REVIEW ARTICLE

THE STORY OF MUSCARINE

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Muscarine, first isolated from the fly agaric (*Amanita muscaria*), is one of the foundation stones of modern pharmacology. It was, indeed, among the first substances known to possess an action which more or less faithfully reproduces some of the effects of stimulation of the autonomic nervous system. The free base is almost certain to be a valuable heuristic tool—it has, for instance already given clear confirmation[1] of the presence of atropine-like actions, originally recorded by Tedeschi[2], in the anti-cholinesterase drug 284C51. But unless an abundant natural source is found* muscarine is not likely to be available in reasonable quantities until it can be synthesised, and its synthesis is unlikely to precede the discovery of its structure. It is surprising, therefore, to find that this small molecule, which has played such an important role, has defied all but the most recent attempts to unravel its structure.

Early History

The earliest attempts to isolate the active toxic principles of the fly agaric, and of other fungi, were made by Braconnot[4–6] and by Schrader[7]. Braconnot was unable to relate the poisonous properties of any fungus to the acrid principle he obtained. Schrader looked for the active principle in the red-coloured material which was the only part he found toxic to birds. Vauquelin[8] suspected that the toxic substance was in the fatty contents of the mushroom. Letellier[9] believed that there were two active principles—the "acrid" and the "narcotic"—but both he and Braconnot were more concerned with fungi in general than with the fly agaric. Although Letellier did not succeed in isolating either the acrid or the narcotic principle in a pure state he found that the acrid principle was easily destroyed by boiling, by drying, by alcohol, by alkali, and by dilute acid; the narcotic principle—which he called amanitine—was resistant to these treatments. His acrid principle was, therefore, unlike muscarine which is stable in acid or alkaline solution and which is usually extracted in alcohol. As he attributed the narcotic properties of fungi to amanitine, and believed that amanitine was the toxic principle of *A. phalloides*, it is unlikely that he used the name amanitine for the substance eventually called muscarine. Indeed, the name amanitine is now applied to one of the main toxic principles of *A. phalloides*[10]. Eventually it became apparent that it was chiefly choline that Letellier had obtained[11]; probably the choline was contaminated with other active principles of the fungi investigated.

The first successful researches into the active principles of the fly agaric were those of Schmiedeberg and Koppe[12] who obtained a deliquescent

*Eugster[3] has apparently found such a source in *Inocybe patouillardi*.

syrupy base which stopped the isolated frog heart in diastole. This base was produced by precipitation with potassium bismuth iodide or with potassium mercuric iodide. They called it muscarine, but according to Harnack it was still mixed with choline.

Soon after this Harnack[11] isolated the aurichloride of a material which he called amanitine after Letellier but which he eventually showed to be choline, one of the most abundant constituents of the fly agaric. Harnack also obtained some muscarine aurichloride to which he gave the empirical formula $C_5H_{12-14}O_2N.AuCl_4$. As Harnack's aurichlorides of muscarine and of choline were similar in appearance, and as he had 8 g. of the so-called muscarine for analysis, his material was more probably choline aurichloride contaminated with muscarine aurichloride[13]. Furthermore, Harnack's muscarine was equal in potency on the frog heart to that prepared by Schmiedeberg and Koppe, which he had already claimed to be mainly choline.

Schmiedeberg and Harnack[14] were the first to obtain comparatively pure muscarine. They thought that it was a hydrated betaine aldehyde and proposed for it the structure: $Cl^-Me_3N^+CH_2CH(OH)_2$.

"*Synthetic Muscarine*"

Schmiedeberg and Harnack claimed to have confirmed this structure by synthesis. By oxidising choline with nitric acid, they obtained a material which they thought had the required empirical formula—although it really had one H atom less—and which they believed to have the structure proposed for muscarine. As this "synthetic muscarine" behaved pharmacologically like natural muscarine, the structure proposed by Schmiedeberg and Harnack for muscarine was accepted until Boehm[15] showed that the synthetic and natural substances, although biologically much alike, were not identical. The synthetic compound, or pseudo-muscarine, was much the weaker in most pharmacological tests. It also possessed some actions not present in the natural alkaloid and not antagonised by atropine: it had, for instance, a strong curare-like effect. Other differences also soon became apparent: Meyer[16] found that synthetic muscarine was much more potent than natural muscarine as a miotic drug in birds and that the relative potency was reversed in mammals. He also confirmed Boehm's observation of a curare-like effect in synthetic muscarine and its absence in the natural base.

Nothnagel[17] repeated the so-called synthesis of muscarine by the method of Schmiedeberg and Harnack and confirmed their structure for synthetic muscarine. He recognised that choline nitrous ester was formed in this synthesis but considered that it was an intermediate product in the formation of synthetic muscarine.

Here was a very unsatisfactory situation: two bases, which had obviously different biological actions, could not be distinguished by chemical methods. This situation might never have arisen had Schmiedeberg and Harnack, or Nothnagel, determined the nitrogen content of their synthetic compound!

The true identity of synthetic muscarine was not known until Ewins[18]

showed that the product obtained on oxidising pure choline with nitric acid was choline nitrous ester—or choline nitrite, $Cl^{-}Me_3N^{+}CH_2CH_2$-ONO. Dale[19] showed that this substance was very much less active than acetylcholine as a depressor agent in the cat.

Isolation of Muscarine

Varying success has attended other attempts to isolate natural muscarine. Inoko[20] extracted it from *Amanita pantherina*. Nothnagel[17], who accepted the structure proposed by Schmiedeberg and Harnack, claimed to have isolated 500 mg. of the platinum salt of muscarine from many hundredweights of the fresh fungus; but King doubted the purity of this material. Harmsen[21], and Honda[22] also isolated some muscarine, but they were more interested in its pharmacology than in its chemical structure. Heinisch and Zellner[23], Zellner[24], and Küng[25] failed to obtain muscarine.

King[13] eventually obtained the first really pure muscarine, which he crystallised as the aurichloride from a mixture of muscarine and choline aurichlorides. The method depended upon the solubility of muscarine in absolute ethanol, its non-precipitation by basic lead acetate, and its precipitation by aqueous and by alcoholic mercuric chloride and by phosphotungstic acid. King found that there was twenty times as much choline as muscarine in the fly agaric. From assays on the toad heart and rabbit gut, he estimated that each kilogram of fresh *A. muscaria* contained about 16 mg. muscarine chloride; he crystallised 80 per cent of this. He did not give a melting point. King's muscarine, which he said was not adsorbed by charcoal, had a molecular weight of about 210, was stable to boiling in decinormal acid or alkali, and was about equal in potency to Honda's preparation in stopping the frog's heart in diastole.

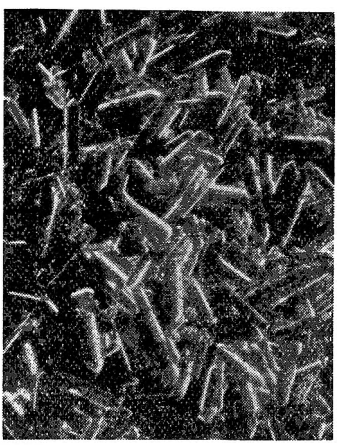


Fig. 1. Crystals of muscarine chloride (× 165) prepared by Dr. S. Wilkinson.

It was, however, more potent than other earlier samples, being twenty times more potent than the materials isolated by Schmiedeberg and Koppe and by Harnack; it was five times more active than acetylcholine, and seven times more active than arecoline, on isolated rabbit gut. These results are supported by others on a recently isolated, highly pure sample of muscarine chloride (Fig. 1) prepared by Dr. S. Wilkinson of the Wellcome Research Laboratories; it was about four or five times more active than acetylcholine on isolated rabbit auricles in the absence of an anticholinesterase drug. When the relative potencies were determined in the presence of neostigmine, muscarine and acetylcholine were about equal (Mogey, unpublished work). Fraser[26] obtained similar results

although muscarine and acetylcholine did not give parallel dose-response curves. Thus, presumably, muscarine is not hydrolysed by cholinesterase; as muscarine is also stable in alkali it cannot be a choline ester.

King's muscarine aurichloride crystallised as large delicate leaflets quite unlike choline aurichloride crystals. It therefore resembled the sample prepared by Nothnagel—the biological potency of which has not been recorded—but was totally unlike the material, crystallising as long or short prismatic shapes, which Harnack regarded as muscarine, and which he could not distinguish in crystal form from choline.

Another step in the elucidation of the structure of muscarine was taken by Kögl and his colleagues[27]. They treated the fresh fungi with ethanol, and removed fats with ether and other impurities with charcoal. Choline was adsorbed on Permutit, and other substances were precipitated by suitable adjustment with mercuric chloride. The muscarine was precipitated from acetone as the reineckate and converted to the chloride. Their yield of base was 2·8 mg./kg. in one experiment and 1·3 mg./kg. in another.

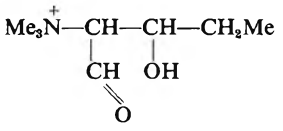
Properties of Muscarine

By this time a good deal had been published about the chemical and physical properties of muscarine. It was known to be soluble in ethanol and in water, slightly soluble in chloroform, and insoluble in ether; according to King, it was not adsorbed by charcoal or kaolin, and was stable in alkaline solution; Kögl and others[27] stated that it soon decomposed in acid solution but Ewins[28] has recorded that the muscarinic activity of extracts of *A. muscaria* is not appreciably reduced by boiling in acid or in alkali, and King[13] confirmed this. Kögl, Salemink, Schouten, and Jellinek[29] now state that muscarine is stable to boiling in acid or in alkali.

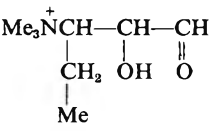
Although the presence of an aldehyde group has been denied[30], Kögl, and others[27] concluded—from positive reactions with Schiff's reagent and the nitroxyl reagent of Angeli-Rimini—that there was such a group in muscarine. As a benzoyl derivative was obtained, they believed that there was also a hydroxyl group. Trimethylamine was produced by the action of silver oxide: the base was therefore thought to contain a trimethylammonium group. Muscarine is optically active*; therefore there is probably at least one asymmetric carbon atom present. The chloride had a molecular weight of 195·5 according to Kögl and Veldstra[31] or about 210 according to King. Kögl and Veldstra gave the melting point of the aurichloride as 115–117°†. Kögl and his colleagues[27] concluded that the earlier formula of Schmiedeberg and Harnack was too small; they proposed $C_8H_{18}O_2N$, and suggested that muscarine was structure (I) or possibly (II).

* $[\alpha]_D^{20}$ + 1·57° (water; chloride; Kögl and others[27]). $[\alpha]_D^{25}$ + 8·1° (ethanol; chloride; Kuehl, Lebel and Richter[34]), $[\alpha]_D^{20.5}$ + 6·7° (water; reineckate; Eugster and Waser[35]).

† Later, however, Eugster and Waser[35] gave 121–121·5° as m.p., Kuehl and others[34] gave it as 116–119° and Kögl and others[29] as 120–121° for the same salt.



(I)



(II)

Pfeiffer[32] preferred the second formula on the basis of the distances between the nitrogen and the oxygen atoms.

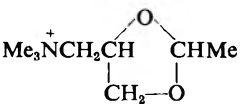
Kögl and Veldstra synthesised the first of their proposed structures, but the synthetic material had only about one-forty-thousandth of the activity of natural muscarine. Kögl and Veldstra, however, suggested that the discrepancy in activity between their synthetic compound and natural muscarine might be explained by a very weak activity in all but one of the isomers present in the synthesised racemate. According to Kögl, Salemink, and others[29] van der Laan[33] failed to resolve the various isomers. But what other compound shows such stereospecificity? If a completely inert material is mixed with an equal weight of an active material, A, the mixture will have half the activity of A. Common ratios for the potencies of optical isomers are 20 (tubocurarine), 17 (adrenaline), and 4 (methadone): 40,000 is surely too much; and this, moreover, is the ratio of the synthetic racemate, prepared by Kögl and Veldstra, and consisting of the presumed active and inactive isomers, to the natural alkaloid. Furthermore the stereoisomers of simple ammonium compounds show no marked differences in muscarinic activity[36].

The formula put forward by Kögl and Veldstra is, therefore, unlikely to be correct. But it is still quoted in some textbooks as the accepted structure of muscarine.

More Synthetic Approaches

Other attempts to synthesise molecules which might be identical with that of natural muscarine have also failed. Because muscarine was supposed to be a hydrated betaine aldehyde, it was reasonable to expect betaine aldehyde, $Cl^{-}Me_3N^{+}CH_2CHO$, to show some muscarine-like actions. It was synthesised by Berlinerblau[37], and later by Fischer[38], but Meyer[16] found that betaine aldehyde and muscarine were quite different pharmacologically and that the former was inactive on the pigeon's pupil. Bode[39], Nothnagel[17] and Ewins[28] all failed in their attempts to make muscarine by synthetic means. None of the synthetic compounds was as active as the natural alkaloid and none was without the nicotine-curare type of action, which is absent in muscarine[19].

Of the compounds recently synthesised, none has excited more interest than the acetal derivative of Fourneau and his colleagues[40]. They prepared 2268F (III), and other related compounds, because of Fourneau's hypothesis that the difference between Kögl's synthetic compound and natural muscarine might be due to the formation "d'une liaison interne du type acetal".



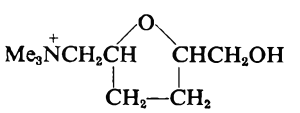
(III) 2268F.

This compound is more potent than acetylcholine in many respects and is practically devoid of nicotinic actions; it is one of the most potent muscarinic substances known, being even more active than carbachol, which, unlike acetylcholine, is not sensitive to destruction by cholinesterase.

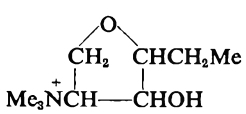
But 2268F is not muscarine: in large doses (6 mg./kg.) it produces, for instance, a pressor response in atropinised, anaesthetised cats[41], an action not present in muscarine according to Ambache[42]. It also stimulates the frog's rectus abdominis; we now know that muscarine also has this action[26] but large doses are needed; the effect is antagonised by tubocurarine. Compound 2268F also has a weak ganglion-stimulating effect, an action also possessed by muscarine[43–44]; this ganglion stimulation is easily blocked by atropine.

The molecule of 2268F is, furthermore, too small for it was based on Kögl's formula for muscarine, $C_8H_{18}O_2N$, which would give a molecular weight of 195·5 for the chloride. The molecular weight assigned by King to muscarine (about 210), suggests that Kögl's formula is one $-CH_2$ group too small. Addition of this $-CH_2$ group gives the empirical formula $C_9H_{20}O_2NCl$ (mol. wt. 209·5) which is, in fact, the formula ascribed to muscarine chloride by Eugster and Waser[35]. Kuehl and others[34] and Kögl, Salemink, and others[29] agree with this formula.

Dr. Wilkinson has written to us to say he obtained trimethylamine on Hofmann degradation of pure crystalline muscarine chloride. The trimethylamine was identified conclusively by comparison of X-ray powder photographs of the aurichloride and authentic trimethylamine aurichloride; Wilkinson thus concluded that muscarine contains a trimethylammonium group. Indeed he stuck firmly to this conclusion even after Eugster and Waser[35] (see below) had doubted the presence of this group. In 1952, Wilkinson established $C_9H_{20}O_2N$ as the empirical formula for muscarine and, concluding from the infra-red absorption spectrum that there is a tetrahydrofuran ring present in the molecule, he prepared, in 1954, two compounds (IV) and (V) for model experiments relating to the structure of muscarine.



(IV)



(V)

Wilkinson's synthetic compounds

Each compound was found by Dr. P. Fraser also of the Wellcome Laboratories to have less than one-thousandth ($\frac{1}{1000}$th) of the activity of natural muscarine on the isolated rabbit ileum.

The possibility that muscarine is an alkoxytrimethylammonium compound was suggested by Rogers, Bovet, Longo, and Marini-Bettolo[45].

They based their suggestion on the fact that certain compounds of the $R{\cdot}CH_2ON^+Me_3X$ type had marked muscarine-like actions and because they are decomposed by alkali to give trimethylammonium and the corresponding aldehyde, R·CHO. This reaction was held to resemble the Hofmann degradation of muscarine to trimethylamine and $\alpha\beta$-dihydroxyvaleric acid; silver oxide oxidised the aldehyde. Furthermore, Schiff and Angeli-Rimini reactions, believed by these authors—after Kögl and others[27]—to be given by muscarine, are also given by alkoxytrimethylammonium compounds. Rogers and others concluded that more such compounds, and particularly *n*-amyloxytrimethylammonium ($C_8H_{20}ON$), should be examined. This alkyloxytrimethylammonium type of structure is not, however, supported by the stability of muscarine free base to hydrolysis; even after drastic acid or alkaline hydrolysis no evidence for the formation of aldehydes could be obtained.

