RESEARCH PAPERS

CHROMATOGRAPHIC STUDIES OF THE EFFECT OF INTRAVENOUS INJECTIONS OF TYRAMINE ON THE CONCENTRATIONS OF ADRENALINE AND NORADRENALINE IN PLASMA

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Received April 20, 1960

Intravenous injections of tyramine, increased the concentrations of adrenaline and noradrenaline in the plasma of heparinised blood withdrawn from the lower aortae of chloralosed cats, which had been rested for 30–40 minutes after induction of lasting ganglion block with hexamethonium and exclusion of the adrenals from the circulation. Both the pressor response to tyramine and this increase in catechol amine was abolished either by pretreatment of the animal with reserpine or by the intramuscular injection of cocaine. Only one tenth of the increase in adrenaline and noradrenaline in the plasma of the lower aorta reached the inferior vena cava, and there was only a very small accompanying rise in the catechol amine content of plasma from blood taken from the base of the carotid arteries when the tyramine injected did not exceed 120 μg./kg.

It is now well known that postganglionic sympathetic denervation of a tissue greatly reduces or abolishes its responsiveness to tyramine. For example, tyramine fails to dilate the denervated pupil[1] and no longer causes vasoconstriction in the denervated forelimb[2] or contraction of the denervated nictitating membrane[3] in cats. Reserpine also reduces or abolishes the pressor action of tyramine[4] and depletes[5] the chromaffin tissue in the walls of large blood vessels[6] of their stores of noradrenaline-like material. The pressor action of tyramine is re-established after treatment with reserpine by prolonged infusion of noradrenaline[7]. It was predominantly these facts which led Burn and Rand to advance the hypothesis that at least part of the pressor activity of tyramine might be attributed to a release of noradrenaline-like material from the walls of the blood vessels. The object of the present work has been to make direct test of this hypothesis, since it rested on indirect evidence alone, and to measure the changes in the plasma concentrations of catechol amines which result from the intravenous injection of tyramine.

EXPERIMENTAL

Methods

Male, female or neuter cats were used. Anaesthesia, induced with ether, was maintained by the intravenous injection of 7 ml./kg. of 1·0 per cent chloralose in 0·9 per cent aqueous sodium chloride. The trachea was cannulated, the adrenals were excluded from the circulation and the mean arterial pressure was recorded in every experiment. Heparin, 500 units/kg. was used throughout as anticoagulant. In five experiments

of series 1 and in all other experiments records were made of the contractions of the right nictitating membrane in response to rectangular stimuli of 0·5 milliseconds duration delivered to the right cervical sympathetic chain at rates up to 30/second. Hexamethonium bromide 10 mg./kg. intravenously, supported by 10 mg./kg. given subcutaneously, blocked transmission in the superior cervical ganglion for the duration of these experiments. The supporting dose of hexamethonium was doubled in those three experiments of group 1 in which block of the superior cervical ganglion was not demonstrated.

Series 1. *Eight experiments.* Intravenous injections were made through a glass cannula tied into the right femoral vein. Samples of arterial blood were withdrawn through a polythene cannula inserted into the aorta through the right femoral artery so that the tip lay 0·5 to 2·0 cm. above the bifurcation. Mean arterial pressure was recorded from a carotid artery.

Series 2. *Four experiments.* Injection and aortic cannulae as in series 1. An additional cannula for arterial sampling was introduced into the left carotid artery, the tip reaching a point within 1 cm. of its origin. Mean arterial pressure was recorded from the left femoral artery.

Series 3. *Three experiments.* The cannula for intravenous injections was inserted into the right external jugular vein. Mean arterial pressure was recorded from the left carotid artery. Polythene cannulae for the collection of blood samples were introduced into the lower aorta (as in series 1) and through the right femoral vein to extend for 1·0 to 2·5 cm. above the junction of the common iliac veins.

Series 4. *Two experiments.* As for series 1 except that each animal received reserpine 1 mg./kg. by intramuscular injection 36 hours before the experiment. Experiments began with the withdrawl of control blood samples 30–40 minutes after ganglion block had been established (see above). Further blood samples were withdrawn ten minutes later during a pressor response to an intravenous injection of tyramine. The whole process was repeated 20 minutes after the pressor effect of the first dose of tyramine had disappeared. Plasma from blood collected from each site before and between responses to tyramine was separately pooled. That from blood collected during tyramine effects was similarly but separately treated. Blood samples varied from 4 to 8 ml. and were taken into cooled heparinised tubes. The plasma was removed without delay. Adrenaline and noradrenaline were separated by ascending paper chromatography, using phenol-hydrochloric acid as solvent in an atmosphere of carbon dioxide, from protein free extracts of plasma, and eluates were prepared for bioassay as previously described[8].

Noradrenaline was assayed on the blood pressure of rats anaesthetised with 1·5 ml. 15 per cent urethane per 100 g. intraperitoneally, and treated with 0·5 mg. hexamethonium intravenously and 1·0 mg. subcutaneously shortly before the assay began.

Adrenaline was always assayed by inhibition of the responses of the rat's quiescent uterus to a fixed dose of acetylcholine[10] and was also assayed on the mean arterial pressure of the rat in many experiments of group 1.

Drugs

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

Results

The plasma of lower aortic blood contained only low concentrations of adrenaline and noradrenaline when taken from acutely adrenalectomised chloralose cats which had been rested for 30–40 minutes under the full and lasting ganglion blocking action of hexamethonium bromide. These concentrations were very markedly increased during pressor responses to intravenous injections of tyramine hydrochloride (Table I). Both the pressor effect of tyramine and the raised concentrations of adrenaline and noradrenaline which it caused in lower aortic blood were abolished or greatly reduced either by the intramuscular injection of 10 mg./kg. cocaine hydrochloride or pretreatment of the cats with reserpine (Table I).

TABLE I

COMPARISON OF THE EFFECT OF TYRAMINE ON THE MEAN ARTERIAL PRESSURE AND THE CONCENTRATIONS OF (−)-ADRENALINE AND (−)-NORADRENALINE IN PLASMA SEPARATED FROM BLOOD WITHDRAWN FROM THE LOWER ABDOMINAL AORTAE OF CATS UNDER CHLORALOSE ANAESTHESIA AND TREATED WITH HEXAMETHONIUM

Experiment		Tyramine HCl µg./kg. i.v.	µg./100 ml. plasma Resting		µg./100 ml. plasma after tyramine		Rise in blood pressure after tyramine mm. Hg
Series	No.		Adren.	Noradren.	Adren.	Noradren.	
I	1	100	0·03	0·09	6·0	5·5	71
	2	100	<0·10	<0·80	5·0	6·0	76
	3	100	0·07	0·41	7·2	3·0	68
			0·06	<0·03	0·05	<0·3	2 after cocaine
	4	100	<0·06	<0·75	6·5	2·4	82
			0·32	<0·20	0·9	0·4	4 after cocaine
	5	100	0·80	1·20	10·5	6·5	108
			1·10	0·84	2·0	1·3	28 after cocaine
	6	120	0·04	0·93	8·6	2·1	46
			2·68	1·00	1·6	0·9	10
	7	140	0·40	0·27	8·9	1·1	98
			0·92	<0·20	0·8	<0·2	12 after cocaine
Cats pretreated with reserpine							
VI	1	100	0·43	1·57	0·4	1·6	4
	2	100	0·32	0·98	0·4	1·2	6

Whereas intravenous injections of tyramine caused a large increase in the concentrations of adrenaline and noradrenaline in plasma from lower aortic blood they evoked little change in that drawn from a carotid artery close to its origin (Table II). Only a fraction of that adrenaline and noradrenaline liberated by tyramine into the lower aortic blood returned from the leg and tail to be found in blood drawn from the inferior vena cava a little above its origin (Table II).

Discussion

The trace amounts of catechol amine found in the blood of chloralosed cats, which had been rested for 30–40 minutes after exclusion of the

adrenal glands from the circulation and induction of a lasting block to transmission in autonomic ganglia, did not differ significantly from the normal concentrations of these amines in blood withdrawn from man by venipuncture[11–14]. Neither did the site of withdrawal of these resting samples much affect the concentrations of amines they contained (Tables I and II). These, and the blood samples taken from man, contained much more noradrenaline than adrenaline. It is therefore probable that a lasting block of transmission in sympathetic ganglia does not prevent a slow leak of catechol amine from the region of the terminations of adrenergic fibres. This last statement does however rest on the assumption that the low concentrations of adrenaline and noradrenaline found yield adequate substrate concentrations for the *o*-methylating enzyme system[15].

TABLE II

COMPARISON OF THE EFFECTS OF TYRAMINE ON THE MEAN ARTERIAL PRESSURE AND ON THE CONCENTRATIONS OF (−)-ADRENALINE AND (−)-NORADRENALINE IN PLASMA SEPARATED FROM BLOOD WITHDRAWN FROM THE LOWER AORTA, NEAR THE ORIGIN OF THE CAROTID ARTERY, AND FROM THE LOWER PART OF THE INFERIOR VENA CAVA

Experiment		Tyramine HCl mg./kg.	μg./100 ml. plasma								Rise in blood pressure after tyramine mm./Hg
			Lower aorta				Carotid artery				
			Resting		After tyramine		Resting		After tyramine		
Series	No.		Adren.	Nor-adren.	Adren.	Nor-adren.	Adren.	Nor-adren.	Adren.	Nor-adren.	
II	1	188	0·65	<0·9	9·9	—	0·02	<0·8	0·35	<0·9	42
	2	100	0·34	1·4	4·2	2·6	0·06	1·8	0·45	1·3	46
	3	115	0·84	<0·3	9·5	4·0	0·11	<0·9	1·03	<0·4	88
	4	146	1·48	<0·9	11·8	5·7	0·21	<0·5	1·19	<0·9	51
			Lower aorta				Inferior vena cava				
III	1	165	0·57	<0·4	16·7	8·4	1·26	<0·3	7·3	<0·4	48
	2	130	0·07	<0·04	6·1	4·3	0·10	<0·1	0·7	<0·1	43
	3	100	0·34	1·4	4·2	2·6	0·21	1·5	2·1	1·3	44

Intravenous injections of tyramine caused very considerable increase in the concentrations of adrenaline and noradrenaline in the plasma of blood withdrawn from the lower aorta but evoked only small change in the levels of these amines in plasma taken from the base of a carotid artery (Table II). Blood plasma therefore gained in catechol amine whilst in transit from the arch to the bifurcation of the aorta. The gain in adrenaline exceeded that in noradrenaline. This fact may indicate a different origin for the tyramine-liberated amine and that found normally in plasma. There are two possible sites of origin of the tyramine liberated base which require exclusion before a third is sought. First, the stores of a material described as noradrenaline-like by Schmiterlow[6] and accepted as noradrenaline by Burn and Rand[7,8]. The fact that reserpine depletes these arterial stores of noradrenaline[7,8] and abolishes the pressor effect of tyramine is not evidence that the amines liberated by tyramine emerge from them, because reserpine depletes other stores of their catechol amine[7,8] also. If the material in the arterial stores is solely noradrenaline it could not explain the appearance of adrenaline in aortic plasma. The

second site from which adrenaline and noradrenaline may have been liberated by tyramine is from the blood cells themselves, for Weil-Malherbe and Bone[12] have shown that the red cells, for instance, contain more adrenaline, but less noradrenaline, than plasma. Whatever the source of the amines which appear in the aortic blood during a pressor response to tyramine, the concentrations resulting in the arterial blood are certainly sufficient to have peripheral constrictor effect (Table I) and only a small fraction of the catechol amine present in the arterial blood returns to the inferior vena cava from the legs and tail.

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

References

1. Burn and Tainter, *J. Physiol.*, 1931, **71**, 169.
2. Burn, *J. Pharmacol.*, 1932, **46**, 75.
3. Fleckenstein and Burn, *Brit. J. Pharmacol.*, 1953, **8**, 69.
4. Carlsson, Rosengren, Bertler and Nilsson, *Psychotropic Drugs*, edit. Garattini and Ghetti, Elsevier Publishing Company, Amsterdam, 1957, pp. 363–372.
5. Burn and Rand, *Lancet*, 1957, **273**, 1097.
6. Schmiterlow, *Acta physiol. scand.*, 1948, 16, Suppl. 56.
7. Burn and Rand, *J. Physiol.*, 1958, **144**, 314.
8. Burn and Rand, *Brit. J. Pharmacol.*, 1958, **13**, 471.
9. Lockett, *ibid.*, 1957, **12**, 86.
10. Gaddum, Peart and Wogt, *J. Physiol.*, 1949, **108**, 467.
11. Lund, *Acta pharm. tox. Kbh.*, 1950, **6**, 137.
12. Weil Malherbe and Bone, *Lancet*, 1953, **2**, 974.
13. Wiel Malherbe and Bone, *Biochem. J.*, 1954, **58**, 132.
14. Euler and Schmitterlow, *Acta physiol. scand.*, 1947, **13**, 1.
15. La Brosse, Axelrod and Ketty, *Science*, 1958, **128**, 593.

INTERMEDIATES FOR THE SYNTHESIS OF ANALOGUES OF ADRENAL CORTICAL HORMONES*

Trans-6-OXO-6,7,8,9-TETRAHYDRO-4,5-BENZINDANE AND 1,6-DIOXO-6,7,8,9-TETRAHYDRO-4,5-BENZINDANE

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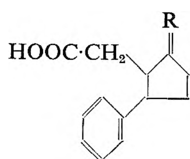
Received May 19, 1960

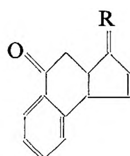
Previous assignments on mechanistic grounds of the stereochemistry of *trans*-6-oxo-6,7,8,9-tetrahydro-4,5-benzindane have been confirmed by degradation of a precursor to the known *trans*-2-carboxycyclopentane acetic acid. 1,6-dioxo-6,7,8,9-tetrahydro-4,5-benzindane has been shown to suffer autoöxidation in alkaline solution to yield 6-hydroxy-1-oxo-4,5-benzindane.

TOWARDS the synthesis of oxygenated 6,7,8,9-tetrahydro-4,5-benzindanes, the cyclisation of 2-phenylcyclopentane acetic acid (I,R = H_2) and its 5-oxo derivative (I,R = O) has been investigated. Starting material (I,R = O) was synthesised essentially by the method of Robinson[1], and on Wolff-Kishner reduction, furnished *trans*-2-phenylcyclopentane acetic acid (I,R = H_2). The stereochemistry of this was demonstrated by submitting (I,R = H_2) to destructive ozonolysis when the known *trans*-2-carboxycyclopentane acetic acid[3] resulted. Previous assignments[2,4,5] of *trans* stereochemistry to (I,R = H_2) are thus directly confirmed.

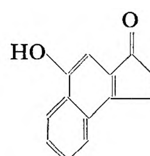
Cyclisation of (I,R = H_2) was smoothly accomplished with anhydrous hydrogen fluoride to give *trans*-6-oxo-6,7,8,9-tetrahydro-4,5-benzindane (II, R = H_2)[4,5]. Likewise under the same conditions, 5-oxo-2-phenylcyclopentane acetic acid (I,R = O) yielded 1,6-dioxo-6,7,8,9-tetrahydro-4,5-benzindane (II,R = O). The acid (I,R = O) was shown to be stable under the alkaline conditions used in the Wolff-Kishner reduction and is also assigned a *trans* configuration; for obvious reasons, however, this does not necessarily apply to the tricyclic product (II, R = O) resulting on ring closure. The constitution of the cyclised products (II,R = H_2 and O) was confirmed by reduction of the carbonyl groups followed by dehydrogenation. 4,5-benzidane[6] was obtained in both cases.



(I)

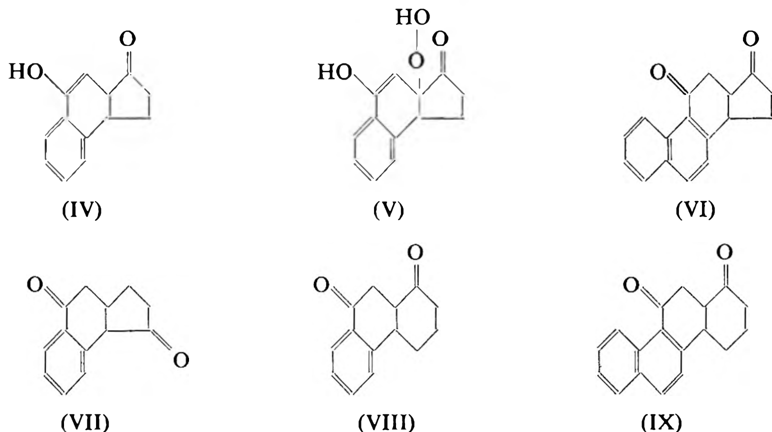


(II)

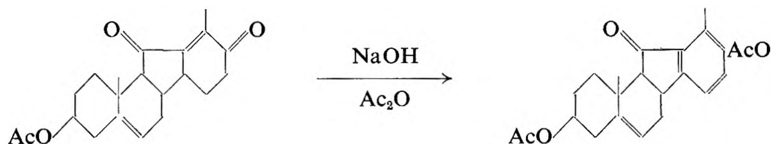


(III)

* Cf. Cowell and Mathieson, *J. Pharm. Pharmacol.*, 1957, **9**, 549; Coles, Linnell, Mathieson and Shoukri, *J. chem. Soc.*, 1954, 2617.



Since under the usual conditions—sodamide in liquid ammonia—neither α-tetralone nor 6-oxo-6,7,8,9-tetrahydro-4,5-benzindane (II,R $=H_2$) would react with sodium acetylide, it was hoped in the case of (II,R = O) to react only the ketone at C (1) with this reagent. Analogues of 11-oxo steroids would then have been available. Instead, the compound suffered dehydrogenation and an almost quantitative yield of 1-oxo-6-hydroxy-4,5-benzindane (III) resulted. This almost complete conversion of (II,R = O) to (III) precludes disproportionation of any intermediate form such as the monoenolic dihydronaphthalene (IV). Involvement of oxygen moreover was indicated by the fact that (II,R = O) was stable in alkaline solution provided all oxygen was excluded from the system. When shaken with oxygen in 0·5N sodium hydroxide, two atoms of gas were absorbed for each mole of the compound and the resulting solution gave tests indicative of peroxides. Since neither *trans*-6-oxo-6,7,8,9-tetrahydro-4,5-benzindane (II,R $= H_2$) nor the isomeric 1-oxo compound underwent oxidation in this wise, the course of aromatisation may be one of hydroperoxide formation involving attack at C(8) (cf. V); activation both by the enol from C(6) and by the C(1) ketone being necessary. Breakdown of the hydroperoxide would then follow to yield in this case the substituted naphthalene (III). Oxidation of the identically constituted naphthindane (VI)[7] provides a similar example of this type of reaction and undernoted transformation of an aetiojervine derivative[8] may likewise be of a similar type.



The susceptibility of such a system to oxidation of this kind was demonstrated with the compounds shown above, (VII), (VIII) and (IX), all of which readily absorbed oxygen in alkaline solution. Uptake of gas, however, did not cease at two atoms per mole and as much as 10–15 moles were recorded. No definite products could be isolated from the resulting

solutions and oxidation of the third ring is probably involved in the case of (VIII) and (IX). Examination of the ultra-violet absorption spectra of the crude products in these two cases indicated an extension of conjugation from the simple benzene chromophore originally present.

1,9-Dioxo-1,2,3,4,4a,9,10,10a-octahydrophenanthrene (VIII) was synthesised by cyclisation of the previously described[11] 6-oxo-2-phenylcyclohexylacetic acid. Our constants of the diketone (VIII) are in agreement with those found by Nasipuri[12] who recently synthesised this compound independantly by an identical route.

Experimental

Trans-2-*phenylcyclopentane acetic acid* (I,R = H_2). 1-Oxo-3-phenylcyclopentane-2-acetic acid[2] (20 g.) in diethylene glycol (200 ml.) was refluxed for 45 minutes under nitrogen with potassium hydroxide (16 g.) and 50 per cent w/v hydrazine hydrate (22 ml.). Sufficient water was then distilled off to raise the temperature of the solution to 195° and this temperature was maintained for 2 hours. On working up in the usual manner there resulted (17 g. of a dark viscous liquid which was purified by slowly passing an ethereal solution through a charcoal column. The resulting colourless gum crystallised from light petroleum (b.p. $< 40°$ at $- 30°$ to give colourless needles m.p. 40–40·5° (lit.[4] cites b.p. 115–125°/ 0·02 mm.). (Found: C, 76·5; H, 7·9·calc. for $C_{13}H_{16}O_2$, C, 76·9; H, 7·9 per cent.)

The *anilide* of the above acid crystallised from benzene/light petroleum (b.p. 60–80°) in colourless needles m.p. 82–84°. (Found: C, 81·6; H, 7·5; N, 5·0. $C_{19}H_{21}ON$ requires C, 81·2; H, 7·4; N, 4·9 per cent.)

The *amide* crystallised from benzene/light petroleum (b.p. 60–80°) in colourless needles m.p. 80–80·5°. (Found: C, 76·9; H, 8·3; N, 6·9. $C_{13}H_{17}ON$ requires C, 76·7; H, 8·4; N, 6·9 per cent.)

Ozonolysis. Ozone was bubbled through the above acid (1 g.) in glacial acetic acid (20 ml.) for 5 hours; 3 per cent hydrogen peroxide solution (50 ml.) was evaporated almost to dryness on the water bath, a further 25 ml. hydrogen peroxide solution was then added and the process repeated. The residue was then added and the mixture left overnight. The solution was dissolved in aqueous sodium carbonate (20 ml.) and neutral material extracted with ether. Acidification of the alkaline layer and ether extraction yielded *trans*-2-carboxycyclopentane acetic acid crystallising from light petroleum (b.p. $< 40°$) m.p. 62–64° (lit.[3] cites m.p. 66°).

Trans-6-oxo-6,7,8,9,-tetrahydro-4,5-benzindane (II, R = H_2). 2-Phenylcyclopentane acetic acid (700 mg.) was dissolved in anhydrous hydrogen fluoride (30 ml.). After 10 days, evaporation of the reagent gave a neutral residue crystallising from ethanol in needles m.p. 88–89° (yield 90 per cent) (lit.[5] cites m.p. 79–82°).

λmax in ethanol 248 mμ (ϵmax 13,400), 293 mμ (ϵmax 2,000), νmax in carbon tetrachloride 1,695 cm.$^{-1}$.

The oxime crystallised from ethanol in colourless needles m.p. 183–189 (lit.[5] cites m.p. 181°). The 2,4-dinitrophenylhydrazone crystallised from

xylene in dark red plates m.p. 246–247°. λmax (main band in chloroform) 387 mμ (εmax 28,100) (lit.[5] cites m.p. 242–244°).

1,6-*Dioxo*-6,7,8,9-*tetrahydro*-4,5-*benzindane* (II, R = O). 1-Oxo-3-phenylcyclopentane-2-acetic acid (4·2 g.) was treated with hydrogen fluoride as described above: a neutral residue was obtained which crystallised from light petroleum (b.p. 80–100°) in colourless needles (2·35 g.) m.p. 107–109° after sublimation at 120°/0·2 mm. (Found: C, 78·0; H, 6·0. $C_{13}H_{13}O_2$ requires C, 78·0; H, 6·0 per cent.) λmax in ethanol 248 mμ (εmax 12,100), 293 mμ (εmax 1800). νmax in carbon tetrachloride 1,760 $cm.^{-1}$. (C = O in 5 membered ring) 1,705 $cm.^{-1}$ (aryl ketone). The *dioxime* crystallised from aqueous ethanol in long needles m.p. 171–173°. (Found: C, 67·8; H, 6·1; N, 12·1. $C_{13}H_{14}O_2N_2$ requires C, 67·8; H, 6·1; N, 12·2 per cent.)

