamine still produced its strong depressor effect (Fig. 2, E and M). For comparison the Figure also shows the effect of unincubated kidney extract without added histidine (at J) as well as the effects of 2 mg. histidine sample 9 and 10 (at K and L) an amount equivalent to that added to 1 ml. kidney extract. The fact that the kidney extract incubated with histidine sample 10 which contained the high impurity did not produce a fall in arterial blood pressure was shown to be due to the histamine-destroying activity of the extract. This destruction occurred rapidly as was evident when kidney extract was kept with histidine No. 10 at room temperature (18°) for periods varying between 25 sec. and 20 min. (at F–I). Thus the ability of the kidney extract to destroy histamine was not abolished by the pretreatment with kaolin. Therefore the failure to detect formation of histamine by these extracts does not exclude the possibility that histamine was formed but subsequently destroyed.

Discussion

The finding that commercial samples of histidine contain histamine, generally in small amounts, but occasionally in large quantities has to be taken into account when investigating the enzymic formation of histamine by tissue extracts with histidine as substrate.

From the present results it would appear that the early report by Holtz, Heise and Spreyer (1937–1938) on the formation of histamine by cat liver is attributable to impurities of histamine in the samples of histidine used as substrate by these authors, since they did not examine the histamine content of their histidine samples, but only compared the effects on the arterial blood pressure of liver extract incubated with and without histidine. In the present experiments, it was shown that, if a histidine sample containing a high amount of histamine was used as substrate, the histamine was still present after 18 hr. incubation, but no evidence for newly formed histamine was obtained. If it had not been known that the histidine sample, used as substrate, was contaminated with a large amount of histamine, this finding might have suggested formation of histamine.

Since the kidney extracts used in the present experiments were able to destroy histamine, the kaolin treatment did not fully remove the histaminase known to abound in cat kidney (Waton, 1956) and this fact explains why kidney extracts in contrast to liver extracts, no longer contained detectable amounts of histamine after incubation for 18 hr. with histamine contaminated histidine. Holtz and Heise who detected histamine in 5

out of 30 samples of cat kidney extracts after treatment with kaolin and incubation with histidine for 18 hr. were probably more successful in removing the histaminase from at least some of their kidney extracts, but their five positive results cannot be taken as evidence for the presence of histidine decarboxylase in cat kidney.

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THE INFLUENCE OF LIPID ON THE PAPER CHROMATOGRAPHIC BEHAVIOUR OF SYMPATHOMIMETIC CATECHOLAMINES IN PLASMA EXTRACTS

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Inconsistent results have been obtained during the separation and estimation of catecholamines in extracts of rabbit plasma and the cause traced to displacements of R_F values by lipid. An extraction technique which overcomes this is described. The possibility of misinterpretation of results after chromatographic separation on paper of the catecholamines from biological extracts is discussed.

Using a modified version (Vogt, 1952) of the recommendations of Crawford and Outschoorn (1951) for the quantitative separation of catecholamines from biological extracts and tissue fluids by paper chromatography, inconsistent recoveries, high blank activities and assay results diverging from parallelism were encountered in this laboratory when estimating concentrations of noradrenaline, adrenaline and isoprenaline in rabbit plasma. The inconsistent results were obtained with the eluates from the paper chromatograms of all three amines. It seemed that, with the phenol-hydrochloric acid solvent system used, neither the formation of lactyl-noradrenaline or lactyl-adrenaline (R_F values 0·57 and 0·80 respectively; Lockett, 1954), nor the interference by 5-hydroxytryptamine (5-HT) or dopamine (R_F values 0·51 and 0·37; Vogt, 1959) were major causes of the irregularities. As the extraction techniques convert all the amines to hydrochloride salts and the developing solvent contained hydrochloric acid, possible multiple spot formation (West, 1959; Beckett, Beaven and Robinson, 1960) was eliminated since the use of the same acid in salt and solvent system resulted in compact spots in the chromatography of divers bases (Munier, 1952). The R_F values of these catecholamines have been shown to be dependent on many factors (Roberts, 1963) it was decided, therefore, to investigate the effects of plasma as a source of explanation of the results obtained.

Methods

Preparation of the Extracts for Chromatography

Blood was collected from the femoral arteries of heparinised (500 u./kg.) rabbits under urethane anaesthesia (6·0 ml./kg. of a 20 per cent w/v solution in 0·8 per cent w/v NaCl injected intravenously via a marginal ear vein) and was then centrifuged at 3000 r.p.m. for 10 min. The plasma was removed and aliquots mixed with 0·1 ml. of a solution of noradrenaline adrenaline and isoprenaline in distilled water (500 μg. each amine per ml.), before preparation of an extract for chromatography by the methods of Vogt (1952), Peacock (1960) or that now described.

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The plasma was shaken in a large conical flask with six times its volume of ice-cold acid-ethanol (1·0 ml. N HCl/litre absolute ethanol) and left for 1 hr. at −20° to complete precipitation of proteins. The mixture was centrifuged (3000 r.p.m. for 3 min.), the precipitate washed with more acid-ethanol (5–10 ml.), re-centrifuged and the combined supernatants transferred to a suitable R.B. Quickfit flask. The solution was then distilled at 0·5–1·0 mm. Hg (bath temperature 30–35°). The moist residue was shaken vigorously with 4·0 ml. ether : benzene mixture (5 : 2) plus 4·0 ml. 0·01N hydrochloric acid and the resultant mixture transferred to a centrifuge tube. The flask was washed with a further 2·0 ml. 0·01N hydrochloric acid and the total 10·0 ml. centrifuged at 3000 r.p.m. for 5 min. The "milky" upper layer and any interfacial "cream" was removed and the clear aqueous layer remaining was mixed with an equal volume of absolute ethanol, saturated with sodium chloride, and distilled as before. The residue was washed thoroughly with acetone : ethanol (1 : 1) to a total volume of 1·0 ml. and the resultant solution centrifuged (3000 r.p.m., 5 min.), and the supernatant chromatographed on paper.

In some experiments the residues were taken up in distilled water (0·5 ml.) and applied to the paper as an aqueous solution.

Chromatography

Chromatograms were developed by the ascending technique using phenol containing 15 per cent v/v 0·01N hydrochloric acid and apparatus and conditions previously described (Roberts, 1963). The amines were located by spraying the papers with a solution of potassium ferricyanide (0·44 g.) in sodium hydroxide (100 ml., 0·05N). Because of the dependence of the R_F values of the catecholamines on factors such as temperature and distance of solvent flow (Roberts, 1963) extracts were compared with each other on a single sheet of paper and a quantitative relationship between the chromatograms was not sought.

For the recovery experiments, the papers were sprayed with an aqueous solution of ascorbic acid (Crawford and Outschoorn, 1951) before use, and the air inside the chromatography tanks was displaced by carbon dioxide (Vogt, 1952). After development the papers were washed thoroughly with benzene to remove the phenolic solvent and the amines eluted overnight using techniques essentially similar to those described by Crawford and Outschoorn (1951). Distilled water only was used as the eluant since I have found solutions of sodium dihydrogen phosphate to give rise to "blank" activity.

Biological Assay of Eluates

In addition to using the rat uterus, rat colon and rat blood pressure, after urethane or pentobarbitone anaesthesia, for the biological estimation of the eluates containing sympathomimetic catecholamines, extensive use was made of the blood pressure of pithed rats both before and after the potentiation by cocaine (2·5 mg./kg.) administered intravenously.

Results and Discussion

Displacement caused by Plasma

Of 10 experiments, in each of which 2·5, 5 and 10 ml. samples of plasma containing the reference amines were extracted using the method of Vogt (1952) and chromatographed as spots, 6 showed displacements in R_F values as shown in Fig. 1, three resulted in a concentration of the amines around the adrenaline R_F value and one produced an upward migration, greater than shown in the figures, for all three amines. Similar experiments in which the extracts were applied as strips 5 cm. long again resulted in definite, but less marked, shifts in R_F values proportional to the volume of plasma used.

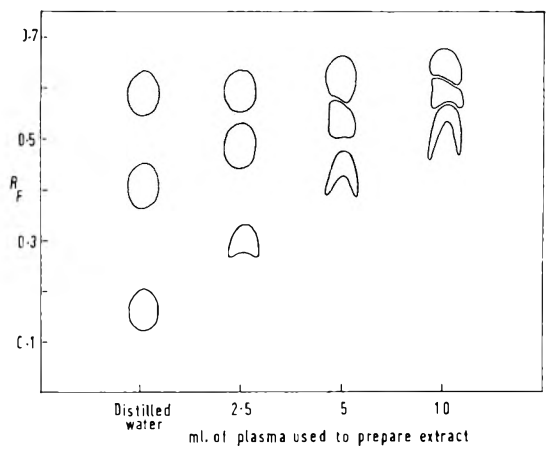

FIG. 1. Displacement of the R_F values of noradrenaline (lower spots), adrenaline (middle spots) and isoprenaline (upper spots) caused by lipid when chromatographed from plasma extracts (Vogt, 1952) compared with values obtained from aqueous solution. Developing solvent, phenol containing 15 per cent v/v 0·1N hydrochloric acid.

The figure indicates that the use of aqueous solutions of catecholamines for reference chromatograms for plasma extracts would give misleading results in that much noradrenaline and adrenaline, being displaced, would not be taken up in their eluates, their biological activity then appearing as "blank" activity at the surrounding R_F value levels. The use of "adjusted" aqueous R_F values (Crawford and Outschoorn, 1951) would be equally misleading. Usually, reference chromatograms are developed from plasma extracts to compensate for any alteration in R_F values, but often any suitable volume of plasma is used, and even when the volumes of the control and test plasma samples are the same, the practice of applying the reference extract as a spot (i.e. a greater local concentration of the interfering substance) and the test extract as a strip, invalidates the control.

Applying a reference extract as a strip uses valuable space on the chromatography paper but an attempt to locate the catecholamines

present in a plasma extract applied as a series of 10 spots, by using a reference extract prepared from 1/10th of the volume of the test sample and applied as a single spot, was unsuccessful.

Protein Precipitation

The course of protein precipitation by acid-ethanol over 24 hr. was followed by treating aliquots of the supernatant with an equal volume of 10 per cent aqueous trichloroacetic acid and comparing the turbidities produced. It was impossible to obtain complete removal of proteins by this method but the conditions described on page 580 gave optimal precipitation.

Lipid Extraction

Crawford and Outschoorn (1951) have described their final extracts as "faintly yellow" and "lipid-like" and I have found this to apply to extracts prepared by the method of Vogt (1952). Papers held to the light showed well-defined grease-spots at the application areas; this indicated that lipid rather than residual protein was responsible for the displacement of the R_F values and that some form of lipid extraction before application to the paper might stabilise the chromatographic behaviour of the catecholamines. An extraction of this kind is included in the method of Peacock (1960) where the supernatant, after acid-ethanol protein precipitation, is evaporated to 5–10 ml. and the lipids removed with ether. Unfortunately when this method was tried, the residual solution after evaporation was miscible with ether presumably as a result of azeotropic mixture formation. When benzene was added to the mixture the acid-ethanol was displaced from solution in the ether. This is in agreement with the results of Lockett (personal communication) who found that a mixture of benzene and ether was the most satisfactory solvent for extracting lipid from human plasma with minimum loss of catecholamine.

The extraction method I have described results in constant R_F values for the catecholamines when they are chromatographed as spots from extracts prepared from 1 to 10 ml. volumes of plasma. These values were higher than those from aqueous solution, but when the amines were chromatographed from 0·9 per cent sodium chloride the R_F values were similarly elevated and therefore saline solutions were used to produce reference chromatograms. By applying the extracts as strips 10 cm. long it was possible to use extracts from plasma volumes of up to 100 ml. without significant change in R_F values. Furthermore, blank activity between the spots was largely eliminated, biological assays were parallel and the grease-spots and "clogging" of the paper at the application areas were no longer evident. The recoveries of 5μg. of each amine were seldom greater than 60 per cent but were relatively constant for extracts prepared from a wide range of plasma volumes.

The Influence of Lipid

The influence of lipid was confirmed by adding varying amounts of lipid, extracted from rabbit plasma, to acetone-ethanol solutions of the

catecholamines when the R_F values were again displaced in proportion to the amount of lipid added. When lipid-free extracts were prepared from volumes of plasma in excess of 10 ml. and applied as spots, displacement of the amines was again observed even when the final residues were applied to the paper from aqueous solution. It was also found that chromatography, using distilled water, of extracts of plasma (1–25 ml.) prepared by the method of Vogt (1952), resulted in constant R_F values up to 10 ml. of plasma after which increased migration, particularly of noradrenaline, was observed. Much larger shifts occurred as the plasma volumes were increased. The interfering substance(s) must be soluble in both distilled water and acetone-ethanol but the phenomenon has not been investigated further.

The unsatisfactory results initially obtained may be explained by the inadequacy of the reference chromatograms, because the variable displacements of the amines could result in variable recoveries and leave unknown amounts of amines to appear as blank activity. When displacement is severe, the contamination of one amine with another will give rise to lack of parallelism in biological assays. There also exists the possibility of new active substances and metabolites (Roberts and Lockett, 1961) being contaminated or falsely "identified" because of the difference between the R_F values obtained on the reference and test chromatograms of the naturally occurring catecholamines. Similar results were obtained with cat plasma.

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A DIRECT TITRIMETRIC METHOD FOR THE DETERMINATION OF SOME ORGANIC BASES IN PHARMACEUTICAL PREPARATIONS

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A rapid titrimetric method using an extractive end-point has been developed for the determination of atropine, codeine, methadone and pethidine in pharmaceutical preparations. Chloroform is added to the organic base dissolved in pH 2·8 buffer solution so that the ratio of chloroform to aqueous phase lies between 2 to 1 and 4 to 1. Titration is carried out with sodium dioctylsulphosuccinate using Dimethyl Yellow screened with Oracet Blue B as indicator; at the end-point the chloroform phase changes colour from green to pink. The method is accurate to ±1 per cent and has been applied to a number of injection, eye-drop, syrup and tablet preparations. Since little sample-preparation is required, most preparations may be assayed within 10 min., whilst the codeine content of complex compound tablets can be determined in 20 min.

Volumetric methods using extractive end-points with anionic surface-active agents as titrants have been described for the determination of certain tertiary bases (usually containing a terminal *N*-substituted side chain) in pharmaceutical preparations (Carkhuff and Boyd, 1954; Pellerin, Gautier and Demay, 1962). The extension of this type of titration to certain cyclic amines, notably codeine, has been investigated.

Experimental

Many cyclic amines form insoluble complexes with sodium lauryl sulphate or sodium dioctylsulphosuccinate but when Carkhuff and Boyd's method was used in an attempt to determine these compounds poor and premature end-points were obtained. Previous experience had shown us that better end-points could sometimes be obtained by replacing the mineral acid used in Carkhuff and Boyd's method with a weak acid such as acetic acid. This proved to be the case with codeine, the use of acetic acid giving stoichiometric end-points. In practice the use of an acetate buffer, pH 2·8, has been found to be convenient and satisfactory.

Detection of the end-point as recommended by Carkhuff and Boyd, by the colour of the drop of chloroform formed on shaking at the surface of the aqueous phase, is unsatisfactory in the titration of cyclic amines. As the ratio of chloroform to aqueous phase is progressively decreased below about 1·5 to 1, the volume of standard titrant required to titrate a given quantity of amine decreases. No such variation in the volume of titrant occurs if the ratio of chloroform to aqueous phase is maintained between 2 to 1 and 4 to 1; there is no drop formation and the end-point is

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judged by a change in colour of Dimethyl Yellow (the indicator used by Carkhuff and Boyd) from yellow to pink in the bulk of the chloroform. This colour change is greatly improved if the indicator is screened with Oracet Blue B. Under these conditions the titration may be conveniently carried out in a 500 ml. flask, provided that vigorous swirling is employed throughout.

Factors that might influence the results obtained have been examined. The volume of buffer solution used can be varied between 1 and 10 ml. without effect; above 10 ml., end-points are less sharp and titration values are slightly high. The ratio of chloroform to aqueous phase may be varied from 4 to 1 down to 1·5 to 1, whilst the volumes of chloroform and aqueous phases may lie between 20 to 150 ml. and 10 to 100 ml. respectively, provided that the correct ratio is maintained. A small titration blank is obtained and should always be allowed for; many batches of chloroform B.P. have been examined for use in this titration and all have given a blank that varies from about 0·13 ml. of titrant when 60 ml. of chloroform is used to 0·22 ml. when 150 ml. is used. The titration can be carried out equally well in daylight or under various forms of artificial lighting, and laboratory temperature variations between 15 and 26° have been shown to be without effect. The titrant appears to be very stable; no significant change in concentration was detectable over a period of 7 weeks.

Method

Reagents. Buffer solution pH 2·8: Dissolve anhydrous sodium acetate (4 g.) in water (approximately 830 ml.), add glacial acetic acid (approximately 155 ml.) until the pH reaches 2·8 and then dilute to 1 litre with water.

Sodium dioctylsulphosuccinate: Add "Manoxol O.T. 60 per cent solution" (15 ml.) to water (300 ml.) and warm to dissolve; cool to room temperature, then dilute to 2 litres with water. Store in an amber-glass bottle. Standardise by titration of codeine phosphate, atropine sulphate, pethidine hydrochloride or methadone hydrochloride, the purity of which has been determined by the official method of the British Pharmacopoeia. Then $E = \frac{A \times B}{C}$ where E = "mg./ml. equivalent"—the weight (mg.) of organic base equivalent to 1 ml. of sodium dioctylsulphosuccinate solution. A = weight (mg.) of organic base titrated. B = per cent purity of the organic base as determined by the official method of the British Pharmacopoeia. C = titre (ml.).