Further Analysis

Although muscarine contains a quaternary nitrogen, Eugster and Waser[35] could not show that it was a trimethyl quaternary grouping, for—unlike Kögl and others[27] and Wilkinson—they obtained volatile bases but no trimethylamine on Hofmann degradation. From the negative colour tests, and the infra-red absorption spectrum, they concluded that there is neither aldehyde nor ketone group in the molecule. On oxidation with chromic acid they got acetic acid and no $\alpha\beta$-dihydroxyvaleric acid, which Kögl and his colleagues did obtain.

The results obtained by Eugster and Waser thus differ radically from those of Kögl and others[27]. If the C_9 formula is correct, and if there are no double or triple bonds in muscarine, it must of necessity contain one ring[46].

As muscarine was completely inert to periodate oxidation it was assumed by Kuehl and colleagues[34] that there are no vicinal hydroxyl groups or adjacent hydroxyl and ketone groups. Acetylation gave a monoacetyl derivative which possessed, according to infra-red spectroscopy, one ester-group; as muscarine was obtained again on deacetylation there was no internal rearrangement of the molecule on acetylation. There is thus one, but not two, hydroxyl groups. Chemical reagents failed to demonstrate an aldehyde group and no carbonyl group was detected by infra-red spectroscopy. The Zeisel methoxyl test was negative—therefore no methyl ether is present. The inertness of the second oxygen suggested that it might be an ether. Kuehl and colleagues obtained no trimethylamine on Hofmann degradation of natural muscarine under a variety of conditions including those described by Kögl and others[27]. Neither did they detect any acid substances after these procedures. All they got was unchanged muscarine.

Eugster[47] repeated that on oxidation of muscarine with chromic acid he obtained acetic acid only, and claimed that Hofmann degradation with silver oxide gave no trimethylamine though fusion with potassium hydroxide gave an unspecified amount. He showed that any structure which would give rise to a carbonyl group in acid solution is impossible. As muscarine is, furthermore, not sensitive to reduction or hydrogenation

by sodium amalgam, lithium aluminium hydride, sodium borohydride, or hydrogen and platinum it cannot be an acetal derivative as was assumed by Fourneau. It is possible that a ring structure might help to stabilise the acetal grouping, but it is unlikely that it would give the negative carbonyl reaction with dinitrophenylhydrazine reported by Eugster and Waser.

Kögl, Salemink, and others[29] have modified some of the original claims of Kögl and others[27] and Kögl and Veldstra[31]. They now state that muscarine is stable to boiling for 3 hours in 2N HCl or 3N NaOH or for 8 hours in HCl at pH 4 or below pH 1; that as tests with phenylhydrazine, dinitrophenylhydrazine and semicarbazide all gave negative results there can be no carbonyl group; that negative results were also obtained with Schiff's reagent and with the nitroxyl reagent of Angeli-Rimini whereas earlier Kögl and others[27] had reported positive results with these reagents; that—like their previous results, but unlike the more recent work of Eugster and of Kuehl and colleagues—treatment, under very vigorous conditions, of a large quantity of muscarine (100 mg.) with silver oxide yielded trimethylamine. The muscarine isolated by Kögl, Salemink, and colleagues[29] was one-quarter as active as acetylcholine on the Straub frog heart; Fraser[26] reported that Wilkinson's muscarine was two-thirds as active as acetylcholine on the Hartung-Clark frog-heart preparation.

As Kögl, Salemink, and colleagues[29] discovered acetylcholine in their extracts of *A. muscaria* it is possible that the 1931 sample of muscarine was contaminated with this, and that it was the acetylcholine which disappeared on boiling and which gave the reactions leading to the erroneous conclusion that muscarine contained a carbonyl group. But this does not explain how Wilkinson (private communication) and Kögl, Salemink, and others[29] obtained a good yield of trimethylamine whereas Kuehl and colleagues[34] and Eugster[47] did not. The discrepancy is more likely to be due to the degradative procedure, Kögl, Salemink and colleagues[29] using vigorous conditions and much muscarine, whereas the American authors used gentler conditions. Eugster only obtained small quantities of trimethylamine after fusion of muscarine with potassium hydroxide; under similar conditions he obtained material chromatographically similar to trimethylamine from morpholine compounds.

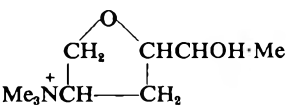
So muscarine is $C_9H_{20}O_2N$. It contains no aldehyde or ketone—as Scelba[30] claimed before Kögl and others[27] stated that some such group was present. It has one ring in its structure and no double bond. It is dextrorotatory. There is only one hydroxyl group—a secondary alcohol*—and the second oxygen function is probably an ether although not a methyl ether; it may be in a tetrahydrofuran ring (Dr. S. Wilkinson, private communication and ref. 29). Eugster and Waser[35] could not

* That this hydroxyl group is a secondary alcohol was first suggested by Eugster[47] because muscarine gave a positive response to the iodoform test. He therefore suggested that the side chain –CHOH·Me was present in muscarine. In reality the positive iodoform test was given by the other oxygen of the tetrahydrofuran ring and so did not prove the presence of the –CHOH·Me group. The evidence for a secondary alcohol group therefore rests on the X-ray crystallographic data[49] (see below).

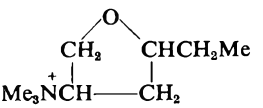
confirm that there are three methyl groups on the nitrogen, but Wilkinson (unpublished work), Eugster[47], and Kögl, Salemink and colleagues[29] decided that muscarine contains a trimethylammonium grouping.

The nature of the oxygen functions in the molecule is one of the most interesting points. It now seems fairly clear that one is in a hydroxyl group and that the other is most probably an ether but not a methyl or ethyl ether; infra-red absorptiometry seems to indicate that it is in a tetrahydrofuran ring. The nature of this atom may not be important, however, for cells may not be able to distinguish clearly between an ether, an acetal, or a carbonyl oxygen: choline ethyl ether[19], 2268F, and acetylcholine all have high muscarinic activities. The relative position of the oxygen is probably more important. Many active compounds have one oxygen function about 3–4 Ångstrom units away from the nitrogen and the second about 1–2 Å further off, e.g. 2268F and acetylcholine.

Eugster[47] has recently suggested that muscarine is the trimethylammonium salt of 2-(1-hydroxyethyl)-4-aminotetrahydrofuran (VI).



(VI) Structure of muscarine after Eugster[47]

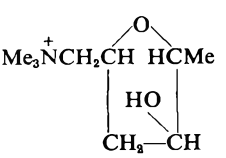


(VII) Eugster's desoxymuscarine

He synthesised the related desoxy compound (VII) from which, on degradation with silver oxide, he obtained almost a 47 per cent yield of trimethylamine, whereas from natural muscarine he obtained practically none. It seemed to him, therefore, that the introduction of the hydroxyl group—as in his proposed structure for muscarine—produced some special orientation preventing the production of trimethylamine. Thus Eugster also took shelter in the last refuge of the organic chemist—stereospecificity—and so joined the company of those who had seriously tackled this problem before him. Eventually he showed that his proposed structure was incorrect[48].

The Structure of Muscarine

From the infra-red absorption spectrum Kögl, Salemink and colleagues[29] concluded that muscarine contains a tetrahydrofuran ring, thus settling the nature of the second oxygen function. By the action of hydrogen iodide followed by hydrogenation they obtained trimethylhexylammonium iodide thus showing the carbon skeleton of muscarine. They suggested that muscarine is the quaternary trimethylammonium salt of 2-methyl-3-oxy-5-(aminomethyl)-tetrahydrofuran (VIII)



(VIII) Muscarine according to Kögl, Salemink and colleagues[29]

X-ray crystallographic data were held to confirm this structure. Details of the bond distances and angles in the muscarine ion are shown in Figure 2. The standard deviation of the measurement of the bond lengths was 0·08 Å and that for the angles was 6°. The values are therefore all within normal limits.

In the tetrahydrofuran ring of Figure 2 all the atoms except C(3) are in the one plane. C(3) lies on the same side of this plane as do C(1) and C(6) whereas O(2) is on the opposite side. The sequence C(3), C(2), C(1), N, and C(7) is virtually in a single plane; O(2) is only 0·05 Å away from this plane and the mean distance of C(3), C(2), C(1), N, and C(7) from the plane is 0·06 Å[49]. Thus O(2) can be regarded as being in the plane of the chain enumerated.

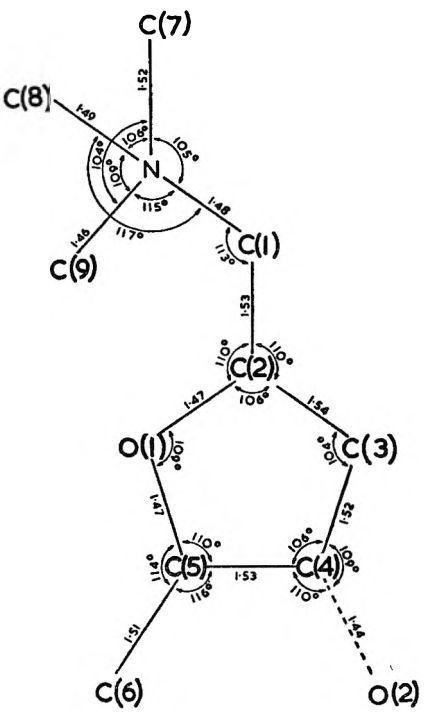


Fig. 2. Bond distances (in Å) and angles in the muscarine ion. (By kind permission of Dr. F. Jellinek and *Acta crystallographica*.)

The compound with this structure has been synthesised[50,51]. The racemate aurichloride had a melting point of 69–72°; the m.p. of natural muscarine aurichloride is 120°–121°. The synthetic racemic chloride has the same R_F value as the natural chloride. The infra-red absorption spectra of the natural and synthetic products showed a general identity although there were some differences in fine detail—particularly in C–H bands—which were taken to indicate that the synthetic material was a mixture of stereoisomers. The pharmacological activity of the synthetic material[50], tested on the frog heart, was one-third that of natural muscarine. The resolution of another synthetic racemate has apparently been tackled and the promised results[52] are awaited with interest.

Conclusion

Although the structure now proposed seems to be fairly well established there are still some slight difficulties about accepting the results unreservedly. Why do different workers obtain different results—some getting trimethylamine and $\alpha\beta$-dihydroxyvaleric acid, and some getting neither of these—on subjecting muscarine to Hofmann degradation? Can this be due solely to the vigour of the reactions? Since trimethylamine was apparently obtained by Eugster from dimethylmorpholine, can the

detection of trimethylamine from muscarine prove the presence of a trimethylammonium group? How can the tetrahydrofuran ring give a positive iodoform test, which seems to indicate the presence of a –CHOH·Me group: does the ring become hydrolysed at the oxygen? The infra-red absorption spectrum suggests that a tetrahydrofuran ring is present, but does it rule out all other ring structures?

The latest structural proposal by Kögl and his colleagues is, nevertheless, probably correct. Before final acceptance, however, the synthetic compound—satisfactorily resolved—and natural muscarine should be shown to be identical chromatographically, pharmacologically and by infra-red spectroscopy. Such a series of identities is necessary for convincing proof, because of past difficulties in elucidating the structure of this fascinating molecule.

Note added in Proof

Corrodi, Hardegger and Kögl[53] have recently stated that their claim[52] to have synthesised a mixture of muscarine and its diastereoisomers cannot be substantiated. They now believe that this product contained little or no muscarine but that it was a racemic mixture of allomuscarine. All derivatives of allomuscarine showed the same R_F values, and had nearly the same infra-red spectra, as the corresponding derivatives of muscarine. The mixture was only $\frac{1}{200}$ as active as natural muscarine on the frog heart. Thus the biological test has again proven its value—it should have been tried earlier.

Two other syntheses have also been reported. One of (±)-muscarine[54] yielded muscarine chloride m.p. 148–152° and a tetrachloroaurate m.p. 79–83° (see footnote † p. 148, and p. 154), and the other of L-muscarine[55] gave muscarine chloride m.p. 179–180°. Previously reported melting points for natural muscarine chloride were 181–182°[35] and 180–181°[29]. No biological results for the (±)-muscarine have been published; the L-muscarine was equal in activity to natural muscarine on the frog heart.

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RESEARCH PAPERS

THE DETERMINATION AND EXCRETION OF POLYHYDROXY (CATECHOLIC) PHENOLIC ACIDS IN URINE

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The Mitchell reaction (ferrous sulphate) has been applied to the determination of catecholic phenolic acids and pyrogallol in urine. A technique has been described for the determination of pyrogallol in the presence of catecholic phenolic acids. The separation of catecholic phenolic acids by paper chromatography has been examined. A study has been made of the excretion of these substances in urine.

The present paper is concerned with the determination in urine of certain catecholic phenolic substances, namely pyrogallol and gallic, 3:4-dihydroxybenzoic and caffeic acids. Although certain aspects of the pharmacology of tannic acid have been investigated[1–5], the group has received little attention, probably because of a lack of suitable analytical techniques.

The determination is by means of the Mitchell reaction[6,7]. The purple colour so produced is specific for this group of substances and the intensity follows Beer's Law up to 500 μg. The intensity of colour is not however proportional on a molecular basis when different reacting substances are compared. As a result, 3:4-dihydroxybenzoic acid has been used as the general standard. A preliminary separation by means of ether is necessary since the reaction cannot be applied directly to urine. Hot acid-hydrolysis is also an essential preliminary since phenolic acids are mainly excreted as conjugates which may not react and are not soluble in ether.

When the reaction is applied to urine extracts, there is some development of a non-specific yellow colour—this may amount to the equivalent of 20 mg./day expressed as 3:4-dihydroxybenzoic acid.

Catechol, which is poorly soluble in ether, and which reacts in the Mitchell reaction, could not be recovered and hence is not included in the final results. The methoxy phenolic acids like ferulic, vanillic and syringic acids, which also occur in human urine, are not included since the methoxy group is stable under the conditions of hydrolysis employed.

An attempt has been made to determine the individual reacting phenolic substances. Pyrogallol has been determined by reason of its ready volatility in hot ethanol vapour. Paper chromatography has been employed to separate the acids. A complete separation of gallic acid was achieved but caffeic and 3:4-dihydroxybenzoic acids could be partially separated only.

The Determination of Total Catecholic Phenols and Phenolic Acids

Reagents. (1) Ferrous sulphate reagent (Mitchell). Ferrous sulphate ($FeSO_4 \cdot 7H_2O$) 0·1, sodium potassium tartrate 0·5 g., and water to 100 ml.

(2) 10 per cent (w/v) ammonium acetate. (3) 2N aqueous ammonia solution (approximate). The concentration of the ammonia solution should be so adjusted that the pH of the final reaction mixture is 7·8.

TABLE I

THE RECOVERY OF CATECHOLIC PHENOLS AND PHENOLIC ACIDS ADDED TO 10 ML. URINE

	Quantity added, µg.	Recovery, per cent
Initial content of urine—18 mg./l.		
A. Gallic acid	50	89
	100	109
	250	104
	500	98
Initial content of urine—9·8 mg./l.		
B. 3:4-Dihydroxybenzoic acid ..	50	87
	100	89
	250	94
	500	97
Initial content of urine—12·5 mg./l.		
C. Caffeic acid	50	85
	100	88
	250	91
	500	96
Initial content of urine—14·5 mg./l.		
D. Pyrogallol	50	89
	100	91
	250	93
	500	97

Hydrolysis. 10 ml. of urine and 1 ml. of 10N hydrochloric acid are heated in a glass tube with a ground glass stopper in a boiling water bath for 1 hour.

Extraction. The cooled mixture is extracted three times with 40 ml. quantities of ether in a glass stoppered measuring cylinder, the ether

TABLE II

THE DETERMINATION OF PYROGALLOL IN THE PRESENCE OF GALLIC AND 3:4-DIHYDROXYBENZOIC ACID—REMOVAL OF PYROGALLOL BY VOLATILISATION

Pyrogallol, µg.	Gallic acid, µg.	Gallic acid recovered, µg.
50	150	156
100	200	192
150	200	208
Pyrogallol, µg.	**3:4-Dihydroxybenzoic acid, µg.**	**3:4-Dihydroxybenzoic acid recovered, µg.**
50	150	158
100	200	210
150	200	208

extracts being separated with a teat pipette. On the addition of ether, the mixture is shaken vigorously for 2 minutes.