4,5-*Benzindane*. The above dioxobenzindane (1·6 g.) in toluene (60 ml.) was reduced with zinc amalgam (20 g.) in water 25 ml. and concentrated hydrochloric acid (35 ml.). From the organic layer there was recovered a mobile colourless oil, 6,7,8,9-tetrahydro 4,5-benzindane[9] (0·85 g.) b.p. 72°/0·05 mm. This was refluxed for 4 hours with 30 per cent palladium on charcoal. Extraction with ether yielded a mobile oil with a blue fluorescence. The ultra-violet spectrum of this was identical with that of an authentic sample of 4,5-benzindane. A picrate was obtained in orange-red needles, m.p. 107–108°: this gave no depression on admixture with an authentic sample.

Autoöxidation experiments. (i) 1,6-Dioxo-6,7,8,9-tetrahydro-4,5-benzidane (II, R = O) (1·04 g.) in ethanol (250 ml.) was added to a 5 per cent aqueous solution of potassium hydroxide (250 ml.) and allowed to stand for 4 hours. Acidification of the light yellow solution gave a crystalline residue (1·02 g.) m.p. 280–284°. A sample of this (390 mg.) was chromatographed on a column of alumina grade I (76 g.). After placing on the column with benzene, 44 mg. of a gum was eluted (20 per cent in benzene) followed by a single peak (340 mg.) m.p. 289–291°: this gave no depression on admixture with an authentic specimen of 6-hydroxy-1-oxo-4,5-benzindane (III) prepared by ring closure of 1-oxo-3-phenylcyclopentene-2-acetic acid. The acetate crystallised in colourless prisms m.p. and mixed m.p. with an authentic sample[10] 163–164°. The ultra-violet absorption spectra of the two samples of (III) were identical: λmax in ethanol 223, 262, 290, 362 mμ (εmax 27,600, 37,500, 4,500, 5,800).

(ii) The above diketone (30 mg.) in 0·5N sodium hydroxide in ethanol (10 ml.) was shaken with oxygen in a microhydrogenation apparatus of conventional design. Oxygen (3·07 c.c. at N.T.P.) was steadily absorbed in 14 minutes when uptake ceased. This corresponds to 2 atoms oxygen per mole of diketone used. When uptake of gas had ceased the solution gave a blue colour with acidified starch iodide paper and gave a positive perchromic acid test.

(iii) When the above experiment was repeated with nitrogen in place of oxygen, starting material 1,6-dioxo-6,7,8,9-tetrahydro-4,5-benzindane was recovered quantitatively.

(iv) When ethanol alone was used as a solvent no uptake of oxygen occurred and starting material was again recovered unchanged.

1-*Oxo*-3-*phenylcyclohexane*-2-*acetic acid.* 6-Oxo-2-phenylcyclohexene-1- acetic acid (II) (4 g.) in ethanol (60 ml.) was hydrogenated at 5 per cent palladium on strontium carbonate (800 mg.). The residue (3·9 g.) obtained on evaporation of the solvent was crystallised from benzene/light petroleum (b.p. 40–60°) then from aqueous acetic acid in fine colourless needles m.p. 140–141° (lit.[11] cites m.p. 136–137°). Low melting fractions were converted to the above form by warming to 50° for 3 hours under nitrogen with N sodium hydroxide. (Found: C, 72·9; H, 6·9. Calc. for $C_{14}H_{16}O_3$, C, 72·4; H, 6·9 per cent.)

The semicarbazone crystallised from ethanol in fine colourless needles m.p. 206–207° (lit.[11] cites m.p. 192–193°).

The 2,4-*dinitrophenylhydrazone* crystallised from aqueous acetic acid in yellow needles m.p. 242° (darkens at 235°). (Found: C, 58·5; H, 4·9; N, 13·7. $C_{20}H_{20}O_6N_4$ requires C, 58·3; H, 4·9; N, 13·6 per cent.

1,9-*Dioxo*-1,2,3,4,9,10,11,12-*octahydrophenanthrene.* The above acid (1 g.) was allowed to stand for 5 days in anhydrous hydrogen fluoride (30 ml.). Evaporation yielded a residue which sublimed at 100–105°/0·2 mm. Crystallised from aqueous methanol this gave colourless needles m.p. 150–151°. Found: C, 79·3; H, 6·7. $C_{14}H_{14}O_2$ requires C, 78·5; H, 6·6 per cent.) νmax 1,730 cm.$^{-1}$, 1,708 cm.$^{-1}$ in carbon tetrachloride: λmax 249 mμ (ϵ11,000) in ethanol. The *bis* 2,4-*dinitrophenylhydrazone* crystallised from nitrobenzene in crimson needles m.p. 290° (decomp.). (Found: N, 19·8. $C_{26}H_{22}O_8N_8$ requires N, 19·5 per cent.)

Acknowledgements. We thank Professor D. Ginsberg for a sample of compound (VII) and Dr. D. Nasipuri for a sample of the octahydrochrysene (IX).

REFERENCES

1. Robinson, *J. chem. Soc.*, 1938, 1390.
2. Nun and Rapson, *ibid.*, 1949, 1051.
3. Linstead and Cook, *ibid.*, 1934, 956.
4. Winternitz, Mousseron and Rouzier, *Bull. soc. chim. Fr.*, 1953, **20**, 190.
5. Amiel, Loffler and Ginsberg, *J. Amer. chem. Soc.*, 1954, **76**, 3625
6. Modi, Ph.D. Thesis, London, 1952.
7. Koebner and Robinson, *J. chem. Soc.*, 1941, 566.
8. Fried and Klingsberg, *J. Amer chem. Soc.*, 1953, 75, 4929.
9. Kon, *J. chem. Soc.*, 1933, 1081.
10. Martin and Robinson, *ibid.*, 1943, 497.
11. Dutta and Banerjee, *Science and Culture*, 1947, **12**, 408.
12. Nasipuri and Roy, *J. chem. Soc.*, 1960, 1571.
13. Downes, Gill and Lions, *Austral. J. Sci.*, 1948, **10**, 147.

A TECHNIQUE FOR THE EVALUATION OF EMULSION STABILITY

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Received May 2, 1960

An economical sampling and statistical treatment has been developed for screening the stability of experimental fat emulsion formulations. Coalescence or progressive increase in mean globule diameter with passage of time was interpreted to indicate physical instability. The test technique attempts to efficiently extract reliable data from a relative minimum of man hours and materials per test formulation. The counting and measuring of 100–200 globules per determination was considered to be adequate.

THE search for a satisfactory method of determining the physical stability of emulsions has been of interest for many years. Sumner[1] and Becher[2] have correlated the literature on this subject. Where time is important in the evaluation of stability of a series of experimental emulsion formulations, and the cost of technical assistance must be kept nominal, no single procedure has been available that would accurately screen a number of formulations in a short time. In an attempt to formulate an intravenous emulsion of vegetable oil in an aqueous solution of glucose, a method that would be both reliable and practical was sought. Using the coalescence, or lack of it, of the fatty globules as a criterion of emulsion stability, a limited number of the dispersed globules were counted and their diameters measured at definite time intervals and a simple statistical analysis promptly applied to the data as they accumulated.

In the past it has been recommended that a large number of globules, 2,000 or more, should be counted for each determination. Such a procedure is extremely lengthy and tedious. This study set out to ascertain whether or not a much smaller number of globules could be counted without impairing the validity of the evaluation of emulsion stability.

EXPERIMENTAL

Preparation of the Emulsions

To illustrate the evaluation procedure, an emulsion of the following composition was used (Formulation 48): sesame oil, 25 per cent; polyoxyethylene sorbitan trioleate (Tween 85, Atlas), 1 per cent; 5 per cent glucose in water for injection, to 1,000 ml. Sesame oil was pre-sterilised first by heating in an oven for 90 minutes at 170°. Glucose was then dissolved in water for injection and polyoxyethylene sorbitan trioleate and sesame oil added. This mixture was run through the Eppenbach Colloid Mill at 0·005 inches clearance for 5 minutes and then autoclaved at 121·2° with a gauge pressure of 15·3 pounds (30 pounds, absolute) for 15 minutes.

Technique of the Measurements

A drop of the undiluted emulsion was placed on a microscope slide with a glass stirring rod and a small amount of dry amaranth added. After uniform distribution of the colour throughout the sample, giving a contrast between the internal and external phases of the emulsion, the slide, covered with a cover slip, was placed beneath the microscope. A few minutes for drying out was allowed. A Spencer research microscope was used with a Filar Micrometer Eyepiece, Bausch and Lomb catalogue number 31–16–50. This micrometer is equipped with a movable cross hair, the traverse of which can be measured by rotating a drum marked off in 100 subdivisions. By using the 8 mm. (×20 inital magnification) Spencer objective and the ×12·5 ocular of the micrometer attachment, a final magnification of ×250 is obtained.

After bringing the field into focus, an area was chosen at random between the two static lines of the micrometer and the globules were counted and their diameters measured. Accurate measurements were made by revolving the drum. For example, if upon calibration with the 8 mm. objective, one division of the micrometer was equivalent to 42·5 μ and the reading on the drum was 5, then 42·5 × 0·05 or 2·125 μ was the diameter of the globule.

Sampling and Statistical Evaluation

Figure 1 illustrates the sampling technique practiced upon the formulation. This work outline was used to extract the maximum amount of information from the work hours available. This procedure enables one to test the stability of one lot over a period of 3 weeks, to test the reproducibility of four lots manufactured at various intervals of time, as well as to test the reliability of the two technicians against one another.

Slide samples should be prepared and coded by a third person to insure unbiased recording. The sample coding scheme used consistently in the figures and Table I is an identification system provided for this paper and was not used to identify samples during the collection of the data. Sample identification by means of tables of random numbers is recommended. The short statistical treatment used for our daily data involved the use of an automatic calculator that could accumulate products of multiplication and a mimeographed work sheet that was prepared from the techniques outlined by Bliss and Calhoun[3]. This work sheet and summary of results is shown with an illustrative test calculation in Figure 2. This work form provides for a test of significance between the means of the two groups (row 1), an F test for comparable group variances (rows 2, 3), calculation of group mean diameters, standard deviation of group data, and standard error calculation for the group means. The format is arranged to provide means for checking the accuracy of calculations. If desired, the expected range of globule size present per sample (P = 0·05) can be calculated as the mean diameter ± 2 × standard deviation. In calculating the ratio of the group variances or the F test, the larger variance (1·8006) is always in the numerator; the critical value (P = 0·05)

for this determination can be obtained from a 2·5 per cent point table for the F distribution.

The sampling design and the statistical format were chosen to qualify and concisely summarise each day's results as soon as possible after the data had been recorded. This test design need not be fully completed before any calculation and evaluation may be made; the screening can be stopped after the first week of testing if the results are unfavourable. Manufacturing procedure or formulation or both can then be changed and a new stability screen begun.

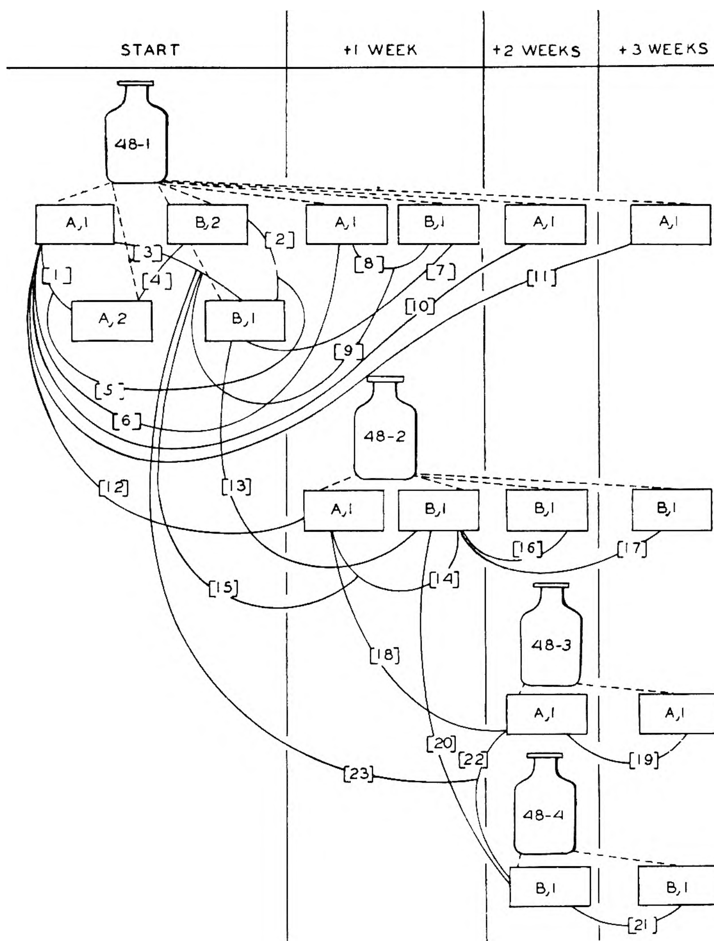

FIG. 1. Sampling scheme and test comparisons made of 4 lots of Formulation 48, with technicians A and B. The figures in brackets indicate the reference numbers for the statistical comparisons summarised in Table I.

After the results of the first day have been calculated, it is necessary to determine the optimum number of globules that should be counted per sample to detect an arbitrary 10 per cent shift in globule diameter.

TABLE I

PROGRESSIVE STATISTICAL EVALUATION OF FORMULATION 48

Fig. 1 ref. no.	Test comparison	Mean globule size, μ ±1 standard deviation	Significance between groups; observed P
1	(48–1)(A,1)(0) *vs.* (48–1)(A,2)(0)	4·12(195)[a] ± 1·34 *vs.* 4·07(203) ± 1·34	>0·50
2	(48–1)(B,1)(0) *vs.* (48–1)(B,2)(0)	4·22(200) ± 1·14 *vs.* 4·31(200) ± 1·36	0·50–0·25[b]
3	(48–1)(A,1)(0) *vs.* (48–1)(B,1)(0)	4·12(195) ± 1·34 *vs.* 4·22 (200) ± 1·14	0·50–0·25[b]
4	(48–1)(A,2)(0) *vs.* (48–1)(B,2)(0)	4·07(203) ± 1·34 *vs.* 4·31(200) ± 1·36	0·10–0·05
5	(48–1)(A,1,2)(0) *vs.* (48–1)(B,1,2)(0)	4·10(398) ± 1·34 *vs.* 4·26(400) ± 1·26	0·10–0·05
6	(48–1)(A,1)(0) *vs.* (48–1)(A,1)(7)	4·12(195) ± 1·34 *vs.* 4·24(175) ± 1·42	0·50–0·25
7	(48–1)(B,1)(0) *vs.* (48–1)(B,1)(7)	4·22(200) ± 1·14 *vs.* 4·06(200) ± 1·37	0·25–0·10[b]
8	(48–1)(A,1)(7) *vs.* (48–1)(B,1)(7)	4·24(175) ± 1·42 *vs.* 4·06(200) ± 1·37	0·25–0·10[b]
9	(48–1)(A,1 : B,1)(0) *vs.* (48–1)(A,1 : B,1)(7)	4·17(395) ± 1·24 *vs.* 4·14(375) ± 1·39	>0·50
10	(48–1)(A,1)(0) *vs.* (48–1)(A,1)(14)	4·12(195) ± 1·34 *vs.* 4·11(104) ± 1·26	>0·50
11	(48–1)(A,1)(0) *vs.* (48–1)(A,1)(21)	4·12(195) ± 1·34 *vs.* 4·19(100) ± 1·40	>0·50
12	(48–1)(A,1)(0) *vs.* (48–2)(A,1)(0)	4·12(195) ± 1·34 *vs.* 4·09(200) ± 1·29	>0·50
13	(48–1)(B,1)(0) *vs.* (48–2)(B,1)(0)	4·22(200) ± 1·14 *vs.* 3·87(201) ± 1·27	0·005–0·001[c]
14	(48–2)(A,1)(0) *vs.* (48–2)(B,1)(0)	4·09(200) ± 1·29 *vs.* 3·87(201) ± 1·27	0·10–0·05
15	(48–1)(A,1 : B,1)(0) *vs.* (48–2)(A,1 : B,1)(0)	4·17(395) ± 1·24 *vs.* 3·98(401) ± 1·28	0·05–0·025[c]
16	(48–2)(B,1)(0) *vs.* (48–2)(B,1)(7)	3·87(201) ± 1·27 *vs.* 4·01(101) ± 1·12	0·50–0·25
17	(48–2)(B,1)(0) *vs.* (48–2)(B,1)(14)	3·87(201) ± 1·27 *vs.* 4·08(100) ± 1·24	0·25–0·10
18	(48–2)(A,1)(0) *vs.* (48–3)(A,1)(0)	4·09(200) ± 1·29 *vs.* 4·20(100) ± 1·77	>0·50[b]
19	(48–3)(A,1)(0) *vs.* (48–3)(A,1)(7)	4·20(100) ± 1·77 *vs.* 4·09(100) ± 1·49	>0·50
20	(48–2)(B,1)(0) *vs.* (48–4)(B,1)(0)	3·87(201) ± 1·27 *vs.* 3·42(100) ± 1·18	0·005–0·001[c]
21	(48–4)(B,1)(0) *vs.* (48–4)(B,1)(7)	3·42(100) ± 1·18 *vs.* 3·68(100) ± 0·90	0·10–0·05[b]
22	(48–3)(A,1)(0) *vs.* (48–4)(B,1)(0)	4·20(100) ± 1·77 *vs.* 3·42(100) ± 1·18	<0·001[c]
23	(48–1)(A,1 : B,1)(0) *vs.* (48–3)(A,1)(0) + (48–4)(B,1)(0)	4·17(395) ± 1·24 *vs.* 3·81(200) ± 1·55	0·005–0·001[bc]

[a] Number in parentheses indicates the number of globules counted.
[b] Significant F test calculated, P = 0·05.
[c] Group mean diameters considered to be significantly different.

These simple calculations have been outlined by Snedecor[4]. Thus 1 week later, the number of globules counted can either be increased or decreased at the desire of the investigator. For Formulation 48, the sample size should range between 100–200 globules. Counting more, decreases the efficiency; counting less, decreases accuracy.

An alternative screening procedure is indicated where a full 3 weeks' stability evaluation is routinely required for each test formulation. By restricting the sampling design to only two lots prepared concurrently, with both technicians reading one sample per each lot at the time of preparation, at +1 week, at +2 weeks, and at +3 weeks, a balanced design is produced that can be treated at the end of that period by the classical statistical techniques outlined by Cochran and Cox[5].

DISCUSSION

A formulation that does not fail during the statistical evaluation based on this flexible sampling scheme should then be considered to be worthy of a pharmacological evaluation and further stability testing utilising control chart analysis over a period of months to years. The accumulated results for Formulation 48 are summarised in Table I. The test comparisons are coded as follows: (48–2) (B, 1) (0) translates to (Formulation 48—lot 2) (technician B made the observations, this is the first reading that he has made on this lot this day) (age of the test emulsion in days—here freshly prepared).

Within the limits of this experimental procedure, Formulation 48 displayed excellent physical stability; however, significant variations were

noted in globule size between the different lots prepared as shown by comparisons 13, 15, 20, 22, and 23 of Table I. This indicates that the reproducibility of lots by this manufacturing procedure may be a problem. The degree of efficiency achieved here with counts of 100–200 globules appears adequate for preliminary stability screening. With Formulation 48, higher counts quantitated variations that were of little practical significance. The sampling techniques used here appear to be both efficient and reliable, yet they require only a relative minimum of labour and materials per test formulation.

Group 1 Identification: (48-1)(A,1)(0)[a]				vs.	Group 2 Identification: (48-1)(A,2)(0)			
Globule size, μ	Mid-range, n	Observed frequency, f	nf	n^2f	n	f	nf	n^2f
0–1	0·5	0	0	0	0·5	0	0	0
1–2	1·5	3	4·5	6·75	1·5	3	4·5	6·75
2–3	2·5	26	65·0	162·50	2·5	35	87·5	218·75
3–4	3·5	86	301·0	1053·50	3·5	80	280·0	980·00
4–5	4·5	39	175·5	789·75	4·5	42	189·0	850·50
5–6	5·5	19	104·5	574·75	5·5	24	132·0	726·00
6–7	6·5	13	84·5	549·25	6·5	11	71·5	464·75
7–8	7·5	8	60·0	450·00	7·5	6	45·0	337·50
8–9	8·5	1	8·5	72·25	8·5	2	17·0	144·50
9–10	9·5	0	0	0	9·5	0	0	0
10–11	10·5	0	0	0	10·5	0	0	0
Column totals:		195	803·5	3658·75		203	826·5	3728·75
Designations:		N_1	$S(x_1)$	$S(x_1^2)$		N_2	$S(x_2)$	$S(x_2^2)$
Mean diameter: $S(x_1)/N_1 = 4{\cdot}12$					$S(x_2)/N_2 = 4{\cdot}07$			

Row	Term	D.F.[b]	Sum of squares	Mean squares	F	Observed P
1	Between groups	1	$[x^2]_g = (C_1 + C_2) - C = 0{\cdot}240$	$A = [x^2]_g/1 = 0{\cdot}240$	$A/s^2 = 0{\cdot}133$	> 0·50 (< 3·84)[c]
2	Within group 1	$N_1 - 1 = 194$	$[x_1^2] = S(x_1^2) - C_1 = 347{\cdot}918$	$s_1^2 = [x_1^2]/N_1 - 1 = 1{\cdot}7934$	$s_1^2/s_2^2 = 1{\cdot}0039$	> 0·50 (< 1·20)[c]
3	Within group 2	$N_2 - 1 = 202$	$[x_2^2] = S(x_2^2) - C_2 = 363{\cdot}714$	$s_2^2 = [x_2^2]/N_2 - 1 = 1{\cdot}8006$		
4	Within groups	$N - 2 = 396$	$[x^2] - [x^2]_g = [x_1^2] + [x_2^2] = 711{\cdot}632$	$s^2 = [x^2] - [x^2]_g/N - 2 = 1{\cdot}7970$		
5	Total	$N - 1 = 397$	$[x^2] = S(x^2) - C = 711{\cdot}872$	$C_1 = T_1^2/N_1 = 3310{\cdot}832$		
6	Corr.	1	$C = T^2/N = 6675{\cdot}628$	$C_2 = T_2^2/N_2 = 3365{\cdot}036$		

$T = T_1 + T_2 = S(x_1) + S(x_2) = 803{\cdot}5 + 826{\cdot}5 = 1630{\cdot}0$

$S(x^2) = S(x_1^2) + S(x_2^2) = 3658{\cdot}75 + 3728{\cdot}75 = 7387{\cdot}50$ $\quad N_1 + N_2 = N = 398$

Calculations check: $[x_1^2] + [x_2^2] + [x^2]_g = [x^2] = 711{\cdot}872$

Standard deviation: $\sqrt{s_1^2} = s_1 = 1{\cdot}3392$; $\sqrt{s_2^2} = s_2 = 1{\cdot}3418$

Standard error of the mean: $s_1/\sqrt{N_1} = 0{\cdot}0959$; $s_2/\sqrt{N_2} = 0{\cdot}0942$

[a] Groups are coded for speed and clarity of identification; translation: (Formulation 48, lot 1) (globules counted by person A; this is his first test sample at this time) (age of the emulsion in days; here the emulsion is freshly prepared).

[b] D.F. designates degrees of freedom.

[c] Figure in parenthesis is the critical value, P = 0·05.

FIG. 2. Concise work sheet for statistical treatment with illustration for use.

Acknowledgements. The authors would like to acknowledge the assitance of Mr. Charles J. Olson and Mr. John G. Haeberle, technicians.

REFERENCES

1. Sumner, *Clayton's The Theory of Emulsions and Their Technical Treatment*, 5th Edn, J. A. Churchill Ltd., London, 1954.

2. Becher, *Emulsions: Theory and Practice*, Reinhold Publishing Corp., New York, 1957.
3. Bliss and Calhoun, *Outline of Biometry*, Yale Co-op Corp., New Haven, Conn., 1954, p. 87.
4. Snedecor, *Statistical Methods*, 5th Edn, Iowa State College Press, Ames, Iowa, 1956, p. 275.
5. Cochran and Cox, *Experimental Designs*, John Wiley and Sons, Inc., New York, 1950, p. 91.