Screened Dimethyl Yellow indicator: Dissolve Dimethyl Yellow (15·0 mg.) and Oracet Blue B (15·0 mg.) in chloroform, B.P. (500 ml.).

Sample preparation. Syrup, linctus and simple aqueous preparations. Titrate directly the quantity of preparation specified in Tables III and V, diluted to 25 ml. with water.

Tablets (containing lactose basis). Dissolve the number of powdered tablets specified in Tables III and V in water (25 ml.), warming gently if

necessary. Add pH 2·8 buffer solution (5 ml.) and continue by the general procedure below, beginning with the words "a known volume of chloroform . . .".

Compound Codeine Tablets. Add N sodium hydroxide (20 ml.) to the powdered tablets (2·5 g.) contained in a 150 ml. separator; shake for 2 min. then extract by shaking with one 50 ml., two 20 ml. and one 10 ml. portions of chloroform, washing each extract in turn with the same 10 ml. of water. To the combined chloroform extracts add water (25 ml.), pH 2·8 buffer solution (5 ml.) and continue by the general procedure below, beginning with the words "and screened Dimethyl Yellow indicator (5 ml.)".

General procedure. To a solution of the active material in a total volume of 25 ml. contained in a 500 ml. conical flask, add pH 2·8 buffer solution (5 ml.), a known volume of chloroform (between 60 and 120 ml.) and screened Dimethyl Yellow indicator (5 ml.). Titrate with sodium dioctylsulphosuccinate solution, adding the titrant fairly rapidly until nearing the end-point and swirling vigorously throughout. Then add the titrant dropwise, again swirling vigorously; after each addition allow the two phases to separate and then gently swirl for about 5 sec. The end-point is detected by a colour change from green to pinkish-grey in the bulk of the chloroform. Carry out a blank determination under the same conditions; the difference between the two titration values is equivalent to the amount of base present.

Results and Discussion

Of the substances examined, codeine phosphate (3·5 to 38 mg.), atropine sulphate (6 to 30 mg.), pethidine hydrochloride (7 to 24 mg.) and methadone hydrochloride (7 to 31 mg.) are all satisfactorily titrated. Hyoscine, aneurine, morphine, procaine, pilocarpine, lignocaine and dexamphetamine also titrate, but the end-points are very sluggish. The pink dimethyl yellow-sodium dioctylsulphosuccinate complex formed at the end-point tends to concentrate at the interface between the chloroform and aqueous phases and is best extracted into the bulk of the chloroform by very gentle swirling. It is for this reason that the titration technique described under "General Procedure" has been adopted. It has proved satisfactory even to operators new to the method. The precision of the titration, as applied to codeine phosphate (between 3·5 and 38 mg.) by two operators on different days, is shown in Table I. Possible interference from commonly occurring tablet excipients, bacteriostats, etc., was examined by titrating codeine phosphate in their presence (Table II).

TABLE I

Precision of the titration when applied to codeine phosphate, B.P.

	No. of determinations	Mean	Standard deviation
Analyst A*	9	3·95	0·026
Analyst B	26	3·96	0·015

* Analyst A had no previous experience of the method.

TABLE II

INTERFERENCE FROM OTHER MATERIALS IN THE TITRATION OF 36·3 MG. OF CODEINE PHOSPHATE, B.P.

Material	Quantity	Codeine phosphate found (mg.)	Recovery per cent
Syrup, B.P.	10 g.	36·2; 36·2	99·7; 99·7
Lactose, B.P.	1 g.	36·4; 36·1	100·3; 99·4
Stearic Acid, B.P.C.	10 mg.	36·5; 36·2	100·6; 99·7
Solution for Eye-drops, B.P.C.	10 ml.	36·3; 36·5	100·0; 100·6
Starch, B.P.	20 mg.	36·3; 36·5	100·0; 100·6
*Sodium Chloride, B.P.	0·1 g.	37·0; 36·7	101·9; 101·2
0·5 per cent w/v Phenol, B.P.	20 ml.	36·3; 36·3	100·0; 100·0
0·5 per cent w/v Cresol, B.P.	20 ml.	36·4; 36·6	100·4; 100·9
Salicylamide	1 g.	36·7; 36·6	101·2; 100·9
Caffeine, B.P.	0·75 g.	36·4	100·3
†Soluble Compound Codeine Tablets B.P. Add. 1960, but containing no codeine	2·5 g.	36·3; 36·3	100·0; 100·0
†Polyvinylpyrrolidone	10 mg.	36·5; 37·1	100·6; 102·3
Gelatin, B.P.	10 mg.	Titrates with emulsification of the two phases	

* End-point sluggish; this quantity should not be exceeded.
† Chloroform extract made from 20 ml N sodium hydroxide.

TABLE III

APPLICATION TO SIMPLE CODEINE PREPARATIONS

Preparation	Quantity taken	Codeine phosphate content found by		Results expressed as
		Proposed method	Official method	
Syrup of Codeine Phosphate B.P.C. 0·5 per cent w/v	6 g.–9·5 g.	0·495; 0·500; 0·495; 0·501; 0·495 (Mean = 0·497)	0·49	Per cent w/v of monohydrate
Linctus of Codeine B.P.C. 0·375 per cent w/v	9 g.–11 g.	0·372; 0·372 (Mean = 0·372)	0·369	Per cent w/v of monohydrate
Codeine Phosphate Tablets B.P. 16·2 mg./tab.	2 tablets	16·7; 17·0; 16·7; 16·8 (Mean = 16·8)	16·9	mg./tab. of $1\frac{1}{2}$ H_2O
Codeine Phosphate Tablets B.P. 32·4 mg./tab.	1 tablet	33·2; 32·6; 32·7 (Mean = 32·8)	32·8	mg./tab. of $1\frac{1}{2}$ H_2O

TABLE IV

APPLICATION TO COMPOUND CODEINE TABLETS

Preparation	Declared mg./tab.	mg./tab. Codeine Phosphate B.P. found by	
		Proposed method	Official method
Soluble Compound Codeine Tablets B.P. Add. 1960	8·1	1. 7·6; 7·6 2. 7·7; 7·7; 7·7 3. 7·1; 7·3 4. 7·8; 7·9 5. 9·3; 9·3†	8·0 7·6 7·3 7·7 9·3; 9·0
Compound codeine tablets (modified formula)	8·1	1. 8·1; 8·1	8·0
Compound Codeine Tablets B.P.	8·1	1. 8·2; 8·2 2. 8·1; 8·4	— —
Soluble compound codeine tablets (modified formula)	8·1	1. 8·1; 8·0	—
*Codeine, caffeine and aspirin tablets	16·2	1. 16·1; 16·1	—

* Weight of powdered tablets equivalent to two tablets used in the determination.
† Rejected batch.

TABLE V

APPLICATION TO THE DETERMINATION OF OTHER ORGANIC BASES IN MISCELLANEOUS PHARMACEUTICAL PREPARATIONS

Preparation	Quantity taken	Declared	Compound found by		Results expressed as
			Proposed method	Alternative method	
Injection of Atropine Sulphate B.P.	25 ml.	0·65	0·67; 0·68	0·67*	mg./ml. Atropine Sulphate B.P.
Hypodermic tablets of atropine sulphate ..	30 tablets	0·65	0·61	0·62†	mg./tab. Atropine Sulphate B.P.
Eye-drops of atropine sulphate	2 or 3 ml.	1·00	1·00; 0·99; 0·99	0·99§	per cent w/v Atropine Sulphate B.P.
Atropine Sulphate Tablets B.P.	40 tablets	0·65	(*a*) 0·67; 0·68 (*b*) 0·68; 0·67 (*c*) 0·69	0·66* 0·62* 0·69†	mg./tab. Atropine Sulphate B.P.
Methadone Tablets B.P. ..	5 tablets	5·0	4·89; 4·89	4·87‡; 4·93‡	mg./tab. Methadone Hydrochloride B.P.
Methadone Tablets—rejected batch	5 tablets		4·43; 4·56; 4·41	4·45†	mg./tab. Methadone Hydrochloride B.P.
Methadone Injection B.P.	3 ml.	1·00	1·03; 1·02; 1·04; 1·03	1·03*	per cent w/v Methadone Hydrochloride B.P.
Pethidine Tablets B.P. ..	1 tablet	25·0	23·9; 23·8; 23·9	23·9* 23·9†	mg./tab. Pethidine Hydrochloride B.P.
Pethidine Injection B.P. ..	Dilute 2 ml. to 50 ml. with water; take 10 ml.	50·0	50·7; 50·5; 50·9; 51·1; 51·1	50·8*	mg./ml. Pethidine Hydrochloride B.P.

* Official method of the British Pharmacopoeia.
‡ Ultra-violet spectrophotometric method.
† Tetraphenylboron method (Johnson and King, 1962).
§ Laboratory-prepared sample.

Table III lists the results obtained by the proposed method and other methods on simple codeine preparations, and Table IV gives results for a number of compound tablets. Table V shows the results obtained for the determination of other organic bases, by the proposed and alternative methods, in various pharmaceutical preparations. Linctus, syrup, simple tablet, injection solution and aqueous eye-drop preparations may be assayed within 10 min., and the codeine content of compound tablets can be determined in 20 min.

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SULPHAMOYLBENZO-1,2,3,4-THIATRIAZINE 1,1-DIOXIDES: A NEW CLASS WITH ORAL DIURETIC ACTIVITY

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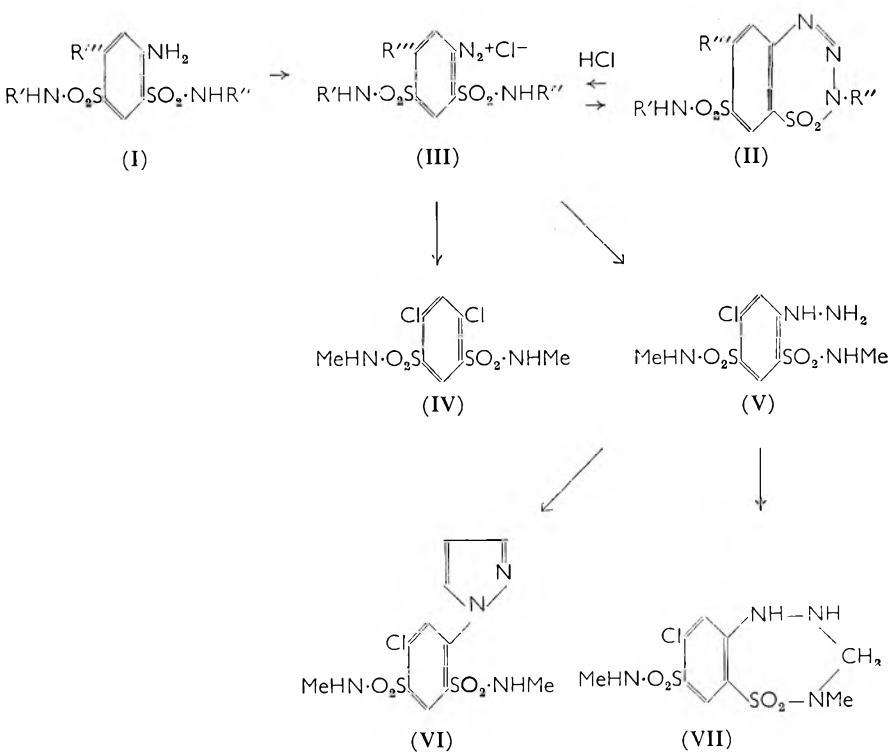
A series of sulphamoylbenzo-1,2,3,4-thiatriazine 1,1-dioxides has been prepared. While some of them were found to cause diuresis in rats on oral administration, none was more active than chlorothiazide.

During the past three years a search has been made in these laboratories for new orally active diuretic agents. One line of approach has been to synthesise analogues of known diuretics, introducing structural features which were sufficiently novel for there to be a reasonable chance that the products might possess, in addition to diuretic activity, the advantage of producing a low relative potassium ion excretion.

During one phase of this work, some compounds related to chlorothiazide were prepared in which the amino-group of the active diuretic 4-amino-6-chlorobenzene-1,3-disulphonamide (Sprague, 1958) (I; R′ = R″ = H, R‴ = Cl) was modified in various ways.



This compound was readily diazotised with nitrosyl sulphuric acid, but the product was unstable, nitrogen being evolved at 0–5°; on diluting the reaction mixture no precipitate was formed. By sharp contrast, diazotisation of the *N*-substituted sulphonamides (I; R′ = R″ = Me, R‴ = Cl), (I; R′ = H, R″ = Me, R‴ = Cl) and (I; R′ = H, R″ = $-CH_2$·Ph, R‴ = Cl) under similar conditions yielded stable diazonium salts which, on diluting the reaction mixture with water, were precipitated as pale-cream crystalline plates having properties conforming with the benzo-thiatriazine structure (II).

As expected, a solution of the benzo-1,2,3,4,-thiatriazine 1,1-dioxide (II; R′ = R″ = Me, R‴ = Cl) in concentrated hydrochloric acid behaved as if it were a solution of the corresponding diazonium salt (III). For example, on heating the solution at 95°, the dichloro-compound (IV) was formed. Also, treatment of the solution with stannous chloride gave the hydrazino-compound (V), characterised as its *N*-acetyl derivative. The hydrazino-compound (V) also gave the expected cyclic derivatives (VI) and (VII) when treated with tetraethoxypropane in ethanolic hydrochloric acid, and with formaldehyde in alcoholic sodium hydroxide, respectively.

By contrast with the parent amine (I; R′ = R″ = Me, R‴ = Cl), the hydrazine (V) had little or no diuretic activity in the rat. This could have been due to the fact that the hydrazine was more strongly basic than the parent amine. Modification of the basicity of the hydrazino- group in two different ways—by *N*-acetylation and by conversion to the cyclic derivative (VII)—also gave inactive compounds; the pyrazol-1-yl derivative (VI), however, had approximately one-quarter the activity of chlorothiazide.

The benzo-1,2,3,4-thiatriazine 1,1-dioxide (II; R′ = R″ = Me, R‴ = Cl, Brit. Pat., 1959) was approximately as active a diuretic in rats as chlorothiazide; the compound also resembled chlorothiazide in the ion-excretion pattern (K^+, Na^+ and Cl^-) it produced. Similar biological results were obtained with the corresponding unmethylated sulphonamide (II; R′ = H, R″ = Me, R‴ = Cl) and with the trifluoromethyl compound (II; R′ = R″ = Me, R‴ = CF_3). Replacement of the methyl group R″ in position-2 in the benzo-1,2,3,4-thiatriazine 1,1-dioxide (II; R′ = H, R″ = Me, R‴ = Cl) by a benzyl group reduced the activity to one-tenth of that of the 2-methyl compound.

The fact that these position–3 nitrogen isosteres of the corresponding derivatives of chlorothiazide should also be active diuretics is of considerable theoretical interest; similar nitrogen isosteres of certain quinazolone diuretics analogous to chlorothiazide have likewise been shown by Gadekar and Frederick (1962) to have diuretic activity.

One of the foregoing compounds (II; R′ = R″ = Me, R‴ = Cl) has recently been prepared by Childress (1962) and found to have a statistically significant diuretic activity in rats.

Experimental

6-Chloro-2-methyl-7-methylsulphamoylbenzo-1,2,3,4-thiatriazine 1,1-dioxide (II; R′ = R″ = Me, R‴ = Cl). 5-Chloro-2,4-di-(methylsulpha-

moyl)aniline (S. African Pat., 1958) (3·13 g.) was dissolved in concentrated sulphuric acid (40 ml.). To this solution was added with stirring at 0–5° a solution of sodium nitrite (0.7 g.) in concentrated sulphuric acid (20 ml.). The solution was stirred for 2 hr. at 0–5°, poured on to ice (100 g.), diluted at 0–5° to 325 ml., and rapidly filtered through a sintered-glass funnel. The filtrate was now diluted to 900 ml., whereupon a pale orange crystalline precipitate was formed. This solid (2·53 g., m.p. 165–167°) was filtered off, washed with water, and dried at room temperature *in vacuo* over silica gel. Found: Cl, 11·3; N, 17·35; S, 19·8. Calc. for $C_8H_9ClN_4O_4S_2$: Cl, 11·0; N, 17·3; S, 19·7 per cent.

6-*Chloro*-2-*methyl*-7-*sulphamoylbenzo*-1,2,3,4-*thiatriazine* 1,1-*dioxide* (*II; R′ = H, R″ = Me, R‴ = Cl*). 5-Chloro-2-methylsulphamoyl-4-sulphamoylaniline (S. African Pat., 1958) (31·6 g.) was dissolved in concentrated sulphuric acid (416 ml.). To this solution was added with stirring at 0–5° a solution of sodium nitrite (7·3 g.) in concentrated sulphuric acid (206 ml.). The solution was stirred for 30 min., poured into water (625 ml.) at 0–5°, and filtered. The clear filtrate was poured into water (9 litres), and the buff precipitate (17 g., m.p. 166–170°) was filtered off, washed with water, and dried at room temperature *in vacuo* over silica gel. Found: C, 26·5; H, 2·3; Cl, 11·0; N, 18·3; S, 20·1. $C_7H_7ClN_4O_4S_2$ requires C, 27·0; H, 2·26; Cl, 11·4; N, 18·0; S, 20·6 per cent.

2-*Benzyl*-6-*chloro*-7-*sulphamoylbenzo*-1,2,3,4-*thiatriazine* 1,1-*dioxide* (*II; R′ = H, R″ = CH_2Ph, R‴ = Cl*). 6-Chloro-3,4-dihydro-3-oxo-7-sulphamoyl-1,2,4-benzothiadiazine 1,1-dioxide (Close, Swett, Brady, Short and Vernsten, 1960) (10 g.) was dissolved in dry dimethylformamide (30 ml.), and sodium hydride (1·5 g., 50 per cent oil dispersion) added in portions. The solution was heated to 70°, and benzyl bromide (5·5 g.) added. The solution was heated at 70° for 1 hr., cooled, and poured on to ice. The crude material was recrystallised from ethanol/water to give a product with a m.p. of 250–252°. This material was hydrolysed with 20 per cent sodium hydroxide solution to give 2-benzylsulphamoyl-5-chloro-4-sulphamoylaniline, m.p. 155–160°. Found: C, 41·7; H, 4·1; N, 11·2. $C_{13}H_{14}ClN_3O_4S_2$ requires C, 41·6; H, 3·74; N, 11·1 per cent.