Removal of ether. The ether is allowed to evaporate spontaneously at room temperature.

Development of the colour. The residue is dissolved in 0·5 ml. of water and 0·5 ml. of ferrous sulphate reagent added. Then 10 ml. of 10 per cent

ammonium acetate solution are added, followed by 1 ml. of 2N ammonia solution. The mixture is allowed to stand at room temperature for 20 minutes and then read against a blank at 560 mμ (Unicam Spectrophotometer S.P.350).

Standard. A standard containing 250 μg. of 3:4-dihydroxybenzoic acid is set up at the same time.

The Determination of Pyrogallol

Pyrogallol is determined by difference. An ethereal extract is prepared as described above. This is evaporated to dryness in an all glass still *in vacuo* (water pump), a water bath heated to near boiling point being

TABLE III

THE DISTRIBUTION OF CATECHOLIC PHENOLIC ACIDS ON PAPER (WHATMAN NO. 1) AFTER CHROMATOGRAPHY (*n*-BUTANOL/ACETIC ACID/WATER)

Strip No.	Per cent acid located		
	Gallic acid	3:4-Dihydroxybenzoic acid	Caffeic acid
5	11	—	—
6	89	—	—
7	—	39	—
8	—	61	31
9	—	—	69

employed. 80 ml. of ethanol are added and the evaporation repeated. This process is repeated twice. Pyrogallol is thereby removed by volatilisation.

Reacting phenolic acids are then determined in the residue as described above. The difference between this result and that obtained above is taken to be representative of the pyrogallol content.

Separation of Catecholic Phenolic Acids by Paper Chromatography

Whatman No. 1 filter paper (width 15 cm.) is used and development is with the organic phase of *n*-butanol/acetic acid/water (40/10/50) by the descending technique. A beaker containing the aqueous phase is placed

TABLE IV

THE RECOVERY OF GALLIC ACID AND 3:4-DIHYDROXYBENZOIC ACID FROM 10 ML. URINE AFTER SEPARATION ON PAPER

Quantity added, μg.	Quantity recovered, μg.	Recovery, per cent
Gallic acid		
1000	910	91
500	440	88
200	185	93
3:4-*Dihydroxybenzoic acid*		
500	450	90
250	210	84
100	85	85

at the bottom of the tank. Two parallel lines, 3 cm. apart, are drawn horizontally across the paper and just below the level of the trough. The extract obtained from at least 10 ml. of urine is dissolved in 10 ml. of ethanol and applied to the paper between the parallel lines. Development

is allowed to proceed until the solvent front has advan ced 30 cm. from the upper line (overnight). After drying, the paper is divided into 10 equal parts between the point of origin and the limit of the solvent front. Each strip is then extracted with cold ethanol overnight. The alcoholic extracts are evaporated to dryness and the Mitchell reaction applied to the residues.

TABLE V

CATECHOLIC PHENOLS AND PHENOLIC ACIDS IN HUMAN URINE

A, Daily exretion—as 3:4-*dihydroxybenzoic acid* 46 to 116 mg./day (8 cases)

B, "pyrogallol" excretion

	"Pyrogallol" (mg./day)	Total (mg./day)
1.	8·2	46
2.	10·6	84
3.	8·4	108
4.	7·6	92

C. Variations in excretion throughout 24 hours

Time of excretion	Total excreted (mg.)	Excretion (mg./hr.)
2 a.m. to 7 a.m.	14·4	2·8
7 a.m. to 10 a.m.	17·6	5·9
10 a.m. to 2 p.m.	20·0	5·0
2 p.m. to 10 p.m.	41·6	5·2
10 p.m. to 2 a.m.	12·9	3·2

D. The distribution of individual phenolic acids

	Per cent		
	A	B	C
Gallic acid	28	31	22
3:4-Dihydroxybenzoic acid ..	56	55	58
Caffeic acid	16	14	20

RESULTS

The data in Table I indicate that gallic, caffeic and 3:4-dihydroxybenzoic acids and pyrogallol added to urine can be determined quantitatively by the procedure described.

From Table II it can be seen that pyrogallol can be determined accurately in the presence of the reacting phenolic acids by the use of the difference technique.

The data in Table III show the zones occupied by the three reacting phenolic acids after separation by paper chromatography. Material occupying strips 5 and 6 and reacting to the Mitchell reaction is assumed to represent gallic acid. In the case of 3:4-dihydroxybenzoic and caffeic acids, there is some overlapping. For the purposes of the present experimental work, it will be assumed that reacting material located on strips 7 and 8 represents 3:4-dihydroxybenzoic acid whereas that located on strip 9 represents caffeic acid. It is quite possible that a complete separation could be achieved by using a longer time of development.

As shown in Table IV gallic and 3:4-dihydroxybenzoic acids added to urine can be determined quantitatively after separation by paper chromatography. Pyrogallol is not detectable after such treatment.

Excretion in Urine

Data are recorded in Table V from the examination of urine. The daily excretion appears to be about 100 mg./day (V*A*) and the greater part appears to be excreted during the day, suggesting that these substances are of dietary origin (V*C*). Dietary examinations would suggest that tea

TABLE VI

THE EXCRETION OF CATECHOLIC PHENOLIC ACIDS IN URINE AFTER THE ADMINISTRATION OF TANNIC ACID IN MG./DAY

A. Oral route (1·5 *g.**)

Day 1	Day 2	Day 3 (administered)	Day 4
38	51	149	70
106	95	185	90
34	78	168	85

B. Barium enema (1200 *ml.*) *containing* 2 *per cent tannic acid*

Day 1	Day 2	Day 3 (enema)	Day 4	Day 5
47	43	—	275	98
—	34	160	25	—
40	54	102	43	56
43	38	49	43	—
37	36	39	50	51

* 1·5 g. tannic acid was the total oral dose. Larger doses were difficult to tolerate.

infusions are responsible for most of the reacting material and that the intake is about 1 g./day. This would suggest that absorption rates are not particularly high.

More direct data on absorptive rates are recorded in Table VI. Administration of tannic acid by mouth (VI*A*) resulted in demonstrable increases in excretion but absolute absorptions are quite small. The data recorded in VI*B* are from patients receiving barium enema containing 2 per cent tannic acid for the X-ray examination of the colon. Absorptions are generally low.

The separation of phenolic acids by paper chromatography would suggest that 3:4-dihydroxybenzoic acid is the predominant reacting substance.

The presence of pyrogallol (V*B*), a relatively more toxic substance, is of some interest. It is probably derived from gallic acid by decarboxylation in the alimentary tract.

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THE PERSISTENT ANTAGONISTIC ACTION OF *N*-ALLYL-1-(*P-CYCLO*HEXYLOXYPHENYL)ETHYLAMINE TO ANALGESIC AGENTS

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N-Allyl-l-(*p-cyclo*hexyloxyphenyl)ethylamine, given one to two days beforehand, reduced the amount of l-(*p-cyclo*hexyloxyphenyl)ethylamine entering the adrenal glands of rats after an intraperitoneal dose by about 5 μg./g. without affecting non-specific accumulation. The effect was not observed with a closely similar, but analgesically inactive amine. The cerebral cortex acquired increased ability to accumulate the amine but heart remained unaffected. The reduced ability of the adrenal to accumulate the amine was restored at the same time that the analgesic antagonistic activity of the allylamine had begun to wane.

Antagonism of morphine analgesia by nalorphine was mimicked in rats by *N*-allyl-l-(*p-cyclo*hexyloxyphenyl)ethylamine[1]. The effect was slow to develop, taking some twelve hours, and then persisted for several days. A formal chemical resemblance to dibenamine, whose persistent adrenergic blocking action has been attributed to absorption by, and slow release from, body fat[2], prompted an investigation of the distribution and release of the amine in rat tissues, and a search for any changes that could parallel its prolonged effect.

Methods and Materials

(1-^{14}C)-*N*-Allyl-l-(*p-cyclo*hexyloxyphenyl)ethylamine hydrochloride, m.p. 162–164° with 26,200 counts/min./mg., and the corresponding primary amine, m.p. 176–178°, with 30,200 counts/min./mg., both at infinite thickness using a G.E.C. EHM2 mica end window counter, were prepared by published methods[1,3]. Albino rat tissue samples were dried at 100° and powdered. They were counted at infinite thickness on polythene discs[4], recording 2,000–2,500 counts (approximate error 2 per cent) for any tissue showing marked activity. Where necessary kieselguhr was added to the powder, usually the adrenal, as an inert diluent to give sufficient mass for infinite thickness.

Table I shows that any deviation produced by this procedure was about the same as the counting error. Animals were killed and bled out by excision of the heart under ether anaesthesia.

Results

Distribution of the labelled allylamine in male rat tissues at ten minutes after giving 20 mg./kg. intraperitoneally or 10 mg./kg. intravenously resembled that of labelled 1-(*p-cyclo*hexyloxyphenyl)-ethylamine found in previous experiments[3]. Perirenal fat gave low counting rates, approximately three times background and equivalent to 5–10 μg./g. tissue.

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There was little activity in gastrocnemius muscle, plasma or whole blood. One hour after the dose the tissue concentrations were little changed, but only traces of activity remained in the nineteen tissues examined at twenty-four hours after the dose. Rather more than half the injected counts appeared in urine within twenty-four hours but little further

TABLE I

THE EFFECT ON COUNTING RATE OF DILUTION OF TISSUE SAMPLES WITH KIESELGUHR

Tissue	Dilution per cent	Observed count/min. ± S.D.	Expected count/min.
Kidney	0	288 ± 6	—
	40	178 ± 4	169–176
	60	115 ± 5	112–118
Cerebral cortex	0	198 ± 5	—
	50	113 ± 5	96–102
Heart	0	218 ± 5	—
	50	120 ± 5	106–116

excretion occurred by this route. Faeces were not examined. The only tissues found to show evidence of residual activity were those of adrenal and kidney. After lapse of four days after the dose, these tissues, when dried, gave about two counts/min. above a background of 8–10 counts/min.

The allylamine (20 mg./kg. intraperitoneally) or dibenamine (20 mg./kg. intraperitoneally) given two days previously, or nalorphine (20 mg./kg. intraperitoneally) given fifteen minutes previously had no obvious influence on the amount of 1-(*p-cyclo*hexyloxyphenyl)ethylamine entering tissues,

TABLE II

THE INFLUENCE OF ANTAGONISTS ON THE DISTRIBUTION OF 1-[^{14}C]-1-(*p-cyclo*HEXYLOXYPHENYL)ETHYLAMINE IN RAT TISSUES EXPRESSED AS THE RATIO TO CEREBELLAR CORTICAL CONCENTRATION

Tissue	Control Ratios	Allylamine 1 day	Allylamine 2 days	Allylamine 3 doses at 6, 4 and 2 days	Dibenamine 2 days	Nalorphine 15 mins.
Cerebral cortex	1·60 ± 0·05 (9)	1·80*	1·65	1·82*	1·83*	1·50
Thalamus	0·99 ± 0·12 (5)	1·23	1·11	—	1·15	—
Hypothalamus	0·80 ± 0·11 (4)	0·94	0·84	—	1·04	0·81
Dorsal root ganglia	0·47 ± 0·07 (4)	0·45	—	—	—	0·33
Adrenal	3·58 ± 0·32 (6)	2·63*	2·78*	2·54*	2·82*	2·90*
Thyroid	1·90 ± 0·57 (6)	—	1·93	—	1·14	2·32
Heart	1·85 ± 0·26 (6)	—	1·87	1·42	1·42	—

Doses of all drugs were 20 mg./kg., i.p. Rats were killed ten minutes after the dose of amine. Antagonists were given at times stated before the amine. Number of results for estimation of control ratios in parentheses. Allylamine = *N*-Allyl-1-(*p-cyclo*hexyloxyphenyl)ethylamine.

* Indicates ratios falling outside two S.D. of controls.

as assessed at ten minutes after dosage, when compared with previous results in the same strain of rat[3]. When the tissue distributions were expressed relative to a reference tissue however, a limited disturbance in the adrenal and cerebral cortex became apparent. Since plasma activity was always too low to count accurately and gave widely divergent ratios,

cerebellar cortex was chosen as the reference tissue. This could be reasonably expected to have little bearing on development of analgesia. Table II shows that in spite of considerable variation in tissue activities between individual rats (cerebellar cortical concentrations varied from 20–45 μg./g. wet tissue: mean, 31·4 ± 9·1 S.D.), the ratios to cerebellar cortical concentrations were reasonably constant in animals without drug pretreatment, for the tissues studied. Cerebellar cortical concentrations in rats previously given allylamine were rather higher but not significantly so (range 17–56 μg./g. wet tissue: mean, 41·2 ± 10·0 S.D.). In three

TABLE III

WET: DRY RATIOS OF RAT TISSUES AFTER TREATMENT WITH PHENYLETHYLAMINES

Tissue	1-(*p-cyclo*Hexyloxyphenyl)-ethylamine		*N*-Allyl-1-(*p-cyclo*hexyloxy-phenyl)-ethylamine
	After saline (6)	24 hr. after *N*-allylamine (5)	(4)
Cerebral cortex	4·96 ± 0·12	4·90 ± 0·13	4·89 ± 0·07
Cerebellar cortex	4·53 ± 0·06	4·50 ± 0·09	4·49 ± 0·07
Heart	4·12 ± 0·11	4·18 ± 0·14	4·16 ± 0·09
Adrenal	3·36 ± 0·14	3·31 ± 0·23	3·29 ± 0·18

Number of results used for estimation of control ratios in parentheses.
Doses were 20 mg./kg., i.p.
Tissues were taken ten minutes after the last dose of amine.

experiments under slightly different conditions (see Table II) the adrenals gave a ratio between two and three standard deviations lower than the controls. Dibenamine or nalorphine pretreatment in single experiments had a similar effect. Conversely the ratio to cerebral cortex tended to increase. Heart, thyroid, and a few gross dissections of the central nervous system showed no comparable change. The effect was not traceable to a fluid shift in the tissues. In spite of a marked increase in haematocrit after the amine there was little change in this strain of rat in the wet: dry ratios between treated and control animals (Table III).

To confirm the result, sixteen female albino rats of a different strain, weighing 225 g., were divided into two equal groups. Females were chosen since their adrenals are larger than in males. One group received the allylamine, 20 mg./kg. intraperitoneally, on each of two successive days, while the other group received saline. One pair from each group received labelled 1-(*p-cyclo*hexyloxyphenyl)ethylamine, 20 mg./kg. intraperitoneally, at 1, 2, 7 and 14 days after the last dose of allylamine. They were killed ten minutes after the dose, and the adrenals, apex of the heart, cerebral cortex and cerebellar cortex dissected rapidly and prepared for counting. A scintillation assembly was used instead of the mica end window counter. There was rather greater variation in the wet: dry ratios of the tissues compared with the previous result and a clearer picture emerged by calculating ratios on a dry weight basis. Some justification for this procedure can be derived from the following points which indicate that the amine is mainly associated with formed elements of the tissues rather than dissolved in cellular fluids. It was not possible to derive a reasonably

constant ratio by comparison with plasma activity and the amine virtually disappeared from blood within 2–5 minutes after intravenous injection. It was not absorbed from a subcutaneous depot, the tissue blackening and eventually sloughing as a hard mass, an observation consistent with the failure to obtain a subcutaneous LD50 value[5]. This absorption by tissue to give a spurious concentration gradient was observed *in vitro* when slices

TABLE IV

RESTORATION OF CONCENTRATION RATIO BETWEEN TISSUE AND CEREBELLAR CORTEX FOR 1-(*p-cyclo*HEXYLOXYPHENYL)ETHYLAMINE AFTER PRETREATMENT WITH THE CORRESPONDING *N*-ALLYLAMINE

Tissue	1-(*p-cyclo*Hexyloxyphenyl)ethylamine					1-(*p-iso*Propyloxyphenyl) ethylamine	
	Control Ratios ± S.D.	1 day	2 days	7 days	14 days	Control Ratios ± S.D.	1 day
Cerebral cortex	1·69 ± 0·12 (4·88 ± 0·09)	1·88 (4·96)	1·65 (4·74)	1·78 (4·90)	1·65 (4·99)	1·61 ± 0·02	1·70 ± 0·27
Heart	1·62 ± 0·15 (3·99 ± 0·12)	1·62 (4·04)	1·62 (3·96)	1·54 (4·01)	1·72 (4·15)	0·83 ± 0·12	0·91 ± 0·51
Adrenal	2·80 ± 0·30 (3·22 ± 0·16)	1·94 (3·09)	2·52 (3·12)	2·73 (3·15)	2·66 (3·33)	1·36 ± 0·07	1·39 ± 0·54

Ratios are referred to dry weights of tissue. In the treated series they are the mean of two results. Control ratios were estimated in a group of eight rats. Treated rats received the allylamine on two successive days before receiving the primary amine. Times refer to lapse after the last dose of allylamine. All doses were 20 mg./kg., i.p. Tissue wet : dry ratios are in parentheses.

of cerebral cortex took up the amine from salines independent of interference with energy generating processes by addition of metabolic inhibitors[6]. Table IV shows that the ratios for adrenal and cerebral cortex had the expected disturbance at one day after completing the allylamine dosage, that the cerebral cortex had recovered within two days, and the adrenal within seven days. The chemically similar but analgesically inactive 1-(*p-iso*propyloxyphenyl)-ethylamine[5] revealed no similar disturbance in two groups of four animals tested at one day following treatment with the allylamine. The heart remained unaffected in each instance.