SOME 2, 3-DISUBSTITUTED 3*H*-4-QUINAZOLONES AND 3*H*-4-THIOQUINAZOLONES

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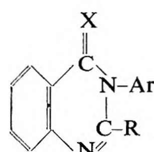
Received May 17, 1960

Some 2-alkyl-3-aryl-3*H*-4-quinazolones (I; X = 0) have been prepared and a number of them converted into the thioquinazolones (I; X = S). A new method for the synthesis of 3-alkyl-2-aryl-3*H*-4-quinazolones (VI) has been developed and has been employed for the synthesis of representative derivatives.

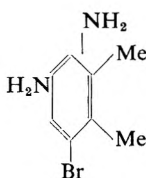
Biological study of the above compounds revealed that some of them, and in particular the 2-alkyl-3-halophenyl-3*H*-4-quinazolones are potent anticonvulsant agents.

Our interest in quinazolones stemmed from the discovery of Gujral, Saxena and Tiwari[1] that certain 2-alkyl-3-aryl-3*H*-4-quinazolones (I; X = 0) and in particular the 2-methyl-3-*o*-tolyl (I; R = Me, Ar = *o*-Me. C_6H_4, X = 0) and 2-ethyl-3-phenyl (I; R = Et, Ar = Ph, X = 0) derivatives were potent hypnotic agents superior to allobarbitone in the assay. We therefore began in 1955 a systematic study of quinazolones of this type, preparing them by the general method of Grimmel, Guenther and Morgan[2] in which an *o*-acylamidobenzoic acid is condensed with an aromatic amine in the presence of phosphorus trichloride. The compounds listed in Table I which includes, *inter alia* a few derivatives already described in the literature[3-5], were largely synthesised in this way.

In addition to phosphorus trichloride we have found other condensing agents to be effective in the synthesis of the quinazolones (I). Benzenesulphonyl chloride in pyridine previously used by Brewster and Ciotti[6] for the preparation of amides, proved highly satisfactory. Somewhat lower yields followed the use of phosphorus trichloride in pyridine, but reaction was more rapid. Dicyclohexylcarbodiimide in molar proportion in tetrahydrofuran solution at room temperature for 5 hours gave yields of quinazolones (I) of about 50 per cent.



(I)



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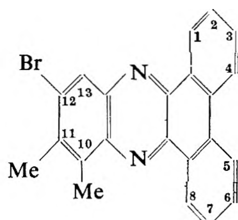

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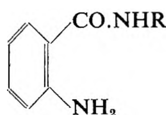

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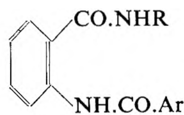

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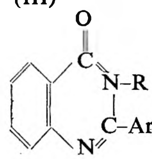


(VI)

Biological study of early members of the series, for which we are indebted to Dr. A. David and Dr. G. Bianchi revealed the pronounced central depressant activity of 3-(4-bromophenyl)-2-methyl-3*H*-4-quinazolone. Extending this observation we prepared 3-(4-bromo-2,3-dimethylphenyl)-3*H*-4-quinazolone. 4-Bromo-2,3-dimethylaniline, required for this purpose has not been described in the literature and was obtained by bromination of 2,3-dimethylacetanilide in acetic acid at 10°, followed by hydrolysis with hydrochloric acid (cf. 7). The orientation of the bromine atom in the acetanilide was established by nitration when 4-bromo-2,3-dimethyl-6-nitroacetanilide was obtained, identified by reduction and hydrolysis to the diamine (II) which passed into 12-bromo-10,11-dimethyldibenzo[*a*,*c*]-phenazine (III) on reaction with phenanthraquinone. 6-Bromo-2-methyl-3-*o*-tolyl-3*H*-4-quinazolone[8] was prepared in order to determine the effect upon biological activity of a bromo-substituent in the benzene ring of the heterocyclic nucleus. In addition a series of new 2-alkyl-3-aryl-3*H*-4-thioquinazolones (I; X = S) (Table II) were prepared from the corresponding 4-oxo-derivatives by the general method of Leonard and Curtin[9].

We next turned our attention to the synthesis of the related 3-alkyl-2-aryl-3*H*-4-quinazolones (VI). 3-Methyl-2-phenyl-3*H*-4-quinazolone (VI; Ar = Ph, R = Me), the only member of this type described in the literature, was prepared by Korner[10] (*a*) by heating 2-benzamido-*N*-methylbenzamide at 230° to 250° and (*b*) by direct methylation with methyl iodide in a sealed tube at 120° of 2-phenyl-3*H*-4-quinazolone, which was itself prepared by the action of boiling aqueous potassium hydroxide on 2-benzamidobenzamide.

Initial attempts to prepare compounds of type (VI) by condensing *N*-alkyl-2-aminobenzamides (IV) with a benzoic acid in the presence of phosphorus trichloride proved unsuccessful. Somewhat better results followed the cyclisation of *N*-alkyl-2-benzamidobenzamides (V) with phosphorus trichloride in pyridine, but the yields of 3-alkyl-2-aryl-3*H*-4-quinazolones (VI) obtained were unsatisfactory. We ultimately found that the required quinazolones (VI) were readily prepared in good yield from the corresponding *N*-alkyl-2-aminobenzamides (IV), which we obtained by reaction between isatoic anhydride and the appropriate amine[11]. Aroylation of the benzamides (IV) was preferably accomplished with the benzoyl chloride in aqueous ethanolic sodium acetate solution when the *N*-alkyl-2-benzamidobenzamides (V) recorded in Table III were obtained. The use of pyridine as solvent for this reaction proved less satisfactory as in the condensation of anthranilamide (IV, R = H) with *p*-bromobenzoyl chloride, for example, substantial quantities of 2-*p*-bromobenzamidobenzonitrile were formed in addition to the usual aroylated product[12]. Conversion of the intermediate *N*-alkyl-2-benzamidobenzamides (V) into the quinazolones (VI) was effected by boiling the compounds with 5 per cent aqueous sodium hydroxide solution (cf. [13,14]) or by heating them to 260° for 1 to 2 hours. The former method of cyclisation was preferred for compounds (V; R = H or Me), but was less satisfactory with ethyl and higher alkylamides. In many cases both methods gave mixtures of product and starting material from which the

quinazolones were obtained by extraction with light petroleum or by precipitation from ethereal solutions as the hydrochloride. 3-Methyl-2-*o*-tolyl-3*H*-4-quinazolone (VI; R = Me, Ar = *o*-Me.C_6H_4) was prepared by direct methylation of 2-*o*-tolyl-3*H*-4-quinazolone with methyl sulphate in alkaline solution. 3-(2,3-Dihydroxypropyl)-2-phenyl-3*H*-4-quinazolone (VI; R = CH_2.CHOH.CH_2OH, Ar = Ph) was obtained by the condensation of 2-phenyl-3*H*-4-quinazolone with 2,3-epoxypropan-1-ol ("glycidol") in ethanolic solution employing pyridine as catalyst.

Biological results have been reported[15].

EXPERIMENTAL

The following examples illustrate the methods used for the preparation of compounds listed in Table I.

3-*p-Bromophenyl-2-methyl*-3H-4-*quinazolone* (I; R = Me, Ar = *p*-Br. C_6H_4, X = O).

(*a*) To a stirred mixture of *p*-bromoaniline (34·4 g.) and acetylanthranilic acid (35·8 g.) in toluene (250 ml.) at room temperature, a solution of phosphorus trichloride (8 ml.) in toluene (50 ml.) was added slowly. After the addition was complete the pasty mixture was heated for 2 hours at reflux temperature. After cooling, 15 per cent sodium carbonate solution was added and the toluene removed by distillation in steam. The residual *product* (27 g.) was collected and purified by crystallisation from 95 per cent ethanol. The hydrochloride, prepared by addition of concentrated hydrochloric acid (9 ml.) to a solution of the base (20 g.) in warm 95 per cent ethanol (300 ml.) had m.p. ca *c*, 260° (decomp.) after crystallisation from 95 per cent ethanol.

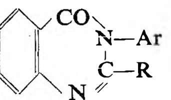
(*b*) To a solution of acetylanthranilic acid (9·0 g.) and *p*-bromoaniline (8·9 g.) in tetrahydrofuran (100 ml.) was added a solution of dicyclohexylcarbodiimide (11·4 g.) in tetrahydrofuran (50 ml.) and the mixture allowed to stand at room temperature for 5 hours. Acetic acid (1·5 ml.) was added and after 2 hours the separated dicyclohexylurea was filtered off. The filtrate was evaporated to dryness at reduced pressure and the residue dissolved in ethyl acetate (150 ml.). The ethyl acetate was shaken with 2N hydrochloric acid when the *hydrochloride* (9 g.) of the product separated and was collected. It had m.p. ca 260° (decomp.) and was identical with the product described under (*a*). The ethyl acetate solution was washed with N sodium bicarbonate solution, then with water and concentrated to ca. 50 ml. to yield, 2-*acetamido*-4′-*bromobenzanilide* (1·5 g.), m.p. 215–216°. Found: C, 53·5; H, 4·0; N, 8·7. $C_{15}H_{13}O_2NBr$ requires C, 54·1; H, 3·9; N, 8·4 per cent.

3-(2,4-*Dichlorophenyl*)-2-*methyl*-3H-4-*quinazolone* (I; R = Me, Ar = 2, 4-Cl_2.C_6H_3, X = O)

(*a*) A solution of acetylanthranilic acid (17·9 g.) in pyridine (30 ml.) was treated in one portion with benzenesulphonyl chloride (17·8 g.) followed by 2,4-dichloroaniline (16·2 g.), added in portions with shaking. The mixture was heated on the steam bath for 2 hours. After cooling and dilution with water the resultant gum solidified on trituration with ethanol.

TABLE I

2-ALKYL-3-ARYL-3H-4-QUINAZOLONES



R	Ar	Base (B) hydrochloride (H)	M.p. °C.*	Formula	Found per cent				Required per cent			
					C	H	N	Cl	C	H	N	Cl
Me	*p*-Anisyl	H	ca. 240 (*d*)	$C_{16}H_{15}O_2N_2Cl$	63·0	5·1	8·7	11·9	63·5	5·0	9·3	11·7
Me	*o*-Phenetyl	B	115–116	$C_{17}H_{16}O_2N_2$	73·0	6·1	9·7	—	72·9	5·8	10·0	—
		H	ca. 215 (*d*)	$C_{17}H_{17}O_2N_2Cl$	64·1	5·5	9·3	11·6	64·4	5·4	8·9	11·2
Me	*m*-Phenetyl	B	130–132	$C_{17}H_{16}O_2N_2$	73·0	5·9	9·6	—	72·9	5·8	10·0	—
		H	ca. 225 (*d*)	$C_{17}H_{17}O_2N_2Cl$	64·3	5·5	8·7	11·1	64·4	5·4	8·9	11·2
Me	*p*-Phenetyl	H	ca. 240 (*d*)	$C_{17}H_{17}O_2N_2Cl$	64·1	5·2	—	11·3	64·4	5·4	—	11·2
Me	3,4,5-Trimethoxyphenyl	B	150–152	$C_{18}H_{16}O_4N_2$	65·8	5·5	—	—	66·2	5·6	—	—
		H	ca. 250 (*d*)	$C_{18}H_{19}O_4N_2Cl$	59·7	5·1	7·6	9·8	59·6	5·3	7·7	9·8
Me	2,3-Xylyl	B	172–173	$C_{17}H_{16}ON_2$	77·4	6·4	10·3	—	77·3	6·1	10·6	—
		H	ca. 240 (*d*)	$C_{17}H_{17}ON_2Cl$	68·1	5·7	—	11·3	67·9	5·7	—	11·8
Me	2,4-Xylyl	B	100–102	$C_{17}H_{16}ON_2$	77·1	6·3	10·8	—	77·3	6·1	10·6	—
		H	ca. 240 (*d*)	$C_{17}H_{17}ON_2Cl$	68·2	5·8	8·9	11·6	67·9	5·7	9·3	11·8
Me	2,5-Xylyl	B	125–127	$C_{17}H_{16}ON_2$	77·2	6·1	—	—	77·3	6·1	—	—
Me	2,6-Xylyl	H	ca. 215 (*d*)	$C_{17}H_{17}ON_2Cl$	67·5	5·9	9·7	11·6	67·9	5·7	9·3	11·8
Me	3,4-Xylyl	B	134–136	$C_{17}H_{16}ON_2$	77·6	6·0	—	—	77·3	6·1	—	—
Me	*p*-Fluorophenyl	B	133–134	$C_{15}H_{11}ON_2F$	70·7	4·4	—	7·5*a*	70·9	4·5	—	7·6*a*
		H	ca. 280 (*d*)	$C_{15}H_{12}ON_2FCl$	61·4	4·3	9·6	—	61·9	4·2	9·6	—
Me	*o*-Chlorophenyl	B	130–132	$C_{15}H_{11}ON_2Cl$	66·7	4·2	10·3	—	66·5	4·1	10·3	—
Et	*o*-Chlorophenyl	B	124–126	$C_{16}H_{13}ON_2Cl$	66·9	4·6	9·7	11·8	67·5	4·6	9·8	12·4
Me	*m*-Chlorophenyl	B	133–135	$C_{15}H_{11}ON_2Cl$	66·0	4·0	10·2	—	66·5	4·1	10·3	—
Me	*o*-Bromophenyl	B	147–148	$C_{15}H_{11}ON_2Br$	57·4	3·6	8·8	25·1*b*	57·1	3·5	8·9	25·3*b*
		H	ca. 220 (*d*)	$C_{15}H_{12}ON_2ClBr$	51·7	3·5	8·1	32·4*d*	51·2	3·5	8·0	32·8*d*
Me	*m*-Bromophenyl	B	134–136	$C_{15}H_{11}ON_2Br$	57·4	3·5	9·0	25·1*b*	57·1	3·5	8·9	25·3*b*
		H	ca. 260 (*d*)	$C_{15}H_{12}ON_2ClBr$	51·7	3·5	8·2	32·2*d*	51·2	3·5	8·0	32·8*d*
Me	*p*-Bromophenyl	B	170–172	$C_{15}H_{11}ON_2Br$	57·3	3·5	8·7	25·5*b*	57·1	3·5	8·9	25·3*b*
		H	ca. 260 (*d*)	$C_{15}H_{12}ON_2ClBr$	51·6	3·6	8·1	33·4*d*	51·2	3·5	8·0	32·8*d*
Et	*p*-Bromophenyl	B	170–172	$C_{16}H_{13}ON_2Br$	58·3	4·0	8·3	24·0*b*	58·4	4·0	8·5	24·3*b*
Pr	*p*-Bromophenyl	B	139–141	$C_{17}H_{15}ON_2Br$	59·6	4·5	8·2	22·8*b*	59·5	4·4	8·2	23 3*b*
Me	*p*-Iodophenyl	B	178–180	$C_{15}H_{11}ON_2I$	50·0	3·1	7·8	35·3*c*	49·7	3·1	7·7	35·1*c*
		H	ca. 265 (*d*)	$C_{15}H_{12}ON_2ClI$	44·9	3·0	—	40·4*d*	45·2	3·0	—	40·7*d*
Me	2,4-Dichlorophenyl	B	151–152	$C_{15}H_{10}ON_2Cl_2$	59·2	3·5	9·1	23·0	59·0	3·3	9·2	23·3
		H	ca. 250 (*d*)	$C_{15}H_{11}ON_2Cl_3$	52·6	3·3	7·9	31·5	52·7	3·2	8·2	31·2
Me	2,5-Dichlorophenyl	B	161–163	$C_{15}H_{10}ON_2Cl_2$	59·2	3·5	9·1	23·3	59·0	3·3	9·2	23·3
		H	ca. 244 (*d*)	$C_{15}H_{11}ON_2Cl_3$	53·0	3·2	8·3	30·6	52·7	3·2	8·2	31·2
Me	4-Bromo-2,3-xylyl	B	168–170	$C_{17}H_{15}ON_2Br$	58·9	4·1	8·4	23·0*b*	59·5	4·4	8·2	23·3*b*

* All hydrochlorides melted with decomposition over a range of several degrees.
a = Fluorine
b = Bromine
c = Iodine
d = Total halogen

Crystallisation from ethyl acetate-light petroleum (b.p. 60–80°) furnished the product in needles (9·6 g), m.p. 151–152°. The hydrochloride separated from methanol in long needles, m.p. 242–250°.

(*b*) A mixture of acetylanthranilic acid (17·9 g.) and 2,4-dichloroaniline 16·2 g.) in pyridine (50 ml.) was stirred vigorously and treated slowly dropwise with phosphorus trichloride (6·9 g.). After the addition was complete the mixture was heated on the steam bath for 1 hour. It was then cooled and diluted with water. The solid product was washed by decantation, dissolved in ethanol (100 ml.) and treated with a slight excess of hydrogen chloride. The *hydrochloride* (25 g.) separated rapidly from the solution; it had m.p. 240–250° and was identical with the compound described in (*a*)

4-*Bromo*-2,3-*dimethylacetanilide*

A solution of bromine (27·5 ml.) in acetic acid (50 ml.) was added dropwise with stirring to a solution of 2,3-dimethylacetanilide (81·5 g.) in acetic acid (150 ml.) cooling being applied to keep the temperature at 10°. Stirring was continued for a further hour after the addition was complete, when the solids were collected and washed with acetic acid. The *product* crystallised from aqueous ethanol in long colourless needles (82 g.), m.p. 158–160°. Found: C, 49·8; H, 5·2; N, 6·2; Br, 32·9. $C_{10}H_{12}ONBr$ requires C, 49·6; H, 5·0; N, 5·8; Br, 33·0 per cent.

4-*Bromo*-2,3-*dimethylaniline*

A solution of the foregoing compound (81 g.) in ethanol (600 ml.) was treated with concentrated hydrochloric acid (120 ml.) and the mixture heated under reflux for 6 hours. The hydrochloride (77·5 g.), m.p. 268° (decomp.), separated on cooling and was collected. Basification of the hydrochloride with N sodium hydroxide (1 litre) followed by distillation in steam yielded 4-*bromo*-2,3-*dimethylaniline*, which crystallised from light petroleum (b.p. 60–80°) in plates, m.p. 32–34°. Found: C, 47·7; H, 5·1; N, 7·0; Br, 40·1. $C_8H_{10}NBr$ requires C, 48·0; H, 5·1; N, 7·0; Br, 40·0 per cent.

4-*Bromo*-2,3-*dimethyl*-6-*nitroacetanilide*

A hot solution of 4-bromo-2,3-dimethylacetanilide (12·1 g.) in acetic acid (19·2 ml.) was added gradually with stirring to a mixture of acetic acid (7·2 ml.) and fuming nitric acid (21·6 ml.) and the resultant mixture heated on the steam bath for 30 minutes. After cooling and pouring into water (500 ml.) the solids were collected and crystallised from 95 per cent ethanol. The *product* separated in pale-yellow needles of m.p. 207–209°. Found: N, 10·1. $C_{10}H_{11}O_3N_2Br$ requires N, 9·8 per cent.

4-*Bromo*-2,3-*dimethyl*-6-*nitroaniline*

The foregoing acetaniline (10 g.) was heated under reflux with 40 per cent sulphuric acid (400 ml.) for 90 minutes. The solution was cooled, diluted and basified with ammonia solution. The *product* crystallised from 50 per cent ethanol in flat golden-brown needles, m.p. 147–149°. Found: N, 11·1. $C_8H_9O_2N_2Br$ requires N, 11·4 per cent.

5-*Bromo*-3,4-*dimethyl*-o-*phenylenediamine* (II)

A solution of the foregoing nitroamine (2·5 g.) in 50 per cent ethanol (300 ml.) was heated on the steam bath and sodium hydrosulphite (dithionite) (10 g.) added in portions with shaking over 1 hour. The ethanol was removed under reduced pressure when the product (1·5 g.) crystallised on cooling. It had m.p. 85–87° after crystallisation from light petroleum (b.p. 60–80°). Found: N, 12·8; Br, 37·1. $C_8H_{11}N_2Br$ requires N, 13·0; Br, 37·2 per cent.

The diamine (0·5 g.) was characterised by condensation with phenthraquinone (0·4 g.) in hot acetic acid (30 ml.). The product (0·6 g.) separated from chloroform in yellow needles, m.p. 257–259°. Found: N, 7·0; Br, 20·8. $C_{22}H_{15}N_2Br$ requires N, 7·2; Br, 20·6 per cent.

The method used for the preparation of the compounds listed in Table II is illustrated by the following example:

3-o-*Bromophenyl*-2-*methyl*-3H-4-*thioquinazolone* (I; R = Me, Ar = *p*-Br. C_6H_4, X = S)

A mixture of 3-*p*-bromophenyl-2-methyl-3*H*-4-quinazolone (47·2 g.) and phosphorus pentasulphide (33·3 g.) in xylene (400 ml.) was stirred and heated under reflux for 2 hours. It was then cooled and treated with 10 per cent sodium hydroxide solution (280 ml.) added in portions. The xylene was then removed by distillation in steam. The residual product (47·3 g.) was purified by crystallisation from 95 per cent ethanol when it separated in yellow-brown needles of m.p. 190–192°.

The following three experiments illustrate the methods used for the preparation of the *N*-alkyl-2-benzamidobenzamides recorded in Table III.

2-*Benzamido*-N-*ethylbenzamide* (V; R = Et, Ar = Ph)

To a well stirred solution of 2-amino-*N*-ethylbenzamide (32·8 g.) in 50 per cent aqueous ethanol (700 ml.) containing hydrated sodium acetate (54·4 g.), benzoyl chloride (28·2 g.) was added in portions over 15 minutes. The reaction was completed by heating on the steam bath for 30 minutes. The product was purified by crystallisation from aqueous ethanol.

2-p-*Bromobenzamido*-N-*ethylbenzamide* (V; R = Et, Ar = *p*-Br.C_6H_4)

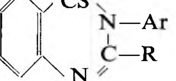
2-Amino-*N*-ethylbenzamide (32·8 g.) was added in small portions with stirring over 30 minutes to a solution of *p*-bromobenzoyl chloride (43·8 g.) in warm pyridine. Reaction was completed by heating on the steam bath for 15 minutes. The mixture was then cooled slightly and poured with stirring into N hydrochloric acid (1,500 ml.). The solids were collected, washed with dilute sodium carbonate solution, then with water and purified by crystallisation from 95 per cent ethanol.

2-p-*Bromobenzamidobenzamide* (V; R = H, Ar = *p*-Br.C_6H_4)

(*a*) Preparation in aqueous ethanol containing sodium acetate, as described for 2-benzamido-*N*-ethylbenzamide except that the reaction was carried out at 60–65°, gave a 60 per cent yield of product m.p. 224–226°.