2-Benzylsulphamoyl-5-chloro-4-sulphamoylaniline (18·3 g.) was dissolved in a mixture of glacial acetic acid (490 ml.) and water (245 ml.). To this solution was added 2N sulphuric acid (61 ml.), followed at 0–5° by a solution of sodium nitrite (3·42 g.) in water (49 ml.). The reaction mixture was diluted with water (600 ml.), and the precipitated solid washed with water, dried, and recrystallised from methanol to give pale yellow prisms (10·4 g.), m.p. 158° (decomp.). Found: C, 40·4; H, 3·9; Cl, 8·9. $C_{13}H_{11}ClN_4O_4S_2$ requires C, 40·4; H, 2·9; Cl, 9·2 per cent.

2-*Methyl*-7-*methylsulphamoyl*-6-*trifluoromethylbenzo*-1,2,3,4-*thiatriazine* 1,1-*dioxide* (*II; R′ = R″ = Me, R‴ = CF_3*). 2,4-Di(methylsulphamoyl)-5-trifluoromethylaniline (Yale, Losee and Bernstein, 1960) (20 g.) was dissolved in a solution of concentrated sulphuric acid (246 ml.) in water (56 ml.). To this solution was added with stirring at 0–5° a solution of sodium nitrite (3·9 g.) in concentrated sulphuric acid (56 ml.), and the

clear solution was further diluted with water (3 litres). The precipitated buff solid (18 g., m.p. 148–149°) was filtered off, washed with water, and dried at room temperature *in vacuo* over silica gel. Found: N, 15·4; S, 17·8. $C_9H_9F_3N_4O_4S_2$ requires N, 15·7; S, 17·9 per cent.

1,5-*Dichloro*-2,4-*di*(*methylsulphamoyl*)*benzene* (*IV*). 6-Chloro-2-methyl-7-methylsulphamoylbenzo-1,2,3,4-thiatriazine 1,1-dioxide (20 g.) was dissolved in concentrated hydrochloric acid (150 ml.), and the solution heated on a steam-bath until no further solid was precipitated. The solid was filtered off, washed with water, dried, and recrystallised from ethanol/water to give pale pink prisms, m.p. 184–186°. Bourdais and Meyer (1961) have prepared this compound by a different method and quote m.p. 186°. Found: Cl, 20·9; N, 8·45; S, 19·5. $C_8H_{10}Cl_2N_2O_4S_2$ requires Cl, 21·4; N, 8·4; S, 19·3 per cent.

5-*Chloro*-2,4-*di*(*methylsulphamoyl*)*phenylhydrazine* (*V*). 6-Chloro-2-methyl-7-methylsulphamoylbenzo-1,2,3,4-thiatriazine 1,1-dioxide (24·8 g.) was dissolved in cold concentrated hydrochloric acid (220 ml.). To this solution was added stannous chloride (100 g.) in concentrated hydrochloric acid (100 ml.). This solution was kept at room temperature for 48 hr. The crystalline substituted phenylhydrazine which formed was filtered off, suspended in water, again filtered off, washed with water, dried, and recrystallised from water to give a cream solid (15·5 g., m.p. 234–236°). Found: Cl, 11·0; N, 17·25; S, 19·8. $C_8H_{13}ClN_4O_4S_2$ requires Cl, 10·8; N, 17·0; S, 19·5 per cent.

5-Chloro-2,4-di(methylsulphamoyl)phenylhydrazine (14·7 g.) was dissolved in hot 2N hydrochloric acid (100 ml.), the solution cooled to 20°, and acetic anhydride (15 ml.) added, followed by sodium acetate (100 g.) in water (100 ml.). The precipitated white *N*-acetyl derivative was filtered off, washed with water, and recrystallised from water to give colourless prisms (10 g., m.p. 197–198°). Found: C, 32·4; H, 4·25; Cl, 9·8; N, 14·9; S, 17·3. $C_{10}H_{15}ClN_4O_5S_2$ requires C, 32·5; H, 4·05; Cl, 9·6; N, 15·1; S, 17·4 per cent.

5-*Chloro*-2,4-*di*(*methylsulphamoyl*)-1-*pyrazol*-1′-*ylbenzene* (*VI*). 5-Chloro-2,4-di(methylsulphamoyl)phenylhydrazine (0.92 g.) was suspended in 2N hydrochloric acid (2 ml.) and ethanol (20 ml.). Tetraethoxypropane (1 ml.) was added, and the solution heated at reflux for 2½ hr. The white solid formed on cooling was recrystallised from ethanol, m.p. 203–205°. Found: Cl, 10·0; N, 15·15; S, 17·6. $C_{11}H_{13}ClN_4O_4S_2$ requires Cl, 10·1; N, 15·4; S, 17·6 per cent.

7-*Chloro*-2,3,4,5-*tetrahydro*-2-*methyl*-8-*methylsulphamoylbenzo*-1,2,4,5-*thiatriazepine* 1,1-*dioxide* (*VII*). 5-Chloro-2,4-di(methylsulphamoyl)-phenylhydrazine (3.28 g.) was suspended in industrial methylated spirit (200 ml.). Sodium hydroxide (1 ml. of a 4 per cent solution) and aqueous formaldehyde (0·75 ml. of 40 per cent solution) were added, and the now green solution was heated at reflux for 30 min. The solution was evaporated to dryness *in vacuo*, and the residue recrystallised from water to give a golden solid, m.p. 210–212°. Found: Cl, 10·5; N, 16·7; S, 18·7. $C_9H_{13}ClN_4O_4S_2$ requires Cl, 10·4; N, 16·4; S, 18·8 per cent.

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THE MORPHOLOGY AND HISTOLOGY OF THE SEEDS OF *STRYCHNOS NUX-VOMICA*, LINN., AND ITS ADULTERANTS, *STRYCHNOS NUX-BLANDA*, HILL, AND *STRYCHNOS POTATORUM*, LINN.

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The morphology and histology of the seeds of *Strychnos nux-vomica*, Linn., *Strychnos nux-blanda*, Hill., and *Strychnos potatorum*, Linn., are described. The diagnostic features by which the two adulterants *S. nux-blanda* and *S. potatorum* can be distinguished from the pharmacopoeial drug *S. nux-vomica*, are noted.

Various authors have commented upon the presence of seeds of *Strychnos nux-blanda*, Hill, and *Strychnos potatorum*, Linn., in samples of *Strychnos nux-vomica*, Linn. Reports indicate that the amount of *S. nux-vomica* exported from India has more than doubled in the last 50 years, in the year 1913, for example, the quantity exported was 1,200 tons, in 1956, 2,700 tons (Chopra, 1949; Younken and Pratt, 1956). It is also apparent that adulterations of *S. nux-vomica* with one or both of the adulterants still persists, such samples having been investigated as recently as July, 1961 (Wallis, private communication).

With the exception of a description of the tree by Hill (1917) and a note on the histology of the seed by Small (1913) there is no record of a complete examination of the seed of *S. nux-blanda*, similarly the only accounts of the seed of *S. potatorum* are those of Basu and Kirtaker (1818) and Dymock (1885) and these do not include the histology.

It was decided that a complete examination of the two known adulterants should be made and the characters by which they may be distinguished from the seed of *S. nux-vomica*, investigated.

In the literature referring to the seed of *S. nux-vomica* there was a number of conflicting statements about the macroscopical and microscopical characters. In addition, a microscopical examination revealed certain structures which have not previously been described. A re-examination of *S. nux-vomica* seeds was therefore necessary.

Plant Materials

The seeds of *S. nux-vomica* were obtained from five commercial sources: those of *S. nux-blanda* were obtained direct from the Forest Research Institute and College, Dehra Dun, India, and the seeds of *S. potatorum* were obtained from the Conservator of Forests, Colombo, Ceylon, with a small sample from the Owens College Museum, Manchester.

Recorded Dimensions

The macroscopical measurements are recorded as four figures, those in bold type indicate the range of size between which approximately two-thirds of the sample fall. These are obtained by a statistical analysis of

not less than 500 measurements and represent the summation and difference of the standard deviation and the mean. The first and fourth figures are respectively the minimum and maximum values observed.

The microscopical measurements are recorded as three figures. The figure in bold type denotes the statistical mode of not less than 250 measurements whilst the first and third figures are respectively the minimum and maximum values observed.

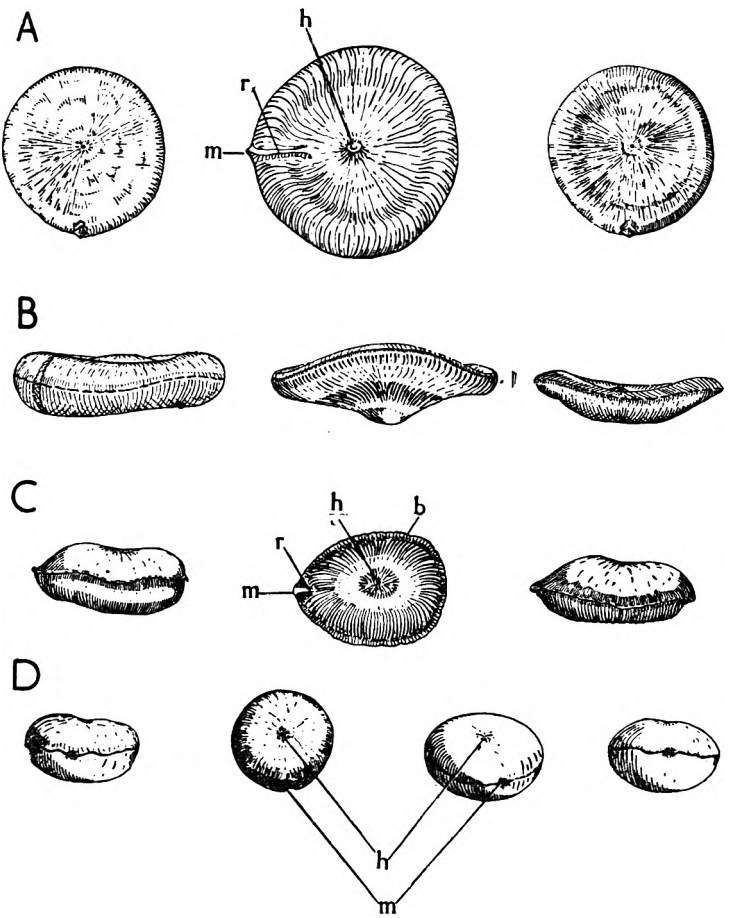

FIG. 1. *Strychnos nux vomica*, Linn. seed. A, Surface views. B, Lateral views, of entire seeds. *Strychnos nux blanda*, Hill, seed. C, Surface and lateral views of seeds. *Strychnos potatorum*, Linn. seed. D, Surface and lateral views of entire seeds; All × 1. m, micropyle; r, ridge; h, hilum; b, lateral ridge.

EXPERIMENTAL

Macroscopical Characters of the Dried Ripe Seed of Strychnos nux-vomica, *Linn.*

The macroscopical characters of the seed of *S. nux-vomica* are well known. The illustrations of the entire seeds in Fig. 1A and 1B serve as

a direct comparison with those of *S. nux-blanda*, Fig. 1C, and *S. potatorum*, Fig. 1D.

The dimensions of the samples examined were as follows:

Entire seed (Figs. 1A, 1B): diameter, 17·0—**18·5** to **23·1**—29·5 mm.; thickness, 2·2—**3·5** to **5·3**—7·0 mm.; weight, 0·8—**1·3** to **2·1**—2·7 g.

Horizontally split seed (Fig. 2A): disc shaped cavity, diameter, 13·1—**17·5** to **20·0**—25 mm.; thickness, 0·1—**0·9** to **1·5**—1·9 mm.; *Area of fusion* of endosperm halves. Width 1·2—**1·6** to **2·0**—3·5 mm.

Embryo (Fig. 2D): length, 5·0—**6·5** to **8·0**—9·0 mm.; width cotyledons at widest point, 2·5—**3·0** to **4·0**—4·5 mm.; length of radicle, 2·0—**3·0** to **3·5**—4·5 mm.

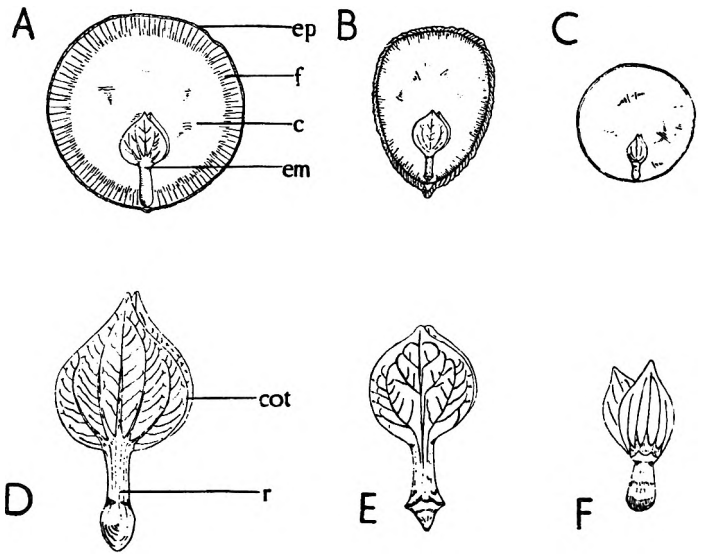

FIG. 2. *Strychnos nux vomica*, Linn. seed. A, Horizontally split seed; D, embryo. *Strychnos nux blanda*, Hill. seed. B, Horizontally split seed; E, embryo. *Strychnos potatorum*, Linn. seed. C, Horizontally split seed. F, embryo. Seeds × 1. Embryos × 5. ep, epidermis; f, area of fusion of two endosperm halves; c, area of central cavity; em, embryo; cot, cotyledons; r, radicle.

Macroscopical Characters of the Dried Ripe Seed of Strychnos nux-blanda *(Hill)*

The overall shape of the seed approximates to an ellipsoidal disc, 16·2—**17·2** to **20·0**—23·0 mm. long and 11·0—**13·3** to **15·8**—17·0 mm. wide. A few of the seeds are almost flat on one or both sides but the majority are concave on both upper and lower surfaces.

The margin is keel-shaped, due to the presence of a wide ridge which extends laterally from the otherwise rounded edge. Figure 1C shows the surface and lateral views of the seed. The seed is 5·6—**6·1–7·2**—7·8 mm. thick, and weighs 0·4—**0·9** to **1·3**—1·4 g.

Situated on the margin of the seed, at the subacute end, the lateral ridge has a marked labiate protuberance which indicates the position of the micropyle. A raised ridge runs from this marginal prominence to the hilum.

The surface is buff coloured with brown-black patches and is densely covered with appressed hairs arranged along the approximate radius with the hair tip directed toward the periphery.

In the horizontally split seed, the endosperm has the same shape as the seed and is hard and horny in texture, the colour is a translucent white or greyish white.

Lying centrally and in the horizontal plane of the endosperm is a disc shaped cavity which is 15·0—**17·1** to **18·0**—19·5 mm. long, on a line running through the micropyle and the centre of the seed, and 10·0—**12·2** to **14·1**—16·0 mm. wide, on a line running through the centre of the seed and at right angles to the first dimension. In the entire seed the cavity is 0·1—**0·6** to **0·8**—1·0 mm. wide at the centre.

(The centre of the seed in the above dimensions is taken as that point positioned immediately beneath the hilum and midway between the two endosperm discs.)

In the split seed (Fig. 2B) the cavity is surrounded by the area of fusion of the two endosperm halves this extends from the edge of the cavity to the testa and is 1·1—**1·5** to **2·0**—2·2 mm. wide.

Within the cavity lies the embryo, radially directed, with the radicle in a cylindrical channel leading from the central cavity to the micropyle.

The embryo is 5·0—**6·0** to **7·0**—7·5 mm. in length, greyish white and as shown in Fig. 2E consists of two superposed straight, broadly ovate, leafy cotyledons 2·0—**3·0**–**4·0**—5·0 mm. wide at their widest point showing pinnate venation with from five to seven principal veins. The radicle is 1·5—**2·0** to **2·5**—3·0 mm. in length and terete in section with a conical apex.

The dried seeds are odourless, the soaked seeds develop a slight earthy odour after 3 days. The taste is bland and mucoid.

Macroscopical Characters of the Dried Ripe Seed of Strychnos potatorum, *Linn.*

The seed is sub-spherical 9·5—**10·0** to **11·8**—13·2 mm. in diameter and 3·0—**6·4** to **8·2**—9·0 mm. thick, the weight is 0·41—**0·52** to **0·88**—1·13 g.

Some specimens show a shallow concavity on the hilar side but most are smoothly curved. The margin is rounded except for a fine ridge which, as shown in Fig. 1D divides the seeds into two almost equal parts. At one point on the margin is a small depression which marks the position of the micropyle. The hilum is situated in the centre of one of the curved surfaces of the seed and occurs as a small circular cavity.

The surface is pale buff in colour, a few specimens exhibiting shiny black patches which, on examination, proved to be fragments of the pulp of the berry.

The seeds are covered with closely appressed hairs which are set radially with their tips directed towards the periphery. This trichomatous layer is enclosed by an adherent endocarpic layer which gives to the seeds a finely rugulose appearance.

The horizontally split seed shows that the endosperm has the same shape as the seed and is hard and horny in texture. The colour is a translucent white.