DISCUSSION

The prolonged effect of the allylamine could not be related to absorption and retention by any of nineteen representative rat tissues, nor would this hypothesis explain the relatively long lag in onset of analgesic antagonism in this species. Agarwal and Harvey[7] thought that the prolonged effect of dibenzyline, a dibenamine-like drug, was not explicable on this basis. Though there was no good evidence for retention of the allylamine in tissues for periods exceeding twenty four hours, with the doubtful exceptions of adrenal and kidney, the method of assay was not sufficiently sensitive to detect amounts that could well have physiological importance. The concentrations required to give a count of twice background exceeded 3 μg./g. wet tissue, equivalent to 0·01 μmole/g. Moreover activity could be mediated by breakdown products not incorporating the labelled atom. Nevertheless, ignoring the last possibility, if it be assumed that the fall in ratio for the adrenal arises by blockade of specific receptors, an effect

being obscured by non-specific accumulation, then the amount of allylamine so associated at twenty four hours after the dose, as calculated from the observed decrease in ratio, should be about 5 μg./g., corresponding to a count roughly three times background and therefore easily detectable. Present results seem more consistent with a hypothesis that the amine alters tissues in the adrenal and possibly the cerebral cortex without retention to produce a slowly reversible change. The fair parallel between the restoration of the normal concentration ratio in the adrenal and return of sensitivity to the analgesic activity of the primary amine[1] suggests that these changes are related in some obscure manner to generation of analgesic antagonism. Possible reasons for an increased ratio in the cerebral cortex cannot be profitably discussed but it is of interest that the allylamine in no way antagonised the depression of spontaneous activity induced in rats by the analgesic primary amine.

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THE PERCUTANEOUS ABSORPTION OF SULPHANILAMIDE

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A test has been designed suitable for the comparison of vehicles and bases as "carriers" of sulphanilamide through the intact skin of rabbits. The blood levels attained were taken as a measure of percutaneous absorption. Statistical analysis of the results showed that the differences in efficiency of the vehicles and bases tested were highly significant.

We have recently reviewed the methods which have been adopted for the evaluation of drug release from topical applications[1]. The sulphonamides penetrate the intact healthy skin only slowly and the amount absorbed into the bloodstream is small. The value of the *in vitro* methods which have been suggested for the estimation of drug release is doubtful since no membrane, either artificial or natural, exists which will simulate the properties of the intact healthy skin.

A number of *in vivo* methods which have been proposed are of interest. Strakosch and Clark[2] applied various sulphonamide ointments to guinea pigs and carried out biopsies on the treated skin. They were unable to demonstrate significant differences between different types of ointments and showed that an increase in concentration of sulphanilamide from one per cent to ten per cent did not greatly increase the concentration found in the skin, although the duration of application did so. Also, an increase in concentration of sulphanilamide over one per cent had little effect on tissue levels. A concentration of up to 6 mg. per cent sulphanilamide was obtained in skin biopsies. Repeated applications of a five per cent sulphathiazole ointment over half the body surface of infants under treatment for skin infections were shown to give blood concentrations of 2 to 4 mg./100 ml.[3]. Zondek, Bromberg and Shapiro went so far as to suggest that the percutaneous absorption of sulphanilamide might be useful where the oral route of administration is not possible[4]. Using rabbits in their experiments they obtained blood concentrations of sulphanilamide of 1 mg./100 ml. in one hour to 8 mg./100 ml. in three and four hours from 0·5 g. sulphanilamide applied as a solution in acetone, glycerol, liquid soap and water. Woodward and others[5] determined sulphathiazole levels in blood samples, catheterised urine samples, at intervals after the application of ointment to the clipped intact skin of rabbits, and carried out localisation tests by analysing definite areas of skin. Among the observations made was one that localisation from a five per cent sulphathiazole ointment equalled that from one of twenty per cent. Clark, Strakosch and Nordlum[6], by the use of iontophoresis, attempted to increase the penetration of sulphonamides into the skin. They found that penetration was essentially equal by iontophoresis and from wet dressings but was less from an ointment base. Fuller, Hawking, and Partridge[7] determined the rate of absorption from standard wounds

in rabbits by estimating the excretion of drug in urine, but they did not concern themselves with the intact skin. Strakosch and Clark[8] and Clark[9], by means of tissue analysis, determined the rate of penetration of sulphonamides into the intact skin of guinea pigs and were unable to show correlation of penetration and the type of ointment base used. Increase in time of application increased penetration but the addition of a solubilising and a wetting agent did not.

In view of the somewhat conflicting and inconclusive results relating to the percutaneous absorption of the sulphonamides, and in spite of the suggestion by Hawking and Lawrence[10] that for all practical purposes the action of the sulphonamides applied to the skin is limited to the surface and superficial layers, this study was undertaken to determine the extent, and to compare the degree, of absorption of sulphanilamide from a number of different vehicles and common ointment bases.

The most promising method for this work was considered to be a technique whereby sulphanilamide, following its cutaneous application, was detected in the bloodstream of the animal. This method provided conclusive proof of its absorption since the sulphanilamide applied in a suitable vehicle must necessarily penetrate the skin and enter the bloodstream in order to be detected and simple penetration or lodgement within the appendages may be discounted. The blood level of sulphanilamide estimated at suitable time intervals would allow a comparison of the efficiency of various vehicles and ointment bases as "carriers" for the drug and since the study of blood concentrations has been a feature of sulphonamide therapy, and the sulphonamide drugs are uniformly distributed in the animal body, this seemed to be the logical approach.

Previous workers in this field have in general provided insufficient results for complete statistical examination. The series of experiments recorded in this paper were designed with a view to obtaining sufficient results to make such an analysis.

Methods

Choice of Sulphanilamide

Sulphanilamide, *p*-aminobenzenesulphonamide, was chosen as a rapidly absorbed sulphonamide with a solubility in water of 400 mg./100 ml. at 15°, and 1,500 mg./100 ml. at 37°, and a solubility in serum of 1,970 mg./100 ml. It is soluble in ethanol, acetone, glycerol, hydrochloric acid and sodium and potassium hydroxides. It is insoluble in ether and benzene and soluble in chloroform to the extent of 25 mg./100 ml. and in propylene glycol to the extent of 10,000 mg./100 ml. The acid dissociation constant (pKa) is 10·43.

Choice of Vehicle

Seven common constituents of ointment bases were tested for their ability to modify the absorption of sulphanilamide and their respective efficiencies were compared. They were liquid paraffin, white soft paraffin, lard, woolfat, propylene glycol, and water in the form of a five per cent carboxymethyl cellulose gel.

Four ointment bases were tested. They were Hydrous Ointment B.P., a water-in-oil emulsion, Emulsifying Ointment B.P., and two oil-in-water emulsions, Hydrous Emulsifying Ointment B.P., and a cetomacrogol emulsifying wax base of the following composition: Cetomacrogol Emulsificans B.P.C. 14 g., liquid paraffin 8 g., and water to 100 g.

Concentration of Sulphanilamide

Although concentration is stated to increase penetration, it has been reported that an increase in strength above five per cent is not significant.

TABLE I

COMPARISON OF PERCUTANEOUS ABSORPTION OF SULPHANILAMIDE IN THE RABBIT FROM DIFFERENT VEHICLES AND BASES

	Total amount sulphanilamide observed in the blood of 6 individual rabbits over 8 hours (each value is the sum of sixteen samples), mg./100 ml.						Total amount sulphanilamide observed in 6 rabbits over 8 hours
	Rabbit number						
	1	2	3	4	5	6	
Liquid paraffin	9·39	9·53	7·71	5·53	6·21	6·32	44·69
White soft paraffin	11·57	11·72	12·02	10·02	9·65	8·85	63·83
Woolfat	8·08	6·84	8·00	6·06	5·61	5·24	39·83
Lard	14·14	16·05	14·54	16·24	13·03	11·92	85·92
Ethyl oleate	12·98	15·60	8·92	6·53	7·89	11·12	63·04
Propylene glycol	17·17	12·19	8·64	8·69	8·80	9·55	65·04
5 per cent methyl cellulose	4·46	4·47	4·64	7·67	7·19	5·93	34·36
Hydrous Ointment B.P.	7·39	12·54	12·29	12·44	9·68	10·56	64·90
Emulsifying Ointment B.P.	7·62	9·08	8·45	6·27	7·71	5·57	44·70
Hydrous Emulsifying Ointment B.P.	5·69	5·72	6·28	6·56	6·58	9·88	40·71
Cetomacrogol emulsifying wax base	8·82	8·30	9·01	8·76	6·97	8·43	50·29

The concentration of sulphanilamide used in all applications was ten per cent weight-in-weight, a strength recommended as a maximum useful concentration[8].

Design of Test

The experiments were designed to give sufficient results for a statistical analysis. All tests were made on six rabbits from the same litter since litter-mates usually yield results less variable than those obtained from animals selected at random from an animal population. Rabbits used in each test consisted of three males and three females between 2·5 and 3·5 kg. weight.

No. of rabbits	Base	No. of tests on each base
6	A	2
6	B	2
6	C	2

The design of the tests made on six rabbits is shown above. The three bases, A, B, and C, were applied in random order thus avoiding any cumulative effect which might have occurred.

Experimental Details

Six Copenhagen White rabbits, litter-mates, weighed and sexed, were used in each test. Food, but not water, was withheld for eighteen hours

before the test since it was found that by this procedure the animals seldom if ever urinated or defaecated while the test was in progress; thus the task of assaying excretory products was avoided. On the day before the test the fur was removed from the skin of the back and sides of each animal with electric clippers, taking care not to damage the skin. The area

TABLE II

COMPARISON OF PERCUTANEOUS ABSORPTION OF SULPHANILAMIDE IN THE RABBIT FROM DIFFERENT VEHICLES AND BASES

	Mean blood levels of sulphanilamide observed in 6 individual rabbits over 8 hours, mg./100 ml.						Mean blood level of sulphanilamide observed in 6 rabbits over 8 hours, mg./100 ml.
	Rabbit number						
	1	2	3	4	5	6	
Liquid paraffin	0·59	0·59	0·48	0·35	0·39	0·39	0·47
White soft paraffin	0·72	0·73	0·75	0·63	0·60	0·55	0·66
Woolfat	0·50	0·43	0·50	0·38	0·35	0·33	0·41
Lard	0·88	1·03	0·91	1·02	0·82	0·75	0·89
Ethyl oleate	0·81	0·98	0·56	0·41	0·49	0·70	0·66
Propylene glycol	1·07	0·76	0·54	0·54	0·55	0·60	0·68
5 per cent methyl cellulose	0·28	0·28	0·29	0·48	0·44	0·37	0·36
Hydrous Ointment B.P.	0·46	0·78	0·77	0·78	0·60	0·66	0·68
Emulsifying Ointment B.P.	0·48	0·56	0·53	0·39	0·48	0·35	0·47
Hydrous Emulsifying Ointment B.P.	0·36	0·36	0·39	0·41	0·41	0·62	0·42
Cetomacrogol emulsifying wax base	0·55	0·52	0·56	0·55	0·44	0·53	0·52

under test was marked by a dermograph pencil using a 6 × 4 inch template placed on the dorsal thoracico-lumbar region of the rabbit. The rabbits were then placed in restraining boxes to prevent any oral absorption and a 0·2 ml. blood sample was withdrawn from the marginal ear

TABLE III

COMPARISON OF PERCUTANEOUS ABSORPTION OF SULPHANILAMIDE IN THE RABBIT FROM DIFFERENT VEHICLES AND BASES

	Table of efficiencies	
	Total amount of sulphanilamide absorbed by six rabbits, mg.	Mean individual rabbit response, mg.
Lard	85·92	14·32
Propylene glycol	65·04	10·84
Hydrous Ointment B.P.	64·90	10·82
White soft paraffin	63·83	10·64
Ethyl oleate	63·04	10·51
Cetomacrogol emulsifying wax base	50·29	8·38
Emulsifying Ointment B.P.	44·70	7·45
Liquid paraffin	44·69	7·45
Hydrous Emulsifying Ointment B.P.	40·71	6·79
Woolfat	39·83	6·64
5 per cent methyl cellulose	34·36	5·73

vein of each animal as a blank for assay purposes. About 30 g. of vehicle or base, containing ten per cent sulphanilamide, was applied to the marked area and inuncted for a period of three minutes. Liquid preparations were applied with a brush. Reapplications, in both instances, were at fifteen minute intervals. At intervals of thirty minutes, after the initial

application, over a period of eight hours, 0·2 ml. blood samples were withdrawn. Thus each completed test involved the withdrawal of sixteen blood samples from each of the six rabbits giving a total of ninety-six samples for one base, and for the complete experiment on three bases, each applied twice, a total of five hundred and seventy-six blood samples. On completion of a single test, the rabbits had the application removed and the area thoroughly washed with warm, soapy water. After several washings, their backs were rinsed and dried. Before a further test, the rabbits were allowed at least one week's rest and their blood was then tested for absence of sulphanilamide.

Estimation of Sulphanilamide in Blood Samples

Sulphanilamide in blood was estimated by King's micro-modification of the method of Bratton and Marshall[11]. All the solutions were assayed at 540 mμ, the wavelength of maximal absorption for sulphanilamide[12].

Results

The results are summarised and shown in Tables I, II and III; the results of the statistical analysis are given in Table IV.

In Table I the results are shown for six individual rabbits; the values obtained in each of the sixteen blood samples withdrawn over the eight

TABLE IV

Analysis of variance

Source of variance	Sum of squares	Degrees of freedom	Variance
Between vehicles or bases ..	397·834	10	39·7834
Residual	214·332	55	3·8969

$$F = \frac{39{\cdot}7834}{3{\cdot}8969} = 10{\cdot}21$$

F = 3·7 corresponds to P = 0·001.

The Standard Deviation corresponding to the residual variance of 3·8969 is 1·97.
This gives a Standard Deviation for means of samples of six equal to 0·81.

$$\frac{1{\cdot}97}{\sqrt{6}} = 0{\cdot}8059 \simeq 0{\cdot}81.$$

hours were added together and this sum was entered in the Table. The total amount absorbed by each rabbit can thus be seen. These individual totals were then added together and the response of the six rabbits to the base under examination was found.

Table II was calculated from Table I. The total amount absorbed by the individual rabbits over the eight hours was divided by sixteen, i.e. the number of blood samples withdrawn, and the mean blood level for the duration of the test obtained. The average of the blood levels found for the individual rabbits was then taken for each vehicle and base and this figure was used as an index of the efficiency of the particular vehicle or base tested. These values are represented graphically in Figure 1. Figure 2 represents graphically the mean blood level over eight hours found in the individual rabbits for each of the bases tested.

Table III was also obtained from Table I by dividing the total amount absorbed by the six rabbits over the eight hours by six, thus giving the mean value of an individual rabbit response.

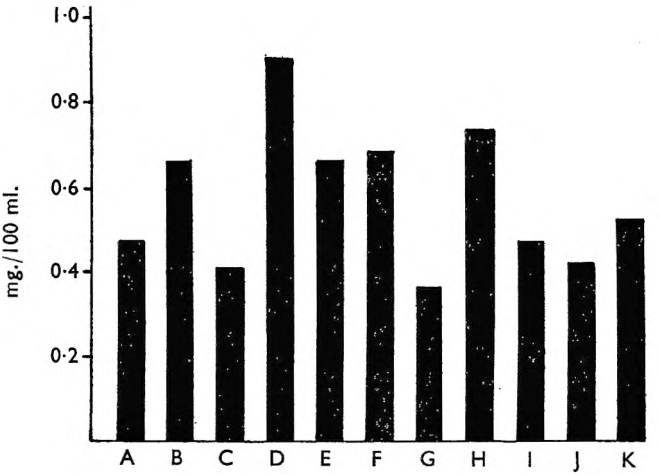

FIG. 1. The mean blood level of sulphanilamide observed in rabbits following the application of the selected vehicles and bases.