TABLE II

2-ALKYL-3-ARYL-3*H*-4-THIOQUINAZOLONES

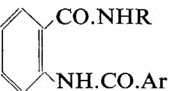


R	Ar	Base (B) hydrochloride (H)	M.p. °C.	Formula	Found per cent C	Found per cent H	Found per cent S	Found per cent	Required per cent C	Required per cent H	Required per cent S	Required per cent
Me	*o*-Tolyl	B	121–123	$C_{16}H_{14}N_2S$	72·3	5·1	11·9	10·6*a*	72·1	5·3	12·0	10·5*a*
		H	228–230	$C_{16}H_{15}N_2SCl$	63·2	5·3	10·8	11·8*b*	63·4	5·0	10·6	11·7*b*
Me	*p*-Fluorophenyl	B	128–130	$C_{15}H_{11}N_2SF$	67·1	4·2	11·5	—	66·6	4·1	11·9	—
Me	*p*-Chlorophenyl	B	183–185	$C_{15}H_{11}N_2SCl$	62·4	3·9	11·3	12·3*b*	62·8	3·9	11·2	12·4*b*
Me	*o*-Bromophenyl	B	174–176	$C_{15}H_{11}N_2SBr$	54·9	3·5	9·3	23·8*c*	54·4	3·4	9·7	24·1*c*
Me	*p*-Bromophenyl	B	190–192	$C_{15}H_{11}N_2SBr$	54·2	3·3	10·2	—	54·4	3·4	9·7	—
Et	*p*-Bromophenyl	B	168–170	[illegible]$H_{13}N_2SBr$	56·0	3·8	8·6	22·9*c*	55·7	3·8	9·3	23·1*c*

a = Nitrogen
b = Chlorine
c = Bromine

TABLE III

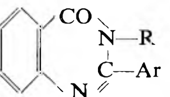
N-ALKYL-2-BENZAMIDOBENZAMIDES



R	Ar	M.p. °C.	Formula	Found per cent C	Found per cent H	Found per cent N	Found per cent Br	Required per cent C	Required per cent H	Required per cent N	Required per cent Br
H	*p*-Bromophenyl	224–226	$C_{14}H_{11}O_2N_2Br$	52·5	3·4	9·1	25·1	52·7	3·5	8·8	25·0
Me	*o*-Tolyl	167–170	$C_{16}H_{16}O_2N_2$	71·7	6·0	10·1	—	71·6	6·0	10·4	—
Me	*p*-Chlorophenyl	162–164									
		190–192	$C_{15}H_{13}O_2N_2Cl$	62·5	4·5	9·9	—	62·4	4·5	9·7	—
Me	*o*-Bromophenyl	170–172	$C_{15}H_{13}O_2N_2Br$	54·2	3·9	8·4	—	54·0	3·9	8·4	—
Me	*p*-Bromophenyl	165									
		197–199	$C_{15}H_{13}O_2N_2Br$	53·6	4·1	8·3	—	54·0	3·9	8·4	—
Et	Phenyl	158–160	$C_{16}H_{16}O_2N_2$	71·6	6·0	10·6	—	71·6	6·0	10·4	—
Et	*p*-Bromophenyl	174–176	$C_{16}H_{15}O_2N_2Br$	55·1	4·8	8·0	23·0	55·3	4·4	8·1	23·0
Bu^n	Phenyl	125–127	$C_{18}H_{20}O_2N_2$	72·7	6·5	8·9	—	72·9	6·8	9·4	—

TABLE IV

C-ALKYL-2-AMYL-3*H*-4-QUINAZOLONES



R	Ar	Base (B) Hydrochloride (H)	M.p. °C.	Formula	Found per cent				Required per cent			
					C	H	N	Cl	C	H	N	Cl
Me	Phenyl	B	136–138	$C_{15}H_{12}ON_2$	75·7	5·3	12·0	—	76·3	5·1	11·9	—
Me	Phenyl	H	ca. 208 (*d*)	$C_{15}H_{13}ON_2Cl$	66·5	4·6	10·3	13·2	66·1	4·8	10·3	13·0
Et	Phenyl	B	130–132	$C_{16}H_{14}ON_2$	76·7	5·5	11·2	—	76·8	5·7	11·2	—
Et	Phenyl	H	ca. 205 (*d*)	$C_{16}H_{15}ON_2Cl$	67·1	5·2	—	12·4	67·0	5·3	—	12·3
Bu	Phenyl	B	116–118	$C_{18}H_{18}ON_2$	78·0	6·6	10·0	—	77·7	6·5	10·1	—
2,3-Dihydroxypropyl	Phenyl	B	179–181	$C_{17}H_{16}O_3N_2$	69·1	5·4	9·7	—	68·9	5·5	9·5	—
Me	*o*-Tolyl	B	107–109	$C_{16}H_{14}ON_2$	76·4	5·8	11·4	—	76·7	5·7	11·2	—
Me	*o*-Tolyl	H	ca. 205 (*d*)	$C_{16}H_{15}ON_2Cl$	67·1	5·2	9·9	12·1	66·7	5·3	9·8	12·4
Me	*p*-Chlorophenyl	B	170–172	$C_{15}H_{11}ON_2Cl$	66·4	4·1	10·5	13·3	66·5	4·1	10·3	13·1
Me	*o*-Bromophenyl	B	154–156	$C_{15}H_{11}ON_2Br$	57·6	3·6	9·1	25·1*a*	57·1	3·5	8·9	25·3*a*
H	*p*-Bromophenyl	B	313–315	$C_{14}H_9ON_2Br$	55·9	3·1	9·4	26·4*a*	55·8	3·0	9·3	26·5*a*
Me	*p*-Bromophenyl	B	170–172	$C_{15}H_{11}ON_2Br$	57·6	3·7	8·9	25·3*a*	57·1	3·5	8·9	25·3*a*
Et	*p*-Bromophenyl	B	122–124	$C_{16}H_{13}ON_2Br$	58·4	3·9	8·7	24·1*a*	58·4	4·0	8·5	24·3*a*
			138–140									

a = Bromine

(*b*) Preparation in pyridine as described for 2-*p*-bromobenzamido-*N*-ethylbenzamide gave a mixture which was separated by extraction with chloroform to give the required product in 40 per cent yield, m.p. 224–226° together with 30 per cent of the chloroform-soluble 2-*p*-bromobenzamidobenzonitrile, m.p. 185–187°. Found: C, 56·0; H, 3·3; N, 8·9. $C_{14}H_9ON_2Br$ requires C, 55·8; H, 3·0; N, 9·3 per cent.

*Preparation of 3-alkyl-2-aryl-3*H*-4-quinazolones* (VI)

The following examples illustrate the methods used for the preparation of 3-alkyl-2-aryl-3*H*-4-quinazolones in Table IV.

3-*Methyl-2-o-tolyl-*3H-4-*quinazolone* (VI; R = Me, Ar = *o*-Me.C_6H_4)

(*a*) *N*-Methyl-2-(*o*-methylbenzamido)benzamide (26·7 g.) was heated under reflux for 1 hour with 5 per cent sodium hydroxide solution (300 ml.) containing ethanol (50 ml.). The product (56 per cent) solidified on cooling and was crystallised from light petroleum (b.p. 60–80°), then from aqueous ethanol.

(*b*) 2-*o*-Tolyl-3*H*-4-quinazolone (4 g.) was dissolved in N sodium hydroxide (80 ml.) and methyl sulphate (7·2 ml.) added dropwise with stirring. After the addition was complete a further 20 ml. of N sodium hydroxide was added and stirring continued for 1½ hours at room temperature followed by heating on the steam bath for 15 minutes. The *product* (50 per cent) separated on cooling and was purified as in (*a*). The *hydrochloride* crystallised from ethanol.

3-*Butyl-2-phenyl-*3H-4-*quinazolone* (VI; R = Bu, Ar = Ph)

2-Benzamido-*N*-butylbenzamide (29·6 g.) was heated under reflux for 3 hours with 5 per cent sodium hydroxide solution (400 ml.) and ethanol (400 ml.). The solid (27 g.) which separated on cooling was a mixture of the required product and starting material. Extraction with ether followed by treatment of the extract with hydrogen chloride furnished the hydrochloride of the product which was collected. This was dissolved in ethanol, basified with sodium carbonate solution to yield the required *base* (9 g.) and purified by crystallisation from 95 per cent ethanol. Unchanged starting material was recovered by concentration of the ethereal extract.

2-p-*Bromophenyl-3-methyl-*3H-4-*quinazolone* (VI; R = Me, Ar = *p*-Br.C_6H_4)

2-*p*-Bromobenzamido-*N*-methylbenzamide (66·6 g.) was finely powdered and heated with stirring at 250–260° for 1 hour. After cooling, the residue was crystallised from ethanol to furnish the product in 68 per cent yield.

3-(2,3-*Dihydroxypropyl*)-2-*phenyl*–3H-4-*quinazolone* (VI; R = CH_2.CHOH.CH_2OH, Ar = Ph)

To a suspension of 2-phenyl-3*H*-4-quinazolone (44 g.) in boiling ethanol (800 ml.) was added 2,3-epoxypropan-1-ol (40 ml.) followed by

pyridine (0·5 ml.) and heating continued for 30 minutes after a homogeneous solution was obtained (5 hours). Most of the ethanol was distilled off, the cooled residue diluted well with water and the product isolated with chloroform. After removal of the chloroform the residual solid was purified by crystallisation from ethanol to yield the product (30 g.) in colourless needles.

2-p-*Bromophenyl*-3-*methyl*-3H-4-*thioquinazolone*

A stirred suspension of 2-*p*-bromophenyl-3-methyl-3*H*-4-quinazolone (44·1 g.) in xylene (400 ml.) was treated with phosphorus pentasulphide (37·4 g.) and the mixture heated under reflux for 2 hours. After cooling 10 per cent sodium hydroxide solution (275 ml.) was added cautiously in portions. The xylene was then removed by distillation in steam and the residual *product* (42 g.) purified by crystallisation from a large volume (2 litres) of ethanol, separated in yellow hair-like crystals, m.p. 167–169°. Found: C, 55·0; H, 3·3; S, 10·2. $C_{15}H_{11}N_2SBr$ requires C, 54·4; H, 3·4; S 9·7 per cent.

REFERENCES

1. Gujral, Saxena and Tiwari, *Ind. J. med. Res.*, 1955, **43**, 637.
2. Grimmel, Guenther and Morgan, *J. Amer. chem. Soc.*, 1946, **68**, 542.
3. Gujral, Sareen and Kohli, *Ind. J. med. Res.*, 1957, **45**, 207.
4. McLamore, P'an and Laubrach, *Abstracts* 133*rd Meeting of Amer. chem. Soc.*, 12(M).
5. Rani, Vig, Gupta and Narang, *J. Ind. chem. Soc.*, 1953, **30**, 331.
6. Brewster and Ciotti, *J. Amer. chem. Soc.*, 1955, **77**, 6214.
7. Hinkel, Ayling and Walters, *J. chem. Soc.*, 1934, 283.
8. Salimath, Patel and Shah, *J. Ind. chem. Soc.*, 1956, **33**, 140.
9. Leonard and Curtin, *J. org. Chem.*, 1946, **11**, 341.
10. Korner, *J. prakt. Chem.*, 1887, **36**, 155.
11. Staiger and Wagner, *J. org. Chem.*, 1953, **18**, 1427.
12. Mitchell and Ashby, *J. Amer. chem. Soc.*, 1945, **67**, 161.
13. Thompson, *ibid.*, 1951, **73**, 5841.
14. Stephen and Wadge, *J. chem. Soc.*, 1956, 4420.
15. Bianchi and David, *J. Pharm. Pharmacol.*, 1960, **12**, 501.

THE INFLUENCE OF SEX ON THE CATABOLISM OF GRISEOFULVIN

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Received May 13, 1960

Blood griseofulvin levels have been determined on rats given a single oral, subcutaneous, intraperitoneal or intravenous dose and on guinea pigs and human volunteers after oral administration. In rats the blood levels of females were higher than those of males: this applied to all four routes of administration. No differences in blood levels between males and females were observed in guinea pigs or human volunteers. *In vitro* studies have shown that liver slices obtained from male rats destroy griseofulvin more rapidly than those from females; the sex difference did not apply to rabbits or guinea pigs.

THE influence of sex on the toxicities and pharmacological activities of some drugs is well recognised and has been reviewed recently[1]. This influence, however, is often found to apply only to a single species. In some instances its dependence on hormones has been demonstrated[2–7].

In earlier studies on the biological disposition of griseofulvin, we observed that the blood levels obtained after oral dosing were higher in female albino rats (WAG strain) than in males. We decided to investigate this finding further and to extend our studies to another strain of rats and to other species.

MATERIALS AND METHODS

Estimation of Griseofulvin

Blood samples and solutions from the liver-incubation experiments were assayed in duplicate by the spectrophotofluorometric method of Bedford, Child and Tomich[8]. Blood samples (3 to 5 ml.), were obtained from anaesthetised rats and guinea pigs by direct cardiac puncture and from human volunteers by venipuncture. Heparin, 50 I.U. in 0·1 ml. physiological saline to 4 ml. blood, was used to prevent clotting.

Blood Level Experiments

Single 10 mg. tablets of griseofulvin were administered orally to male and female guinea pigs weighing 280 to 320 g., and single oral doses of 1 g. (4 × 250 mg. tablets) to human male and female volunteers. Male and female albino rats, WAG strain, weight range 125 to 175 g., received griseofulvin by the oral, intraperitoneal, subcutaneous or intravenous route. The doses used, mg./kg., were 50 and 100 orally, 25 intraperitoneally, 100 subcutaneously and 20 intravenously. The griseofulvin was presented orally or intraperitoneally as an aqueous suspension in 0·5 per cent Tween 80, subcutaneously as a suspension in arachis oil and intravenously as a solution in 75 per cent *NN*-dimethylformamide.

TABLE I

BLOOD GRISEOFULVIN LEVELS IN MALE AND FEMALE RATS GIVEN SINGLE DOSES BY THE ORAL, INTRAVENOUS, SUBCUTANEOUS OR INTRAPERITONEAL ROUTE

(each value is the mean ± S.E.* for 6 rats)

Route	Dose mg./kg.	Sex	Hours after administration												
			1/12	¼	½	1	2	3	4	5	6	7	8	12	16
			Blood griseofulvin concentrations (μg./ml.)												
Oral	50	male	—	—	—	—	0·9 ± 0·1	—	1·3 ± 0·3	—	0·7 ± 0·1	—	0·6 ± 0·1	0·1	0
		female	—	—	—	—	1·9 ± 0·2	—	2·3 ± 0·1	—	2·2 ± 0·3	—	1·7 ± 0·2	0·2	0·1
	100	male	—	—	—	—	1·7 ± 0·2	—	2·5 ± 0·6	—	1·7 ± 0·2	—	2·1 ± 0·4	0·6 ± 0·4	0
		female	—	—	—	—	2·4 ± 0·2	—	3·8 ± 0·3	—	2·9 ± 0·2	—	2·4 ± 0·2	0·8 ± 0·1	0·2
Intra-venous	20	male	7·6 ± 0·4	5·6 ± 0·3	4·3 ± 0·3	3·0 ± 0·6	1·4 ± 0·4	0·4 ± 0·1	0·1	—	—	—	—	—	—
		female	10·2 ± 0·4	7·6 ± 0·6	5·4 ± 0·3	4·4 ± 0·3	2·5 ± 0·4	1·6 ± 0·3	1·2 ± 0·2	0·9 ± 0·2	0·5 ± 0·1	0·4 ± 0·1	—	—	—
Intraperi-toneal	25	male	0·5 ± 0·1	1·2 ± 0·1	0·7 ± 0·1	0·6 ± 0·1	0·3	0·4 ± 0·2	0·2	—	—	—	—	—	—
		female	0·7 ± 0·1	1·7 ± 0·1	1·7 ± 0·1	1·0 ± 0·1	0·9 ± 0·2	0·8 ± 0·1	0·6 ± 0·1	—	0·4 ± 0·1	—	—	—	—
Subcu-taneous	100	male	—	—	—	—	0·3	0·3	0·4 ± 0·1	—	0·2	—	0·2	—	—
		female	—	—	—	0·7 ± 0·2	0·9 ± 0·1	1·0 ± 0·2	1·5 ± 0·2	—	0·7 ± 0·1	—	0·4 ± 0·1	0·3	—

* Standard errors not quoted for mean values less than 0·4 μg./ml.

Incubation of Griseofulvin with Liver Slices

Albino and piebald rats, WAG and PVG strains, respectively, guinea pigs and rabbits were used in these experiments. The animals were killed by a blow on the head; their livers were immediately removed and placed in ice-cold Krebs's solution. The livers were thinly sliced with a Stadie-Riggs tissue slicer, and approximately 200 mg. of the slices were incubated at 37° for 2 hours with 10 ml. Krebs's solution containing griseofulvin (10 μg./ml.). Immediately before and during incubation the solutions were oxygenated with a mixture of 95 per cent oxygen and 5 per cent carbon dioxide. After 2 hours incubation the fluids were decanted and their griseofulvin contents determined. The liver slices were blotted dry and weighed. The results are expressed as amounts of griseofulvin destroyed by 200 mg. of liver in 2 hours.

Results

Blood Levels in Rats

The blood levels of griseofulvin at various times after a single oral dose are given in Table I. At both dose levels, 50 and 100 mg./kg., the female rats gave higher and more prolonged blood levels than the males; the lower dose in females and the higher dose in males gave approximately equal blood levels.

It has previously been shown that griseofulvin disappears rapidly from the blood of male rats after intravenous injection[9]. The blood levels of griseofulvin in male and female rats after a single intravenous dose are given in Table I. Again the females gave higher and more prolonged levels than the males.

Blood levels after subcutaneous injection of the arachis oil suspension and after intraperitoneal injection of the aqueous suspension are also given in Table I. Griseofulvin was not detectable in the blood after a subcutaneous injection of an aqueous suspension at a dose of 100 mg./kg., whereas this dose of the drug in arachis oil gave measurable levels lasting for several hours. Griseofulvin was absorbed rapidly after intraperitoneal injection of 25 mg./kg., peak levels being attained in 15 to 30 minutes. As after oral dosing, the blood levels after subcutaneous or intraperitoneal injection were higher in the female rats than in the males.

Blood Levels in Guinea Pigs and Human Volunteers

Blood griseofulvin levels obtained in guinea pigs and human volunteers given single oral doses are shown in Figures 1 and 2. There were no differences in the blood levels of male and female guinea pigs. In both sexes peak values were reached at 3 hours: at 24 hours griseofulvin was no longer detectable (Fig. 1).

In the human volunteers peak levels were attained in 4 hours, and griseofulvin was still detectable 26 hours after dosing. After a single oral dose of 1 g. there were no differences in blood levels between men and women. These results are shown in Figure 2.

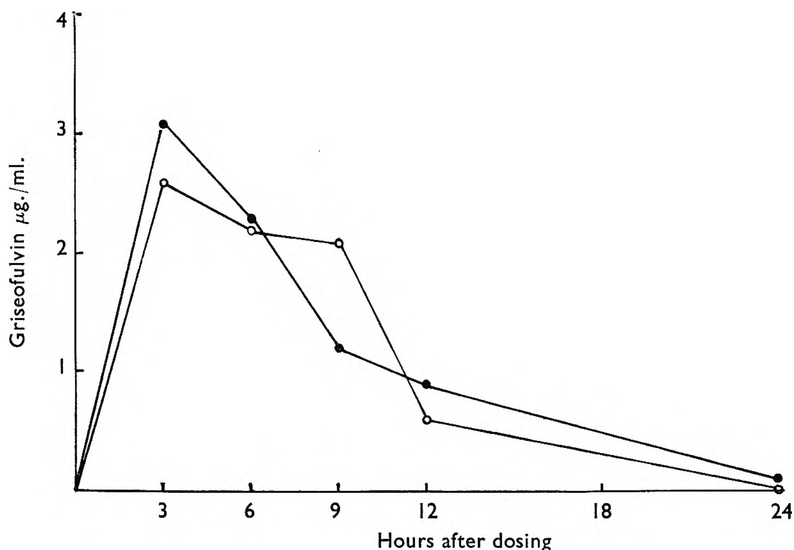

FIG. 1. Blood griseofulvin levels in guinea pigs after a single oral dose of 33 mg./kg. Each point is the mean for three animals.

Female = O—O Male = ●—●

Incubation of Griseofulvin with Liver Slices

The results obtained are given in Table II. Liver slices from adult male WAG rats destroyed griseofulvin more rapidly than those from adult females of the same strain ($P < 0{\cdot}001$). Similar but less significant differences between males and females were obtained with weanlings of the same strain ($P < 0{\cdot}02$) and with adult rats of the PVG strain ($P < 0{\cdot}02$).

No sex differences were recorded in the rates of griseofulvin catabolism when the livers of rabbits or guinea pigs were used.

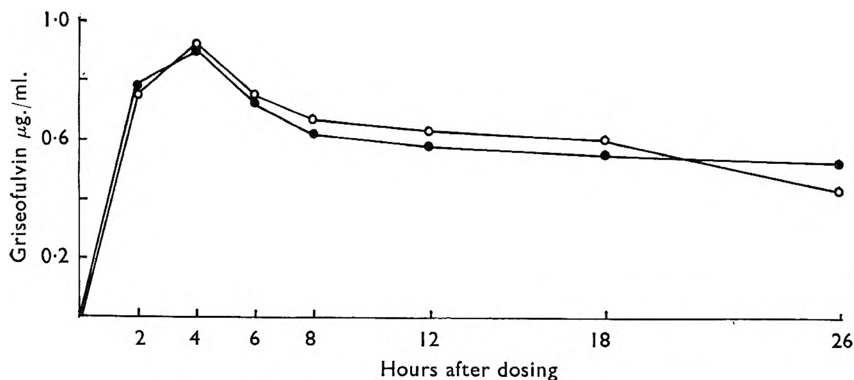

FIG. 2. Blood griseofulvin levels in human volunteers after a single oral dose of 1 g. Each point is the mean for six volunteers.

Female = O—O Male = ●—●

TABLE II

In vitro CATABOLISM OF GRISEOFULVIN BY LIVER SLICES

Species and strain	μg. griseofulvin destroyed in 2 hours by 200 mg. liver		Probability of difference between males and females being due to chance
	Male	Female	
Adult WAG rats	55·4 ± 2·8* (21)†	36·2 ± 2·7 (21)	P < 0·001
Weanling WAG rats	57·7 ± 2·8 (6)	45·7 ± 2·7 (6)	P < 0·02
Adult PVG rats	49·2 ± 4·6 (5)	32·8 ± 4·6 (5)	P < 0·02
Adult rabbits	42·5 ± 5·4 (8)	48·4 ± 7·6 (8)	P > 0·5
Adult guinea pigs	45·3 ± 0·8 (6)	47·8 ± 4·2 (6)	P > 0·5

* Standard error. † Number of observations.

Discussion

The experiments described above show that female rats have higher blood griseofulvin levels than males, and this difference can be observed after either oral or parenteral administration. Presumably the difference does not arise from different degrees of gastrointestinal absorption. The results of the parenteral experiments, particularly those by the intravenous route, suggest that the differences in blood level between the two sexes reflect different rates of catabolism.

The results of the *in vitro* experiments are consistent with this view. In common with other drugs showing differences in toxicity or pharmacological activity between the sexes, griseofulvin appears to show this difference in only one species, in this instance the rat.

References

1. Weston Hurst, *The Evaluation of Drug Toxicity*, Churchill Ltd., 1958, p. 12.
2. Brodie, *J. Pharm. Pharmacol.*, 1956, **8**, 1.
3. Culliford and Hewitt, *J. Endocrinol.*, 1957, **14**, 381.
4. Dubois, Doull, Salerno and Coon, *J. Pharmacol.*, 1949, **95**, 79.
5. Edgren, *Experientia*, 1957, **13**, 86.
6. Holck, Kanân, Mills and Smith, *J. Pharmacol.*, 1937, **60**, 323.
7. Hewitt, *Brit. J. Exp. Path.*, 1956, **37**, 32.
8. Bedford, Child and Tomich, *Nature, Lond.*, 1959, **184**, 364.
9. Bedford, Busfield, Child, MacGregor, Sutherland and Tomich, 1960, *A.M.A. Archives of Dermatology*, in press.

THE COLORIMETRIC DETERMINATION OF SANTONIN IN ARTEMISIA

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Received May 5, 1960

A new colorimetric method for the estimation of santonin in artemisia using hydroxylamine and ferric chloride has been evolved and compared with a gravimetric and a volumetric method. Improvements in the method of extraction of santonin have also been proposed.

SEVERAL species of artemisia are grown on a commercial scale in the north-western region of West Pakistan and their unexpanded flowerheads are utilised in the manufacture of santonin which is used as an anthelmintic and is official in the British Pharmacopoeia[1].