Lying centrally and in the horizontal plane of the endosperm is a disc shaped cavity less than 0·1 mm. wide at the centre and 8·0—**9·0** to **11·1**—12·0 mm. in diameter. In the split seed (Fig. 2C) this is surrounded by the area of fusion of the two endosperm halves this extends from the edge of the cavity to the testa and is 0·25—**1·00**—1·25 mm. wide.

Within the cavity lies the embryo, radially directed, with the radicle in a cylindrical channel leading from the central cavity to the micropyle.

The embryo is 3·0—**3·5** to **4·0**—4·5 mm. in length, greyish white in colour and (Fig. 2F) consists of two superposed straight, narrowly ovate, leafy cotyledons, 1·5—**2·0**–**2·5**—3·0 mm. wide at their widest point, showing palmate venation with from three to five principal veins. The radicle is 0·75—**1·0**—1·5 mm. long, terete and shortly clavate.

The dried and soaked seeds are odourless, the taste is bland.

Histology of the Seed of S. nux-vomica

The principal histological characters of *S. nux-vomica* are well known; this re-examination revealed certain characters not previously reported. These are reported in detail with the microscopical dimensions of all the structures.

The testa consists of one integument, having two cell layers, an outer trichomatous epidermis and an inner layer of ground tissue Wallis (1951).

Trichomatous epidermis (Fig. 3A ep.). The dimensions of the base of the trichomes are R, 65—**80**—100 μ, T, 40—**60**—85 μ, L, 45—**65**—87 μ. In longitudinal sections the anticlinal walls are strongly thickened and are pierced by tubular pits which are sometimes branched. Transverse sections of the upper region of the base (Fig. 3B) and the central part of the base (Fig. 3C) are shown.

A transverse section of the basal periclinal wall (Fig. 3D) indicates a slight tapering of the trichome base and a decrease in the sinuosity of the anticlinal walls. The section has a retiform appearance caused by the irregularly protuberant nature of the cell wall.

The trichome limb is composed of 6—**10**—15 ribs, 3—**10**—12 μ in diameter (Fig. 3F), the entire limb is 18—**24**—45 μ in diameter and 600—**950**—1290 μ in length. The ribs exhibit a varying degree of anastamosis as illustrated in the partially disintegrated trichomes in Fig. 3E, Fig. 3G a-f shows the variation that exists in the anastamosis of the trichome tips.

On the *rounded acute margin* of the seed the trichomes are not appressed but are arranged vertically directed away from the seed centre.

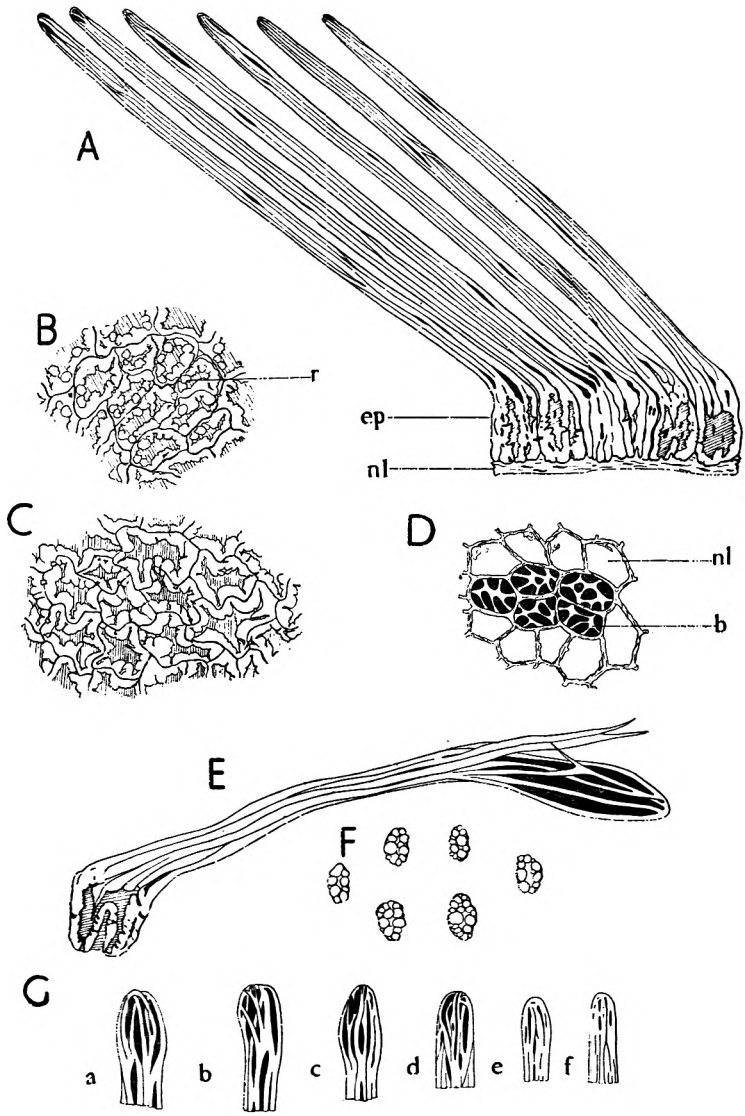

FIG. 3. *Strychnos nux vomica*, Linn. seed. A, Longitudinal radial sections of testa. B, Transverse section of upper half of trichome base. C, Transverse section of lower half of trichome base. D, Transverse section of basal periclinal wall of trichome base; E, Trichome with partially disintegrated tip; F, Transverse section of trichome limb; All × 100. G, a to f; trichome tips showing varying degrees of anastamosis, × 200. ep, epidermis; nl, ground tissue; r, ribs on secondary wall; b, basal periclinal wall.

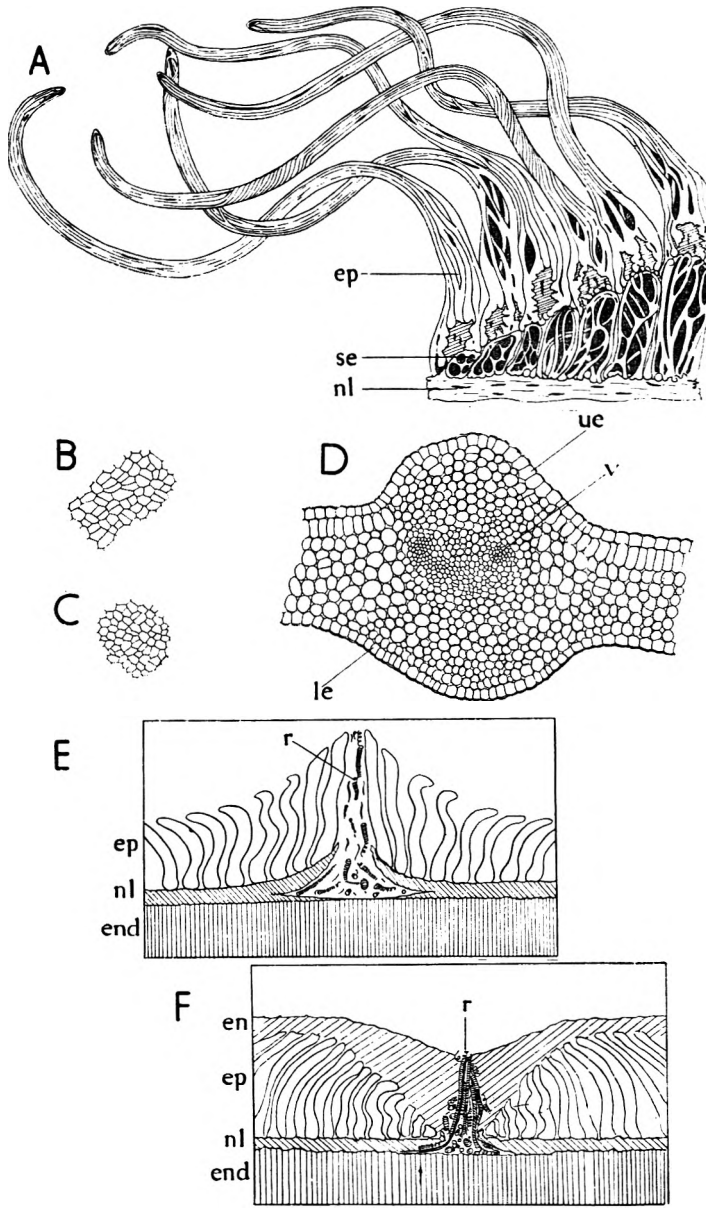

FIG. 4. *Strychnos nux vomica*, Linn. seed. A, Longitudinal tangential section of testa in region of ridge. B, Surface view, upper epidermis of cotyledon. C, Surface view, lower epidermis of cotyledon. D, Transverse section of midrib and lamina of cotyledon; All × 100. E, Diagram to show distribution of tissue in hilar region. *Strychnos potatorum*, Linn. seed. F, Diagram to show distribution of tissue in hilar region. ep, epidermis; se, subepidermal layer; nl, ground tissue; ue, upper epidermis; le, lower epidermis; v, vascular tissue; r, raphae; end, endosperm; en, endocarp.

Sections were taken as follows:

R = A longitudinal section made in a radial direction perpendicular to the surface. T = A transverse section made parallel to the surface. L = A longitudinal section perpendicular to the surface at a right angle to the radius.

The ground tissue of the testa has functioned as a nutrient layer (Wallis, 1951) and is represented by a brown band of flattened parenchyma, R, 12—**25**—32 μ (Fig. 3A, nl.) (Fig. 4A, nl.). In transverse section they appear as ill defined polygonal cells T, 29—**55**—70 μ (Fig. 3D, nl.).

The *narrow ridge* which runs from the hilum to the micropyle is composed of an epidermal and a sub-epidermal layer. The trichomes of the epidermis in this region (Fig. 4A, ep.) are similar to those covering the remainder of the seed but the hairs are irregularly arranged with more conical bases and partially chordaceous limbs. The sub-epidermal cells (Fig. 4A, se.) are lignified and appear as extensions of the trichome bases. The anticlinal walls are ribbed and are pierced by longitudinal slit-like pits. In longitudinal section they appear to be reticulate in nature, the lumen being traversed by anastamosing and branching lignified rods. The height varies according to their position in the ridge. Those at the highest point of the ridge are 182—**220**—280 μ, decreasing gradually towards the lateral limits of the ridge.

The hilum is marked by the trichome limbs being raised from the appressed to the vertical position (Fig. 4E, ep.). The vertical trichomes surround the raphae (Fig. 4E, r) which is a narrow vascular strand composed of ruptured spiral vessels. This vascular strand traverses the epidermis and then extends into the ground tissue for a short distance on all sides.

The endosperm of the seed is found adjacent to the ground tissue of the testa, and is composed of simple parenchyma (Fig. 6A). The outermost layer consists of radially elongated polyhedral cells, R, 33—**52**—68 μ; T, 10—**16**—20 μ; L, 10—**18**—20 μ; the outer walls of which are thickened. Within this outer layer are two to three layers of cells which are almost isodiametric, R, 25—**40**—45 μ; T, 20—**30**—40 μ; L, 20—**35**—45 μ. The remainder of the endosperm is composed of large polyhedral cells, R, 42—**60**—100 μ; T, 35—**55**—75 μ; L, 35—**60**—75 μ. The cell walls are 20—**25**—30 μ thick. The cells of the endosperm show fine perforations in the walls; these are described by Tschirch and Oesterle (1900) and other authors as containing the plasmodesmal strands which unite the protoplasts of neighbouring cells.

The cell content is an oil plasma the aleurone grains of which are irregularly shaped, show one or more globoids and are 15—**30**—50 μ in diameter.

The embryo is composed almost entirely of small, thin walled parenchyma. In transverse section (Fig. 4D) the lamina is up to 180 μ thick and shows partial cellular differentiation into upper and lower epidermis and vascular tissue. In surface view the upper epidermal cells (Fig. 5B) have almost straight anticlinal walls and are 20—**25**—40 μ long and 8—

15—20 μ wide. The lower epidermal cells in surface view (Fig. 4C) are polygonal in outline and isodiametric, 5—**10**—17 μ. The cells of the embryo are filled with fixed oil and contain a few scattered aleurone grains.

Histology of the Seed of Strychnos nux-blanda, *Hill*

The testa of the seed consists of one integument having two layers, an outer trichomatous epidermis and an inner layer of ground tissue.

The trichomatous epidermis is composed of strongly lignified cells having a short prismatic base extended to form an appressed hair which is directed at an angle to the base and radiates towards the margin of the seed (Fig. 5A, ep.).

The dimensions of the base are R, 90—**100**—116 μ; T, 52—**63**—80 μ; L, 50—**69**—80 μ. In longitudinal section the anticlinal walls appear thickened and are pierced by simple ovoid pits and scattered groups of fine slit-like pits. The simple ovoid pits are arranged with the long axis in a vertical direction; the slit-like pits are irregularly arranged and appear on the cell wall as fine striations in surface view and as serrulations in section.

The cell lumen is sometimes traversed by lignified rods which have their origin in the basal periclinal or anticlinal walls and terminate in the trichome limb. The apex of the base is irregularly tapered and the basal periclinal wall is coarsely papillate, the protuberances extending into the ground tissue.

A transverse section of the upper region of the base (Fig. 5B) shows the anticlinal walls to be slightly sinuous in outline with small circular structures and protuberances on the secondary cell wall, these latter structures are the basal portions of the trichome ribs (described later). The central region of the trichome base shows, in transverse section (Fig. 5C), a marked decrease in the number of protuberances on the secondary wall. The basal periclinal wall has a fine retiform appearance in transverse section (Fig. 5D) caused by the coarsely papillose nature of the wall.

The circular structures and protuberances found in the transverse section of the upper region of the base are extended longitudinally as lignified ribs which are drawn into a fascicle to form the trichome limb. Each limb is composed of 6—**10**—12 ribs, 4—**8**—16 μ in diameter, with a crenulate outer margin surrounding a small lumen (Fig. 5G).

The entire trichome limb is 16—**20**—40 μ in diameter and 700—**1100**—1,380 μ in length.

The ribs show a degree of anastamosis and branching similar to that for *S. nux-vomica*. Each fascicle is surrounded by a thin cellulose membrane which retains the ribs in close proximity, in many trichomes this membrane is ruptured and the ribs separate along their length (cf. Small, 1913), recombining only at the trichome tip (Fig. 5A, ep.). This results in the trichome limbs having a loosely interwoven appearance and exhibiting a variation in apparent diameter proportional to the degree of separation.

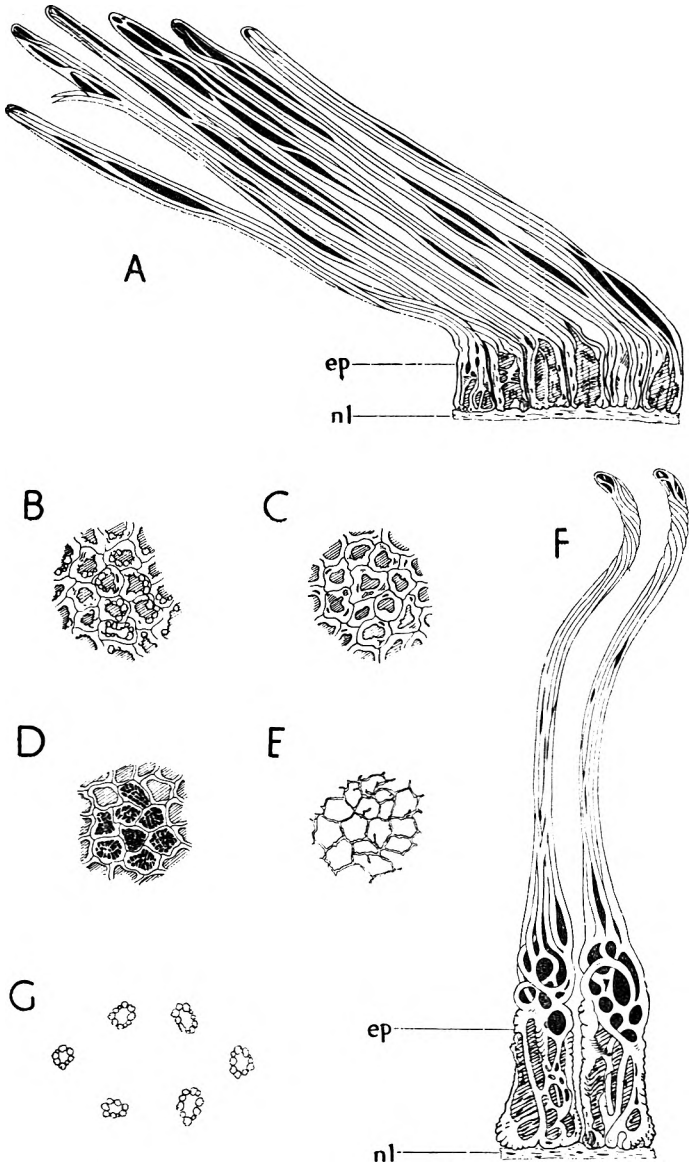

FIG. 5. *Strychnos nux blanda*, Hill, seed. A, Longitudinal radial section of testa. B, Transverse section of upper half of trichome base. C, Transverse section of lower half of trichome base. D, Transverse section of basal periclinal wall of trichome base. E, Transverse section of ground tissue of testa. F, Longitudinal radial section of epidermis in region of ridge. All × 200. ep, epidermis; nl, ground tissue.