A Liquid paraffin.
B White soft paraffin.
C Woolfat.
D Lard.
E Ethyl oleate.
F Propylene glycol.
G 5 Per cent methyl cellulose gel in water.
H Hydrous Ointment B.P.
I Emulsifying Ointment B.P.
J Hydrous Emulsifying Ointment B.P.
K Cetomacrogol emulsifying wax base.

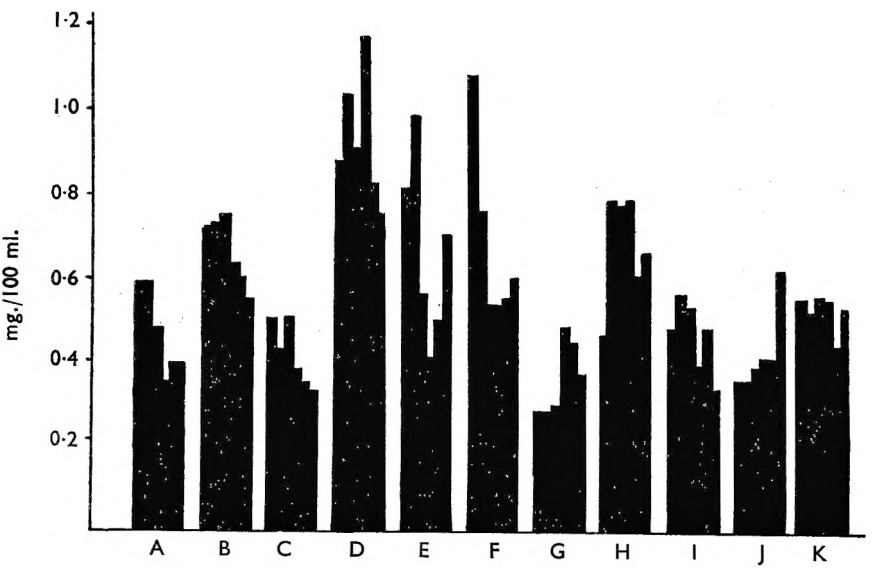

FIG. 2. The blood level of sulphanilamide observed in individual rabbits following the application of the selected vehicles and bases. Six animals were used with each base. See Figure 1 for key.

Table IV shows the analysis of variance carried out on the results of the series of experiments. The Standard Deviation corresponding to the residual variance of 3·8969 is 1·97 giving a standard deviation for means of samples of six equal to 0·81. This value may be used as a rough measure for significant differences between the vehicles and bases in Table III. A difference between the vehicles and bases in the mean individual rabbit response of less than 0·81 is unlikely to be significant. A difference in this response of more than 0·81 is likely to be significant. It will be seen from Table III that the vehicles and bases fall into six groups. the differences between each group being significant.

Discussion

A statistical analysis of the experimental results has been made. An analysis of variance of the results in Table I showed that the differences in the bases as efficient "carriers" of sulphanilamide were very highly significant ($F = 10{\cdot}21$ where $F = 3{\cdot}7$ for $P = 0{\cdot}001$). On this basis it is possible to arrange the vehicles and bases in several groups. Within a group, the vehicles or bases are equally efficient, but there are significant differences between the vehicles or bases in different groups. The difference in response between individual rabbits was insignificant.

Sulphanilamide, when applied in various vehicles and bases to the intact, healthy skin of rabbits, penetrates the skin and is absorbed into the blood stream where it can be detected in measurable quantities. The amount of sulphanilamide circulating in the bloodstream is small. For example, using lard, which was found to be the most effective vehicle or "carrier," the maximum blood level observed in any one rabbit was 1·03 mg./100 ml. and from the six rabbits tested with this vehicle the mean blood level obtained was 0·89 mg./100 ml. The blood levels obtained from other vehicles and bases were lower and in the case of the five per cent carboxymethyl cellulose gel in water, which was in effect absorption from water, the methyl cellulose being present merely as an aid to application, a mean blood level of 0·36 mg./100 ml. was obtained. For the other vehicles and bases, the mean blood levels varied between 0·41 and 0·66 mg./100 ml.

These findings emphasise that the intact healthy skin of the rabbit presents an effective and almost impermeable barrier to the passage of sulphanilamide applied percutaneously, especially when it is considered that approximately one-fifth of the body area of the animals under test was exposed. Blood levels which have been reported over 3 mg./100 ml. following the application of various topical preparations containing sulphanilamide should be viewed with caution, but in the case of wounded, burned, or diseased skin, where the outer epithelial layers are damaged, systemic absorption is greater and blood levels of this order may be expected. It is interesting to note that for prophylaxis the blood level of sulphanilamide considered adequate is 2 to 3 mg. per 100 ml.[10].

Despite the small amount of sulphanilamide absorbed, differences in the efficiencies of the chosen vehicles and bases as "carriers" could be

clearly distinguished and the method can, therefore, be used for comparative purposes.

Absorption from the methyl cellulose gel showed that water as a "carrier" of sulphanilamide was the least effective vehicle tested. This was borne out by the low absorption from Hydrous Emulsifying Ointment B.P., an oil-in-water emulsion, which has a continuous water phase, whereas Hydrous Ointment B.P., a water-in-oil emulsion, with a continuous oil phase, was approximately one-and-a-half times more efficient as a "carrier." Such a finding had been reported previously[13] and suggests that the absorption of sulphanilamide by way of the sebaceous glands and hair follicles is simplified when the drug is presented to the skin in an oil phase. The skin surface being protected by a greasy layer of waxes is impervious to water and aqueous solutions and absorption of sulphanilamide occurring from the aqueous phase must be due to the partitioning of the drug between the aqueous phase and the fats of the sebaceous glands and hair follicles.

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A NOTE ON THE SUITABILITY OF THE INDIAN FROG (*RANA TIGRINA*) FOR THE BIOLOGICAL ASSAY OF TINCTURE OF DIGITALIS

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The relationship between the log of the dose of tincture of digitalis and the effect, in terms of probits of the mortalities (per cent) among the Indian frog, *R. tigrina*, has been found to be linear. Statistical analysis of the data collected from four experiments performed from July to November has not shown any non-linear relationship. The results obtained by applying the B.P. 2 and 2 quantal response assay on the frog method have been found to be in fair agreement with those obtained by using guinea pigs. The fiducial limits of error of potency of the samples at (P = 0·95) have been found to lie within the limits specified in the B.P. 1953. By using *R. tigrina*, potencies of the standard, substandard and abnormal samples of tincture of digitalis have been satisfactorily determined. The advantage of the frog method is the speed and simplicity of the technique: the disadvantage is the restricted availability of the suitable size of the frog during the five months July to November.

Rana temporaria is not available in India, and it therefore seemed desirable to investigate the suitability of an Indian frog *Rana tigrina* for the frog assay of digitalis in the 1953 British Pharmacopoeia. We have also compared the results obtained with *R. tigrina* with those obtained from the B.P. assay method using guinea pigs.

EXPERIMENTAL

Dose Mortality Relation

Specimens of *R. tigrina* weighing from 25 to 50 g. were divided into five groups of 20 such that the average weight of the frogs in the groups were almost equal. Females were equally distributed in the groups; those with distended abdomens were not used. Apart from these restrictions the selection was random.

A tincture was prepared by method (2) in the B.P. using International Standard digitalis leaf powder. The tincture served as the stock solution and was stored in a refrigerator at 5°. For use the solution was diluted with 0·6 per cent saline and injected into the ventral lymph sac in doses of 0·02 ml./g. The number of frogs in each group which died from the specific effect of digitalis on the heart was determined. Four sets of results during the period July to November were obtained.

Comparison of Frog and Guinea Pig Methods

Frog method. Eighty frogs in groups of 20 were injected in a 2 and 2 quantal response assay as described in the B.P. using dilutions of the stock solution and of test preparation. The estimation of potency ratio of test and standard and the calculation of the fiducial limits of error were

made according to the B.P. recommendation under the "2 and 2 dose assay" procedure.

Guinea pig method. Healthy male animals weighing between 300 and 600 g. were selected in groups of 12 for each experiment, such that the individual weights in each group did not differ by more than 100 g. Each group was then divided into two groups of 6 such that the average weights of both were almost equal. The guinea pigs were anaesthetised by subcutaneous injection of 25 per cent urethane in a dose of 1·8 g./kg. body weight, and the standard and test preparation in 0·9 per cent saline infused

TABLE I

DOSE-MORTALITY DATA ON FROGS

Dose (mg. leaf/g. body weight)	Mortality—number dead/number injected			
	I: July	II: September (2nd week)	III: September (4th week)	IV: November
0·250	2/20	0/20	1/20	1/20
0·375	4/20	4/20	4/20	6/20
0·500	14/20	8/20	7/20	13/20
0·625	17/20	15/20	14/20	18/20
0·750	18/20	16/20	19/20	20/20

into the jugular vein at about 1 ml./6 minutes. No artificial respiration was given. Estimation of test and standard, and calculation of the fiducial limits of error, were made according to the Pharmacopoeial method recommended under "Assays depending upon measurement of the effective dose of each animal", Example III.

RESULTS AND DISCUSSION

Dose-mortality Relationship

Probit analysis. The results of the first four experiments to establish the dose-mortality relationship on frogs are given in Table I. The mortalities in each experiment were converted into percentages and plotted against logarithms of the respective doses. The resultant curves assumed typical sinusoidal shape. The data were analysed and the probits of the percentage mortality were plotted against the logarithms of the doses; an examination of the curves thus obtained revealed that in each case a straight line would fit the observations. The regression of the probit of the percentage mortality on the logarithm of the corresponding dose was estimated in each experiment. The relationships observed as a result of this analysis are given in Table II.

The regression equations in the case of experiments II and IV were obtained by iteration as mortalities of 0·0 per cent and 100 per cent respectively were observed in these cases.

Linearity of regression in the experiments. The assumption of linearity of regression in each experiment was tested by calculating the value of χ^2 according to the following formula.

$$\chi^2 = \Sigma\, nw\,(y-\bar{y})^2 - \frac{[\Sigma\, nw\,(x-\bar{x})\,(y-\bar{y})]^2}{\Sigma\, nw\,(x-\bar{x})^2}$$

The degrees of freedom associated with χ^2 were three in respect of experiments I and III and two for experiments II and IV. The values of χ^2 in the experiments are given below Table II. As the values of χ^2 corresponding to 5 per cent level of probability and 2 and 3 degrees of

TABLE II

ANALYSIS OF THE DATA IN TABLE I

	Dose of digitalis leaf mg./g. body weight	Log dose of digitalis leaf mg./g. body weight	Percentage mortality	Probit	Regression coefficient
I	0·250	−0·60	10	3·7	6·05
	0·375	−0·43	20	4·15	
	0·500	−0·30	70	5·66	
	0·625	−0·20	85	6·05	
	0·750	−0·12	90	6·25	
II	0·250	−0·60	0	—	6·47
	0·375	−0·43	20	4·15	
	0·500	−0·30	40	4·80	
	0·625	−0·20	75	5·65	
	0·750	−0·12	80	5·85	
III	0·250	−0·60	5	3·35	6·26
	0·375	−0·43	20	4·15	
	0·500	−0·30	35	4·62	
	0·625	−0·20	70	5·50	
	0·750	−0·12	95	6·62	
IV	0·250	−0·60	5	3·35	7·87
	0·375	−0·43	30	4·48	
	0·500	−0·30	65	5·40	
	0·625	−0·20	90	6·25	
	0·750	−0·12	100	—	

Values of χ^2 in the four experiments:—I = 2·19; II = 1·37; III = 2·57; IV = 0·56.

freedom were 5·991 and 7·815 respectively, none of the values of χ^2 in the above experiments were significant, showing that there is no evidence of non-linearity of regression in any of the four experiments. Therefore the 2 and 2 quantal response assay method described in the Pharmacopoeia was considered applicable in this case.

TABLE III

COMPARISON OF THE RESULTS OBTAINED BY ASSAYING TINCTURE OF DIGITALIS ON FROGS (*R. tigrina*) AND GUINEA PIGS

Sample No.	Frog method		Guinea pig method		B.P. limits	
	Potency ratio (T/S) per cent	Fiducial limits of error (P = 0·95) per cent	Potency ratio (T/S) per cent	Fiducial limits of error (P = 0·95) per cent	Potency ratio (T/S) per cent	Fiducial limits of error (P = 0·95) per cent
1*	149·3	72·5 to 139·8	134·2	90·6 to 110·4	90 to 110	70 and 140
2	98·6	89·6 to 111·4	96·2	93·0 to 107·4		
3	100·7	86·8 to 113·5	99·6	99·7 to 100·3		
4	77·3	77·5 to 117·9	83·8	78·7 to 127·1		

Sample 1 was obtained locally and was freshly manufactured. Samples 2 and 3 were standard tinctures prepared from International digitalis powder by the B.P. method (2). Sample 4 was a known dilution (80 per cent potency) of the standard.

Comparison of Frog and Guinea Pig Methods

Four samples of tincture of digitalis were assayed against the standard preparations by using the technique described. The results are tabulated in Table III.

It will be seen that the results of the frog method, using *R. tigrina*, in these four experiments compare very favourably with the method using guinea pigs; the limits of error of the estimated potency ($P = 0{\cdot}95$) lie within the tolerances prescribed in the Pharmacopoeia. The frog method also appears to be suitable for assessing the potency of the samples which is beyond the acceptable range recommended in the Pharmacopoeia.

The frog method has the advantage that the technique employed is simpler and less time-consuming than the other B.P. methods, while a disadvantage is that the frogs of required body weight are available only during July to November in the year.

A METHOD FOR THE ESTIMATION OF ADRENALINE AND NORADRENALINE IN URINE.*

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A method for the estimation of sympathin (adrenaline plus noradrenaline) in urine is described. The catecholamines are adsorbed on a cation exchange resin, Amberlite IRC–50, and eluted with acid. The sympathin in the eluate is estimated by a fluorimetric method, in which adrenaline and noradrenaline yield the same intensity of fluorescence so that sympathin estimates are uninfluenced by the proportions of the two amines. Estimates of the adrenaline and noradrenaline are obtained after separation of the amines in the urine extract by paper chromatography. A mean recovery of 82 per cent (±6 per cent S.D. (51)) was obtained when adrenaline or noradrenaline (2–5000 μg.) was added to a urine sample analysed for sympathin content. Estimation of the separated amines from mixtures of adrenaline or noradrenaline (2–15 μg. of either amine) showed a mean recovery of 51 per cent (±9 per cent S.D. (10)). Experimental evidence is presented of the validity of applying appropriate correction factors to analytical results, the correction factors being determined by recovery experiments run concurrently with each series of estimations.
The specificity of the method is discussed briefly.

Most methods for the estimation of adrenaline and noradrenaline in urine involve adsorption of the amines on aluminium hydroxide[1], or on aluminium oxide[2]. After elution, the catecholamines are estimated either by bioassay[1] or by fluorimetry[2]. Attempts to use Lund's[3,4] fluorimetric method of assay following adsorption of the amines on aluminium oxide or hydroxide lead to unsatisfactory results in our hands.

Better results were obtained using the cation exchange resin, Amberlite IRC–50, as adsorbent, and this paper describes a method for the estimation of adrenaline and noradrenaline in urine utilising this material. After elution, the sympathin is estimated by a modification of Lund's fluorimetric method. The term, "sympathin", is used to indicate a mixture of adrenaline and noradrenaline irrespective of the proportions of the two amines in the mixture. Separate estimations of the two amines in the eluates are made by the fluorimetric technique after paper chromatographic separation[5].

The method has been used to study the urinary sympathin excretion of normal rats and of rats subjected to various experimental procedures. This study was undertaken primarily with a view to demonstrating that changes in the urinary sympathin excretion would provide an indication of alteration in the level of sympathico-adrenal discharge. The results of this investigation will be reported elsewhere.

The method has also been used to study the urinary sympathin excretion of normal male infants and of infants suffering from pink disease[6].

* Much of the work reported in this paper formed part of a Ph.D. thesis submitted by W. L. in May 1955, at Edinburgh University.

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Reagents

Amberlite resin IRC–50 (H), analytical grade (The Rohm and Haas Co., Philadelphia, U.S.A., obtained from The British Drug Houses, Ltd.).

L-Ascorbic acid (The British Drug Houses, Ltd.).

(—)-*Adrenaline* (synthetic, Burroughs Wellcome and Co.). A stock solution, 1·00 mg./ml., was prepared by suspending the base in 0·01N HCl, dissolving it by the addition of a slight excess of concentrated HCl and diluting to volume with 0·01N HCl. The solution was stored at 5°.