A number of methods of assay[2–4] of this drug based on the gravimetric estimation of santonin have been described where use is made of a correction factor calculated on the basis of the solubility of pure santonin in the strength of ethanol employed for crystallisation. These corrections appear to be arbitrary and throw doubts on the accuracy of the gravimetric methods. The first method of estimation independent of a correction factor was proposed by Bohme[5] where, after extraction, santonin was determined volumetrically. This method, however, lacked specificity and could only be used for artemisia rich in santonin. Moreover, according to Bohme himself, the results were always higher than the gravimetric method.

Several methods[6–8] based on the colour reactions of santonin have been reported. In Fucci's[6] method, santonin is estimated by the colour of the sodium salt in strongly alkaline solution, but the method does not appear to possess a quantitative basis. Iwayama[7] utilises the colour reaction between an acidic solution of santonin and diazobenzene sulphonic acid, but the assay of artemisia is not described. Yamagishi and his co-workers[8] make use of the reaction with sodium methoxide for quantitative purposes but the results are imprecise because of variations in the sodium methoxide reagent; atmospheric moisture also appears to increase the experimental error.

A need clearly exists for a simple, rapid and accurate method applicable to artemisia of different santonin contents and independent of any arbitrary correction. We have evolved a new method based on Fiegl's[9] colour reaction for esters and lactones which has been applied quantitatively by Hestrin[10] and Wollish and Schmall[11]. The lactone, santonin (I) was treated with alkaline hydroxylamine and the hydroxamic acid derivative (presumably II) so formed was then treated with ferric chloride to give a purple-coloured solution of the ferric hydroxamate derivative (presumably III). The colour was found to be suitable for spectrophotometric determination. The reactions are shown in Figure 1.

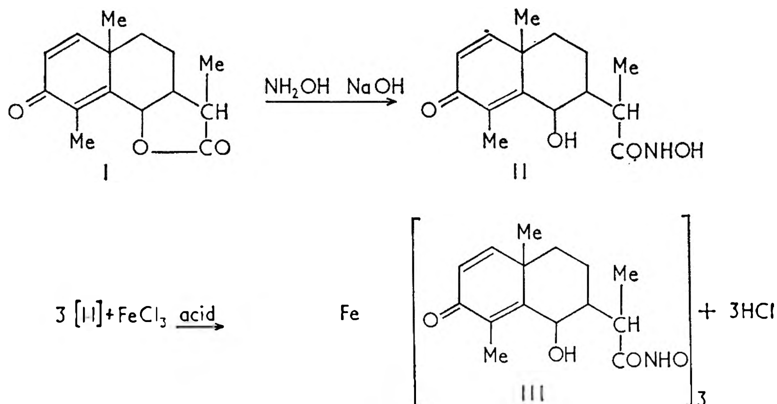

FIG. 1

We extracted santonin from the crude drug by Massagetov's method[2] and after removing the colouring matter, developed the colour in the same manner as on pure santonin. Five different samples of the drug were used for a comparison of Massagetov's gravimetric method *loc. cit.* and Bohme's[5] volumetric method with our modifications.

EXPERIMENTAL

Colorimetric Method.

Apparatus. Unicam Spectrophotometer, SP600; reflux condenser, ground-glass joints; flat bottom flasks 250 ml. ground-glass joints; steam bath; 1,000 ml. separating funnels.

Reagents. Standard solution of santonin B.P. in 50 per cent ethanol (1 mg./ml.); hydroxylamine sodium hydroxide solution: 7·5 g. in 100 ml. N sodium hydroxide freshly prepared; 2·0 per cent ferric chloride solution in water; 2,4-dinitrophenol indicator, 0·1 per cent 2,4-dinitrophenol in 90 per cent ethanol; N sodium hydroxide; N hydrochloric acid; ethanol, 95 per cent v/v 50 per cent v/v and 18 per cent v/v; calcium oxide; chloroform; hydrochloric acid, sp. gr. 1·18; sodium hydroxide solution 4 per cent w/v.

Standard Curve. Measure by pipette 1, 2, 3, 4 and 5 ml. quantities of the standard santonin solution into separate 50 ml. volumetric flasks. Add 2 ml. of hydroxylamine-sodium hydroxide solution and then 5 ml. of standard N sodium hydroxide to each flask. Allow to stand for 5 minutes, add three drops of 0·1 per cent 2,4-dinitrophenol indicator and titrate the solution carefully with standard N hydrochloric acid to a colourless end point. Make each flask up to 50 ml. with water. Place 5·0 ml. of each solution in a dry test tube, add 1 ml. of 2 per cent ferric chloride solution and mix thoroughly by shaking. Read the extinction within 3 minutes on a spectrophotometer at 500 mμ in 1 cm. cell, using water as blank, taking care that the cell is free from air bubbles. Carry out a reagent blank using 2 ml. of 50 per cent v/v ethanol in place of the santonin solution.

The standard curve is shown in Figure 2. Blank readings are usually between 0·007 and 0·010.

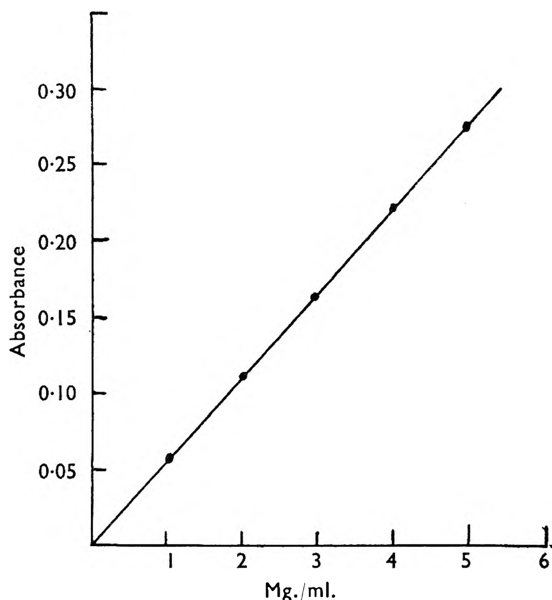

FIG. 2. Standard curve for santonin.

Preparation of sample. Weigh accurately about 2 g. of finely powdered flowerheads, grind them in a mortar with 0·5 g. of calcium oxide and then triturate with 5 ml. of hot water. Transfer the mixture to a 400 ml. beaker with 120 ml. of hot water, bring to the boil and continue boiling for 10 minutes. Filter hot and wash the residue with a further 120 ml. quantity of boiling water. Reject the residue, acidify the warm filtrate with 10 ml. of hydrochloric acid and place the beaker containing the acidified extract on the steam bath for 5 minutes. Cool and extract with 30, 20, 20 and 10 ml. portions of chloroform with vigorous agitation. Shake the combined chloroform extract twice in a separating funnel with 15 ml. of 4 per cent sodium hydroxide solution. Wash the chloroform extract with 10 ml. of water to remove the alkali, and pass through a pledget of cotton wool into a 250 ml. flask. Add 0·1 g. of animal charcoal, connect the flask to a reflux condenser on a water bath and boil gently for 10 minutes. Filter rapidly through a double filter paper (Whatman No. 41), into another 250 ml. flask and wash the first flask with two 5 ml. quantities of chloroform. Distill the chloroform and gently dry the residue. Add 2 ml. of ethanol to the residue and again evaporate to dryness to ensure complete removal of chloroform. Add 20 ml. of 18 per cent v/v ethanol and 0·1 g. animal charcoal. Connect the flask to a reflux condenser on a steam bath and boil gently for 10 minutes. Filter rapidly whilst hot through a double filter paper into a 50 ml. volumetric flask. Wash the flask twice with 5 ml. portions of hot 18 per cent v/v ethanol. Dilute to

TABLE I

COMPARISON OF THE COLORIMETRIC, VOLUMETRIC AND GRAVIMETRIC METHODS FOR THE ASSAY OF SANTONIN

Sample	S. No.	Colorimetric per cent	Melting Point °C.	Volumetric per cent	Melting Point °C.	Gravimetric per cent	Melting Point °C.	Deviation per cent	
								Colorimetric	Volumetric
A	1	1·82	171·0	1·88	171·0	1·82	171·0		
	2	1·85	171·0	1·88	171·0	1·82	171·0		
	3	1·83	171·5	1·88	171·5	1·81	171·0		
	Average	1·83		1·88		1·82		+0·6	+3·3
B	1	2·13	171·5	2·19	171·5	2·09	171·0		
	2	2·17	172·0	2·22	172·0	2·19	173·0		
	3	2·18	172·0	2·22	172·0	2·15	171·5		
	Average	2·16		2·21		2·14		+0·9	+3·3
C	1	2·25	171·0	2·37	171·0	2·25	172·0		
	2	2·25	172·0	2·31	172·0	2·21	173·0		
	3	2·22	171·0	2·25	171·0	2·25	172·0		
	Average	2·24		2·31		2·24		Nil	+3·1
D	1	1·95	172·0	2·00	172·0	1·93	171·0		
	2	1·90	173·0	1·97	173·0	1·90	172·0		
	3	1·95	171·0	1·97	171·0	1·97	172·0		
	Average	1·93		1·98		1·93		Nil	+2·6
E	1	2·86	172·0	2·95	172·0	2·82	172·0		
	2	2·83	172·0	2·92	172·0	2·78	172·0		
	3	2·87	171·0	2·92	171·0	2·86	173·0		
	Average	2·85		2·93		2·84		+0·4	+3·2

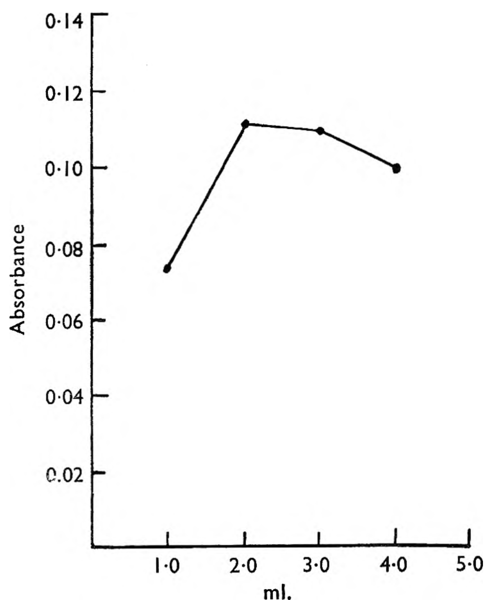

FIG. 3. Effect of varying the amounts of Hydroxylamine sodium hydroxide reagent on the development of colour.

volume with 95 per cent v/v ethanol. Pipette 3 ml. of the solution and develop the colour exactly as described under the method for the standard curve. Measure the extinction, read the concentration of santonin from the standard curve and calculate its percentage in the sample.

Volumetric Method

Weigh accurately about 5 g. of the drug and proceed for extraction as described above varying the quantities of reagents and solvent proportionately. After the complete removal of chloroform, dissolve the residue in 5 ml. of hot ethanol, add 10 ml. of 5 per cent w/v barium hydroxide solution and heat on a water bath for 15 minutes. Cool and filter through filter paper (Whatman No. 41), and wash the residue with 10 ml. of water. Add two drops of phenolphthalein indicator to the filtrate and titrate carefully with 0·5 N hydrochloric acid to a colourless end point. Add 10 ml. of 0·1 N hydrochloric acid by means of a pipette and heat on a water bath for 15 minutes. Cool and titrate with 0·1N sodium hydroxide from a fine burette until the red colour persists for 1 to 2 minutes. Carry out a blank on 10 ml. of the 0·1N hydrochloric acid and calculate the percentage of santonin in the drug as follows,

$$\frac{a \times 246{\cdot}1 \times 100}{5 \times 10{,}000}$$

where a is the difference in the titer between blank and the sample.

Gravimetric Method

Proceed for extraction as described in the volumetric method above to the complete removal of chloroform. Dissolve the residue in 2 ml. of hot ethanol, add 100 ml. of boiling water and then concentrate to 50 ml. on a water bath. Place the flask in a refrigerator (maximum temperature 10°) for 2 days. Filter the crystals of santonin which separate on a sintered glass crucible (No. 4), measure the exact volume of the filtrate, and dry at 100°, to constant weight. To the weight found add 0·0002 g. for each ml. of the filtrate and calculate the percentage of santonin in the sample.

The results on the five samples are summarised in Table I. The melting points of the crystals obtained in the colorimetric, volumetric and the gravimetric methods are also given for each determination.

Discussion

Extraction of Santonin

We chose Massagetov's method of extraction in preference to the methods of Qazilbash[3] and Kassner and others[4], because of its simplicity of operation and economy in time and solvents. The melting point determinations on the extracted santonin agreed closely with those reported by these workers on santonin extracted by their methods. We found, however, that Massagetov's method of extraction needed some improvement. The residue on being dissolved in 95 per cent v/v ethanol still contained coloured impurities which interfered in the colorimetric determination. It was therefore dissolved in hot 18 per cent v/v ethanol and

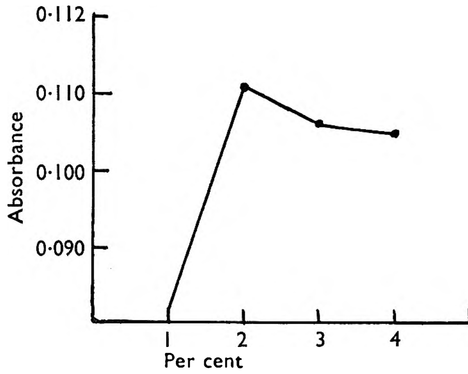

FIG. 4. Effect of varying the amount of Ferric chloride reagent on the development of colour.

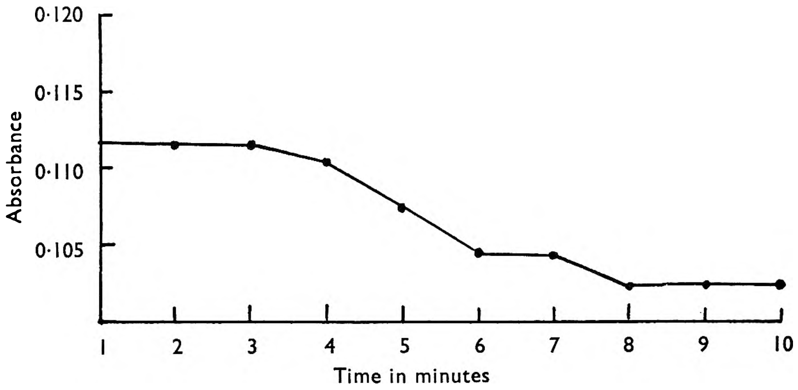

FIG. 5. Stability of the colour.

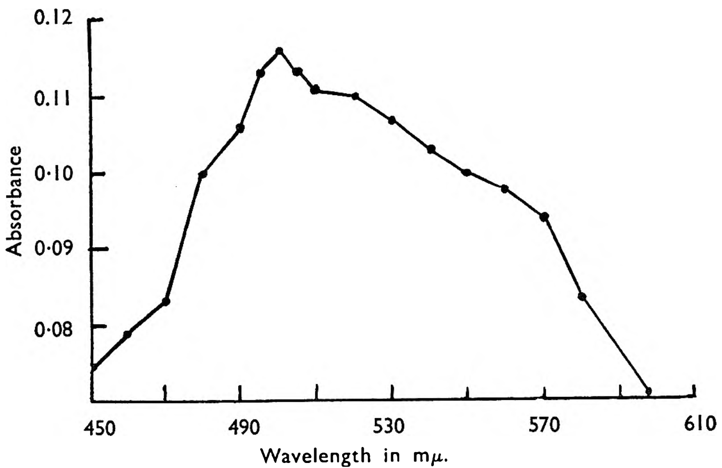

FIG. 6. Absorption curve of the developed colour.

refluxed with a further quantity of charcoal. After hot filtration, the santonin crystallised out partially but could be taken up in solution by addition of 95 per cent v/v ethanol, this time giving a clear colourless solution. On being made up to volume by water so that the final strength of ethanol was approximately 50 per cent v/v, santonin remained in solution and it was possible to develop colour on an aliquot of this solution.

Development of Colour

For the conditions affecting the development of colour from santonin we were guided by those adopted by Wollish and Schmall[8] for pantoyl lactone. The effect of varying the amounts of hydroxylamine reagent showed that 2·0 ml. of this reagent gave the maximum colour intensity (see Fig. 3). Similarly we varied the amount of ferric chloride and found that 1 ml. of 2·0 per cent ferric chloride gave the best colour as is shown in Figure 4. With the quantities of these reagents fixed, we investigated the stability of the developed colour and found that maximum intensity was reached immediately on addition of the ferric chloride and remained constant for 3 minutes. Thereafter fading took place as shown in Figure 5. The absorption curve of the developed colour was then determined and the maximum located at 500 mμ as shown in Figure 6.

The initial colorimetric determinations were made on 5 g. of the drug. After fully establishing the conditions and standardising the procedure, the quantity of the drug was reduced to 2 g. with proportionate reductions in the quantities of the reagents and solvents. The results were almost identical with those found on 5 g. of the drug.

From the standard curve it is seen that Beer's Law is obeyed with up to 5 mg. of santonin in the aliquot of the final solution.

Results and Conclusions

Table I shows that the results on the five samples by the proposed colorimetric method compare well with those given by the gravimetric method (mean deviation less than 1·0 per cent). The volumetric method gives a larger deviation, between 2·6 and 3·3 per cent. In agreement with the findings of Bohme[5] results tend to be high. Also, the low quantity of santonin in relation to the final alkali titration, would necessitate the use of a larger quantity of the drug. For artemisia deficient in santonin, the volumetric method is of little use.

Apart from the general merits of the colorimetric method over the gravimetric estimation, the former requires no arbitrary correction. It is economical in solvents since extraction can be made on smaller quantities of the drug. Also, the colorimetric method much reduces the total time for the determination from the 2 days required for complete crystallisation in the gravimetric method.

The main advantage of the colorimetric method over the existing ones lies in its ability to determine santonin in quantities as low as 1 mg. in the final aliquot and can be employed for the assay of low-grade artemisia for which, so far, no other suitable method is available.

Other lactones like artemisin, α-santonin and ψ-santonin present in artemisia are also determined by the colorimetric method. This, however, is of little consequence as these lactones are present in minute quantities and are known to have anthelmintic properties similar to santonin[12].

Acknowledgement. The authors wish to thank Mr. S. M. I. Shah, Pakistan Industrial Development Corporation Laison Officer at Parachinar, West Pakistan for his assistance in the supply of artemisia.

REFERENCES

1. *British Pharmacopoeia*, 1958, 573.
2. Massagetov, *Arch. Pharm.*, 1932, **270**, 392, *Quart. J. Pharm. Pharmacol.*, 1932, **5**, 681.
3. Qazilbash, *J. Pharm. Pharmacol.*, 1951, **3**, 105.
4. Kassner, Johnson and Terry, *ibid.*, 1953, **5**, 245.
5. Bohme, *Arch. Pharm.*, 1942, **280**, 89.
6. Fucci, *Clin. Med. ital.*, 1935, 660.
7. Iwayama, *J. pharm. Soc. Japan*, 1952, **72**, 719.
8. Yamagishi, Nakamura and Nakajima, *ibid.*, 1954, **74**, 1354.
9. Fiegl, Anger, and Frehden, *Mikrochemie*, 1934, **15**, 9.
10. Hesterin, *J. biol. Chem.*, 1949, **180**, 249.
11. Wollish and Schmall, *Analyt. Chem.*, 1950, **8**, 1033.
12. Fourneau, *Pharmacie*, 1943, **1**, 127.

MEVALONIC ACID AS A PRECURSOR IN THE BIOGENESIS OF DIGITOXIGENIN

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Received April 20, 1960

(±)-Mevalonic acid-2-^{14}C has been injected into a growing plant of *Digitalis lanata* L. to test if this acid serves as a precursor in the biosynthesis of cardenolides. After nine days a portion of the leaves was extracted and the constituents submitted to paper chromatography followed by autoradiography. The carbon 14 was detected in the lipid fraction and in the cardiac glycoside spots, but mostly in an area containing an as yet unidentified compound. Of the two glycosides forming the major glycoside area, one was predominant and, as after Mannich hydrolysis and chromatography, glucose, digitoxose and digitoxigenin were identified, this corresponds to lanatoside A. The aglycone fraction contained the radioactivity.

Since Kennedy's review[1] on the discovery of mevalonic acid lactone (3-hydroxy-3-methyl-pentane-5-lactone) and its possible role in sterol synthesis, a great deal of interest has been directed to this subject. Tavormina, Gibbs and Huff[2] demonstrated that mevalonic acid is converted efficiently to cholesterol by cell-free liver preparations and found that as much as 43·4 per cent of the radioisotope from (±)-mevalonic-2-^{14}C acid could be recovered as cholesterol after incubation with homogenates of rat liver. Tavormina and Gibbs further showed[3] that, in the utilisation of mevalonic acid to form cholesterol, the carboxyl group is lost during the conversion since the isotope labelling of the resulting cholesterol was negligible when carboxyl-labelled mevalonic acid was used, the radioactivity in these experiments being recovered mainly as carbon dioxide. The evidence indicates that mevalonic acid is the direct source of the isoprenoid units in squalene and sterol[4].

As the structures of the cardenolides are chemically closely related to the sterols, it appears likely that they also should derive from mevalonic acid. To test this hypothesis, mevalonic acid-2-^{14}C was fed to *Digitalis lanata* and allowed to metabolise for 9 days, and the selective incorporation of the radioactive carbon into steroids, including the main cardioactive glycoside, studied.

Experimental

Materials, Reagents and Procedures

Reference materials. Digitoxin U.S.P. reference standard and digoxin U.S.P. reference standard. *Paper.* Whatman No. 1, cut to 16 cm. × 57 cm. *X-ray film.* Kodak medical X-ray film, no-screen.

Solvent systems. For chromatography of sugars and glycosides, solvent system 1: butanol : glacial acetic acid : water (4 : 1 : 1). Solvent system 2, for glycosides : toluene : butanol (3 : 1) saturated with formamide, according to Satch and others[5].

Chromatographic procedures. All paper-chromatographic runs were carried out by the descending technique. In the case of solvent system 2 the filter paper was impregnated with formamide by passing the paper through a solution of formamide and acetone (1 : 4) and then allowing the acetone to evaporate.

Reagents. Mevalonic acid-2-^{14}C, from Tracerlab, Inc. (1·22 mc./mM of free mevalonic acid). Radiologically pure. The trichloracetic acid reagent used for the detection of cardiac glycosides was prepared as described by Jensen and Tennoe[6] modified to the extent that sodium hypochlorite was substituted for chloramine.

The *p*-anisidine hydrochloride reagent used for the detection of sugars was prepared as described by Bliss and Ramstad[7].

Preparation and Extraction of Plant Material

A volume of 0·15 ml. of (±)-mevalonic acid-2-^{14}C, representing 3·3 mg. of mevalonic acid having an activity of 15 μc, was injected into the leaf petiole and the stem of a young plant of *Digitalis lanata* by placing with a hypodermic syringe small drops of the dissolved acid on the petiole and stem and puncturing the veins of the tissue with the needle. Due to negative pressure within the plant, the fluid was sucked in and more solution added on the same area before it dried. As shown by means of a Geiger-Müller tube, the radioactivity spread throughout the whole leaf blade in the course of minutes; in the course of hours or days, it spread throughout the entire plant. After 9 days, 1·47 g. (fresh weight) of leaf was harvested from the injected plant. The leaf material was ground in a mortar with 95 per cent ethanol until a slurry was obtained. The liquid was filtered through cotton into a medicine dropper. This extraction was repeated three more times. The extracts were combined and concentrated at room temperature.

Chromatography of Initial Extractive

The concentrated extract was streaked on a piece of chromatographic paper at a distance of 13 cm. from one end (streak = 6541 c.p.m.). The streaked paper was chromatographed for 16 hours with solvent system 1. The paper chromatogram was allowed to dry thoroughly at room temperature and was then placed on X-ray film for a 45-hour exposure. The developed X-ray film showed the presence of four main radioactive areas on the chromatogram (Table I).