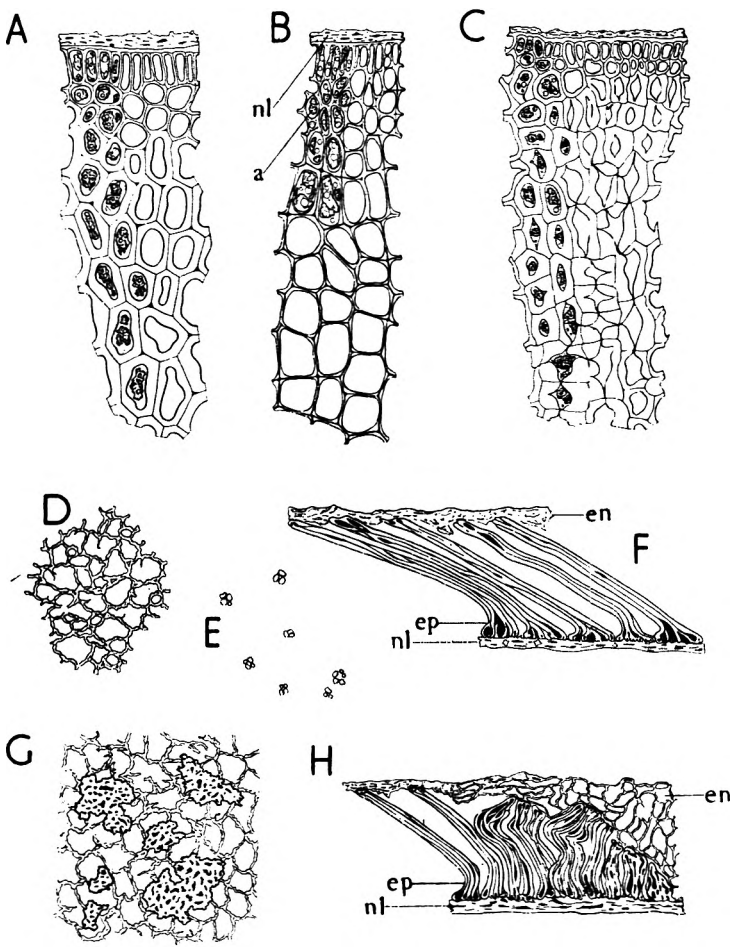

FIG. 6. *Strychnos nux vomica*, Linn. seed. A. Longitudinal radial section of endosperm. *Strychnos nux blanda*, Hill, seed. B, Longitudinal radial section of endosperm. *Strychnos potatorum*, Linn; C, Longitudinal radial section of endosperm. D, Surface view of endocarpic layer. E, Transverse sections of trichome limbs. F, Longitudinal radial section of seed coats. G, Transverse section of basal periclinal walls of trichome bases and adjacent ground tissue. H, Longitudinal radial section through seed coats in hilar region. All × 200. nl., ground tissue; a, aleurone; en, endocarpic layer; ep, epidermis.

The angle which the trichome limb makes with the base is not constant, thus trichomes of the same length may have their tips at varying heights above the seed surface. This factor, in conjunction with the separation of the limbs, described above, imparts the somewhat rough texture to the epidermal surface noted in the macroscopical description.

The trichome limbs in the region of the keel-shaped ridge on the margin of the seed are not appressed but rise perpendicularly from the base and are directed away from the seed centre.

The *narrow ridge* which runs from the micropyle to the hilum (Fig. 1C, r) is composed of trichomes, with different characters to those previously described. The trichome bases in this region are T, 56—**76**—98 μ and R, 240—**290**—340 μ The lower half of the base is typical of the epidermal cells on the rest of the seed, with the exception that the lumen is traversed by a greater number of anastamosing and branching rod-like elements (Fig. 5F). This lower part of the base is surmounted by a compact formation of curving, recurving, anastamosing and branching rods which are finally drawn into a fascicle to form the trichome limb. The trichome limb is not appressed but is gently curved with a recurved chordaceous apex. The entire trichome is 950—**1,100**—1,150 μ in length at the thickest point of the ridge. As the lateral boundaries of the ridge are approached the area of recurving and curving becomes smaller, the longitudinal dimension of the base is decreased, and the degree of recurving of the trichome tip increases until the trichome reverts to the normal epidermal cell type.

The *ground tissue* of the testa is composed of a brown band of flattened parenchyma. R, 8—**14**—23 μ (Fig. 5A, nl.). In transverse section the tissue appears as ill defined isodiametric polygonal cells, T, 25—**53**—63 μ (Fig. 5E). In the region of the keel-shaped ridge on the margin of the seed the ground tissue is less flattened and is composed of six to eight layers of irregularly shaped but distinct cells.

The *hilum* has a similar structure to that described for *S. nux-vomica* (Fig. 4E).

The *endosperm* of the seed is found adjacent to the ground tissue of the testa and is composed of a parenchymatous tissue possessing small intercellular spaces, and with a clearly discernible middle lamella (Fig. 6B).

The outermost layer consists of radially elongated polyhedral cells, R, 24—**44**—60 μ; T, 12—**14**—16 μ; L, 12—**15**—16 μ, the outer walls of which are thickened and the cell contents are as described for the remainder of the endosperm. Within this layer are three or four layers of cells, R, 20—**38**—45 μ; T, 14—**25**—40 μ; L, 14—**25**—40 μ. The remainder of the endosperm is composed of large polyhedral cells, R, 40—**60**—95 μ; T, 38—**50**—60 μ; L, 35—**50**—60 μ.

The cell walls exhibit fine perforations and show little variation in thickness being 3—**5**—8 μ.

The *cell content* is an oil plasma the aleurone grains of which are irregularly shaped, show one or more globoids, are 4—**20**—30 μ in diameter.

The *embryo* is composed almost entirely of small thin walled parenchyma. In transverse section the lamina is up to 150 μ thick and shows partial cellular differentiation into upper and lower epidermis and vascular tissue. In surface view the cells of the upper epidermis have almost straight anticlinal walls and are 18—**23**—29 μ long and 8—**15**—20 μ wide. The cells of the lower epidermis are polygonal and isodiametric, T, 5—**9**—15 μ.

The cells of the embryo are filled with fixed oil and contain a few scattered amorphous aleurone grains.

Histology of the seed of Strychnos potatorum, *Linn.*

Strychnos potatorum has three cell layers surrounding the seed; a discontinuous trichomatous layer bounded on the outer surface by a thin endocarp and on the inner surface by a layer of ground tissue. The trichomatous epidermis and the ground tissue comprise the testa of the seed (Fig. 6F, ep., nl.).

The trichomatous epidermis is composed of strongly lignified cells having a short conical base extended to form an appressed hair which is directed at an angle to the base and radiates towards the margin of the seed (Fig. 6F, ep.).

The base has the dimensions, R, 10—**17**—20 μ; T, 20—**45**—70 μ. In longitudinal section the anticlinal walls are seen to consist of a series of rod-like elements which arise directly from the basal periclinal wall and extend for a short distance to form the base before being drawn into a fascicle to form the trichome limb. The anticlinal walls are pierced by large ovoid pits which are situated between the constituent rods. The basal periclinal wall is irregular and heavily pitted. In transverse section (Fig. 6G) the trichome bases cannot be differentiated as single cells but appear as isolated patches of lignified tissue irregularly distributed and pierced by slit-like pits.

The *trichome limb* is strongly appressed and consists of 3—**4**—6 lignified ribs, enclosed by a thin cellulosic membrane and surrounding a small lumen, the ribs sometimes exhibit a crenulate outer margin, but the majority are smooth (Fig. 6E). The individual ribs are 4—**7**—12 μ in diameter, the entire trichome limb is 12—**16**—32 μ in diameter and 160—**320**—480 μ in length. The ribs anastamose and branch along the length of the trichome limb but not to the same degree as those of *S. nux vomica*. The trichome tip is blunt, rarely pointed and frequently penetrates a short distance into the endocarpic layer.

The *endocarp* is a layer of collapsed parenchyma, R, 10—**20**—30 μ, which lies outside the trichomatous layer and is entire over the whole seed surface (Fig. 6F, en.). In transverse section (Fig. 6D) the cells are parenchymatous with some intercellular pits. They can be divided into two size groups: those polygonal cells having the dimension T, 15—**30**—50 μ, and a smaller group circular in outline and T, 10—**12**—17 μ. The smaller cells are those which receive those trichome tips that penetrate into the endocarpic layer. This penetration results in the finely rugulose appearance of the seed surface referred to under macroscopical characters.

The *ground tissue* of the testa is composed of a brown band of flattened parenchyma, R, 8—**12**—16 μ (Fig. 6F, nl.) and contains a few unidentified prismatic crystals. In transverse section (Fig. 6G) the tissue appears as ill-defined polygonal cells, T, 20—**35**—50 μ. In the region of the fine ridge on the margin of the seed, the tissue is less flattened and consists of two to three layers of irregularly shaped but distinct cells.

As reported under macroscopical characters *the hilum* is found as a small circular depression in the centre of one curved seed surface. A longitudinal section through the hilar region shows that the depression is caused by a change in the trichome formation. As the trichomes approach the hilum they exhibit a lesser degree of appression and become slightly recurved (Fig. 6H, ep.). Nearer the hilum the trichomes lose their curvature, exhibit a greater number of ovoid pits and become progressively shorter, finally, those cells in close proximity to the raphe are short conical cells with thick pitted walls devoid of the typical rod-like elements described previously. The endocarp in the hilar region is less flattened and as the trichomatous layer grows smaller the endocarp shows gradual differentiation into simple parenchyma (Fig. 6H, en.), (Fig. 4F, en.).

The *raphe* is a thin vascular strand composed of small spirally thickened vessels and traverses the endocarp to extend a short distance on all sides into the ground tissue of the testa (Fig. 4F, r.).

The *endosperm* of the seed is found adjacent to the ground tissue of the testa and consists of parenchyma with a clearly discernible middle lamella (Fig. 6C). The outermost layers of cells are radially elongated and polyhedral, R, 21—**34**—45 μ; T, 13—**20**—30 μ; 13—**20**—30 μ, the outer walls are thickened, and the cell content as described below. Within this layer is a single layer having the dimensions R, 18—**30**—45 μ; T, 16—**26**—33 μ; L, 16—**26**—33 μ. The remainder of the endosperm is composed of large polyhedral cells, R, 43—**58**—89 μ; T, 32—**46**—85 μ; L, 32—**43**—83 μ. The walls are 10—**26**—33 μ thick and show a few large funnel-shaped pits which connect with pits in neighbouring cells.

The *cell content* is an oil plasma the aleurone grains of which are irregularly shaped, show one or more globoids and are 3—**12**—20 μ in diameter.

The *embryo* is composed of small thin walled parenchyma. The lamina is up to 130 μ thick in transverse section and shows partial differentiation into upper and lower epidermis and vascular tissue. In surface view the cells of the upper epidermis have straight anticlinal walls and are R, 14—**20**—24 μ; L, 9—**18**—22 μ. The cells of the lower epidermis are polygonal and isodiametric, 4—**8**—12 μ. The cells of the embryo are filled with fixed oil and contain a few scattered aleurone grains.

Discussion

Macroscopical Characters

The examination of the three seeds shows certain characters which will distinguish *S. nux-vomica* from *S. nux-blanda* and *S. potatorum.*

Seed shape. The discoid shape of *S. nux-vomica* contrasts with the ellipsoidal disc of *S. nux-blanda* and the subspherical seed of *S. potatorum.* Both the adulterants show little divergence in general shape or marginal form, but *S. nux-vomica* varies from flat to concavo-convex and exhibits a margin which may be acute or smoothly curved. The slightly keel shaped ridge on the margin of some of the seeds is only slightly elevated in *S. nux-vomica* and contrasts with the broad laterally extended keel-shaped ridge of *S. nux-blanda* and the fine ridge of *S. potatorum.*

Surface characters. The seed surface of *S. nux-vomica* exhibits a sericeous lustre and differs markedly from the scabrous surface of *S. nux-blanda* and the finely rugose appearance of *S. potatorum.*

Dimensions. Various authors report different dimensions for the seed of *S. nux-vomica*; these may be accounted for by the different habitats of the samples measured. Pereira (1850) stated the maximum diameter as 25·4 mm., Moll and Janssonius (1923) gave the maximum as 28·0 mm. The average diameter was given as 22·09 mm. by Bentley and Trimen (1880) and the size range has been given as 13·5–22·0 mm. by Dunstan and Short (1883), 20–25 mm. by Wallis (1951) and 10–30 mm. by Trease (1957). Whilst most of these dimensions fall within the limits given for the sample examined (17·0–18·5 to 23·1–29·5 mm.) they do not give a true indication of the diameter of the seeds in the current commercial samples. The dimensions recorded here show that two-thirds of a sample will have a diameter of between 18·5 and 23·1 mm.

Embryo. The embryos of *S. nux-vomica* and *S. nux-blanda* are similar in shape and size but the apex of the radical is clavate in *S. nux-vomica* and conical in *S. nux-blanda.* The embryo in *S. potatorum* is smaller and exhibits only 3–5 primary veins against the 5–7 found in the other cotyledons.

Histological Characters

The microscopical examination revealed certain characters which can be used to distinguish the three seeds.

The testa. In *S. nux-vomica* the trichomes were first described by Berg (1865). In his description of the basal-cell of the trichome he stated that it showed "spiral like pitting" in the anticlinal wall; later, Greenish (1920) modified this to "oblique pits", and Wallis (1951) described the pits as "sometimes more or less spirally twisted". Other authors have varied their description from "slit like" (Trease, 1957) to "branched slit like" (Moll and Janssonius, 1923) without any mention of obliqueness. In the material examined, the anticlinal walls of the trichome bases were pierced by tubular pits but the pits showed no spiral arrangement and were not oblique. The dimensions of the base have been stated to be R, 75 μ; T, 75 μ (Wallis, 1951) and R, 35 μ; T, 30–35 μ (Moll and Janssonius, 1923), whereas they were observed to be R, 65—**80**—100 μ; T, 40—**60**—85 μ and L, 45—**65**—87 μ. A further anomaly was found in the descriptions of the trichome limb, all authors agreed on its general structure but many disagreed on its linear measurements. These varied from a maximum length of 1,500 μ (Greenish, 1923) to a range of size

600—**800**—1,000 (Wallis, 1951) and 800–1,000 μ (Moll and Janssonius, 1923). In the samples examined, no trichome was observed to be greater than 1,290 μ in length, the mode was 950 μ and the minimum 600 μ.

That part of the epidermis in *S. nux-vomica* and *S. nux-blanda* shows the most divergent structure, is the region of the ridge joining the micropyle and the hilum.

The ridge of *S. nux-vomica* was at one time described as the raphe (Berg, 1865) and more recently as an area in which the trichome limbs are "irregularly directed" (Trease, 1957). The latter statement is correct in that the trichome limbs show a certain degree of sinuosity and are not closely appressed, but the height of the ridge is also increased by the subepidermal layer in *S. nux-vomica* and in *S. nux-blanda* by the extended trichome bases.

The epidermis of *S. potatorum* differs considerably from that of *S. nux-vomica* and *S. nux-blanda*. The trichomes form a discontinuous layer and have a base which bears little resemblance to the prismatic base of the other two seeds. The limb is about one-third as long (160—**320**—480 μ) and there is no variation in the epidermal cell structure excepting those truncated forms found in the hilar region. In addition, *S. potatorum* is the only seed in the group which shows an adherent endocarpic layer.

Endosperm tissue. In all three endosperms there are three cell types varying in size and radiating from the periphery towards the centre. In *S. nux-vomica* and *S. nux-blanda* the layer of cells at the periphery has a palisade-like structure which is radially directed. Small (1913) stated that in *S. nux-vomica* these cells had "more or less triangular ends" whilst those of *S. nux-blanda* were "more usually square ended". This was investigated as a possible means of differentiating between the two endosperms, but the variation within different seeds rendered this character of little value.

In *S. potatorum* the cells of the outer layer are smaller than those of *S. nux-vomica* and *S. nux-blanda* and exhibit a lesser degree of radial extension.

Within this outer layer there is a region where the cell dimension is less in the radial (R) direction than the outer layer but greater in transverse (T) and longitudinal (L) direction. In *S. nux-vomica* this region is two to three cells thick, in *S. nux-blanda* three to four cells thick and in *S. potatorum* consists of only one cell layer.

The remainder of the endosperm in each of the seeds is composed of large slightly elongated polyhedral cells.

The diagnostic feature of the endosperm which is of most value is the variation in the thickness of the cell walls. Those of *S. nux-vomica* are 20—**25**—30 μ and are easily distinguished from those of *S. nux-blanda* which are only 3—**5**—8 μ thick; in *S. potatorum* the walls have a thickness of 10—**26**—33 μ, which is similar to the dimension of *S. nux-vomica* but, in addition, *S. potatorum* exhibits large funnel-shaped pits and the cells appear almost cuboid in section.

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SOME OBSERVATIONS ON THE PHYSICAL AND PHARMACOLOGICAL PROPERTIES OF PICROTOXIN SOLUTIONS

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A slow and spontaneous decrease in pH was observed with freshly prepared aqueous solutions of commercially available picrotoxin and this change was catalysed by the addition of potassium chloride crystals or by contact with a calomel electrode. The pH at which the solution stabilised was dependent upon the original pH of the solvent and the concentration of the solution. Potassium chloride had little effect upon the pH of freshly prepared saline solutions of picrotoxin. There was no difference in pharmacological activity between freshly prepared picrotoxin solutions, solutions after addition of potassium chloride, or solutions which had been stored.

Since freshly prepared solutions of pure samples of the two recognised constituents of picrotoxin (picrotoxinin and picrotin) were found to be electrometrically stable, the factor responsible for the change of pH may be an impurity; an impurity was detected in commercially prepared samples of picrotoxin by paper chromatography.

Picrotoxinin was the active principle and picrotin was relatively inactive. Slight elevation of the pH markedly reduced the activity of picrotoxin solutions thus supporting an earlier suggestion that for clinical reliability, picrotoxin should be prepared in a buffered solution. No evidence was found to support the belief that Picrotoxin Injection B.P. 1958 should be protected from light.