(—)-*Nordrenaline*-(+)-*bitartrate monohydrate* ("Levophed", Bayer Products, Ltd.). A stock solution containing 1·00 mg. base/ml. was prepared in 0·01N HCl. The solution was stored at 5°.

Manganese dioxide (technical powder). 250 g. was treated twice for 0·5 hours and overnight with a litre 12 per cent (v/v) acetic acid with intermittent shaking. The acid was removed by decantation and washing with water until the wash fluid was above pH 4 (Universal Indicator Paper). The MnO_2 was air-dried, reduced to a powder and finally heated, with intermittent stirring, in an evaporating basin over a Meker burner for 2–3 hours. This intense heating was found necessary since, in its absence, the MnO_2 frequently gave rise to troublesome dark brown colloidal suspensions in the course of the amine determinations.

Ethanol. Absolute ethanol refluxed for 4 hours with NaOH (5 g./l.) and twice distilled.

Other reagents were of A.R. quality.

De-ionised water was used throughout.

Methods

pH Determinations were made with a glass electrode unless otherwise stated.

Preparation of columns of ion exchange resin. The Amberlite resin IRC–50 (H) was converted to the sodium form by treatment overnight with excess 8 per cent (w/v) NaOH. Excess alkali was removed by decantation and repeated washings with water until the wash fluid was about neutral (indicator paper). The resin was then stored for at least 2 days under 0·2M sodium phosphate buffer, pH 6·5 with 2 or 3 changes of the fluid in the interval.

A suspension of the treated resin in the same buffer solution was poured into a tapered 11 mm. internal diameter glass tube plugged with glass wool, to give a resin column of 21 cm. (for treatment of 50–100 ml. of urine) or 11 cm. (for treatment of 10–20 ml. of urine). The outflow from the column was controlled by a small screw clip on a short length of narrow bore rubber tubing fitted to the taper of the tube and carrying a short capillary jet at the lower end. The resin was then buffered to about pH 7 by passage of 300 ml. of 0·2N sodium phosphate buffer, pH 6·5, at a flow rate not exceeding 2 ml./min. This was most conveniently carried out by placing the buffer solution in a mercury levelling bulb set about 100 cm. above the column and connected by polythene tubing (2 mm. internal diam.) to a capillary stop-cock inserted through a rubber stopper fitting tightly into the top of the resin-containing tube. The

flow rate, once adjusted by means of the stop-cock, showed negligible alteration throughout the passage of the buffer solution. The prepared column was left overnight if necessary. Immediately before use, 50 ml. of 0·02M sodium phosphate buffer, pH 7, was passed through the column (the pH of the last 10 ml. of the effluent should not exceed 7·5) followed by 25 ml. water.

Adsorption of urinary sympathin. Twenty-four hour specimens of urine were collected in vessels containing 2N H_2SO_4, 0·5 ml. for rat urine, or 5 ml. for human urine to preserve the excreted sympathin[1]. To 10 ml. of rat urine or 50–100 ml. of human urine was added 1 mg. of ascorbic acid/ml. as antoxidant and 2 volumes of water. The mixture was adjusted to approximately pH 6·5 by the slow drop-wise addition, with constant stirring, of 2N NaOH and finally to pH 7 with 0·5N NaOH. The sample, followed by 25 ml. of 0·02M sodium phosphate buffer, pH 7, and then by 25 ml. of water, was passed through a resin column of suitable size at 1·0–1·5 ml./minute (larger column) or 0·5–0·7 ml./minute (smaller column).

Elution of adsorbed sympathin. The adsorbed sympathin was eluted by passage of 2N H_2SO_4 at 0·3–0·5 ml./minute. The eluate was collected in a measuring cylinder containing 2 drops of the acid to acidify the neutral effluent issuing initially from the column and so stabilise any sympathin therein. Elution was continued until the effluent was strongly acidic (tested with short narrow strips of Universal indicator paper). At least 13 ml. or 6 ml. of the acid were required for the larger and smaller columns respectively. The flow was stopped and the resin left in contact with the acid for at least 0·5 hour. The elution was then continued at the same rate as before using 0·01N H_2SO_4 until, in all, 40 ml. of eluate (larger column) or 20 ml. of eluate (smaller column) had been collected. Omission of the stationary phase in the elution resulted in decreased recoveries.

The eluate, usually about pH 1·5, was adjusted to pH 3·5 by the slow dropwise addition, with constant stirring, of 2N NaOH.

It has been found possible for one individual to deal with six samples simultaneously during the adsorption and elution stages of the method.

Preparation of the eluate for the estimation of sympathin. If the concentration of sympathin in the eluate was known to exceed 0·5 μg./ml., the estimation by the fluorimetric method described below could be made using portions of the eluate without further treatment. With lower amounts of sympathin, concentration of the eluate was necessary and this required the removal of at least part of the dissolved salts by the addition of 2 volumes of an ethanol : acetone (1 : 1 v/v) mixture. After standing overnight at 5°, the mixture was filtered or centrifuged and the precipitated salt washed twice with 5 ml. of ethanol : acetone mixture, the washings being added to the main bulk of the solution. The solution was evaporated just to dryness *in vacuo* at less than 35° (external temperature). Care was taken to avoid continued heating of any dried-out residue on the sides of the flask as this resulted in a loss of sympathin, a finding which has also been commented on by Goldenberg and others[7].

The addition of 4 volumes of ethanol : acetone (1 : 1 v/v) mixture to the eluate instead of 2 volumes produced a more complete removal of the dissolved salts. This was necessary when it was desired to make a biological assay in parallel with fluorimetry or a paper chromatographic separation of the adrenaline and noradrenaline prior to their separate estimation.

The dry residue was dissolved in 3 ml. of water for the estimation of the sympathin content by fluorimetry or in 3 ml. of saline for biological assay.

Paper chromatographic separation of adrenaline and noradrenaline in urine extracts. When individual estimates of the adrenaline and noradrenaline were required, preliminary separation of the two amines in the evaporated desalted eluate from the ion exchange column was carried out by paper chromatography. The technique was that of Crawford and Outschoorn[5] with modifications to permit fluorimetric assay of the separated amines.

Just before use, a Whatman No. 1 filter sheet "for chromatography" was washed by descending chromatography with 0·01N HCl for at least 12 hours and dried at room temperature. The sympathin in the evaporated desalted eluate was transferred to the paper with acid-ethanol as described by Crawford and Outschoorn[5]. Preliminary treatment of the paper with ascorbic acid was unnecessary and undesirable since it interfered with the fluorimetry. The chromatogram was developed with a mixture of phenol, distilled from zinc powder, and 0·1N HCl (15 ml./100 g. of phenol), in an atmosphere of nitrogen and not of sulphur dioxide as originally described. Evaporation of the 0·4 per cent $NaH_2PO_4 \cdot 2H_2O$ eluates of the paper strips carrying the separate amines was not required when the fluorimetric method of assay was employed. When biological assays were to be carried out in parallel with fluorimetry, the paper strips were eluted with 0·01N HCl and the eluates evaporated just to dryness *in vacuo* at 35° (external temperature) to remove HCl and traces of phenol. The residues were taken up in saline for assay.

Fluorimetric estimation of sympathin. The method was modified from that of Lund[3,4]. The intensity of fluorescence was measured with a Farrand Fluorometer, Model A (Farrand Optical Co. Inc., New York) using a Corning 5860 filter, which transmits the 365 mμ line of the mercury vapour lamp, as the primary filter and an Ilford gelatin filter No. 625 (Ilford Ltd., London) transmitting between 510 and 590 mμ as the secondary filter.

A measured sample, less than 1 ml. and of pH not less than 3·5, of the solution to be assayed was diluted to 4·00 ml. in a 15 ml. centrifuge tube with 0·4 per cent $NaH_2PO_4 \cdot 2H_2O$ and 1·00 ml. of 1 per cent Na_2HPO_4, $2H_2O$ added. The pH of the mixture should be about 6·5 (indicator paper). Fifty mg. of MnO_2 were added, the tube closed with a clean rubber stopper and the mixture shaken moderately vigorously for 30–35 seconds. The supernatant obtained after centrifuging for 30–35 seconds at 3500 rev./minute was filtered immediately through a double thickness of 4·25 cm. diameter Whatman No. 41 filter paper. One ml. of the filtrate was pipetted without delay into each of two cuvettes. To one cuvette was then added 0·02 ml. of 0·5 per cent ascorbic acid (prepared

fresh daily and kept in a tightly stoppered test-tube). Immediately after inversion of the cuvette twice to mix the solution, 0·20 ml. NaOH (20 g. in 100 ml. of water, stored in a tightly stoppered polythene bottle) was added and the cuvette inverted six times to mix the solution. The fluorescence intensity was measured every half minute from the time of addition of the alkali and the maximum reading recorded. To the other cuvette was added 0·20 ml. of the NaOH solution and the liquid mixed by inversion of the cuvette six times. Twenty minutes later, 0·02 ml. of 0·5 per cent ascorbic acid was added, the solution mixed by inversion of the cuvette twice and the fluorescence intensity measured. This measured the fluorescence of the solution which, unlike that derived from adrenaline and noradrenaline, was stable in alkali in the absence of a reducing agent. It served as the "blank" for the sympathin fluorescence measured in the presence of ascorbic acid.

The presence of substances potentiating or diminishing the fluorescence derived from the sympathin was detected by repeating the estimation on another sample of the test solution to which a known quantity, usually 0·20 μg. of adrenaline or noradrenaline had been added. Potentiation was never encountered from a urine extract and only rarely was there detected material which caused some diminution of the expected additional fluorescence intensity. A correction factor based on the measurement of the fluorescence of the added amine could be applied in such cases but such corrected estimates were always regarded with dubiety.

Before each set of estimations the fluorimeter was calibrated using suitable amounts of a 1 μg./ml. solution of adrenaline or noradrenaline prepared fresh daily in 0·4 per cent $NaH_2PO_4 \cdot 2H_2O$ from the stock 1 mg./ml. solution.

As a stable fluorescing standard for the adjustment and continuous check of the sensitivity setting of the instrument during a series of estimations quinidine sulphate (15 μg. per cent) in 0·1N H_2SO_4 was used.

Estimation of "total" and "free" sympathin in urine. Others[1] have shown that sympathin is excreted in the urine partly in the free state and partly in a biologically inactive form from which the amines could be released by boiling the urine at a pH of about 2 for 20 minutes. Estimation of the sympathin in untreated urine gives a measure of the "free" sympathin while estimation after acid hydrolysis of the urine gives a measure of the "total" sympathin (but see discussion). To assay the "total" sympathin, a portion (10 ml. rat urine; 50–100 ml. human urine) of the urine specimen was adjusted to pH 1·8 with 2N H_2SO_4 and heated in a briskly boiling water bath for 20 minutes, the pH being maintained throughout close to 2 (indicator paper) by the addition of acid. After cooling, the urine was analysed as described above.

Recovery experiments. Estimates of the recovery of sympathin from urine were made. At first, the difference between the sympathin content of a urine sample and the content of a like sample from the same urine to which a known amount of adrenaline and/or noradrenaline had been added was determined. Later, the following method was used. A suitable sample of urine was adjusted to pH 10 with 2N NaOH and

maintained at this pH (indicator paper) during gentle boiling for 20 minutes to destroy the sympathin present. After cooling, the sample was adjusted with 2N H_2SO_4 to pH 4 for "free" sympathin estimation or to pH 1·8 for "total" sympathin estimation. A known amount of adrenaline and/or noradrenaline, about equal to that expected to be

TABLE I

COMPARISON OF THE FLUORESCENCE INTENSITIES DERIVED FROM ADRENALINE AND NORADRENALINE. REAGENT BLANK DEDUCTED

Amount of amine μg.	Fluorescence intensity. Galvanometer reading	
	Adrenaline	Noradrenaline
1·00	82	82
1·00	78	82
1·00	81	—
0·75	61	64
0·75	60	65
0·50	39	41·5
0·50	41·5	40·5
0·25	20·5	22·5
0·25	22	25

present in the experimental urine samples, was added and the sample analysed concurrently with the experimental urine samples. The results for the latter were then corrected by the appropriate factor derived from the recovery estimate.

Both methods gave similar results.

RESULTS

Fluorimetric Estimation of Adrenaline and Noradrenaline in pure Solution

Preliminary experiments showed a linear relation to exist between fluorescence intensity (galvanometer reading) and the amount of amine present with quantities in the range examined (0–1 μg.).

TABLE II

ADRENALINE AND NORADRENALINE MIXTURES. COMPARISON OF THE FLUORESCENCE INTENSITIES WITH THOSE EXPECTED FROM THE SUM OF THE INTENSITIES FROM THE TWO AMINES. REAGENT BLANKS DEDUCTED

Amount of amine in mixture		Fluorescence intensity Galvanometer reading	
Adrenaline μg.	Noradrenaline μg.	Observed	Expected
0·50	—	42·5	—
—	0·50	44	—
0·25	0·25	44	43
0·25	0·50	67	65
0·50	0·25	67	64

Accuracy of single estimates. Calibration curves were constructed from the fluorescence intensity reading for 1·00 μg. and for 0·20 μg. of either amine. With various quantities in the range 0·10–1·00 μg. eighteen single estimates for each amine showed a standard deviation of ±8 per cent for adrenaline and ±6 per cent for noradrenaline from the expected value

on reference to the appropriate (1·00 μg.) calibration curve. Ten single estimates for each amine in the range 0·05–0·20 μg. showed a standard deviation of ± 13 per cent for adrenaline and ± 12 per cent for noradrenaline from the expected value on reference to the appropriate (0·20 μg.) calibration curve. As might be expected, the scatter of replicate estimates was greatest for smallest amounts.

Similar fluorescence intensities from equal amounts of adrenaline and noradrenaline. (Table I.)

Summation of the fluorescence intensities of adrenaline and noradrenaline in a mixture. Table II illustrates that a mixture of the two amines yielded

TABLE III

RECOVERY OF ADRENALINE AND NORADRENALINE ADDED TO URINE

Experiment No. and source of urine	Amine added	Amount added μg.	Recovery per cent
Hydrolysed urine			
1 (rat)	Adrenaline	2	54
	Noradrenaline	2	67
2 (rat)	Adrenaline	2	45
	Noradrenaline	10	49
3 (rat)	Adrenaline	8	41
	Noradrenaline	8	35
4 (human)	Adrenaline	10	49
	Noradrenaline	10	50
5 (human)	Adrenaline	15	56
	Noradrenaline	15	62
	Mean recovery		per cent 49 (adrenaline) 53 (noradrenaline)
Unhydrolysed urine			
1 (rat)	Adrenaline	10	46·5
	Noradrenaline	10	46

a fluorescence the intensity of which was equal, within the limits of experimental error, to the sum of the intensities derived from the individual amines in the mixture.

Paper chromatography. Experiments in which mixtures of adrenaline and noradrenaline in quantities varying between 0·25 and 10·0 μg. of either amine were separated by paper chromatography only and then estimated by fluorimetry showed a mean recovery of 92 per cent (±12 per cent S.D. (19).)

Recovery of Adrenaline and Noradrenaline from Urine

Adrenaline or noradrenaline was added to 10 ml. samples of normal rat urine, the sympathin of which had previously been destroyed. From acid hydrolysed urine the recovery of adrenaline (4–30 μg.) was 82 per cent ± 6 per cent S.D. (8), and that of noradrenaline (3–8 μg.) was 82 per cent ± 8 per cent S.D. (18). From unhydrolysed urine the recovery of noradrenaline (3–8 μg.) was 81 per cent ± 6 per cent S.D. (16).

When added to 100 ml. samples of normal human urine, the recovery of adrenaline (200–5000 μg.) was 81 per cent ± 3 per cent S.D. (6) and that of noradrenaline (1250–5000 μg.) was 84 per cent ± 1·5 per cent S.D. (3). In all these experiments the urine was unhydrolysed. The

endogenous sympathin was not destroyed in those urine samples to which an amount of amine greater than 1000 μg. was added. In calculating the recovery per cent, the amount of endogenous sympathin, being very small in comparison to that of the added amine, was ignored.

The overall recovery for the 51 experiments referred to above was 82 per cent ±6 per cent (S.D.) for adrenaline and noradrenaline added to urine in quantities in the range 3–5000 μg.