TABLE I

MAIN RADIOACTIVE AREAS

Area designation	Nature	R_F value
1	Cardiac glycosides	0·26
2	Unidentified compound, being the most radioactive	0·35
3	Mevalonic acid	0·65
4	Chlorophyll-sterol area	0·85–0·90

A 1·5 cm. strip was cut from the long side of the paper chromatogram and examined for the presence of cardenolides by means of the trichloracetic acid reagent spray. One of the major radioactive areas (R_F, 0·26; 2·5 cm. wide) as well as several minor radioactive areas, gave positive cardenolide reaction with trichloracetic acid reagent. As judged from their R_F values, none of the glycosides gave indication of being a secondary glycoside such as digitoxin or gitoxin.

Elution and Rechromatography of Area 1

Area 1 (major cardiac glycoside area) was cut from the chromatogram and eluted for 48 hours with 50 per cent ethanol. The eluate was concentrated at room temperature and spotted on two chromatographic papers. One paper was chromatographed by use of solvent system 1 (initial spot: 723 c.p.m.). The second paper was chromatographed with solvent system 2 (initial spot: 741 c.p.m.). The results are given in Table II.

Identification of the Major Glycoside in the Eluate and Demonstration that the Labelled Carbon of Mevalonic Acid is Present Only in the Alygcone

Mannich hydrolysis. As indicated in Table II, the radioactive cardiac glycoside area (area 1) consisted of two radioactive cardenolides. The low R_F indicated that they were primary glycosides such as lanatosides A, B or C. In each case, the glycoside having the lower R_F was the major glycoside. It was subjected to a Mannich hydrolysis. The procedure for the hydrolysis was essentially as described by Bliss and Ramstad[7] modified in that the reaction was carried out at 55° for 3 days instead of 21 days at room temperature. The materials subjected to the hydrolysis were: digitoxin U.S.P. reference standard, 3 mg.; digoxin U.S.P. reference standard, 3 mg.; and an aliquot of the eluate from area 1. At the end of the hydrolysis the solvent (acetone) was evaporated from each tube and a volume of 0·5 ml. of water and 0·5 ml. of chloroform was added and the tubes shaken.

TABLE II

RESULTS OF CHROMATOGRAPHY OF ELUATE FROM AREA 1

Solvent system	R_F of cardenolide spot*	Colour under ultra-violet light after treatment with trichloracetic acid reagent
1	0·02 (major spot)	Cream
	0·10	Light blue
2	0·14 (major spot)	Cream
	0·16	Light blue

* Each of the spots were radioactive as established by a 113-hour exposure to X-ray film.

The Sugars

The aqueous layers from the Mannich hydrolysis were drawn off and allowed to concentrate at room temperature. The residue was spotted on Whatman No. 1 paper and chromatographed with solvent system 1. At

the completion of the chromatography the dried chromatograms were sprayed with the *p*-anisidine hydrochloride reagent and heated for 10 minutes at 80–85° and then examined under ultra-violet light. The results are given in Table III.

TABLE III

RESULTS OF CHROMATOGRAPHY OF SUGARS FROM MANNICH HYDROLYSATE

Material hydrolysed	R_F value	Colour under ultra-violet light after treatment with *p*-anisidine hydrochloride reagent
Digitalis lanata		
Eluate from area 1 ..	0·08 (glucose)	Vivid yellow
	0·17 (digitoxose)	Reddish brown
Digitoxin	0·15 (digitoxose)	Reddish brown
Digoxin..	0·16 (digitoxose)	Reddish brown

The two sugars in the eluate from area 1 of the *Digitalis lanata* experiment were identified as glucose and digitoxose from their R_F values and colour reactions with the reagent. Glucose and digitoxose constitute the sugar portion of the major glycosides of *Digitalis lanata.*

The paper chromatogram of the sugars from area 1 was exposed to X-ray film for a period of 21 days. Upon development of the film no radioactivity was noted to reside in the sugar spots.

The Aglycone

The chloroform layers of the Mannich hydrolysis were concentrated and each were spotted on formamide-treated paper. To the chloroform solution of the eluate from area 1 was added a chloroform solution of digitoxigenin. The paper was then chromatographed by use of solvent system 2. The chromatograms were treated with the trichloracetic acid reagent and then inspected under ultra-violet light. The results are given in Table IV.

TABLE IV

RESULTS OF THE CHROMATOGRAPHY OF CHLOROFORM LAYER FROM MANNICH HYDROLYSATE

Material hydrolysed	R_F values	Colour in ultra-violet light after treatment with trichloracetic acid reagent
Digoxin..	0·72 (digoxigenin)	Cream
	0·78 (anhydro form of digoxigenin)	Blue
Digitoxin	0·63 (digitoxigenin)	Yellow
	0·82 (anhydro form of digitoxigenin)	Yellow
Eluate from area 1		
Digitalis lanata ..	0·62 (digitoxigenin)	Yellow

The chromatogram of the eluate from area 1 was exposed to X-ray film for a period of 21 days. The outline of the radioactive area on the chromatogram matched exactly the area of the aglycone as outlined after treatment with trichloracetic acid reagent.

DISCUSSION

The label showed up in the lipidic fraction (sterols) and in the cardiac glycoside spots. The greatest amount of radioactivity was present in

a yet unidentified compound. The major radioactive glycoside area consisted of two glycosides, one of which was predominant. The predominant glycoside, upon Mannich hydrolysis, and chromatography furnished digitoxose, glucose and digitoxigenin and thus corresponds to lanatoside A, the major cardioactive glycoside of *Digitalis lanata*[8]. The aglycone contained all the activity of the glycoside; digitoxose and glucose contained no detectable radioactivity.

The hypothesis that mevalonic acid serves as a precursor in the biosynthesis of the cardiac glycosides has found justification in the experiment with *Digitalis lanata.* That mevalonic acid follows a direct path into the steroid cardenolides and not after the label has entered the general metabolism is supported by the observation that common cell metabolites were not found labelled and also from the fact that the sugar portion of the glycoside was unlabelled.

REFERENCES

1. Kennedy, *Ann. Rev. Biochem.*, 1957, **26**, 119.
2. Tavormina, Gibbs and Huff, *J. Amer. chem. Soc.*, 1956, **78**, 4498.
3. Tavormina and Gibbs, *ibid.*, 1956, **78**, 6210.
4. Popjak, *Ann. Rev. Biochem.*, 1958, **27**, 533.
5. Satoh, Ishii, Oyama, Wada and Okumura, *Chem. Pharm. Bull.*, 1956, **4**, 284.
6. Jensen and Tennoe, *J. Pharm. Pharmacol.*, 1955, **7**, 334.
7. Bliss and Ramstad, *J. Amer. pharm. Ass., Sci. Ed.*, 1957, **46**, 423.
8. Tamm, *Fortsch. organ. Naturstoffe*, 1957, **14**, 124.

THE BIOLOGICAL ASSAY OF *RAUWOLFIA SCHUELI*

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Received April 19, 1960

The biological effects of *Rauwolfia schueli* are essentially the same as those of the pure alkaloid reserpine and the crude compound *R. serpentina* in the rat. No difference in relative potency between the small and large roots of large trees of the *R. schueli* species was apparent when either reserpine or *R. serpentina* was employed as the standard. *R. schueli* was estimated to be about 1/630 as active as reserpine. In terms of reserpine-like activity, this species appeared to be more potent than *R. serpentina* and by comparison with the results of others about as potent as *R. canescens* but more potent than *R. heterophylla*. Within the range of doses selected, blood pressure did not appear to be an adequate index for measuring reserpine-like activity in the rat.

NUMEROUS studies have shown that *Rauwolfia serpentina* is effective in the treatment of hypertension and certain mental disorders. Hypotensive and sedative properties also have been ascribed to *R. canescens* and *R. heterophylla*[1] and to *R. vomitoria*[2]. The preparations tested were either the powdered whole root or some extract thereof. It is the purpose of this study to compare the activity of *R. schueli*, a species peculiar to the northern part of Argentina and Bolivia, with the activity of the pure alkaloid reserpine and the crude compound *R. serpentina*.

MATERIALS AND METHODS

Test Preparations

The test preparations used were *R. schueli* YR-1, a blended batch of small roots of large trees, and *R. schueli* YC-1, a blended batch of large roots of large trees. Samples of these preparations were supplied by E. R. Squibb and Sons. The assumed relative potencies with reserpine as the standard were as follows: YR-1, 1·28 g. reserpine per kg.; YC-1, 1·30 g. reserpine per kg. Each sample was ground to a particle size that could pass a 200 mesh screen.

Standard Preparations

The crystalline alkaloid reserpine was dissolved in glacial acetic acid and enough distilled water added to yield a stock solution of 10 mg. alkaloid per ml. of 10 per cent acetic acid. This solution was stored at 7° and used within 2 weeks. A sample of the crude compound *R. serpentina* also was supplied by E. R. Squibb and Sons. It had an assumed relative potency of 1 g./kg. and was powdered to the same degree of fineness as the test samples, YR-1 and YC-1.

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Experimental Animals

The experimental animals were male albino rats, from 120 to 200 g., taken from the animal stock of the Department of Physiology at the University of Mendoza in Argentina. Each rat was fasted for 18 to 24 hours before dosing.

Methods of Dosing

The rats were divided into 4 groups. The first group received YR-1 or reserpine; the second, YR-1 or *R. serpentina*; the third, YC-1 or reserpine; and the fourth, YC-1 or *R. serpentina*. All preparations were administered at 3 dose levels corresponding in effect to 1, 2 and 4 mg. of reserpine per 100 g. of rat as judged from the assumed relative potencies. This amounted to 781, 1,562 and 3,124 mg./100 g. of rat for YR-1; 769, 1,538 and 3,176 mg./100 g. of rat for YC-1; and 1,000, 2,000 and 4,000 mg./100 g. of rat for *R. serpentina*. There were 16 rats at each dose level for the first group of rats treated (YR-1 and reserpine) and 20 rats at each dose level for the 3 remaining groups.

Because of the high dose volumes required of the 2 test preparations and *R. serpentina*, each dose was divided equally into 2 or 3 portions administered 45 minutes apart. Each preparation was fed by gastric tube after being suspended in a 0·25 per cent agar solution.

Measured Activity

Two types of activity were measured, ptotic activity and decrease in blood pressure. Ptotic activity was scored in whole numbers ranging from 0 to 4 according to a scale devised by Rubin and others[3,4]. Each eye was scored separately and the scores for both eyes totalled. Observations were made 8, 24 and 48 hours after dosing. Blood pressure readings were obtained on the tail by the plethysmographic method of Williams and others[5]. Measurements were taken before dosing, during, and immediately after the 24-hour period subsequent to dosing.

Results

For all preparations, doses and animals tested, the peak ptotic effect was observed 24 hours after dosing. Average 24-hour readings are shown in Table I. At the middle and highest dose, effects still were present after 48 hours for YR-1 and YC-1 and to some extent *R. serpentina*.

TABLE I

AVERAGE 24-HOUR PTOTIC ACTIVITY IN RATS

Group	Preparations	Dose 1	Dose 2	Dose 3	No. of animals each dose
I	YR-1	2·50	4·35	5·92	14
	Reserpine	2·00	4·14	5·07	14
II	YR-1	2·47	4·42	5·23	19
	R. serpentina	2·15	4·10	5·00	19
III	YC-1	3·05	4·33	6·22	18
	Reserpine	2·50	3·94	5·88	18
IV	YC-1	3·05	4·15	5·95	20
	R. serpentina	2·50	3·85	5·65	20

During the first 24 hours, there was a decrease in average blood pressure at each dose for each preparation tested (Table II). But, in no instance was there a consistent decrease in average blood pressure with increasing dose. Sedation, diarrhoea and decrease in body temperature were common to all animals during the 24-hour period subsequent to dosing. During this same period of time, convulsions were observed at the highest dose in 3 rats on YR-1 and in 2 rats on YC-1. In addition, 2 animals died at the highest dose for YR-1 and 3 animals at the highest dose for YC-1.

TABLE II

MEAN BLOOD PRESSURE IN MM. OF HG. BEFORE AND AFTER A 24-HOUR PERIOD SUBSEQUENT TO DOSING

Preparation	Dose 1 Before	Dose 1 After	Dose 2 Before	Dose 2 After	Dose 3 Before	Dose 3 After
YR-1	99·7 (36)*	76·6 (34)	98·4 (36)	83·9 (32)	97·9 (36)	89·4 (32)
YC-1	95·6 (40)	70·1 (37)	98·6 (40)	81·0 (35)	101·0 (40)	70·4 (34)
Reserpine	99·6 (36)	81·2 (27)	97·1 (36)	73·6 (30)	99·9 (36)	79·5 (33)
R. serpentina	99·6 (40)	69·7 (40)	91·5 (40)	71·0 (40)	101·2 (40)	71·8 (39)

* Number of rats.

Relative potencies were estimated by the method of parallel line assays described in Finney[6]. Results are summarized in Table III for 24-hour ptotic activity only. Estimates of relative potency were not obtained from blood pressure readings, because the dosage-response curves were either flat or increasing within the range of doses selected for this study.

TABLE III

RELATIVE POTENCY DETERMINATIONS FOR *Rauwolfia schueli* YR-1 AND YC-1

Test preparation	Standard	Relative potency in g. of reserpine per kg. of test prep.*	95 per cent confidence limits
YR-1	Reserpine	1·603	1·196, 2·142
YR-1	*R. serpentina*	1·533	1·322, 1·787
YC-1	Reserpine	1·557	1·290, 1·868
YC-1	*R. serpentina*	1·550	1·396, 1·720

* 1 kg. of *R. serpentina* is assumed to be equivalent in effect to 1 g. of reserpine.

Although the relative potencies of YR-1 and YC-1 were higher than they initially were assumed to be, both preparations exhibited the same degree of reserpine-like activity. This is shown in Table III. Inasmuch as the relative potencies of YR-1 and YC-1 did not appear to depend on whether the standard was reserpine or *R. serpentina* administered in equivalent doses, the assumption on which equivalent doses were ascertained, that is, 1 kg. of *R. serpentina* is equivalent in effect to 1 g. of reserpine, was indirectly verified. These findings also suggested that the reserpine-like activity of *R. schueli* is greater than that of *R. serpentina.*

Discussion

Various criteria have been used as a basis for estimating the relative potency of rauwolfia compounds. Reputedly, the problem is a difficult one because the onset of action of reserpine is gradual and the dosage-response curve for most of its activity in mammals is notably flat[7]. In the present study, ptotic activity did provide for good estimates of relative potency. However, peak effects were not observed until about 24 hours after dosing.

Earl[7] suggests a unique biological assay of reserpine based on an all-or-none response. He uses a pigeon emesis test and obtains a relatively steep dosage-response curve. There is a possibility that his method will give estimates of relative potency which are subject to less error than those obtained through an evaluation of ptotic activity.

Convulsions and death are effects which previously have not been ascribed to the pure alkaloid reserpine, although in this study, they were observed at the highest dose for YR-1 and YC-1. Presumably, they also could occur at doses higher than 4 mg./100 g. of rat for reserpine considering that the biological equivalence of the *R. schueli* roots was underestimated in selecting the doses. Furthermore, ptotic activity was more sustained at higher doses for YR-1 and YC-1 than it was for reserpine. Of course, convulsions and death also could have been due to the presence of toxic substances in the whole root.

Rubin and others[1] studied the activity of *R. serpentina*, *R. heterophylla* and *R. canescens*. Based on measures of ptotic activity in the rat, they obtained the following reserpine equivalence ratios: *R. serpentina*, 1 : 401; *R. heterophylla*, 1 : 387; and *R. canescens*, 1 : 258. Inasmuch as it has been shown indirectly that *R. serpentina* is about 1/1,000 as active as reserpine, it would appear that the findings of Rubin and his colleagues are not directly comparable to those of the present study. Conceivably, this could be due to a difference in assay techniques. If the equivalence ratios for *R. heterophylla* and *R. canescens* are multiplied by a factor of 1/2·5 in order to adjust for this difference, the following results are obtained: *R. heterophylla*, 1 : 968 and *R. canescens*, 1 : 645. In the present study, the roots of *R. schueli* were about 1/630 as active as reserpine. Thus, it would appear through these adjustments that in terms of reserpine-like activity, *R. schueli* is about as potent as *R. canescens* but more potent than *R. heterophylla*.

Since all of the active principles of *R. schueli* have not been identified, it cannot be stated that the activity of this species is due simply to its reserpine content. For example, Rubin and others[1] used an isotope dilution method for ascertaining the reserpine content of the three species of rauwolfia root which they studied. They compared these results with those obtained from a biological assay in rats and in each instance they found that the activity of the roots was several times greater than that which could be predicted on the basis of their reserpine content alone. Studies on dogs and monkeys by Cronheim and others[8] and Kohli and Mukerji[9] also suggest that reserpine does not account for all of the

activity found in *R. serpentina* or any of its extracts. La Barre[2] found that reserpine is not the only active principle in *R. vomitoria*. He was able to show that the non-reserpine extract of this species had hypotensive effects in dogs, rabbits and rats and tranquillising effects in dogs which were not unlike those of reserpine.

REFERENCES

1. Rubin, Malone, Waugh and Burke, *J. Pharmacol.*, 1957, **120**, 125.
2. La Barre, *Thérapie*, 1958, **13**, 698.
3. Rubin, Diassi, Burke and Wintersteiner, *Chemical and Pharmacological Studies on Rauwolfia serpentina*, Evans Memorial Research Conference, Symposium on Hypotensive Drugs, Boston, Mass., September 14–15, 1953, p. 91.
4. Rubin and Burke, *Fed. Proc.*, 1954, **13**, 400.
5. Williams, Harrison and Grollman, *J. Clin. Invest.*, 1939, **18**, 373.
6. Finney, Statistical Method in Biological Assay, Charles Griffin and Co., London, 1952.
7. Earl, *J. Pharmacol.*, 1955, **115**, 55.
8. Cronheim, Stepp and Brown, *Chemical and Pharmacological Studies on Rauwolfia serpentina*, Evans Memorial Research Conference, Symposium on Hypotensive Drugs, Boston, Mass., September 14–15, 1953, p. 84.
9. Kohli and Mukerji, *Current Sci.*, 1955, **24**, 198.

ISOTONICITY OF FRUCTOSE, GALACTOSE AND MANNOSE SOLUTIONS

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Received April 19, 1960

A haemolytic method has been used for the determination of the isotonic concentrations of dextrose, fructose, galactose and mannose. Fructose, galactose and mannose produce haemolytic effects deviating from those of dextrose. The isotonic concentration in mM per cent of fructose was found to be 20·3; of galactose, 38·9; of mannose, 33·3; whereas that of dextrose is 26 mM per cent.

THE intravenous carbohydrate alimentation by fructose solutions has been recognised since 1954 in *New and Non-official Remedies*, now *New and Non-official Drugs*, for diabetic patients, since it is metabolised or converted into glycogen in the absence of insulin[1,2]. Galactose and mannose have also been given parenterally in several studies of diabetes and sugar metabolism[3,4]. The molecular weights of these monosaccharides are the same as that of dextrose (anhydrous) and it might be reasonably assumed that their similar properties imply similar isotonic concentrations (about 5 per cent). However, as these sugars might be partially permeable to erythrocytes, only by haemolytic tests can their isotonicity be assessed. A quantitative haemolytic method for determining the degree of disintegration of erythrocytes produced by hypotonic solutions was described by Hunter[5]. Husa and others[6–14] used this method for the determination of isotonic coefficients i of various salts and organic medicinal substances including some sugars.

Cadwallader and Husa[11] emphasised the difference between the physico-chemical Van't Hoff's factor (isotonic coefficient i) and the haemolytic one (haemolytic i) of those compounds which are permeable or which affect the erythrocytes in other ways. Grosicki and Husa[7] suggested the use of the i value of sodium chloride as a standard for the evaluation of the haemolytic data of other compounds, as sodium chloride is practically impermeable to erythrocytes; its physico-chemical i value is also its haemolytic i value, and by reference to this the haemolytic i value of any compound at equivalent molar concentrations could be computed. Thus the haemolytic i value could be substituted for the physico-chemical i value in the equation for determining isotonicity using the freezing point method[15]. The isotonic concentration will coincide with the iso-osmotic concentration only where the erythrocytes are not affected by increased permeability, agglutination or even slight haemolysis.

EXPERIMENTAL

Materials

Sodium Chloride B.P.; Dextrose B.P.; D-(−)-fructose, analytical reagent grade, optical rotation of 9·579 per cent solution = − 16·7°; specific rotation = − 87·2°; D-(+)-galactose, analytical reagent grade, optical rotation of 10·04 per cent solution = + 16°, specific rotation = + 79·6°;

D-(+)-mannose, analytical reagent grade, optical rotation of 9·77 per cent solution =+2·6°, specific rotation =+ 14°; water for injection U.S.P. and B.P., boiled before use.

Method

Solutions were made of 0·7 per cent sodium chloride and 10 per cent of each sugar respectively, in water for injection. The solutions were serially diluted. Blood was added in 0·02 ml. portions to 4 ml. of each solution in a test tube. After admixing, the test tubes were set aside at room temperature for 2 hours, and centrifuged at 3,000 r.p.m. for one minute. The light transmission of the supernatant fluid containing the liberated oxyhaemoglobin was determined by a Klett-Summerson photoelectric colorimeter using No. 54 green filter; the sensitivity being increased by a Kipp galvanometer[16]. Two readings were made for each concentration and were then averaged. The amount of haemolysis at the different concentrations of salt and sugars was calculated as a percentage of haemolysis obtained by laking the erythrocytes in a 0·1 per cent sodium carbonate solution which might reasonably be considered as complete haemolysis.

The resistance of the erythrocytes to haemolysis can also be evaluated by determining the maximum concentration of sodium chloride causing 100 per cent haemolysis of the blood of different donors; the lower this concentration, the greater is the erythrocyte's resistance to haemolysis. The haemolytic tests are therefore reproducible only with the same blood but the variations from one healthy donor's blood to another are slight. The effects of these variations could be minimised by averaging the results of the haemolytic tests for each of the compounds of the different donors' blood at equivalent conditions. At concentrations where haemolysis is increased by subsequent dilution, the per cent haemolysis is directly proportional to the hypotonicity, and therefore can be used for computing the haemolytic *i* value and isotonicity.

The *i* value calculation is essentially similar to that suggested by Grosicki and Husa[7] but concentrations are expressed in millimols per cent, in accordance with the following equation:

$$i \text{ (haemolytic) value of compound} = [i \text{ (haemolytic) value of NaCl}] \times \frac{(\text{mM}a + \text{mM}b + \text{mM}c + \text{mM}d + \text{mM}e)}{(\text{mM}f + \text{mM}g + \text{mM}h + \text{mM}i + \text{mM}j)} \quad \ldots \quad (1)^*$$

$$\text{mM per cent of an isotonic solution} = \frac{\text{R}}{i \text{ (haemolytic)}} \times 100 \quad \ldots \quad \ldots \quad (2)^\dagger$$

* *a*, *b*, *c*, *d*, and *e* represent concentrations of sodium chloride in millimols per cent, at 25, 35, 50, 60, and 75 per cent haemolysis whereas *f*, *g*, *h*, *i* and *j* represent the equivalent concentrations of the test compound in millimols per cent, respectively.

† R is the constant osmotic ratio T_f/K_f derived from the freezing point equation of isotonicity[15] which could be rewritten as following: $m = T_f/K_f \times 1/i$; R being a ratio is entirely independent of the freezing point method (and its units) and is equivalent to 0·28 (m = molal concentration, T_f = freezing point depression of blood (−0·52°), K_f = molal freezing point depression constant for water (−1·855°), i = Van't Hoff's factor).

The multiple of mM concentration and the haemolytic i value gives the haemolytic milliosmol units, which should be differentiated from the milliosmol units defined as the multiple of Van't Hoff's factor and millimolar concentration. For isotonicity, the haemolytic milliosmol units should be adopted, and from equation (2) a 0·28 haemolytic-osmolar concentration is equivalent to 28 haemolytic milliosmols per cent, which represents the isotonic concentration with blood serum.