IN investigations of the properties of a series of neutral substances (pH 6–8) having central actions in low concentrations, a new and interesting response of mice to picrotoxin was observed. In the current literature, we found that although aqueous solutions of picrotoxin were widely described as being "neutral to litmus" (B.P. 1958) the pH was often as low as 4·5. Since there was as much as 2 units difference between the initial pH of freshly prepared solution and the final pH readings, experiments were made to determine whether the pharmacological properties varied with changes in pH. Because there was doubt about whether picrotoxin solutions should be protected from light, the effect of tungsten and ultra-violet light on their activity was examined.

Picrotoxin was first isolated in 1812 by Boullay and it was suggested that it was a mixture or a loosely bound combination of two molecules, picrotin and picrotoxinin. Determination of melting and thaw points of a picrotoxinin-picrotin mixture, thermal analysis and X-ray powder photography of the three substances (Hansen and Jerslev, 1954) have confirmed the observation of Cervello (1911) that picrotoxin consists of 54–55 per cent picrotin ($C_{15}H_{18}O_7$) and 45–46 per cent of picrotoxinin ($C_{15}H_{16}O_6$). Picrotoxin is thought to owe its pharmacological activity

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solely to picrotoxinin, while picrotin is reported to be either "saturated and inert" or to have the same yet weaker action as picrotoxinin (Angelico, 1912; Cervello, 1911).

METHODS

Chemical Procedures

Picrotoxin and its derivatives, and solutions of these substances were always stored at 4° in the dark. Crystalline picrotoxin was prepared from the crude drug by T. & H. Smith Ltd. of Edinburgh. Samples of pure picrotoxinin and picrotin were supplied through the kindness of Dr. J. S. E. Holker of the Department of Organic Chemistry, Liverpool University. All glassware was placed in 3 N hydrochloric acid for 8 hr. before use and then rinsed thoroughly with glass distilled water, in which atmospheric carbon dioxide was reduced by the use of soda lime filters. The pH of solutions was adjusted by the addition of 0·01 N hydrochloric acid or 0·01 N sodium hydroxide; pH was determined with a pH meter using conventional glass and calomel electrodes.

Pharmacological Procedures

All injections were made in a volume of 0·1 ml. into the lateral tail veins of randomly bred albino male mice weighing 20–25 g. The tail veins were dilated by warming the mice for 3 min. at 37°; the room temperature was 22–23°. To reduce the variance, mice were transferred to the laboratory at least 24 hr. before use and the animals were kept in groups of 2–6 per cage (Mackintosh, 1962). After injection, the mice were placed beneath 2 litre beakers for observation.

Two responses of mice to picrotoxin may be used as "end-points" (Ramwell and Shaw, 1963). With small doses (7·5 to 25 μg./mouse) the time interval was recorded between the injection and the assumption of a flaccid posture (F.P.) in which the mouse lay full length with its head lowered and stretched out on to the front paws; the hind legs were not properly co-ordinated and tended to be placed awkwardly with respect to the body. The assumption of this posture coincides with the appearance of a short burst of low frequency, high voltage activity in the electroencephalogram. The second "end-point", which was observed with higher dose levels, was the more conventional one, in which the time interval between injection and the first clonic convulsion was measured (convulsion time, C.T.); this response was only observed with doses of picrotoxin greater than 100 μg./mouse. The flaccid posture end-point was always detectable before the first clonic convulsion. There was an inverse linear relationship between the logarithm of the dose and the times elapsing before these two responses.

Exposure of Aqueous Solutions of Picrotoxin to Light

The activity of solutions of picrotoxin of concentrations 25 and 200 mg./100 ml. was tested on the flaccid posture and convulsion time responses, before and after exposure to either tungsten or ultra-violet light

(254 and 366 mμ). The solutions were prepared in distilled water and exposed for varying time intervals to either intense tungsten light in acid-washed pyrex glassware, or to ultra-violet light in silica cells.

Chromatography

For identification of the constituents of picrotoxin single length chromatograms were run in toluene : acetic acid : water (10 : 7 : 3) using Whatman 3 MM paper. After equilibration for 3 or 16 hr. the chromatograms were run for 2·5–3 hr. at 25·5°; 200 μg. of material was applied to each paper. Two colour reactions were employed for identification of the separated substances:

(*a*) Hydroxylamine. The chromatograms were sprayed with hydroxylamine (approximately 5 per cent in 85 per cent ethanol (v/v) and then heated for 3–5 min. at 80°.

(*b*) Silver nitrate. Chromatograms were dipped through a solution of 0·1 N silver nitrate, made just cloudy with 2 N ammonium hydroxide. They were then heated at 45° for approximately 5 min, dipped through 0·5 N sodium hydroxide and left for 10 min. at room temperature.

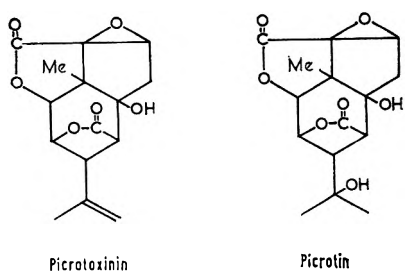
RESULTS

Since the solvent of Picrotoxin Injection U.S.P. 1955 is saline and that of Picrotoxin Injection B.P. 1958 is water, and because aqueous and saline solutions behave differently with respect to pH, the presentation of the results is facilitated if the data obtained using these two solvents is reported separately.

Change in pH of Aqueous and Saline Solutions of Picrotoxin

The pH of aqueous solutions of picrotoxin was found to be proportional to the concentration, and the pH of the water; the pH of the solutions was also modified by contact with calomel electrodes.

Although picrotoxin is widely regarded as a neutral substance, especially in view of the chemical formula (Fig. 1), aqueous solutions were always more acid than the solvent in which they were prepared (Fig. 2). Thus a 3 mg./ml. solution equivalent to Picrotoxin Injection B.P. 1958 attained a final pH, when determined by conventional electrodes, of 5·25 when distilled water of pH 7·15 was used, and with distilled water of pH 4·5 (lower pH limit of "Water of Injection" B.P. 1958) a final pH of 4·44 was recorded.



Picrotoxinin Picrotin

FIG. 1. The composition of picrotoxin (Conroy, 1952).

When measured electrometrically, the pH of freshly prepared solutions rapidly decreased. The change of pH during the 5 min. contact period was rapid, approximately 80 per cent of the change occurring in the first minute (Fig. 3). The slow spontaneous change in pH of freshly prepared solutions was found to be catalysed by the calomel electrode (a crystal of potassium chloride or sodium chloride had an equivalent effect). Whether the fall in pH was spontaneous or catalysed by potassium chloride, the final values were comparable.

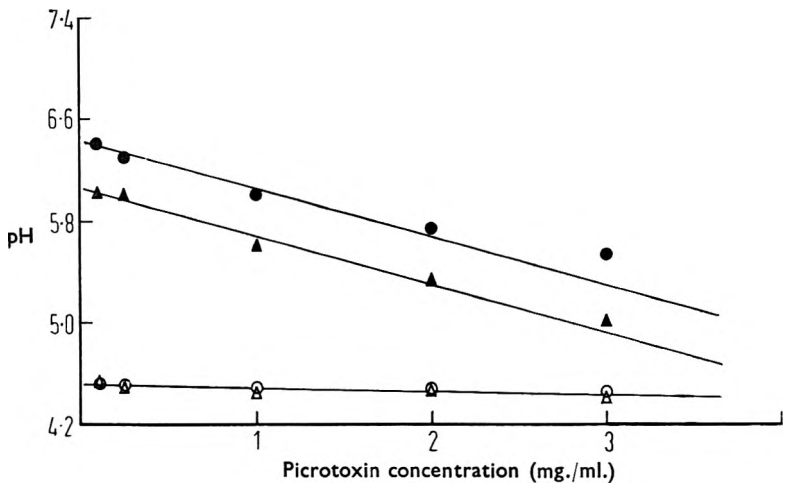


FIG. 2. The pH of aqueous and saline picrotoxin solutions after contact for 5 min. with glass and calomel electrodes.

Aqueous solutions ▽ Solvent pH 4·5 ▲ Solvent pH 7·3 Saline solutions ○ Solvent pH 4·5 ● Solvent pH 7·3

The pH of solutions in 0·9 per cent saline was also proportional to the concentration of picrotoxin (Fig. 2), and not unexpectedly, the slow spontaneous change in pH which was observed with freshly prepared aqueous solutions, did not occur when the solvent was saline. Generally, the final pH was always lower than the pH of the solvent and the final pH readings were close to those of the equivalent aqueous solutions, i.e. the slight leak of potassium chloride from the calomel electrode or the addition of crystals of sodium chloride had little catalytic activity owing to the saline medium. In consequence, though the initial pH readings of Picrotoxin Injection B.P. 1958 and Picrotoxin Injection U.S.P. 1955 are likely to be different on preparation, providing the pH of the solvents is similar, the solutions may attain the same pH after storage.

Response Times of Mice to Different Solutions of Picrotoxin

Since the pH of aqueous solutions of picrotoxin changed soon after preparation, it was necessary to determine whether there were associated changes in potency. Also, the relative pharmacological activity of aqueous and saline solutions was compared.

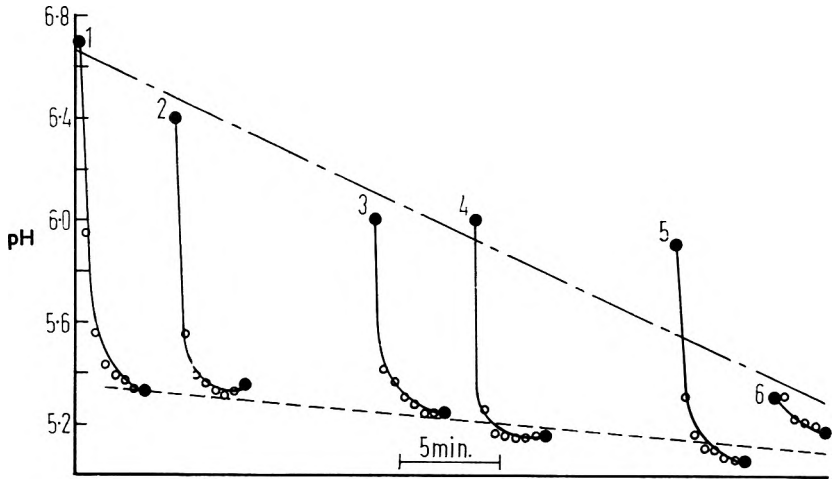


FIG. 3. A 1 mg./ml. solution of picrotoxin was prepared in distilled water (pH 5·72) and divided into 6 aliquots; each aliquot was allowed to stand at room temperature (22°) for a specified time after which the change of pH was measured for 5 min. using glass and calomel electrodes, and then the solution was discarded. The initial pH values of each aliquot (measured within a few sec. of immersion of the electrodes in the solution) decreased almost linearly from 6·7 to 5·5 units over a period of 7 hr. The "final" pH attained is of the same order, whether the fall is allowed to proceed spontaneously or is catalyzed.

Sample 1 tested 0 hr after preparation.
" 2 " 1 " " "
" 3 " 3 " " "
" 4 " 4 " " "
" 5 " 6 " " "
" 6 " 7 " " "

The response times of mice to freshly prepared solutions and to solutions at their final or "acid" pH value were determined. The pH measurements were always made on an aliquot of the original solutions. No significant difference ($P \leqq 0{\cdot}01$) in pharmacological activity could be detected between "acid" and freshly prepared solutions of picrotoxin when both response times were measured; neither was any significant difference found between the activities of freshly prepared aqueous and saline solutions (0·25 mg./ml.) (Table I).

TABLE I

SUMMARY OF RESULTS OF RESPONSES OF MICE TO FRESHLY PREPARED AND "ACID" AQUEOUS PICROTOXIN SOLUTIONS, AND ALSO TO FRESHLY PREPARED SALINE SOLUTIONS

	Concentration mg./ml.	pH	Mean F.P.* (sec.) ± s.e.	Mean C.T.† (sec.) ± s.e.
Freshly prepared solution	0·25 aqueous	6·8	86 ± 5·16	0
	2·00 aqueous	6·4	36 ± 1·38	61 ± 3·24
	0·25 saline	6·6	86 ± 4·32	0
"Acid form" solution	0·25 aqueous	5·1	89 ± 1·50	0
	2·00 aqueous	5·95	32 ± 0·96	61 ± 2·76

* Flaacid posture. † Convulsion time.

Picrotoxin solutions stored in non-acid washed glassware tended to become alkaline. Dilute solutions (0·25 mg./ml.) of picrotoxin in water, when adjusted to pH 9·5 with 0·01 N sodium hydroxide, were 66 per cent as active as the unadjusted solutions. Full activity was restored by neutralising the solution. However, 0·25 mg./ml. picrotoxin solutions of pH 10·5 were pharmacologically inactive and furthermore no activity was detectable after neutralisation (Table II).

TABLE II

SUMMARY OF RESULTS OF RESPONSES OF MICE TO "NEUTRAL", "ALKALINE" AND "NEUTRALISED" PICROTOXIN SOLUTIONS

		pH	Mean F.P. (sec.) ± s.e.
0·25 mg./ml.	controls	6·0	100 ± 3·76
" "	alkaline	9·5	168 ± 10·77
" "	"neutralised"	6·0	107 ± 6·68
0·25 mg./ml.	alkaline	10·5	0
" "	"neutralised"	6·0	0

Response Times of Mice to Solutions of Picrotoxinin and Picrotin

Picrotoxinin solutions (0·25 mg./ml. and 1.00 mg./ml.) were prepared in distilled water of pH 7·3 and the response times of mice to these solutions were tested. There was no significant difference between the activity of these solutions and solutions of picrotoxin of twice the concentration (Table III). Solutions of picrotin (2·0 mg./ml.) caused neither convulsions, nor the assumption of a flaccid posture, but a decrease in motor activity associated with slight ataxia was observed.

TABLE III

RESPONSES OF MICE TO AQUEOUS SOLUTIONS OF PICROTOXIN, PICROTOXININ AND PICROTIN

	Conc. mg./ml.	Mean F.P. (sec.) ± s.e.	Mean C.T. (sec.) ± s.e.
Picrotoxin	0·25	86 ± 3·16	0
	2·00	36 ± 1·38	61 ± 3·24
Picrotoxinin	0·125	87 ± 3·00	0
	1·00	30 ± 2·20	59 ± 3·50
Picrotin	2·00	0	0

Stability of Aqueous Solutions of Picrotoxin to Light

Bryan and Marshall (1948) considered that the unreliability of picrotoxin in the treatment of barbiturate overdosage "might be due to inconstant loss of analeptic activity during preparation and storage of picrotoxin solutions". The British Pharmacopoeia (1958) recommends that Picrotoxin Injection should be stored protected from light, but after exposing 0·25 and 2·0 mg./ml. solutions of picrotoxin to tungsten light for 24 and 96 hr., or ultra-violet light for 24 and 48 hr., we could detect no significant decrease in potency when using the flaccid posture and convulsion time tests (Table IV).

TABLE IV

EFFECT OF EXPOSURE OF PICROTOXIN SOLUTIONS TO TUNGSTEN AND ULTRA-VIOLET LIGHT

Dose of picrotoxin μg. injected in 0·1 ml.	F.P. (sec.)		C.T. (sec.)		P
	Controls ± s.e.	Experimental ± s.e.	Controls ± s.e.	Experimental ± s.e.	
		Tungsten light			
		(*a*) 24 hr.			
25	86 ± 2·63	96 ± 5·30	—	—	N.S.
200	—	—	45 ± 1·64	51 ± 7·23	N.S.
		(*b*) 96 hr.			
25	92 ± 3·53	98 ± 6·49	—	—	N.S.
200	—	—	49 ± 4·10	54 ± 3·72	N.S.
		Ultra-violet light (254 and 366 mμ)			
		(*a*) 24 hr.			
25	100 ± 3·76	98 ± 5·33	—	—	N.S.
200	—	—	61 ± 3·56	66 ± 5·14	N.S.
		(*b*) 48 hr.			
25	100 ± 3·76	101 ± 3·39	—	—	N.S.
200	—	—	68 ± 4·42	64 ± 3·64	N.S.

N.S. indicates value for P not less than 0·01. Between 7 and 10 animals per group were used.

Chromatography

Application of 200 μg. of picrotoxin to a single length chromatogram resulted in separation of the compound into four fractions with R_F of 0·84, 0·72, 0·25 and 0·07 respectively. Chromatograms of picrotoxinin and picrotin were also prepared.

The same four spots were identified with both hydroxylamine and silver nitrate colour reactions. When the spots were eluted and tested for pharmacological activity by the convulsion and flaccid mouse response time tests, activity was found in only one of the eluted fractions and this corresponded to picrotoxinin (R_F 0·25).

DISCUSSION

Picrotoxin is a neutral amaroid obtained from *Cocculus indicus*, a climbing shrub indigenous to the East Indies and Malay Archipelago. The drug is present in the seeds of the berries of the plant; also present are the nitrogen-containing bases menispermine and paramenispermine (Blyth and Blyth, 1920). Picrotoxin is extracted from the berries by boiling the powdered fruits with ethanol. The fatty residue is extracted with hot water and picrotoxin is recrystallised from water or ethanol. Picrotoxin has a bitter taste which is discernible at dilutions of 1 in 80,000. Four samples of picrotoxin, including one from the U.S.A., were obtained from different drug firms and all were found to be equipotent; subsequently, the three British samples were found to have originated from the same wholesale source.

Picrotoxinin and picrotin differ by only the elements of one molecule of water (Fig. 1). It is apparent from infra-red spectroscopy that both compounds contain two lactone groups (Conroy, 1952), which are believed to be stable except in the presence of alkali. Until recently, separation of the two compounds was only possible by chemical means involving

bromination. Using samples prepared by this method, we have confirmed that picrotoxinin is active and that picrotin is almost inactive; picrotoxinin was found to be twice as active as the parent substance.