Separate estimations of adrenaline and noradrenaline in urine. Recovery experiments. A mixture of adrenaline and noradrenaline was added to 10 ml. of rat urine or to 100 ml. of human urine the sympathin of which

TABLE IV

ESTIMATES OF SYMPATHIN IN URINE EXTRACTS COMPARED WITH THE SUM OF THE SEPARATE ESTIMATES OF ADRENALINE AND NORADRENALINE AFTER PAPER CHROMATOGRAPHIC SEPARATION OF THE SAME EXTRACTS. ESTIMATES CORRECTED BY APPROPRIATE FACTORS DETERMINED BY RECOVERY EXPERIMENTS RUN CONCURRENTLY

The urine specimens were from a male infant suffering from pink disease

Treatment of urine	Sympathin μg.	Adrenaline μg.	Noradrenaline μg.	Sum. μg.
Specimen 1				
Hydrolysed ..	41	13·9	26·8	40·7
Unhydrolysed	41	11·2	26·2	37·4
Specimen 2				
Hydrolysed ..	21·5	4·1	15·4	19·5
Unhydrolysed	20	3·7	13·6	16·7

had been destroyed. The urine was extracted, in some cases after acid hydrolysis, and the amines separated by submitted paper chromatography. The recoveries, by fluorimetry, are shown in Table III.

Agreement between urinary sympathin estimates and the sum of the estimates of adrenaline and noradrenaline. In view of the variable recoveries of adrenaline and noradrenaline added to urine, which, in addition were surprisingly low for the separated amines, all analyses embodied recovery experiments in parallel. The analytical values were then corrected by a recovery factor so obtained under the conditions of the particular series of analysis. An indication of the validity of this procedure is given in Table IV in which are recorded the results of several estimations of the sympathin and of adrenaline and noradrenaline in specimens of urine from pink disease in a male infant. In these experiments the sympathin was estimated in part of the extract from the ion exchange column while the remainder of the extract was treated by paper chromatography and the separated amines estimated. The figures in the Table refer to the estimated contents of the 24 hour urine specimens and not to the 100 ml. samples analysed. The agreement between the corrected estimates of the separate amines may be considered satisfactory.

DISCUSSION

The conditions for the production and measurement of the fluorescent derivatives of adrenaline and noradrenaline in the assay of these substances described by Lund[3,4] have been modified so that equal intensities of

fluorescence are obtained from equal amounts of the two amines. Under these conditions, the estimate of the sympathin (adrenaline and noradrenaline) content of a solution is uninfluenced by the proportions of the two amines in the mixture.

The cation exchange resin, Amberlite IRC–50, has been used for the preliminary extraction of urinary sympathin in preference to aluminium hydroxide[8] or aluminium oxide[3]. If adrenaline or noradrenaline is added to the final extract from the oxide, or hydroxide of aluminium there is a considerable diminution of the expected fluorescence. Very rarely was a similar effect seen with extracts from Amberlite IRC–50 but the possible presence of substances modifying the fluorescence was checked in every analysis.

To determine the adrenaline and the noradrenaline in a urine extract, the amines were first separated by paper chromatography. This procedure avoids the interdependence of the estimates of the two amines inherent in such methods as that of Lund[4].

The recovery of adrenaline and noradrenaline added to rat or human urine was about 80 per cent when estimated as sympathin and about 50 per cent when the amines were separately determined. Some variability in the recoveries made it advisable to perform control experiments with each series of analyses and to correct the analytical results accordingly. Experimental evidence in support of this procedure has been presented.

In the estimation of sympathin in urine extracts, it has been assumed that the fluorescence stable in alkali in the absence of ascorbic acid is derived from substances other than adrenaline and noradrenaline and that only these two amines account for the additional fluorescence measured in the presence of ascorbic acid. The good agreement between the sympathin estimates and the sums of the estimates of the adrenaline and noradrenaline after separation by paper chromatography (Table IV) provides some evidence for the validity of this assumption. But the possibility of errors arising from the presence of substances reacting similarly and having similar R_F values to adrenaline and noradrenaline is not thereby excluded. One such substance found in human urine is dopamine, 2-(3′ : 4′-dihydroxyphenyl)ethylamine[9]. With an R_F of 0·44, this substance would be present in the adrenaline (R_F 0·5) fraction of a paper chromatogram of a urine extract. Dopamine, however, gave rise to a fluorescence only about 0·6 per cent the intensity of that from adrenaline and its presence would lead to a significant error of the adrenaline (or sympathin) estimate only if it constituted more than 95 per cent of the mixture of catechol amines. In an experiment with an extract from normal rat urine, the adrenaline and noradrenaline fractions from a paper chromatogram were each assayed against the appropriate (−)-amine by fluorimetry and by three biological tests, namely the rat's blood pressure[5], the isolated rat's uterus[10], and the perfused isolated rabbit's ear[11]. All four estimates for each amine were in agreement within the limits of accuracy of the methods indicating an absence of gross error in the fluorimetric estimates as a result of the presence of substances reacting similarly to adrenaline and noradrenaline.

The available evidence indicates that the fluorimetric method as described gives a reasonably accurate measure of the sympathin in urine extracts obtained by the Amberlite IRC–50 adsorption technique and of the adrenaline and the noradrenaline after separation by paper chromatography.

In the determination of the "total" sympathin in urine, preliminary hydrolysis at pH 2 has been used to release that portion excreted in conjugated form[1]. Euler and Orwén[12] have found that hydrolysis at pH 0 instead of pH 2 leads to a higher estimate for the catecholamines in human urine. As they point out, however, there is no evidence that all the conjugates are split by this treatment. Thus estimates of urinary sympathin after acid hydrolysis can be considered as those of *total* sympathin only in so far as they refer to certain conditions of hydrolysis.

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ESTIMATION OF DIGOXIN AND DIGITOXIN IN *DIGITALIS LANATA*

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Digoxin and digitoxin present in extracts of dried leaves of *Digitalis lanata* have been estimated colorimetrically with xanthydrol after paper chromatographic separation on formamide-impregnated paper, using methyl *iso*butyl ketone-*iso*propyl ether-formamide as developing solvent. The method is reasonably rapid, both glycosides may be estimated on the same chromatograph, and it may be used for the analysis of crystalline glycosides.

THE quantitative estimation of digitoxin in *Digitalis purpurea* using paper chromatographic methods of separation followed by fluorimetric or colorimetric analysis has been investigated by Jensen[1] and by Sellwood[2], but no assay of this type for the glycosides present in *Digitalis lanata* has yet been published.

Freshly gathered leaves of *Digitalis lanata* yield negligible amounts of desglucoglycosides if they are extracted without allowing enzymatic action to occur[3,4] and in order to estimate the potential digoxin or digitoxin content of the plant it is necessary to ensure that enzymatic hydrolysis of the lanatoside precursors takes place. To obtain a rapid assessment of the digitoxin content of a crop, Sellwood[2] allowed freshly gathered leaves of *D. purpurea* to undergo fermentation at 37° in the presence of water for 72 hours before extracting the glycosides with propylene dichloride. In this investigation, however, we have estimated the digoxin and digitoxin content of air-dried leaves of *D. lanata* as used in commerce, enzymatic hydrolysis of the lanatosides having apparently occurred during the long period of drying. Maceration of the dried leaves with several quantities of 20 per cent ethanol followed by chloroform extraction of the aqueous-ethanol solution resulted in complete extraction of digitoxin and digoxin accompanied by a minimum amount of pigment which did not interfere with the chromatographic separation.

Formamide-impregnated paper was used for chromatography to permit greater loading of the paper with the plant extract or glycosides and by using the solvent systems methyl *iso*butyl ketone-*iso*propyl ether-formamide[4] for development, clear separation of the glycosides was obtained without interference from pigment. This system of chromatography is not very sensitive to temperature variations because of the relatively high vapour pressures of the solvents and it is possible to estimate both digoxin and digitoxin on the one chromatograph. If temperatures of 20 to 23° are used, the time of development is only four or five hours and reasonably compact glycoside zones are obtained.

For colorimetric assay the xanthydrol reagent of Arreguine and Pasqualis[5] was chosen. This reagent has been used in modified forms by

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by several workers[2,6–8] for the estimation of cardiac glycosides containing 2:6-desoxyhexoses. It is very sensitive and the colour is developed by immersing the zones of paper containing the glycosides directly in the reagent, thus avoiding elution losses.

Experimental Methods

The leaves of *Digitalis lanata* were air-dried on racks protected from the weather for six to eight weeks. The moisture content of the dried powdered leaves averaged from 11 to 14 per cent.

Method of Extraction

Some preliminary experiments were carried out in which the plant material was extracted with (*a*) 70 per cent ethanol followed by treatment with lead subacetate[9], (*b*) 20 per cent ethanol by stirring for 16 hours, precipitating the pigments with lead subacetate and finally extracting with chloroform[10], (*c*) chloroform-methanol followed by removal of some of the pigments with light petroleum[11]. Methods (*a*) and (*b*) gave results which were considered to be somewhat low and method (*c*) resulted in an extract which was too heavily pigmented. It was finally decided to use 20 per cent ethanol followed by chloroform extraction but without lead treatment. The pigments extracted did not interfere with the chromatographic separation and the details of the process are as follows:

2 g. of dried powdered leaf was shaken with 3 × 25 ml. of 20 per cent ethanol for 3 successive periods of 6 hours. The solvent, after each extraction, was decanted through a Buchner funnel and the residue, after the final filtration, washed with about 10 ml. of 20 per cent ethanol. The clear alcoholic extract was shaken with five successive 20 ml. portions of chloroform and dried over anhydrous sodium sulphate. The chloroform was filtered and the flask and sodium sulphate washed with 3 × 10 ml. of chloroform. The chloroform extract was evaporated to dryness under reduced pressure and the residue in the flask dissolved in a small volume of a mixture of chloroform: methanol (1:1), transferred quantitatively to a weighed test tube and evaporated in a boiling water bath to dryness. 1 ml. of a mixture of chloroform: methanol (1:1) was added and the final solution weighed.

Paper chromatograms prepared from a 90 per cent ethanol extract of the exhausted leaves, and of further chloroform extracts of the 20 per cent ethanol solution showed no detectable amounts of digoxin or digitoxin.

Preparation of Chromatograms

Whatman No. 1 filter paper approximately 6 in. × 16 in. was ruled with a starting line 4 in. from one end. Guide lines to assist in applying the solutions to the chromatograph were ruled 1/12 in. on each side of the starting line. The paper was impregnated by drawing it slowly through a 25 per cent solution of formamide in acetone (about 15 seconds was allowed for this procedure) and then allowed to dry at room temperature

for about 5 minutes before applying the solution. The time needed for the preparation of the paper between dipping and developing was about 15 to 20 minutes.

A weighed quantity of the solution of plant extract (about 30 to 40 mg.) delivered from a tared capillary pipette was spotted on to a section of the starting line (approximately $1\frac{1}{4}$ in. in length) in small quantities at a time so that the solvent did not spread beyond the guide lines to any appreciable extent. Towards the centre of the starting line a secondary quantity

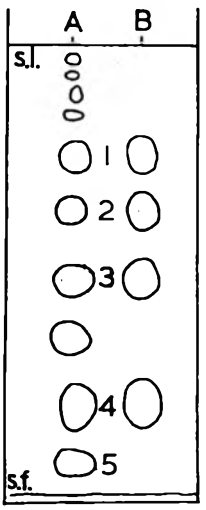

Fig. 1. Paper chromatograph of extract of dried leaves of *Digitalis lanata* on formamide impregnated paper, solvent system: methyl *iso*butyl ketone-*iso*propyl ether-formamide. A, plant extract, B, control glycosides, 1, digoxin, 2, gitoxin, 3, acetyl digoxin, 4, digitoxin, 5, acetyl digitoxin, s.l., starting line, s.f., solvent front.

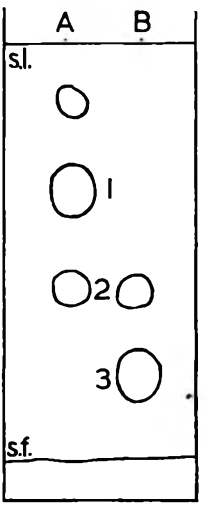

Fig. 2. Paper chromatograph of samples of crystalline digoxin and digitoxin, on formamide impregnated paper, solvent system: methyl *iso*butyl ketone-*iso*propyl ether-formamide. A, digoxin, B, digitoxin, 1, digoxin, 2, gitoxin, 3, digitoxin, s.l., starting line, s.f., solvent front.

of the same extract (approx. 20 mg.) was applied in the same way to a short length of the starting line as a control. Solutions of digoxin and digitoxin (10 to 20 μg. of each) were also spotted on to the line towards the opposite edge of the paper to the extract being estimated.

Development

Methyl *iso*butyl ketone: *iso*propyl ether (100:25 by volume) were shaken with excess formamide and allowed to separate completely, the upper layer being used for development. The atmosphere in the tank was kept saturated by dipping a sheet of filter paper into a layer of the developing solvent on the bottom of the tank. The chromatograms, for quantitative estimations, were developed by the descending method, until

the solvent front reached the end of the paper (about 4 hours at 20° to 23°). At the end of this time the paper was removed and dried in an oven at about 100° for 10 to 15 minutes. This time is not critical and it is not necessary to evaporate all the formamide. The paper was then cut vertically, the portion containing the extract control and the pure glycosides sprayed with 25 per cent antimony trichloride in chloroform and then heated in an oven at about 70° for 2 to 3 minutes only. After this treatment digoxin showed as a greyish-purple band in daylight and as a brownish-orange band under ultra-violet light; gitoxin appeared slightly

TABLE I

ESTIMATION OF CRYSTALLINE GLYCOSIDES ON CHROMATOGRAMS

Glycoside	Per cent recovery	
	Digoxin zone	Combined zones
Digoxin sample A	99·0, 98·3 99·6, 99·5	99·9, 99·8, 99·3
Digoxin sample B	97·1, 97·8 96·6, 96·0	101·0, 99·6, 99·0
	Digitoxin zone	Combined zones
Digitoxin sample A ..	98·8, 98·1, 97·0	99·0, 99·0, 102

yellow in daylight and bright blue under ultra-violet; pure digitoxin appeared rust-red under ultra-violet, but was generally found to be blue in the plant extract. (Fig. 1.)

Two chromatograms were run in the same tank enabling duplicate results to be obtained under the same conditions.

Colorimetric Estimation

Reagents. Xanthydrol 0·125 per cent in glacial acetic acid. This solution was prepared by diluting a 10 per cent methanol solution of xanthydrol with glacial acetic acid. The glacial acetic acid was refluxed with chromic acid for 4 hours, and distilled. The distillate was tested until it did not give a colour with xanthydrol and the subsequent fractions collected and stored in the dark.

Method. The positions of the glycosides being estimated in the unsprayed part of the chromatogram were located from the control and the zones cut out. The rectangular pieces of paper were cut into small strips and placed in a test tube with 10 ml. of the xanthydrol reagent and 0·1 ml. of concentrated hydrochloric acid added and mixed thoroughly. The reaction mixture was then placed in *boiling* water for exactly 1 minute producing a distinct pink colour and immediately cooled in an ice bath for about 5 minutes. The optical density of the solution was then measured using an E.E.L. colorimeter with filter No. 624. Corresponding zones of the blank paper were also cut out and extracted with the reagent as controls in colorimetry.

A standard curve was prepared with pure digitoxose (20, 40, 60, 80 μg.) for each estimation.

RESULTS

Recovery of Glycosides from Chromatograms

Without Development. Known amounts of digoxin and digitoxin were spotted on to filter paper impregnated with formamide. The papers were not chromatographed but dried immediately after spotting and the glycosides in the spots estimated colorimetrically. The recoveries obtained with quantities from 20 to 60 μg. of glycosides were within the range 95 to 100 per cent.

After Development. Weighed amounts of solutions of digoxin an digitoxin (containing quantities of glycosides varying from 20 to 30 mg.) were chromatographed separately according to the method used for plant extracts. Minor glycosidic constituents were present in the samples (Fig. 2) so that colorimetric estimations were made on (*a*) the digoxin or digitoxin zones, and (*b*) on the combined zones present in each chromatogram using pure digitoxose as standard. The results are shown in Table I.

Estimation of Plant Samples

Three batches of leaves were estimated, Sample 1 being taken from a different crop than Samples 2 and 3 which, however, were gathered at different intervals towards the end of the growing season. As shown in Figure 1 a number of glycosides were detected on the chromatograms but only digoxin and digitoxin were estimated. The results are shown in Table II.

TABLE II

DIGOXIN AND DIGITOXIN CONTENT OF *Digitalis lanata* LEAVES

Sample	Digoxin per cent	Digitoxin per cent
1	0·17, 0·16 0·156, 0·165	0·034, 0·038 0·038, 0·041
2	0·125, 0·127, 0·133	0·047, 0·044, 0·055
3	0·128, 0·131, 0·121	0·046, 0·041, 0·052

Acknowledgements. The Authors wish to thank Burroughs Wellcome and Co. (Australia) Ltd. for generous supplies of digoxin and *Digitalis lanata.*

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A NOTE ON THE IDENTIFICATION OF SOME ANTIMALARIAL DRUGS

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Crystal and colour tests are described for 14 antimalarial drugs.