This method for the evaluation of isotonicity is applicable to any of the pharmaceutical injectable compounds including those producing exo-osmotic or endo-osmotic effects on erythrocytes provided that the compounds are non-haemolytic in iso-osmotic concentrations with blood serum.

Experimental and Results

Preliminary Tests

The equivalent per cent haemolysis at each concentration of sodium chloride of the preliminary tests is recorded in Table I. The results confirm those of Grosicki and Husa[7] that the haemolytic tests are sensitive in the range of 0·30 to 0·50 per cent sodium chloride. By plotting the per cent haemolysis against salt concentration a sigmoid curve is obtained. The sensitive range with the monosaccharides (see Table II) is about 1 to 3 per cent and plots produce similar sigmoid curves.

TABLE I

Haemolysis per cent of sodium chloride solutions at various concentrations

Concentration units	Sodium chloride concentrations								
g. per cent	0·15	0·20	0·25	0·30	0·35	0·40	0·45	0·50	0·60
mM per cent	2·56	3·42	4·27	5·13	5·98	6·84	7·69	8·55	10·26
mOsmols per cent	4·76	6·36	7·94	9·54	10·02	12·74	14·20	15·90	19·08
Haemolysis per cent	100	100	100	96	93	76	10	2	0

Haemolysis Tests

Three healthy donors were chosen for the subsequent haemolysis tests which were made at close arithmetic dilutions within the sensitive range of concentrations as following:

Sodium chloride: 0·36, 0·4, 0·44, 0·48, 0·52 and 0·9 g. per cent respectively.

Monosaccharides: 0·2, 0·5, 0·8, 1·2, 1·5, 1·8, 2·1, 2·4, 2·7, 3·0, 3·3, 3·6, 3·9, 4·2, and 4·5 g. per cent respectively.

Haematologic Data of the Blood Donors

Donor Z (female), age 20, erythrocytes = 4,500,000, haemoglobin 90 per cent. The highest concentration of sodium chloride causing about complete haemolysis was 0·4 per cent.

Donor M (male), age 23, erythrocytes = 4,400,000, haemoglobin = 89 per cent. The highest concentration of sodium chloride causing complete haemolysis was 0·36 per cent.

Donor Y (male), age 24, erythrocytes = 4,400,000, haemoglobin = 88 per cent. The highest concentration producing about complete haemolysis was 0·1 per cent.

TABLE II

HAEMOLYSIS* PER CENT PRODUCED BY DEXTROSE, FRUCTOSE, GALACTOSE AND MANNOSE SOLUTIONS

Concentrations per cent			Dextrose	Fructose	Galactose	Mannose
g.	mM	mOsmol.	Per cent haemolysis	Per cent haemolysis	Per cent haemolysis	Per cent haemolysis
1	5·55	5·55	100	92	98	100
2	11·10	11·10	93	10	97	93
3	16·65	16·65	60	2	50	40
4	22·02	22·20	2	0	12	2

* Blood was from the donor of blood for the results in Table I.

Calculation of the Haemolytic i *Value and Isotonicity*

The average readings of light transmission of each blood sample at the concentrations of sodium chloride and monosaccharides used were converted into per cent haemolysis with reference to the total haemolysis obtained by laking each blood sample in 0·1 per cent sodium carbonate. Plotting the per cent haemolysis against the strength of sodium chloride and monosaccharides, respectively, the concentrations producing 25, 35, 50, 60, 75 per cent haemolysis were derived.

The data obtained from the three blood specimens was averaged for each degree of haemolysis produced by the sodium chloride and the dextrose, fructose, galactose and mannose respectively using equation (1). The haemolytic i values of the monosaccharides were computed reckoning the i value of sodium chloride as 1·86[7]. These calculated haemolytic i values at the respective per cent haemolysis are tabulated in Table III, the last column representing the average value which might reasonably be considered as the haemolytic i value.

TABLE III

HAEMOLYTIC i VALUES OF MONOSACCHARIDES

Monosaccharide	At 25 per cent haemolysis	At 35 per cent haemolysis	At 50 per cent haemolysis	At 60 per cent haemolysis	At 75 per cent haemolysis	Haemolytic i value
Dextrose ..	1·014	1·075	1·073	1·099	1·122	1·076
Fructose* ..	1·346	1·346	1·375	1·432	—	1·376
Galactose ..	0·724	0·722	0·718	0·728	0·715	0·721
Mannose ..	0·852	0·836	0·834	0·842	0·845	0·842

* The blood specimen y did not yield to 75 per cent haemolysis within the range of concentrations of fructose studied and therefore the haemolytic i value at this degree of haemolysis was not ascertained. Based on the other two blood specimens, the haemolytic i value for this sugar was 1·408.

Our results for dextrose differ slightly from those obtained by Grosicki and Husa[7], who found the i value to be 1·17. The haemolytic effects of fructose deviated considerably from those of the other monosaccharides and indicate significant exosmosis; in one sample, fructose agglutinated the red blood corpuscles, which might be interpreted as a further effect on

the permeability of the cells. On the other hand, galactose and mannose solutions produce the opposite effect to that of fructose, and even dextrose, on the permeability of erythrocytes indicating a certain degree of endosmosis[7].

From the haemolytic *i* values isotonicity was calculated using equation (2). The results are in Table IV.

TABLE IV

ISOTONIC CONCENTRATIONS OF DEXTROSE, FRUCTOSE, GALACTOSE AND MANNOSE

	Haemolytic *i* value	Isotonic concentrations per cent		
		g.	mM or milliosmols	haemolytic milliosmols
Dextrose	1·076	4·68	26	28
Fructose	1·376	3·65	20·3	28
Galactose	0·721	7·00	38·9	28
Mannose	0·842	6·00	33·3	28

Acknowledgements. We wish gratefully to acknowledge the assistance given by the late Professor F. Rappaport and the colleagues of the Central Pharmacy for Government Hospitals. The preliminary tests were made by Y. Yossifov (graduate thesis, M. Pharm., Hebrew University, Jerusalem).

REFERENCES

1. *New and Non-official Drugs*, 1959, p. 632.
2. Lane and Dodd, *Pediatrics*, 1957, **20**, 668.
3. Barta and Gothy, *Ann. Pediat.*, 1955, **184**, 287.
4. Bhatacharya, *Science*, 1953, **117**, 230.
5. Hunter, *J. Clin. Invest.*, 1940, **19**, 691.
6. Cadwallader and Husa, *Amer. J. Pharm.*, 1957, **129**, 393.
7. Grosicki and Husa, *J. Amer. Pharm. Ass., Sci. Ed.*, 1954, **43**, 632.
8. Easterly and Husa, *ibid.*, 1954, **43**, 750.
9. Hartman and Husa, *ibid.*, 1957, **46**, 430.
10. Thomasson and Husa, *ibid.*, 1958, **47**, 711.
11. Cadwallader and Husa, *ibid.*, 1958, **47**, 705.
12. Ansel and Husa, *ibid.*, 1959, **48**, 516.
13. Zonowiak and Husa, *ibid.*, 1959, **48**, 565.
14. Marcus and Husa, *ibid.*, 1959, **48**, 569.
15. Martin and Cook, *Remington's Practice of Pharmacy*, XI Edn, Mack Publishing Co., Easton, Pennsylvania, 1956, p. 160.
16. Rappaport and Eichorn, *Lab. Digest.*, Nov. 1955.

A PHYTOCHEMICAL SURVEY OF THE HONG KONG MEDICINAL PLANTS

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Received April 26, 1960

332 species of plants from the colony of Hong Kong have been tested for the presence of alkaloids. 38 gave strong positive reactions.

THE Colony of Hong Kong possesses some 2,000 plant species, about 20 per cent of which are used in folk-medicine. Of the 332 medicinal species tested in this survey for the presence of alkaloids, 38 (11·4 per cent) gave strong positive reactions and the presence of alkaloids in these species is indicated. The occurrence in *Zanthoxylum avicennae*[1], *Z. nitidum*[2,3], *Ruta graveolens*[4], *Nelumbo nucifera*[5], *Justicia gendarussa*, *J. ventricosa*, *Anisomeles indica*, and *Murraya paniculata*, has been verified by detailed chemical examination in this laboratory.

The reader is referred to similar surveys carried out in Australia[6], the United States[7,8], Russia[9–13], Argentina[14], North Borneo[15], Papua and New Guinea[16], Malaya[17], and Hawaii[18].

PROCEDURE

Fresh plant-material was obtained from herbalists whose supplies originate from the Island, the New Territories, and outlying islands, including Lan Tao. Tests were performed on two extracts.

Hydrochloric acid extract. Chopped plant material (about 2 g.) was covered with 5 per cent. hydrochloric acid in a specimen tube (1·5 cm. × 7·5 cm.) for 48 hours. Drops of the extract were tested separately with a drop of each of the alkaloid testing reagents.

Prollius extract. Extraction was carried out as for the acid extraction but with Prollius fluid[19] substituted for hydrochloric acid. The decanted extract was allowed to evaporate on a watch-glass and the residue was taken up with two drops of 1 per cent hydrochloric acid. The acid solution was divided into three portions on a glass slide and tested with a drop of each of three alkaloid reagents.

Alkaloid reagents. The alkaloid reagents which were prepared as stated by Henry[19] were: iodine—potassium iodide solution (I); Mayer's reagent (M); silicotungstic acid (S); phosphotungstic acid (P); and Dragendorff's reagent (D).

The strength of precipitate obtained in the tests was judged by eye and was recorded thus: xxxx, very strong precipitate; xxx, strong precipitate; xx, moderate precipitate; x, negligible precipitate; –, no precipitate

Plant extracts which in the main showed xxxx and xxx on testing were considered to contain alkaloids and those plants are designated *A* in

Table 1. Using the method of Douglas and Kiang[17], the following controls were made:

Solution	Concentration (w/v)	Precipitates with reagents				
		I	M	S	P	D
Brucine hydrochloride ..	1 : 100	xxxx	xxxx	xxxx	xxxx	xxxx
	1 : 500	xxx	xxx	xxx	xx	xxx
	1 : 2,500	xx	xx	x	x	x
	1 : 10,000	x	x	–	–	–
Quinine sulphate ..	1 : 100	xxxx	xxxx	xxxx	xxxx	xxxx
	1 : 500	xxx	xxx	xxx	xxx	xxx
	1 : 2,500	xxx	xxx	xx	xx	xxx
	1 : 10,000	xx	xx	x	x	xx

TABLE I*

* (l) refers to leaves; (s), stems; (r), root; (b), bark; (fl.), flower; (fr), fruit; (wp), whole plant or herb (sb), stem bark; (rb), root bark; (rw), root wood; (l, s), leaves and stems.

LIST OF PLANTS EXAMINED

ACANTHACEAE—*Acanthus ilicifolius* L., (r); *Dicliptera chinensis* (Vahl.) Nees. (l); *Hygrophila salicifolia* Nees. (wp); *Hypoestes purpurea* (L.), Soland, (l); *Justicia gendarussa* Burm. f. (l) *A*, *Justicia ventricosa* Wall. (l) *A*, (s) *A*; *Lepidagathis incurva* Don. (wp); *Rhinacanthus communis* Nees. (l), (s).

AGAVACEAE—*Agave angustifolia* Haw?, (wp); *Sansevieria zeylanica* Willd. (l), (r).

AMARANTHACEAE—*Alternanthera sessiles* R. Br., (r), (l, s); *Alternanthera versicolor* Regel. (wp); *Amaranthus spinosus* L. (l), (s), (r).

AMARYLLIDACEAE—*Crinum asiaticum* L. (l) *A*; *Crinum asiaticum* L. var. *Sinicum* Baker (r) *A*; *Curculigo orchidiodes* Gaertin., (r); *Lycoris radiata* Herb., (l); (r) *A*; *Zephyranthes carinata* Herb., (l) *A*; (r) *A*.

ANACARDIACEAE—*Dracontomelon dao* (Blanco.) Merr., (l), (s); *Mangifera indica* L. (l); *Rhus hypoleuca* Champ., (l), (s).

APOCYNACEAE—*Melodinus suavolens* Champ., (fr); *Nerium indicum* Mill. (l), (s); *Strophanthus divaricatus* (Lour.) H. & A., (fr) *A*, (l), (sb) *A*.

AQUIFOLIACEAE—*Ilex asprella* Champ., (l), (s); *Ilex rotunda* Thunb., (sb).

ARACEAE—*Acorus gramineus* Soland, (l), (r); *Aglaonema modestum* Schott., (l), (s); *Alocasia odora* (Roxb.) Koch, (r); *Arisaema japonicum* Bl.? (s), (r); *Epipremnum pinnatum* (L.) Engler., (l), (s); *Lasia spinosa* (L.) Thw., (l), (s); *Pistia stratiotes* L., (wp); *Typhonium divaricatum* (L.) Decne. ,(r) *A*, (l, s); *Typhonium frilobatum* Schott.?, (l, s), (bulb r).

ARALIACEAE—*Acanthopanax trifoliatus* (L.) Merr., (l), (s); *Schefflera octophylla* (Lour.) Harms., (l).

ASCLEPIADACEAE—*Toxocarpus wightianus* H. & A.? (l, s); *Tylophora ovata* Hook., (r) *A*, (l, s) *A*.

BALSAMINACEAE—*Impatiens chinensis* L., (s, l).

BAMBUCACEAE—*Bambusa pervariabilis* McClure? (s); *Bambusa spp.*

BOMBACACEAE—*Gossampinus malabarica* Merr., (l), (b).

BURSERACEAE—*Canarium album* Raeusch., (l), (s); *Canarium pimela* Koenig., (l), (s).

CAESALPINIACEAE—*Bauhinia glauca* Wall., (l), (s); *Caesalpinia crista* L. (seeds); *Caesalpinia vernalis* Champ., (wp); *Cassia occidentalis* L. (l), (r); *Cassia tora* L., (l, s), (r).

CACTACEAE—*Hylocereus undatus* (Haw.) B. & R., (l), (s); *Opuntia dillenii* (Ker.) Haw., (s).

CAPRIFOLIACEAE—*Lonicera confusa* DC., (l), (b); *Sambucus javanica* Reinw.. (l), (s).

CARYOPHYLLACEAE—*Polycarpaea corymbosa* Lam., (wp).

CARICACEAE—*Carica papaya* L., (l).

CHENOPODIACEAE—*Chenopodium ambrosioides* L., (l), s; *Achyranthes aspera* L., (wp).

CHLORANTHACEAE—*Chloranthus glaber* Thunb., (wp).

COMBRETACEAE—*Quisqualis indica* L., (l), (s).

COMMELINACEAE—*Aneilema malabarium* (L.) Merr., (wp); *Commelina nudiflora* L., (l, s); *Cyanotis kewensis* C. B. Clarke, (wp); *Rhoeo discolor* (L'Her.) Hance, (l).

COMPOSITAE—*Artemisia vulgaris* L., (l); *Aster ageratoides* Turcq. (wp); *Bidens pilosa* L., (l, s), (r), (fl); *Blumea balsamifera* DC., (l, s); *Centipeda minima* A. Br. et Aschers., (l, s); *Chrysanthemum indicum* L., (l, s); *Chrysanthemum morifolium* Ram., (l, s); *Crossostephium chinense* (L.) Mak. ex Champ. & Schlecht.. (wp); *Eclipta alba* (L.) Hassk., (wp); *Elephantopus scaber* L., (wp); *Emilia sonchifolia* (L.) DC., (wp) *A*; *Gynura crepidiodes* Benth.?, (wp); *Gynura divaricatus* (L.) DC., (l); *Gynura segetum* Merr., (l, s); *Inula cappa* DC., (l), (sb), (fl); *Laggera alata* Sch.-Bip., (l), (s), (s, l); *Pluchea indica* (L.) Less, (l, s); *Senecio scandens* Ham., (l), (s); *Solidago virgo-aurea* L., (wp); *Taraxacum officinale* Wigg., (l, s); *Vernonia cinerea* (L.) Less., (wp); *Vernonia patula* (Ait.) Merr., (wp); *Xanthium straumarium* L., (wp).

CONNARACEAE—*Rourea microphylla* Planch., (l, s).

CONVOLVULACEAE—*Convolvulus obscura* Ker., (wp); *Ipomoea pes-caprae* (L.) Roth., (l, s); *Merremia gemella* (Burm.) Hall. f., (wp); *Operculina turpethum* S. Manso., (l, s).

CRASSULACEAE—*Kalanchoe laciniata* (L.) Pers., (l); *Kalanchoe pinnata* Pers., (l, s).

CRUCIFERAE—*Capsella bursa-pastoris* Moench., (wp); *Nasturtium montanum* Wall., (wp).

CUCURBITACEAE—*Melothria heterophylla* (Lour.) Cogn., (l, s), (s); *Momordica charantia* L., (wp).

CYATHACEAE—*Cibotiva barometz* (L.) Sm., (s).

CYPERACEAE—*Cyperus alternifolius* L., (l), (fl); *Cyperus malaccensis* Lam. ?, (l, r); *Cyperus rotundus* L., (wp).

DILLENIACEAE—*Tetracera scandens* (L.) Merr., (l).

DIOSCOREACEAE—*Dioscorea hispida* Dennst., (r) *A*.

EBENACEAE—*Diospyros vaccinioides* Lindl., (s), (l), (fr).

ELAEOCARPACEAE—*Elaeocarpus* sp. ? (l), (s).

EUPHORBIACEAE—*Acalypha australis* L., (wp); *Bischofia trifoliata* (Roxb.) Hook., (l), (s); *Bridelia monoica* (Lour.) Merr., (l), (s); *Breynia fruticosa* (L.) Hook. f., (l), (s); *Claoxylon polot* (Burm.) Merr., (l), (s); *Croton crassifolius* Geisel., (wp); *Croton tiglium* L., (l); *Euphorbia antiquorum* L., (l); *Euphorbia hirta* L. var. *typica* L. C. Wheeler, (wp); *Euphorbia thymifolia* L., (l, s); *Glochidion eriocarpum* Cham., (l), (s); *Mallotus apelta* Muell., (l) (s), *A*; *Phyllanthus cochin-chinensis* Spreng., (l, s); *Phyllanthus emblica* L., (l), (sb); *Phyllanthus urinaria* L., (wp); *Ricinus communis* L., (l), (s); *Sapium sebiferum* Roxb., (l, s); *Sauropus rostratus* Miq., (l).

TABLE I—*continued*

GRAMINEAE—*Cymbopogon citratus* Stapf., (l); *Imperata cylindrica* (L.) Beauv., (r), (fl); *Oryza sativa* L.. (budding seed); *Phragmites communus* Trin., (s); *Pogonatherum paniceum* (Lam.) Hack, (wp); *Saccharum officinarum* L., (s).
GUTTIFERAE—*Garcinia multiflora* Champ., (l, s).
HAMAMELIDACEAE—*Liquidambar formosana* Hance, (l), (sb).
HYPERICACEAE—*Cratoxylon ligustrinum* (Spach.) Bl., (l, s); *Hypericum japonicum* Thunb., (wp).
LABIATAE—*Anisomeles indica* (L.) O. Ktze., (l) *A*, (b) *A*?; *Coleus aromaticus* Benth., (l), (s); *Glechoma hederaceae* L., (wp); *Leonurus sibiricus* L., (l, s), (r); *Mentha arvensis* L., (wp); *Mentha rotundifolia* Huds., (l, s); *Ocimum basilicum* L., (l, s); *Orthodon lanceolatum* (Benth.) Kudo, (wp); *Perilla frutescens* (L.) Britton, (l); *Teucrium viscidum* Bl., (wp).
LAURACEAE—*Cassytha filiformis* L., (s) *A*; *Cinnamomum camphora* Sieb., (l), (s); *Cinnamomum japonicum* Sieb., (l), (s); *Litsea cubeba* (Lour.) Pers, (l), (b) *A*; *Litsea glutinosa* C.B. Rob., (l) *A*, (s) *A*; *Litsea rotundifolia* Hemst. var. *oblongifolia* Allen, (l), (sb).
LILIACEAE—*Aloe vera* L. var. *chinensis* (Haw.) Berger, (wp); *Chlorophytum capense* Kuntze, (l), (r); *Cordyline fruticosa* A. Cheval, (l); *Dianella ensifolia* (L.) DC., (l); *Hemerocallis fulva* L., (l), (r).
LOBELIACEAE—*Lobelia chinensis* Lour., (wp) *A*.
LOGANIACEAE—*Gelsemium elegans* Bth., (l, s) *A*; *Strychnos angustiflora* Benth., (l) *A*, (s) *A*.
LYCOPODIACEAE—*Lycopodium cernum* L., (l, s), (r).
LYTHRACEAE—*Lawsonia inermis* L., (l, s).
MAGNOLIACEAE—*Magnolia coco* DC., (l) *A*, (s) *A*.
MALVACEAE—*Abutilon indicum* (L.) G. Don, (l), (fl), (b); *Hibiscus mutabilis* L., (l, s); *Hibiscus rosa-sinensis* L., (l), (s); *Sida acuta* Burm. f., (l, s); *Sida fallax* Walp., (wp); *Sida rhombifolia* L., (l, s).
MELASTOMACEAE—*Melastoma dodecandrum* Lour., (l, s); *Melastoma sanguineum* Sims, (l, s).
MELIACEAE—*Melia azedarach* L., (l). (s).
MENISPERMACEAE—*Stephania hernandifolia* Walp., (l, s) *A*; *Stephania* sp., (wp) *A*; *Thinospora sinensis* Merr., (s).
MIMOSACEAE—*Acacia confusa* Merr.. (l); *Mimosa pudica* L., (wp); *Pithecollobium lucidum* Benth., (l, s).
MORACEAE—*Artocarpus linganensis* Merr., (l), (sb); *Ficus hirta* Vahl., (l), (s); *Ficus hispida* L. f., (l), (s); *Ficus pumila* L., (l), (s); *Ficus pyriformis* Hook. & Arn., (l, s), (tendrils); *Ficus retusa* L., (tendrils); *Ficus wightiana* Benth., (l, s); *Morus alba*, (l, sb).
MYRSINACEAE—*Ardisia crispa* A. DC., (l), (s), (r); *Ardisia punctata* Lindl., (l, s); *Embelia laeta* (L.) Mez., (l, s); *Embelia obongifolia* Hemsl., (l, s).
MYRTACEAE—*Baeckea frutescens* L., (l, s); *Cleistocalyx operculatus* (Roxb.) Merr., (l, s); *Psidium guajava* L., (l, s); *Syzygium jambos* (L.) Alston, (l, s).
PAPILIONACEAE—*Abrus cantoniensis* Hance, (l) *A*, (s) *A*?, (r) *A*; *Crotalaria saltiana* Andr., (l), (s); (l) *A*, (s) *A*; *Desmodium styracifolium* (Osb.) Merr., (wp); *Eriosma chinense* Vogel, (l, s), (rb); *Indigofera suffruticosa* Mill., (wp); *Millettia dielsiana* Harms., (l, s); *Millettia speciosa* Champ., (l), (sb); *Moghania prostrata* (Roxb.) Wang & Tang, (l, s), (r); *Ormosia emarginata* Bth., (l, s) *A*; *Phaseolus lunatus* L., (l, s); *Phyllodium pulchellum* (Bth.) Desv., (l, s) *A*; *Pteroloma triquetrum* Benth., (wp); *Pueraria thunbergiana* (S. & Z.) Benth., (s); *Smithia conferta* Sm., (l, s); *Zornia diphylla* Pers., (wp).
NEPENTHACEAE—*Nepenthes mirabilis* (Lour.) Druce, (l, s).
NYCTAGINACEAE—*Mirabilis jalapa* L., (r) *A*.
NYMPHAEACEAE—*Nelumbo nucifera* Gaertn., (l) *A*, (fr).
OLEACEAE—*Osmanthus fragrans* Lour., (l, s).
ONAGRACEAE—*Jussiaea repens* L., (wp).
ORCHIDACEAE—*Acampe multiflora* Lindl., (l); *Pholidota chinensis* Lindl., (l) *A*, (r).
OXALIDACEAE—*Averrhoa carambola* L., (l), (s); *Oxalis repens* Thunb., (wp).
PALMACEAE—*Areca catechu* Willd?, (wp).
PANDANACEAE—*Pandanus tectorius* Parkinson, (r); *Pandanus* sp., (l).
PASSIFLORACEAE—*Passiflora foetida* L., (l), (s).
PINACEAE—*Pinus massoniana* Lamb., (fl); *Thuja orientalis* L., (l).
PIPERACEAE—*Piper betle* L., (l); *Piper sarmentosum* Roxb., (l), (s).
PLANTAGINACEAE—*Plantago major* L., (wp).
PLUMBAGINACEAE—*Plumbago zeylanica* L., (l), (s).
POLYGALACEAE—*Polygala chinensis* L., (l, s).
POLYGONACEAE—*Polygonum chinense* L., (l, s); *Polygonum hydropiper* L., (l, s); *Polygonum pedunculare* Wall.?, (l, s); *Polygonum perfoliatum* L., (wp); *Polygonum plebeium* R. Br., (wp).
POLYPODIACEAE—*Acrosticum aureum* L., (l, s); *Adiantum fabellulatum* L., (wp); *Athyrium lanceum* (Thunb.) Milde, (l), (s, r); *Odontosoria chinensis* (L.) J. Sm., (wp); *Pteris ensiformis* Burm., (wp); *Pteris semipinata* L., (wp); *Pyrrosia adnascens* (Forst.) Ching, (wp).
PORTULACACEAE—*Portulaca oleracea* L., (l, s), (r); *Portulaca pilosa* L., (wp).
PONTEDERIACEAE—*Eichornia crassipes* Solms., (wp).
PUNICACEAE—*Punica granatum* L., (l, s).
RANUNCULACEAE—*Clematis meyeniana* Walp., (l, s); *Ranunculus cantoniensis* DC., (l), (s).
RHAMNACEAE—*Berchemia racemosa* S. & Z., (l, s).
ROSACEAE—*Duchesnea indica* (L.) Foche, (wp); *Eriobotrya japonica* Lindl., (l); *Prunus mume* S. & Z., (s); *Prunus persica* (L.), Batsch., (l. s); *Pyrus sinensis* Lindl., (s); *Raphiolepis indica* Lindl., (l), (s), (rb) (fl) *Rosa laevigata* Michx., (s), (r); *Rosa wichuraiana* Crep., (l, s); *Rubus parvifolius* L., (l, s), (rb); *Rubus reflexus* Kev., (l, s).
RUBIACEAE—*Adina pilulifera* (Lam.) Franch, (l, b); *Gardenia jasminoides* Ellis, (l), (s); *Hedyotis acutangula* Champ., (l, s); *Hedyotis loganiodes* Benth., (l), (sb); *Lasianthus chinensis* Benth., (l); *Morinda umbellata* L., (l, s); *Mussaenda pubescens* Ait. f., (l, s); *Oldenlandia auricularia* F.-Muell., (l, s) *A*, (r) *A*?; *Oldenlandia hedyotidea* (DC.) Hand.-Mazz., (l), (s); *Paederia scandens* (Lour.) Merr., (l, s); *Psychotria rubra* (Lour.) Poir., (l, s); *Psychotria serpens* L., (wp); *Tricalysia viridiflora* (DC.) Matsum, (l, s).
RUTACEAE—*Atalantia buxifolia* (Benth.) Oliv., (l, s); *Citrus grandis* (L.) Osbeck, (l, s); *Citrus limonia* Osbeck, (l); *Clausena lansium* (Lour.) Skeels, (l), (s), (rb), (rw); *Evodia lepta* (Spreng.) Merr., (l), (sb); *Glycosmis citrifolia* Lindl., (l), (s); *Murraya paniculata* (L.) Jack, (l) *A*, (sb) *A*?; *Ruta graveolens* L., (wp) *A*; *Zanthoxylum avicennae* (Lam.) DC. (l) *A*, (s) *A*, (fr) *A*; *Zanthoxylum nitidum* (Lam.) DC., (l), *A*, (sb), *A*.
SALICACEAE—*Salix babylonica* L., (l, s).
SANTALACEAE—*Henslowia frutescens* Champ., (l), (s).
SAPINDACEAE—*Cardiospermum halicacabum* L., (l, s); *Euphoria longan* (Lam.) Steud., (l), (s); *Sapindus mukorossi* Gaertn., (l), (s).
SAXIFRAGACEAE—*Saxifraga stolonifera* Merrb., (wp).
SAURURACEAE—*Houttuynia cordata* Thunb., (wp); *Saururus chinensis* (Lour.) Baill., (l), (s, r).