Picrotoxin has been described as having some of the properties of an acid (Blyth and Blyth, 1920) and this statement was of interest in view of both the spontaneous and the catalysed fall in pH observed in freshly prepared solutions of picrotoxin. The reason for the fall in pH was not readily apparent from inspection of the structure of the molecule and, furthermore, solutions of pure picrotoxinin and picrotin did not change their pH values when determined electrometrically with calomel and glass electrodes.

The effect of alkali on picrotoxin solutions was not simple, for solutions which had been adjusted to pH 9·5 with 0·01 N sodium hydroxide were less potent, activity being restored on neutralisation, while the activity of solutions adjusted to 10·5 was permanently lost. The addition of alkali to picrotoxinin leads to the formation of picrotoxic acid which is apparently not pharmacologically active. Slater and Wilson (1951) have pointed out that "reversible" opening of the lactone systems in this series is quite common. Further, Holker (personal communication) has suggested that the process leading to loss of activity involves two stages, the first stage being the hydrolysis of the lactone group formed between the 3-hydroxy and 15-carboxyl groups, which is reversible by acid, and the second stage being the formation of an ether link between carbons 3 and 12, which is stable to acid (Fig. 4). This suggestion provides a basis for the explanation of reversible loss of activity below 9·5 and for the permanent loss of activity of solutions with a pH of 10·5 and above. Bryan and Marshall (1948) studied the reasons for the great variation in the dose of picrotoxin required to counteract barbiturate poisoning, and they noted that elevation of the pH was accompanied by a decrease in the activity of

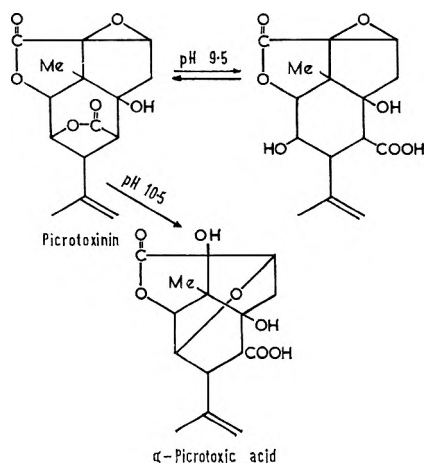


FIG. 4. Suggestion for the formation by alkali of inactive picrotoxic acid from picrotoxin in via an unisolated intermediate.

picrotoxin solutions, e.g. there was anything up to 50 per cent loss of activity between pH 8·5 to 9·0 using a "mouse-awakening" test. They concluded that the rise in pH which led to loss of activity was caused by alkali from the glass container, and to overcome this it was suggested that picrotoxin should be stored in a suitable buffer to maintain the pH below 6·5.

When the pH of freshly prepared picrotoxin solutions decreased, there was no change in potency, such as occurred when similar solutions became alkaline due either to storage in non-acid washed glassware, or the addition of sodium hydroxide. There was no obvious structural basis for the decrease in pH of freshly prepared solutions, and the change in pH was relatively small compared to the concentration used. Consequently it seemed probable that the factor responsible was an impurity. Evidence for this possibility was adduced from paper chromatography of commercially available picrotoxin, as no less than four spots were detected, only one of which possessed pharmacological activity.

A number of analeptics have been widely employed in the treatment of schizophrenia, following the introduction of convulsant therapy by Meduna in 1954. The wide range in the effective convulsant dose of picrotoxin made it less reliable and less extensively used than leptazol, even though the latter possessed the disadvantage of inducing a characteristic terror. The clinical unreliability of picrotoxin was also evident in its use in the treatment of barbiturate poisoning. Since the activity of picrotoxin solutions was markedly reduced by a slight elevation of the pH, this phenomenon may explain the clinical unreliability of the drug. Further, the suggestion that the alkaline nature of some glass containers may be sufficient to cause loss of activity, would appear to be valid, and consequently it is prudent to employ buffered picrotoxin solutions in clinical practice, and also in the laboratory for the accurate determination of biological responses. The presence of alkali may be responsible for the loss of activity previously ascribed to the effect of exposure to light. The spontaneous fall in pH of freshly prepared aqueous solutions did not affect the potency of the active principle picrotoxinin, and it is concluded that the change may be associated with the presence of the impurities that have been demonstrated in commercial samples of picrotoxin.

Acknowledgments. We are grateful to Professor I. E. Bush for his help in the chromatographic separation and detection of the constituents of picrotoxin. Samples of picrotoxin were kindly donated by Abbott Laboratories Ltd., and Kodak Ltd.

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SYNTHESIS OF SOME HISTAMINE DERIVATIVES HAVING POTENTIAL HISTAMINE-LIKE OR ANTIHISTAMINE ACTIVITY

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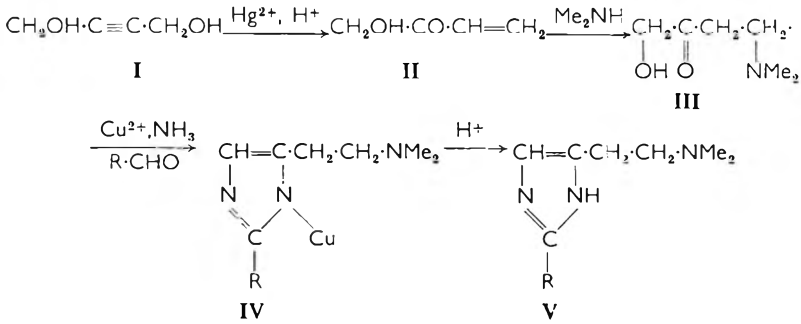
Twelve compounds derived from histamine have been prepared. In these the primary amine hydrogen atoms of histamine are replaced by methyl groups and the imidazole nucleus is substituted in the two position by a range of aliphatic and aromatic residues. Most members of the series have histamine-like pharmacological properties; two compounds have antihistamine activity.

Of the large number of compounds synthesised for possible histamine-like or antihistamine activity few have contained the imidazole nucleus and of these only a small number can be considered to be derivatives of histamine (see, e.g. Protiva, 1955). In view of the competitive antagonism to histamine shown by many antihistamine drugs it seemed that compounds closely related to histamine might possess antihistamine activity. Because many successful antihistamine compounds have an aliphatic chain terminated by a dimethylamino-group, *NN*-dimethylhistamine was chosen as the parent of a series.

Experimental Methods

The parent compound has been synthesised conveniently by Huebner (1951) using the Weidenhagen reaction (Weidenhagen and Herrmann, 1935), a method capable of general application to histamine derivatives substituted in the two position of the imidazole nucleus. In this method but-2-yne-1,4-diol (I) in ethyl acetate solution is isomerised to hydroxymethyl vinyl ketone (II) using a mercuric oxide-trichloroacetic acid-boron trifluoride catalyst (Reppe, 1949). The ketone with dimethylamine solution gives the Mannich-type base (III) which is not isolated but treated with an aldehyde and cupric acetate:ammonia solution on a heated water-bath. In the original Weidenhagen method the resulting precipitate of cuprous imidazole (IV) is separated by filtration, decomposed with hydrogen sulphide and the liberated imidazole derivative (V) obtained by suitable means. We found that improved yields are obtained when the whole of the reaction mixture is treated with hydrogen sulphide to precipitate all the copper as sulphide. This is because the cuprous imidazole compounds of this series are appreciably soluble in ammonia solution. The filtrate is then acidified, evaporated to low bulk, treated with 50 per cent potassium hydroxide solution and the liberated imidazole extracted with n-butanol. The hydrochloride is obtained either by acidification or better by first distilling the base in vacuum and then converting to the salt.



Yields (about 20 per cent) are much lower than those reported by Weidenhagen. This is probably due to the instability of the base III, which readily loses dimethylamine to revert to II, which forms polymeric compounds in ammonia solution (Huebner, 1951).

The compounds listed in Table I have been obtained by this route. The general procedure is exemplified as follows.

2-Ethyl-4(2'-dimethylaminoethyl)-imidazole

To hydroxymethyl vinyl ketone (8 g., 0·11 mole) (obtained from but-2-yne-1,4-diol (32 g., 0·44 mole) by Reppe's method (1949) was added dimethylamine (25 ml. of 33 per cent solution in ethanol, 0·17 mole) with cooling in ice-water. After allowing to stand for 15 min. the mixture was added to a solution of cupric acetate (36 g., 0·18 mole) and propionaldehyde (7 g., 0·12 mole) in ammonia solution (225 ml., s.g. 0·880). The combined solutions were heated on a boiling water-bath for 1 hr. Hydrogen sulphide was then passed in until the copper was completely precipitated, when the suspension was filtered. The filtrate was acidified with 6N hydrochloric acid, evaporated to low bulk and basified with 50 per cent potassium hydroxide. The imidazole base was extracted with n-butanol (6 portions of 20 ml.), the combined extracts dried ($MgSO_4$), the solvent removed and the residue distilled under reduced pressure to give *2-ethyl-4(2'-dimethylaminoethyl)-imidazole* (4·9 g., b.p. 140–142°/1 mm, yield 72 per cent).

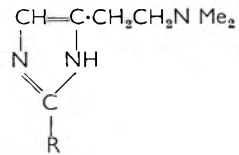
The base was dissolved in ethanolic hydrochloric acid (20 ml., 4N) and an excess of ether added to precipitate *2-ethyl-4(2'-dimethylaminoethyl)-imidazole dihydrochloride* (m.p. 181–183° after crystallisation from isopropanol-ether mixture).

In Table I the equivalent weight recorded was determined by titration with standard silver nitrate for hydrochlorides and with standard acetic-perchloric acid for picrates. Hydrochlorides were crystallised from isopropanol-ether mixture, picrates from aqueous acetone. Melting-points are uncorrected.

Pharmacological Properties and Discussion

The hydrochlorides have been tested for histamine-like and anti-histamine activity on isolated guinea-pig ileum. As reported by Huebner

TABLE I



No.	R	Salt	m.p. °C	Formula	Found per cent				Required per cent				Found equiv. wt.	Required equiv. wt.
					C	H	N	Hal	C	H	N	Hal		
1a	H	di-HCl	192–4[a]	$C_7H_{15}Cl_2N_3$	—	—	—	33·5	—	—	—	33·5	106	106
1b	H	dipicrate	231–2[b]	$C_{19}H_{19}N_9O_{14}$	—	—	21·1	—	—	—	21·1	—	296	299
2a	Me	di-HCl	199–201	$C_8H_{17}Cl_2N_3$	42·1	7·5	18·2	31·6	42·5	7·5	18·6	31·4	114	113
2b	Me	dipicrate	193–4	$C_{20}H_{21}N_9O_{14}$	38·9	3·7	20·5	—	39·3	3·4	20·6	—	304	306
3a	Et	di-HCl	181–3	$C_9H_{19}Cl_2N_3$	44·6	8·0	17·5	29·4	45·0	7·9	17·5	29·6	122	120
3b	Et	dipicrate	154–6	$C_{21}H_{23}N_9O_{14}$	40·6	3·7	20·0	—	40·3	3·7	20·2	—	311	313
4a	Ph·CH_2	di-HCl	174–5	$C_{14}H_{21}Cl_2N_3$	55·2	7·0	13·5	23·4	55·6	7·0	13·9	23·5	151	151
4b	Ph·CH_2	dipicrate	161–2	$C_{26}H_{25}N_9O_{14}$	45·1	3·8	18·3	—	45·4	3·6	18·3	—	346	344
5a	Ph	di-HCl	280–2[c]	$C_{13}H_{19}Cl_2N_3$	54·1	6·9	14·5	24·6	54·2	6·6	14·6	24·7	143	144
5b	Ph	dipicrate	223–4[d]	$C_{25}H_{23}N_9O_{14}$	44·9	3·6	18·7	—	44·6	3·4	18·7	—	336	337
6a	*p*-Cl·C_6H_4	di-HCl	285–6	$C_{13}H_{18}Cl_3N_3$	48·3	5·7	13·3	33·1	48·4	5·6	13·0	33·0	162	161
6b	*p*-Cl·C_6H_4	dipicrate	219–20	$C_{25}H_{22}ClN_9O_{14}$	42·5	3·3	17·8	5·4	42·4	3·1	17·8	5·1	353	354
7a	*p*-Br·C_6H_4	di-HCl	276–8	$C_{13}H_{18}BrCl_2N_3$	42·9	5·2	11·2	—	42·5	4·9	11·5	—	185	183
7b	*p*-Br·C_6H_4	dipicrate	218–9	$C_{25}H_{22}BrN_9O_{14}$	40·3	3·2	16·6	10·6	39·9	2·9	16·8	10·6	377	376
8a	*p*-Me·C_6H_4	di-HCl	296–7	$C_{14}H_{21}Cl_2N_3$	55·2	6·9	13·8	23·5	55·6	7·0	13·9	23·5	152	151
8b	*p*-Me·C_6H_4	dipicrate	213–4	$C_{26}H_{25}N_9O_{14}$	45·9	3·8	18·3	—	45·4	3·6	18·3	—	344	344
9a	*m*-NO_3·C_6H_4	di-HCl	273–4	$C_{13}H_{18}Cl_2N_4O_2$	46·6	5·9	16·5	21·5	46·9	5·4	16·8	21·3	167	167
9b	*m*-NO_3·C_6H_4	dipicrate	221–2	$C_{25}H_{22}N_{10}O_{16}$	42·0	3·2	19·6	—	41·8	3·1	19·5	—	362	359
10a	*p*-Me_2N·C_6H_4	di-HCl	258–9	$C_{15}H_{24}Cl_2N_4$	54·0	7·3	17·4	21·4	54·3	7·3	16·9	21·2	165	166
10b	*p*-Me_2N·C_6H_4	dipicrate	222–3	$C_{27}H_{28}N_{10}O_{14}$	45·3	4·2	19·4	—	45·3	3·9	19·6	—	241	239
11a	*p*-MeO·C_6H_4	di-HCl	287–8	$C_{14}H_{21}Cl_2N_3O$	52·5	6·3	13·0	22·7	52·8	6·6	13·2	22·3	158	159
11b	*p*-MeO·C_6H_4	dipicrate	225 6	$C_{26}H_{25}N_9O_{15}$	44·4	3·5	18·1	—	44·4	3·5	17·9	—	352	352
12a	2-Furyl	di-HCl·H_2O	241–2	$C_{11}H_{20}Cl_2N_3O_2$	44·5	6·6	14·2	23·7	44·6	6·4	14·2	24·0	149	148
12b	2-Furyl	dipicrate	225–6	$C_{23}H_{22}N_9O_{15}$	41·8	3·3	19·0	—	41·6	3·2	19·0	—	331	332

(*a*) Huebner (1951) reports m.p. 184 5°. (*b*) Huebner (1951) reports m.p. 230–2°. (*c*) Huebner (1951) reports m.p. 270 5°. (*d*) Huebner (1951) reports m.p. 218–220°.

(1951) and Huebner, Turner and Scholz (1949), compounds 1a and 4a (Table I) had histamine-like activity, as did 2a, 3a, 8a, 9a, 10a, and 12a, in doses 10 to 100 times those at which histamine itself was effective. Compounds 6a and 11a were found to have a slight antihistamine activity. This is of interest in that a number of commercial antihistamine drugs have a *p*-chlorophenyl or *p*-methoxyphenyl group in the molecule. It is not to be expected that antihistamine activity will be high in these compounds as study of the reported activity of many substances synthesised for antihistamine action shows that a second aromatic or hetero-aromatic substituent is likely to be necessary for high activity.

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A NOTE ON THE DIFFERENTIATION BETWEEN *DEXTRO-* AND *LAEVO*PROPOXYPHENE

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Received June 12, 1963

A method is described for distinguishing between microgram quantities of the (+)- and (−)-isomers of propoxyphene.

Dextropropoxyphene (Darvon), the α-(+)-isomer of 4-dimethylamino-3-methyl-1,2-diphenyl-2-propionoxylbutane, came into use as an analgesic in 1957. Recently *laevo*propoxyphene, the (−)-isomer, has been marketed as an antitussive agent under the name of Letusin. Dextropropoxyphene is said to have an analgesic effect similar to that of codeine, and to be devoid of addictive properties. Internationally, it has been recommended for the same type of restriction as codeine, the *laevo*-isomer being free from control. In the United Kingdom, both isomers are subject to the provisions of Schedule 1 of the Dangerous Drugs Act.

Differentiation between these isomers might therefore become a matter of considerable forensic interest. Such distinction is not possible by ordinary analytical methods, but may, of course, be made polariscopically if sufficient material is available. On the microgram scale, use may be made of the method described by Clarke (1958) for the *N*-methylmorphinan analgesics. This depends on the fact that the racemic form of an alkaloid will usually form crystalline derivatives with certain reagents more easily than will either of the optical enantiomorphs. In the case of propoxyphene, gold chloride yields crystals almost at once with the racemic form, but only after standing for several hours with the (+)- and (−)-isomers.

Practical procedure. The test is carried out as follows, using the hanging microdrop technique of Clarke and Williams (1955). A microdrop of a solution of the test substance in 2N acetic acid is placed on a cover slip, and a microdrop of a 1 per cent solution of one known isomer (say, laevopropoxyphene), made by dissolving the base in 2N acetic acid, added. A microdrop of a 5 per cent solution of gold chloride is now added, and the cover slip sealed, inverted, and examined under the microscope in the usual way. If the test solution contained dextropropoxyphene, the drop will now contain both (+)- and (−)-isomers, and crystals of racemic propoxyphene aurichloride will quickly form; these appear as small, curved irregular needles, sometimes serrated, in bunches or branching chains. If, however, the unknown was laevopropoxyphene, the test drop will contain only this isomer, and crystals will not form for several hours, if at all. If they do, they are usually straight needles, considerably larger than those of the racemic form. Confirmation may be obtained by repeating the test, using dextropropoxyphene as the known isomer. A positive result may be obtained

with a 0·01 per cent solution of the test substance. As the volume of a microdrop is 0·1 μl., the sensitivity of the test is thus 0·01 μg.

Acknowledgements. I wish to thank Messrs. Eli Lilly & Co. Ltd. for supplies of propoxyphene and Miss Sandra Gavin for technical assistance.

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LETTERS TO THE EDITOR

The Metabolism of 5-Hydroxytryptamine in Cultures of *Claviceps purpurea*

SIR,—The metabolism of 5-hydroxytryptamine (5-HT) has been studied extensively in animal tissues, but knowledge of its fate in micro-organisms and plant tissues is scanty. Jannes, Leppanen and Saris (1962) found an unidentified 5-HT metabolite in cells of *Escherichia coli*. 5-Hydroxy-indole-3-acetic acid has been detected by Tyler and Smith (1960) in several species of fungi. *NN*-dimethyl-5-hydroxytryptamine has been isolated from plant sources such as the seeds of *Piptadenia peregrina* (Stromberg, 1954) and the toxic mushroom *Amanita mappa* (Wieland, Motzel and Merz, 1953).

For this study, claviceps mycelia were grown by the methods of Sim and Youngken (1951) and Taber and Vining (1958) from sclerotia of a Secale strain of *Claviceps purpurea* obtained from the Department of Plant Pathology, University of Minnesota. These cultures failed to produce alkaloids with or without the addition of 5-HT, but significant amounts of the added 5-HT disappeared rapidly during 2-4 hr.

Mycelial pads after 14-67 days growth were separated from substrates, washed twice with 50 ml. of saline, and well drained. 5-HT creatinine sulphate (12 mg.) in 50 ml. saline solution was added to the pads in the culture flasks, which were protected from light and shaken at 105 cycles per min. at 20-25° for 1-4 hr. Aliquots of the medium (3 ml.) were analyzed for 5-HT, (Udenfriend, Weissbach and Clark, 1955), at 1, 2 and 4 hr. intervals. An initial 3 ml. aliquot was analyzed just after adding the 5-HT solution and analyses of subsequent aliquots were calculated as a percentage of this initial value. The results are shown in Table I. A marked loss of 5-HT was noted in the medium of cultures of 21–67 days growth. Aliquots from control flasks without mycelia showed no decrease of 5-HT, nor was it demonstrable in those mycelial cultures to which 5-HT was not added.

TABLE I

5-HT REMAINING IN REPLACEMENT CULTURES OF CLAVICEPS AT SHORT INCUBATION PERIODS

Age of mycelia (days)	pH medium at harvest	pH medium end of incubation	Percent of 5-HT remaining (hr. incubation)		
			1	2	4
14	5·8	5·8	104	99	99
21	7·2	6·7	86	66	30
22	7·4	6·8	85	71	57
30	7·5	6·2	66	54	33
33	7·7	7·0	68	49	22
36	8·5	7·7	76	62	28
41	7·7	6·8	40	26	16
44	7·3	6·1	54	34	20
61	8·0	7·0	59	28	9
67	7·6	7·3	60	21	13

Mycelia of similar age (16, 20, 29, 39 days) were then separated from culture substrates by centrifugation at 17,500 × *g* for 10 min. at 3°. They were frozen overnight and allowed to thaw slowly at room temperature to promote cellular disruption. While cold the tissues were homogenized in glass in 0·1 M phosphate buffer, pH 7·0, and the homogenate quickly transferred to chilled nylon centrifuge tubes and centrifuged for 10 min. (600 × *g*) at 3°. Samples of the supernatant (2 ml.) were incubated with 0·4 ml. of 0·1 M phosphate

buffer, pH 7·0, and 0·6 ml. of 5-HT (final concentration 0·025 M). Control vessels omitting the supernatant were similarly prepared. The mixtures were shaken for 1 hr. at room temperature under air. The pH remained at 7·0–7·1. The reaction mixtures were pooled, concentrated to 1-2 ml. under reduced pressure and analyzed by chromatography by the methods of Matthias (1954), using a descending strip method with butanol: acetic acid: water (4:1:5) as the developing solvent. When dried, the strips were sprayed with Ehrlich's reagent (Jepson, 1955). The mycelial extract gave two intense blue fluorescent spots one of which had the same R_F value (0·46) as the single spot given by the control and by a reference spot of 5-HT creatinine sulphate. The second spot, R_F value 0·33, was not identified. The R_F value did not correspond with those of a number of indole reference compounds. It was concluded that it was not identical with any of the metabolites reported thus far by others for animal or plant tissues. The unidentified spot was present in mycelial growth cultures of all ages and was most prominent in those of 16 and 20 days growth. Sufficient quantities of the unknown compound were not obtained for identification. However, R_F values in different solvent systems, colour reactions and ultra-violet absorption peaks were obtained for it as described for indole compounds by Chadwick and Wilkinson (1960), McIsaac and Page (1959) and Keglevic and others (1959).

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Carbohydrate Metabolism and Anaphylaxis

SIR,—A fundamental approach to an understanding of the mechanisms of anaphylactic reactions is the study of factors that make certain species resistant to the process. The rat is well known to be resistant to anaphylaxis and histamine shock. Several conditions like suprarenalectomy (Dews and Code, 1953), hypophysectomy (Molomut, 1953), pretreatment with *Bordetella pertussis* vaccine (Sanyal and West, 1958), insulin injections before challenge (Sanyal, Spencer and West, 1959), facilitate the production of anaphylactic sensitivity. These conditions also produce hypoglycaemia (Sanyal, 1960). It was of interest, therefore, to discover whether conditions which produced hyperglycaemia had a protective effect against the production of systemic anaphylactic shock, in the rat.

When rats were sensitised 14 days previously by simultaneous injections of horse serum and *Bordetella pertussis* vaccine (Sanyal and West, 1958) the fasting blood sugar level was consistently lower than that seen in untreated animals. When a group of ten sensitised animals was subjected to intravenous challenge with horse serum, severe shock was produced and all animals died within the first 4 hr. Another group of 16 similarly sensitised animals, received subcutaneous injections of 2 per cent aqueous solution of alloxan tetrahydrate (300 mg./kg.), 4 days before challenge. This treatment produced glycoscuria and hyperglycaemia within the next few days. On anaphylactic challenge, the shock produced was mild, and 11 animals recovered. In 5 animals, shock was moderate and these animals died in 12 to 24 hr. after challenge.

In a group of rats similarly sensitised 14 days before, 5 ml. of 25 per cent solution of glucose was injected subcutaneously in each rat, at 0, 1, and $2\frac{1}{2}$ hr. It has been reported before (Adamkiewicz and Adamkiewicz, 1960) and confirmed again that such injections produce hyperglycaemia and glycosuria. Another group of similarly sensitised animals received 5 ml. of 8·46 per cent aqueous solution of sodium chloride at similar time periods, and served as control. The solution of sodium chloride injected was isomolar with 25 per cent glucose solution. Anaphylactic challenge was given intravenously 45 min. after the first injection of glucose or saline. All the animals of the control group receiving saline died of severe anaphylactic shock within first 2 hr.; at autopsy, typical haemorrhagic lesions characteristic of anaphylactic shock in this species were found. In contrast, animals that had received glucose, did not develop signs of anaphylactic shock and could be easily distinguished from animals that had received challenge after hypertonic saline. However these animals also died within 24 hr.; at autopsy, changes in intestines were minimal but subdural haematoma, as has been described to be the effect of over dosage of hypertonic solutions (Selye, 1952), were seen in all rats.

Thus in the rat, the state of blood sugar level influences the development of anaphylactic shock.

An interrelationship of allergic conditions and carbohydrate metabolism has been noticed for man (Van Ufford, 1952) and it has been reported also that nocturnal attacks of asthma may be associated with hypoglycaemia (Abrahamsson, 1941).

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The Action of Inhibitors of Catechol-*O*-Methyl-Transferase on the Exploratory Activity of Mice

SIR,—In a previous note we reported that pyrogallol enhanced inhibitory learning in rats (Izquierdo, I. and Merlo, 1963), and we considered it likely that this effect was due to a central adrenergic mechanism.

Using an actograph we have studied the exploratory activity in 77 white mice of 12 to 30 g. weight, before and after intraperitoneal injections of pyrogallol (3, 6, 12·5, 50 and 200 mg./kg.) and of 3,4-dihydroxyphenylacetamide (300 mg./kg.). The drugs were dissolved in 0·1 ml./kg. of distilled water just before injection. Control animals were given the water only.

The actographic records were run for 10 min. before, and for 20 min. after the injections. Only the definite suppression of exploratory activity was considered an inhibition. Responses were evaluated as the number of mice showing suppression at each dose of the drugs.

300 mg./kg. of 3,4-dihydroxyphenylacetamide suppressed exploratory activity of 9 out of 10 mice in which it was tested. The results obtained with pyrogallol are plotted in Fig. 1, in which the ordinates correspond to percentage of mice

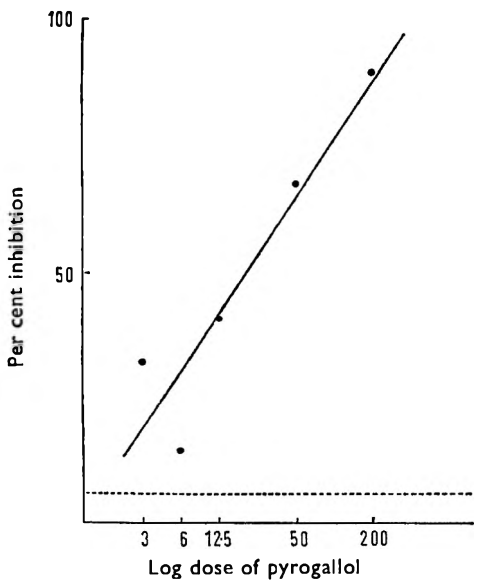

FIG. 1. Inhibition of exploratory activity of mice after pyrogallol. The broken line is the control response.

responding with inhibition, and the abscissae to the doses of pyrogallol in a logarithmic scale. The horizontal broken line corresponds to inhibitions seen in control animals.

Our results seem not to be due to just a non-specific motor impairment: 200 mg./kg. of pyrogallol had no effect upon a rota-rod test performed in 12 mice.

If the decrease of exploratory activity is taken as a measure of habituation (disappearance of the orienting reflex to a new environment), then these results are in agreement with those previously reported in which pyrogallol enhanced habituation as well as extinction in rats (Izquierdo, I. and Merlo, 1963). As both pyrogallol (Axelrod, 1960) and 3,4-dihydroxyphenylacetamide (Carlsson, Lindqvist, Fila-Hromadko and Corrodi, 1962) are inhibitors of catechol-*O*-methyl-transferase, and thereby increase the contents of catecholamines in various encephalic structures (Izquierdo, J. A., Jofre and Dezza, 1963 and unpublished), our data lend further support to the hypothesis of an adrenergic mechanism in the brain, related to inhibition.

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We thank Dr. A. Carlsson for the 3,4-dihydroxyphenylacetamide.

Awakening from Reserpine Sedation by α-Methyldopa

SIR,—Rossum and Hurkmans (1963) found that α-methyldopa, given to mice pretreated with a monoamine oxidase inhibitor, produced central excitation. They suggested that this effect was due to the accumulation of catecholamines liberated from stores within the brain by the metabolic products of α-methyldopa. Their data are consistent with this suggestion. However, they stated that their experiments do not support the hypothesis of Day and Rand (1963) that α-methylnoradrenaline, formed by the metabolism of α-methyldopa, may serve in lieu of noradrenaline as a "false transmitter." The data of Rossum and Hurkmans are concerned only with actions of α-methyldopa exerted on the central nervous system, and are not necessarily applicable to actions of α-methyldopa at peripheral adrenergic junctions. The hypothesis advanced by Day and Rand (1963) was that a metabolic product of α-methyldopa (α-methylnoradrenaline) was able to occupy storage sites in peripheral tissues normally occupied by noradrenaline, and then to be available for release from these stores by stimuli normally leading to a release of noradrenaline. The important evidence for this hypothesis was derived in three ways: that α-methyldopa was able to enter the same metabolic pathways available for dopa, that one of the products, α-methylnoradrenaline was bound in tissues, and that infusion of α-methyldopa enhanced the responses to sympathetic nerve stimulation and to indirectly acting sympathomimetic amines in reserpine-treated animals. The first two points of evidence are derived from the biochemical work of others (cited in Day and Rand, 1963, and in Rossum and Hurkmans, 1963), the last is from our own experimental work. We have been unable to interpret it other than by assuming that α-methylnoradrenaline is able to serve as a transmitter at peripheral adrenergic junctions.

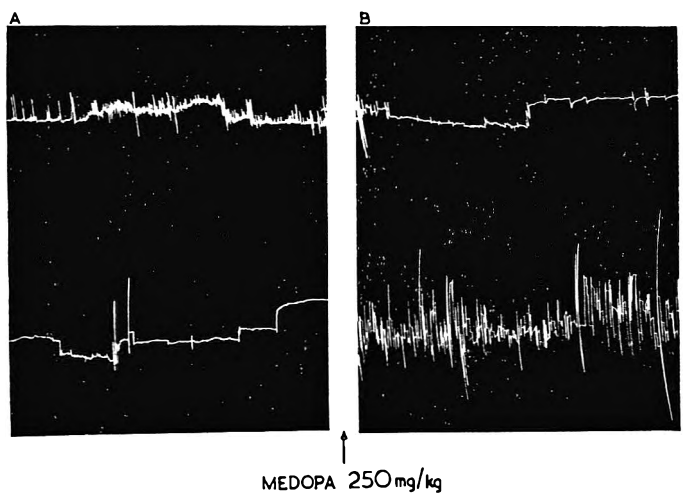

FIG. 1. Motor activity of two young litter-mate rats (55 g.) measured in jiggle cages (Brittain, 1961). Both rats pretreated 16 hrs. before the experiment with reserpine (15 mg./kg.) by intraperitoneal injection. In A both rats were heavily sedated and showed little spontaneous motor activity. Between A and B the rat in the lower record was injected with α-methyldopa (250 mg./kg.). After a period of 2 hr. the treated animal showed a marked increase in motor activity whilst the control (upper record) animal remained sedated.

In reserpine-treated mice, injections of dopa relieve the sedation and replete the stores of central catecholamines (Carlsson, Lindqvist and Magnusson, 1957; and Blaschko and Chruściel, 1960). In reserpine-treated rats which are heavily sedated, injections of α-methyldopa (250 mg./kg., intraperitoneally) caused a gradual increase in activity. The results from one experiment are illustrated in Fig. 1. Injections of α-methyldopa in rats not previously treated with reserpine decreased motor activity.

These observations may be interpreted in the following way. In normal animals, α-methyldopa is metabolised to yield α-methyldopamine and α-methylnoradrenaline. These amines replace the stores of dopamine and noradrenaline in the central nervous system (Carlsson and Lindqvist, 1962). The α-methylated amines may be able to serve in the same roles as dopamine and noradrenaline in the brain, but being less potent, sedation results. In reserpinised animals the stores of catecholamines in the brain are depleted and the animals are heavily sedated. Then, after α-methyldopa, the stores become replenished with the α-methylated amines and the sedation is relieved. It would follow from this explanation that the metabolic products of α-methyldopa may serve as "false transmitters" in the central nervous system as well as at peripheral adrenergic nerve endings. Carlsson and Lindqvist (1962) have also suggested that the α-methylated compounds which replace the catecholamines in the brain after treatment with α-methyldopa may take over the function of the physiological amines.

The excitement which Rossum and Hurkmans observed in mice pretreated with a monoamine oxidase inhibitor and then given α-methyldopa can best be explained by accumulation of catecholamines displaced from their stores but protected from destruction, but this explanation is not incompatible with the displacing α-methylated amines serving as a "false transmitter."

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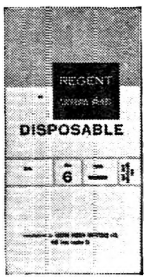

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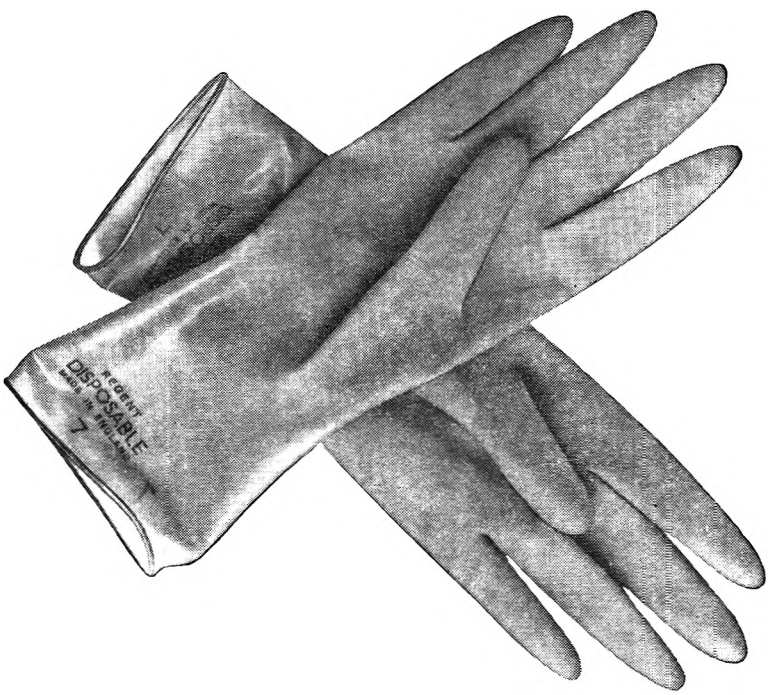

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