TABLE I—*continued*

SCHIZALACEAE—*Lygodium japonicum* Swartz, (wp).
SCHROPHULARIACEAE—*Adenosma glutinosum* (L.) Druce, (fl), (l), (s); *Bacopa monniera* (L.) Wettst., (wp); *Buchnera cruciata* Han., (wp); *Scoparia dulcis* L., (wp).
SELAGINELLACEAE—*Selaginella atroviridis* Spring, (wp); *Selaginella involvens* Spring, (wp).
SMILACACEAE—*Heterosmilax quadichaudiana* Maxim., (l), (r); *Smilax china* L., (l), (s); *Smilax glabra* Roxb., (l), (s).
SOLANACEAE—*Capsicum frutescens*, (l, s); *Datura metel* L., (l) *A*, (s) *A*; *Lycium chinense* Mill., (r).
STERCULIACEAE—*Firmiana simplex* (L.) Wight, (l, s); *Helicteres angustifolia* L., (l), (b); *Pterospermum heterophyllum* Hance, (l), (s); *Sterculia nobilis* Sm., (l).
SYMPLOCACEAE—*Symplocus lansifolia* S. & Z., (l, s).
TAMARICACEAE—*Tamarix chinensis* Lour., (l).
TAXACEAE—*Podocarpus macrophylla* Don., (l), (sb).
TERNSTROEMIACEAE—*Eurya chinensis* R. Br., (wp).
THYMELAEACEAE—*Wikstroemia indica* (L.) C. A. Mey, (l), (s).
TILIACEAE—*Microcos paniculata* L., (l), (sb).
ULMACEAE—*Trema orientalis* (L.) Bl., (l, s).
UMBELLIFERAE—*Centella asiatica* (L.) Urban., (wp); *Hydrocotyl esibthorpioides* Lam., (wp).
URTICACEAE—*Boehmeria nivea* Gaud., (l), (s); *Pouzolzia zeylanica* (L.) Benn., (wp).
VERBENACEAE—*Avicennia marina* (Forsk.) Vierk., (l, s); *Callicarpa nudiflora* H. & A., (l), (s); *Clerodendron crytophyllum* Turcz., (l), (s); *Clerodendron fortunatum* L., (l, s); *Clerodendron fragrans* Vent., (l), (s); *Clerodendron inerme* Gaertn., (l, s); *Lantana camara* L., (l), (s); *Stachytarpheta jamaicensis* L., (l) *A*, (s); *Vitex negundo* L., (l, s); *Vitex trifolia* L. var. *unifoliata* Schauer, (l, s).
VIOLACEAE—*Viola inconspicua* Bl., (wp).
VITACEAE—*Ampelopsis brevipedunculata* (Max.) Koehne., (l, s); *Ampelopsis cantoniensis* (H. & A.) Planch, (l, s); *Vitis flexuosa* Thunb., (l, s), (wp).
ZINGIBERACEAE—*Alpinia* sp.? (r); *Curcuma zedoaria* (Berg.) Rose, (r) *A*.

Acknowledgements. The authors thank Professor J. E. Driver for his interest, Mr. H. C. Tang and Mrs. T. K. Leung (Government Herbarium, Hong Kong) for identification of plant species, the Research Grants Committee of the University of Hong Kong, and Smith, Kline and French Laboratories, Philadelphia, for grants-in-aid.

REFERENCES

1. Arthur, Hui and Ng, *J. chem. Soc.*, 1959, 4007.
2. Arthur, Hui and Ng, *ibid.*, 1959, 1840.
3. Arthur and Ng, *ibid.*, 1959, 4010.
4. Arthur and Cheung, in the press.
5. Arthur and Cheung, *J. chem. Soc.*, 1959, 2306.
6. Webb, *Australian Phytochemical Survey, Bulletins, Commonwealth Sci. Ind. Res. Organisation, Australia*, 1949, No. 241; 1952, No. 268.
7. Willaman and Schubert, *Economic Botany*, 1955, **9**, 141.
8. Wall, Krider, Krewson, Eddy, Willaman, Correll and Gentry, *J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 1.
9. Lazur' evskii and Sadykov, *Trudy Uzbekskogo Gosudarst. Univ., Sbornik. Trudov Khim.*, 1939, **15**, 182.
10. Efros, *Farmatsiya*, 1946, **9**, No. 5, 22.
11. Massagetov, *ibid.*, 1946, **9**, No. 3, 22.
12. Hededus, *Magyar Kem. Lapia*, 1950, **5**, 239.
13. Zolotnitskaya, *Izvest. Akad. Nauk Armyan. S.S.R., Biol., i Sel'khoz Nauki*, 1954, No. 5, 7, 27.
14. Codoni, *Argentina Direct. Forest. Publ. Tech.*, 1949, **9**, 3.
15. Arthur, *J. Pharm. Pharmacol.*, 1954, **6**, 66.
16. Webb, *Pacific Science*, 1955, 430.
17. Douglas and Kiang, *Malayan Pharm. J.*, 1957, **6**, 138.
18. Swanholm, St. John, Scheuer, *Pacific Sci.*, 1959, **13** (3), 295; 1960, **14** (1), 68.
19. *Allen's Commercial Organic Analysis*, 5th Edn, Vol. 7, 1929, (reprinted 1948), p. 6; pp. 20–29.

NEW APPARATUS

A DIFFUSION CELL FOR THE PRODUCTION OF VERY SHARP BOUNDARIES

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Received May 19, 1960

A diffusion cell for the production of very sharp boundaries between aqueous liquids is described, and the Δt correction can be eliminated in most instances. The cell has been tested with substances whose diffusion coefficients are known. Some results for the diffusion of cetomacrogol 1,000 in water are reported.

GENERALLY in diffusion measurements, boundaries between solvent and solution, or between a concentrated and a dilute solution, are formed in sliding cells based on the Neurath type[1], or by flowing a layer of one solution on top of a layer of another[2]. Boundaries are sharpened by flowing out through a pipette tip, or through a slit in the cell placed at boundary level.

A sharp boundary is necessary to reduce the Δt correction to a minimum. The correction must be applied to the observed values of the diffusion coefficient (D′) obtained at different times, t, after the start of diffusion. Δt is defined as the time required for an infinitely sharp boundary to reach the state of the existing boundary when diffusion commences. Longsworth[3] gives:

$$D' = D\left(1 + \frac{\Delta t}{t}\right)$$

A plot of D′ against 1/t will have a slope of $D\Delta t$, and the intercept will be the true diffusion coefficient, D.

Even when Δt is small (20 seconds) a slight uncertainty is introduced in extrapolating to $1/t = 0$. A cell has been designed which virtually eliminated Δt, and which has no moving parts requiring grease to make them leakproof.

EXPERIMENTAL

Apparatus

The cell (Fig. 1) was made from a block of 1 inch thick brass. Two rectangular channels, A_1 and A_2, 0·5 cm. wide, were milled through the block. Brass plates were brazed over A_1, while A_2 was covered with the cell windows, made of optically flat ($\lambda/2$) glass. The windows were gasketted with a rubber resistant to organic solvents. The two channels were joined to one another at the bottom of the cell by a hole 0·5 cm. in diameter. At the top of each channel was a constriction 0·2 cm. diameter (B_1 and B_2). B_2 had a right angle bend before entering A_2.

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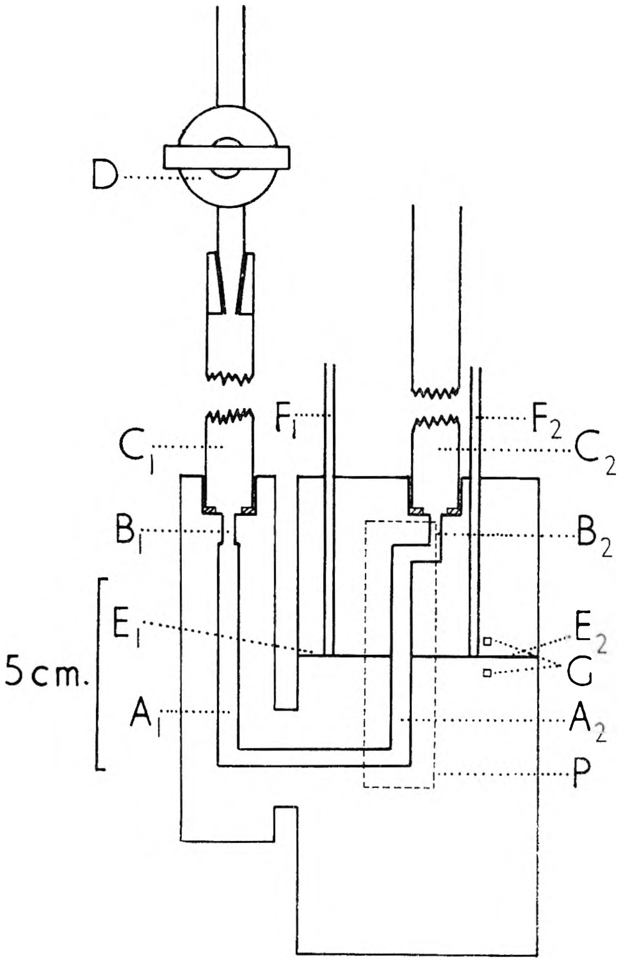

FIG. 1a. Vertical section of diffusion cell.

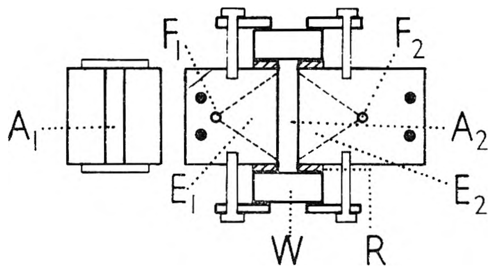

FIG. 1b. Horizontal section of diffusion cell at slit level.

Two reservoirs, C_1 and C_2, screwed into the top of the cell, their ends being seated on rubber gaskets. The top of C_1 was ground to receive a B10 glass joint. A good quality vacuum tap, D, was fused on above the joint.

E_1 and E_2 are two slits opening into the diffusion channel, and were spaced with thin polythene. Liquid could be withdrawn from the diffusion channel via the slits, and removed from the cell up the pipes F_1 and F_2. Two square holes, G, 1×1 mm. in size, were cut in the side of the cell.

The cell was mounted in a thermostat controlled to $25 \pm 0{\cdot}02°$, and fitted with optically flat ($\lambda/2$) windows. The thermostat was bolted to an optical bench.

The Gouy interference method was used to study diffusion: the green line (5461Å) was isolated by interference filters from a mercury vapour lamp, and illuminated a horizontal slit 15 μ wide. An image of the slit was focused through the diffusion cell on to a photographic plate by a lens. All components were mounted on an optical bench, which rested on a girder set on concrete pillars embedded in the floor of a basement laboratory.

Use of Cell

The cell was filled with solution from just below the level of the tap, D, to the middle of the constriction at the top of the diffusion channel A_2, and the tap was closed. After clamping the cell in position in the thermostat and allowing it to come to temperature, a series of photographs of the undeviated slit image and of the interference patterns produced by the square holes was taken. Solvent, or the more dilute solution in the case of a differential diffusion, was run into C_2 by pipette. Solvent and solution met in the constriction, which prevented them from mixing to any great extent, and allowed a crude boundary to be formed. Flow out through one of the slits, (either E_1 or E_2) was started, which lowered the boundary to the middle of the diffusion channel. Solvent was repeatedly added to C_2, and flow through the slit continued. This procedure washed all traces of solute out of the upper part of A_2, which was originally full of solution, and during this operation the boundary sank 2–3 mm. below the level of the slits.

More solvent was added until the level in C_2 was 3–4 mm. below the solution level in C_1. The tap D was opened, causing the boundary to move upwards to the level of the slits. Flow out from the cell was now reduced to about 0·2 ml./minute, and this rate was maintained for 20 minutes, to allow the newly added solvent to come to temperature.

The final stage of the technique was to sharpen the boundary. Liquid was drawn off the cell at an equal rate through both slits, the combined flow rate being raised to between 2 and 6 ml./minute (see later). To start the experiment the flow from the cell was stopped by closing clips placed across the polythene tubes which carried liquid away from the pipes F_1 and F_2. The time at which flow was stopped was taken as zero time, and could be measured with an accuracy of ± 1 second.

A mask of the type described by Gosting[4] was used with the cell. Photographs for evaluating fractional j_m were taken immediately after the experiment had started. The interference patterns produced by diffusion were photographed at timed intervals.

Materials

All materials used were Analar quality, except for the cetomacrogol, which was a commercial grade. All results fitted the theory for the diffusion of a single solute.

Results and Discussion

Table I gives the results of a differential diffusion experiment on barium chloride.

TABLE I

Differential diffusion of barium chloride in water

Concentration difference between solutions* (Δc) = 0·03406M l.$^{-1}$. Mean concentration of two solutions ($\bar{c}$) = 0·04248M l.$^{-1}$. Flow rate = 4 ml./minute. Δt = 1 second

10^4 1/t	11·17	6·390	6·390	4·751	2·822	2·447	1·983
10^5 D′ cm.^{2}sec.$^{-1}$	1·186	1·187	1·187	1·187	1·189	1·187	1·187
10^4 1/t	1·488	1·225	—	—	—	—	—
10^5 D′ cm.^{2}sec.$^{-1}$	1·184	1·186	—	—	—	—	—

* If C_1 and C_2 were the concentrations of two solutions used in an experiment, then $\Delta c = C_1 - C_2$, and $\bar{c} = \frac{c_1 + c_2}{2}$

The diffusion coefficient appears to be constant over a tenfold change in t, while a decrease of D′ with time would have been expected if the boundary had not been sharp. To test if Δt was eliminated, within the limit of experimental error, the slope of the D′ against 1/t plot was calculated by the method of least squares. The slope was $7·34 \times 10^{-6}$, and the intercept, $D = 1·186 \times 10^{-5}$ cm.^{2}sec.$^{-1}$, giving Δt = 1 second. The literature value of D is $1·186 \times 10^{-5}$ cm.^{2}sec.$^{-1}$.

In Table II the results for the diffusion of a number of other substances are given. In all experiments D′ was measured over a tenfold change in t.

TABLE II

Diffusion coefficients of various substances

Substance	$\bar{c}$	Δc	Flow rate
Sucrose	0·75 per cent	1·5 per cent	0·8 ml./minute
Sucrose	0·75 "	1·5 "	1·8 "
Sucrose	0·75 "	1·5 "	3·4 "
Sodium chloride	0·08026M l.$^{-1}$	0·1045M l.$^{-1}$	6·0 "
Potassium chloride	0·1000M l.$^{-1}$	0·2000M l.$^{-1}$	3·8 "
Cetomacrogol	0·669 per cent	0·669 per cent	7·0 "
	10^5 D cm.^{2}sec.$^{-1}$	Δt	10^5 D cm.^{2}sec.$^{-1}$ (literature)
Sucrose ..	0·5158	38	0·5170[6]
Sucrose ..	0·5176	20	0·5170
Sucrose	0·5171	0	0·5170
Sodium chloride	1·489	1	1·490[5]
Potasium chloride ..	1·850	0	1·851[7]
Cetomacrogol	0·0520	1	—

Generally, too slow a flow from the cell results in a Δt value, as shown by the sucrose experiments. The flow rate necessary to reduce Δt until it falls within the limit of experimental error appears to vary from system to system. If sucrose solutions were drawn off the cell too quickly (above 6 ml./minute), considerable mixing appeared in the boundary region, and no worthwhile results could be obtained. A disadvantage of this type of cell is that flow off conditions are critical, and have to be investigated for each type of system studied, e.g., for electrolyte solutions any reasonably fast flow off suffices to eliminate Δt; sucrose requires careful handling, while detergents, like cetomacrogol, can be drawn off quickly. Similar conclusions have been reached using a cell with one slit (Thomas, private communication).

Some experiments were also made on cetomacrogol solutions (Table III). There is little variation of diffusion coefficient with $\bar{c}$, indicating that only small electrical effects are present during diffusion, as would be expected with a non-ionised material. The diffusion coefficient appears to be slightly concentration dependent ($\bar{c}$), and extrapolation to zero concentration gives $D = 5{\cdot}10 \times 10^{-7}$ cm.^{2}sec.$^{-1}$. Δt's of less than two seconds were found in this series of experiments.

TABLE III

DIFFERENTIAL DIFFUSION COEFFICIENTS OF CETOMACROGOL IN WATER

$\bar{c}$ per cent	0·300	0·669	0·673	0·762	1·303
Δc per cent	0·400	0·669	0·601	1·016	0·897
10^7 D (cm.^{2}sec.$^{-1}$) ..	5·13	5·20	5·18	5·24	5·31

Acknowledgements. I should like to thank Dr. L. Saunders for his interest, and Mr. P. Barden for his accurate work in making the apparatus.

REFERENCES

1. Neurath, *Science*, 1941, **93,** 431.
2. Saunders, *J. chem. Soc.*, 1953, 519.
3. Longsworth, *J. Amer. chem. Soc.*, 1947, **69,** 2510.
4. Gosting, Hanson, Kegeles, and Morris, *Rev. Sci. Instruments*, 1949, **20,** 209.
5. Vitagliano and Lyons, *J. Amer. Chem. Soc.*, 1956, **78,** 1549.
6. Gosting and Morris, *ibid.*, 1949, **71,** 1998.
7. Gosting, *ibid.*, 1950, **72,** 4418.

BOOK REVIEW

THE PHARMACOLOGY AND CLINICAL USE OF DIURETICS. By Carroll A. Handley and John H. Moyer. Pp. viii + 194 (including Index). Blackwell Scientific Publications, Ltd., Oxford, 1959. 48s.

The authors of this book, together with their colleagues have been occupied in the pharmacological and clinical study of diuretics for several years. They emphasise in their preface, that the book, being concerned mainly with their own work, is in no way a review of the literature. For this reason the list of references is short, there being 112, of which 39 refer to the work of the authors themselves. The first of two introductory chapters summarises the methods of renal control of water and electrolyte balance and includes a short consideration of the actions of aldosterone and ACTH. The second describes some of the clinical conditions that benefit from treatment with diuretics, and also contains a discussion of the various factors leading to the formation of oedema, and the methods available for its control.

Emphasis is placed upon clinical rather than animal pharmacology since most of the authors' work appears to have been carried out using hospital patients. The method of evaluating the potency of the diuretics involved a study of patients maintained on controlled diets, in order that exact water and electrolyte balances could be established. Against this background, water and sodium chloride excretions, together with changes in body weight were determined for the various diuretics. Dose-response curves capable of statistical analysis could then be obtained. The form of the dose-response curves differed for the various classes of diuretics, and the significance of these results is discussed. The influence of the route of administration on the potency of each diuretic was investigated and a study was made of the rate of their excretion. After a brief summary of the biochemistry and pharmacology of each class of diuretics there is a detailed account of the authors' results together with a note of the optimum dose schedules employed. The book is concluded by a summary of the treatments used in various clinical conditions and a list of special diets employed by the authors. There is also a short addendum describing the more recent derivatives of chlorothiazide.

With one or two exceptions, the graphs and figures are clear and understandable. The book suffers from rather too many typographical errors, but the presentation and binding is good. This book is neither a text-book, nor is it designed for the research worker; it will, however, serve as an adequate introduction to those who wish to employ diuretics in clinical pratice.

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