Since the identification of quinine as the active constituent of cinchona bark, many attempts have been made to produce a substitute that would combine an enhanced plasmodicidal effect with diminished toxicity. Of the many hundred substances which have been tested for antimalarial activity only about a dozen have come into clinical use. These are all

TABLE I

Substance	Reagent	Crystals	Sensitivity (μg.)
Primaquine (Avlon, 6-methoxy-8-(4-amino-1-methylbutylamino) quinoline diphosphate)	Picric acid	Rosettes of curved needles	0·1
	Styphnic acid	Rosettes of rods	0·1
Pentaquine (6-methoxy-8-(5-*iso*-propylaminoamylamino) quinoline phosphate)	Potassium cadmium iodide	Dendrites ON	0·25
	Picrolonic acid	Dense rosettes	0·1
Isopentaquine (6-methoxy-8-(4-*iso*propylamino-1-methyl butylamino) quinoline)	Potassium cadmium iodide	Curved needles	0·1
	Gold cyanide	Dendrites ON	1·0
Rhodoquine (6-methoxy-8-(diethylaminopropylamino) quinoline)	Picric acid	Rosettes of plates	0·25
	Potassium mercury iodide	Rods ON	0·1
Amodiaquine, (Camoquin 7-chloro-4-(3′-diethylamino-methyl-4-hydroxyanilino) quinoline HCl)	Gold cyanide	Snowflake rosettes	0·1
	Potassium cadmium iodide	Burrs of fine needles	0·1
	Picric acid	Rosettes of plates	0·5
Chloroquine (Nivaquine, 7-chloro-4-(4-diethylamino-1-methylbutylamino) quinoline diphosphate)	Styphnic acid	Rosettes of plates	0·5
Hydroxychloroquine, (Plaquenil, 7-chloro-4-(4-(*N*-ethyl-*N*-2-hydroxyethylamino)-1-methylbutylamino) quinoline sulphate	Picric acid	Small irregular plates	0·1
	Styphnic acid	Snowflake rosettes	0·25
Nivaquine C (Sontoquine, 7-chloro-3-methyl-4-(diethyl aminopentylamino) quinoline HCl)	Platinum bromide	Rosettes of irregular needles	0·1
	Gold cyanide	Dense feathery rosettes ON	1·0
Mepacrine (Atabrine, Quinacrine, 2-chloro-5(4-diethylamino-1-methylbutylamino)-7-methoxy acridine HCl)	Potassium tri-iodide (2)	Rosettes of needles, often dense ON	0·5
	Gold cyanide	Clumps of hair-like needles	0·1
Proguanil (Paludrine, N^1-*p*-chlorophenyl-N^5-*iso*propyl diguanidine HCl)	Gold bromide/HCl	Long plates	0·25
	Picrolonic acid	Short rods some in rosettes	0·25
Pyrimethamine (Daraprim, 2:4-diamino-5-(4′chlorophenyl)-6-ethylpyrimidine)	Gold bromide/HCl	Serrated needles	0·1
	Potassium chromate	Irregular blades	0·1
377C54 (2:5 bis(*cyclo*hexylaminomethyl) naphthalene-1:6 diol di HCl)	Potassium tri-iodide (3)	Rosettes of plates ON	0·1
	Ammonium thiocyanate	Rosettes of plates	1·0
Compound 5943 (N^1-3:4-dichlorophenyl-N^5 *iso*propyl diguanide HCl	Gold bromide/HCl	Long plates, often serrated	0·25
	Platinum chloride	Rosettes of irregular rods	0·25

ON (overnight) indicates that the crystals do not usually form until the following day.

basic nitrogenous substances of alkaloidal type, the majority being aminoquinoline derivatives. As they are of low toxicity, few tests for their identification have been described.

This paper describes crystal and colour tests for fourteen of these compounds. Compound 5943 is a derivative of proguanil and is stated to be more active than the parent substance[1]. The substance 377C54

(2 : 5-bis(*cyclo*hexylaminomethyl) napthalene-1 : 6-diol diHCl) has recently undergone clinical trials[2]. Although metachloridine has been used in the treatment of malaria, it is actually a sulphonamide, and has therefore not been included in this study.

Experimental Procedure

Microcrystalline Tests

The hanging microdrop technique developed by Clarke and Williams[3,4] was used. Mepacrine and pyrimethamine were dissolved in 1 per cent acetic acid, the other compounds in 1 per cent hydrochloric acid.

TABLE II

Substance	Colour	Sensitivity (μg.)
Sulphuric acid-formaldehyde test (Marquis)		
Primaquine	Orange	0·5
Pentaquine	Orange	0·5
Isopentaquine	Orange	0·5
Rhodoquine	Orange	0·5
Mepacrine	Bright yellow	0·1
377C54	Yellow-brown	0·5
Ammonium vanadate test		
Primaquine	Purple—orange	0·5
Pentaquine	Purple—orange	0·5
Isopentaquine	Purple—orange	0·5
Rhodoquine	Purple—orange	0·5
Mepacrine	Purple—bright yellow	0·25
377C54	Grey—purple—orange	0·5
Ammonium molybdate test		
Primaquine	Pale blue	1·0
Pentaquine	Pale blue	1·0
Isopentaquine	Pale blue	1·0
Rhodoquine	Pale blue	1·0
Amodiaquine	Green—*blue—green	0·5
Mepacrine	Yellow-green	0·25
377C54	Blue—green—yellow	0·5
Selenium dioxide test		
Primaquine	Greenish yellow	1·0
Pentaquine	Greenish yellow	1·0
Isopentaquine	Greenish yellow	1·0
Rhodoquine	Greenish yellow	1·0
Amodiaquine	Light brown	0·5
Mepacrine	Bright yellow	0·25
377C54	Green—orange brown	0·5

* This blue colour is not seen if drug is in excess.

Pamaquin will be discussed later. The results obtained are shown in Table I. It should be noted that compounds which differ only in their non-functional groups may give crystals that are similar in appearance.

Colour Tests

Colour tests are made with microdrops on opal glass as described previously[3]. Chloroquine, hydroxychloroquine, nivaquine C, proguanil, Compound 5943 and pyrimethamine give no colours with any of the reagents. The results obtained with the other compounds are given in Table II. With Vitali's test most of these substances give indefinite shades of brown and yellow which are valueless for purposes of identification. A further colour test that may be employed is a modification of the diazo test originally described by Sanchez[5,6]. To a microdrop of a saturated solution of *p*-nitroaniline in 2N hydrochloric acid is added a

microdrop of a 10 per cent solution of sodium nitrite, followed by a similar drop of a 1 per cent solution of the drug. Pamaquin, primaquine, pentaquine, isopentaquine, rhodoquine and 377C54 all give an orange colour. A microdrop of 4N sodium hydroxide solution is then added. The 6-methoxy-8-aminoquinoline derivatives all turn purple, 377C54 turns green, while amodiaquine gives a greenish-brown precipitate. The other substances remain colourless.

Discussion

The identification of pamaquin, which is the 2:2′-dihydroxy-1:1′-dinaphthylmethane-3:3′-dicarboxylic acid salt of 6-methoxy-8-(4-diethylamino-1-methylbutylamino)quinoline, calls for special consideration. The test most frequently described for its identification is that in the B.P. 1953 monograph which is the formation of a green colour with the formaldehyde-sulphuric acid reagent. This green colour is, however, due to the acidic, not the basic, part of the molecule, as the free acid gives the same green colour, while pamaquin base with this reagent gives an orange colour similar to that given by the other 6-methoxy-8-aminoquinoline derivatives. The green colour given by pamaquin with the other sulphuric acid reagents is also due to the acid, as the free base gives the same colours as are given by primaquine (Table II).

Pamaquin is insufficiently soluble in dilute acids for the solution to give crystal tests. If a microdrop of a solution of this compound in acetone containing 5 per cent of water is added to a microdrop of a solution of potassium cadmium iodide, small rosettes of needles are formed; similarly the potassium tri-iodide reagent No. 3 gives transparent tablets. But in each case, similar crystals are formed from these reagents with an ethanolic solution of the free acid. Pamaquin base, dissolved in 1 per cent hydrochloric acid, gives amorphous precipitates or oils only with the usual reagents, although in the case of styphnic acid the oil will sometimes crystallise slowly into aggregates of rods.

All the tests described above were carried out on the microgram scale with pure substances supplied by the manufacturers.

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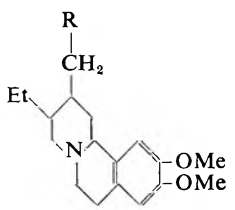
ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

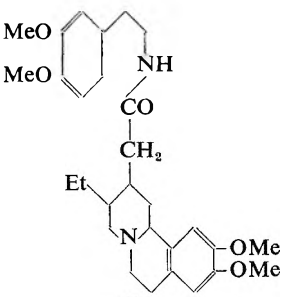
ALKALOIDS

Alkaloids of *Rauwolfia* Species. Studies on *Rauwolfia Cambodiana*. D. A. A. Kidd. (*Chem. Ind.*, 1957, 1013.) Dried roots, collected during March in Bangkhen, Thailand, were extracted with cold and hot methanol. The concentrated extract, acidified with dilute acetic acid, was defatted with *n*-hexane. Basification with ammonia and chloroform extraction gave the bulk of the alkaloids (3·5 per cent). Further basification with sodium hydroxide gave only a minute yield of a second alkaloid fraction: water-soluble alkaloids were precipitated from the mother liquors with ammonium reineckate. The major weakly basic fraction was separated by paper electrophoresis into reserpine (0·01 per cent yield) and a second alkaloid, fluorescent in ultra-violet light, m.p. 211–210), $[\alpha]_D^{12} -111°$). The infra-red spectrum showed two peaks at 5·88 and 6·16 μ consistent with the presence of the ROOC−C=C−O−C−chromophore (compare ajmalicine). J. B. S.

Ipecacuanha Alkaloid, New, and a Partial Synthesis of Emetine. A. R. Battersby, G. C. Davidson and B. J. T. Harper. (*Chem. Ind.*, 1957, 983.) A new alkaloid has been isolated in 0·002 per cent yield from the alkaloid bases of ipecacuanha root, by countercurrent distribution. The alkaloid, $C_{19}H_{27}O_3N$ (two OMe, one C-methyl) showed ultra-violet absorption characteristic of veratrole: the infra-red spectrum indicated the presence of an aldehyde group, confirmed by the reduction of Tollen's reagent, and hydrogenation. The basic strength indicates that the nitrogen is common to two rings. On this evidence and Robinson's proposals for the biogenesis of emetine, structure I, (R = CHO) is advanced for the new alkaloid. This was confirmed by converting the aldehyde, through its oxime, to the nitrile I, (R = CN) and hence to the corresponding acid, identical with that known to have the structure I, (R = CO_2H) from *O*-methylpsychotrine. A partial synthesis of emetine is described from the acid I, (R = CO_2H), the acid chloride of which was condensed with homoveratrylamine to give the amide (II). The latter treated with $POCl_3$ gives *O*-methylpsychotrine, identical with the natural alkaloid, the reduction of which to emetine has already been described. J. B. S.



(I)



(II)

ANALYTICAL

β-Bromallylbarbiturates, Detection of, on Paper Chromatograms. A. S. Curry. (*Acta pharm. tox. Kbh.*, 1957, **13**, 357.) The 5 : 5-disubstituted barbiturates

can be distinguished from the 1 : 5 : 5-substituted barbiturates and the thiobarbiturates by observation of the changes in the ultra-violet absorption spectrum with pH. Identification of the individual members is made by paper chromatography with selective spray reagents for detecting the spots. Thus 5 : 5-disubstituted barbiturates with fully saturated side chains do not react with aqueous solutions of potassium permanganate, but compounds that have an allyl radical in the molecule react immediately. Other barbiturates with side chain radicals also containing a double bond can be divided into two further groups, depending on their speed of reaction with this spray reagent. However because this is a subjective measurement of rate of reaction, it was thought necessary to devise another test to distinguish those barbiturates with β-bromallyl radical in the molecule. Chromatography, using *n*-butanol/5 N ammonia as the solvent and a saturated solution of soluble fluorescein in glacial acetic acid and hydrogen peroxide with a trace of copper acetate, as the spray reagent, was employed. The copper catalyses the hydrolysis of the β-bromallyl barbiturates and the floresceineosin reaction can then be used. With such a method good separation between Noctal, Pernocton and Sigmodal is obtained, and quantities as small as 50 μg. can be detected. M. M.

***Digitalis purpurea*, Chemical and Biological Assay of.** K. B. Jensen. (*Acta pharm. tox. Kbh.*, 1957, **13**, 381.) 25 digitalis leaf specimens of varying origin were assayed biologically by the guinea pig method and the amounts of the individual cardio-active substances were determined chromatographically and fluorimetrically. It was found that there was marked agreement between the percentages that the individual substances constituted of the total amount, in the various specimens analysed, provided that they fulfilled the requirements made in the Scandinavian Pharmacopoeias for Folium Digitalis as to collection and drying. In a few drug specimens dried in a different way the quantitative composition of the glycoside complex was different. The observed uniformity of composition of the glycoside complex in the analysed specimens of Folium Digitalis justifies the presumption that the total glycoside content can be regarded as a measure of the potency of these specimens. A comparison of the results achieved by the biological and the paper-chromatographic and fluorimetric methods showed a marked agreement for most of the drug specimens examined. However, for a few of the specimens the differences were so great that a fixed relationship between the two methods seems unlikely. These differences are assumed to be due to the biological method being not sufficiently specific and including in the assay cardiotoxic substances that do not have the character of cardiac glycosides. It is suggested that the potency of a Scandinavian drug specimen can be estimated by chemical assay of the total content of cardio-active substances—by means of the paper-chromatographic and fluorimetric or an equally specific method. By fermentative decomposition of primary into secondary glycosides before paper chromatography it should be possible to restrict to a practical limit the number of substances that must be determined to give an adequately accurate estimate of potency. M. M.

Mercaptoaneurine and Aneurine, Determination of. B. Buděšínský and E. Vaníčková. (*Československ. Farm.*, 1957, **6**, 308.) Aneurine is precipitated as $(C_{12}H_{18}N_4OS)(BiI_4)_2$ in acid solution and the excess of precipitant is determined complexometrically. A weighed sample (120 to 300 mg.) of aneurine hydrochloride is dissolved in 25 ml. of water and 2 ml. of conc. hydrochloric acid are added, then 5 ml. of a 20 per cent solution of potassium iodide and

exactly 10 ml. of 0·25M potassium iodobismuthite. The volume is made up to 50 ml. and the liquid is filtered through a dry filter. The first 10 ml. of the filtrate are rejected and about 1 ml. of 0·1N sodium thiosulphate and 10 ml. of acetate buffer are added to a 25-ml. quantity of the remainder. This solution is then titrated against 0·05M Complexone III until its yellow colour disappears. A blank experiment is carried out at the same time: 1 ml of 0·05M Complexone III corresponds to 8·432 mg. of anhydrous aneurine hydrochloride. Mercaptoaneurine is determined by potentiometric titration against potassium bromate in acid solution. E. H.

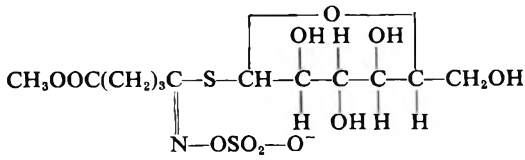
Salicylic Acid and Benzoic Acid in Mixtures, Determination of by Differential Nonaqueous Titration. M. I. Blake. (*J. Amer. pharm. Ass., Sci. Ed.*, 1957, **46**, 287.) For the analysis of ointments containing benzoic and salicylic acids, a 5-g. sample (containing 2–3 m.eq. of benzoic acid and 1–2 m.eq. of salicylic acid) was dissolved in neutralised dimethylformamide with the aid of a magnetic stirrer. The solution was titrated rapidly with 0·1N sodium methoxide, the end points being determined electrometrically. The first end point was due to salicylic acid and the second to benzoic acid. This method was satisfactory for the U.S.P. XV ointment which is made with a macrogol basis. Whitfield's ointment, U.S.N.F. IX, which has a basis of wool fat and soft paraffin did not dissolve readily in the dimethylformamide. In this case good results were obtained by dissolving the ointment in a little chloroform and adding the dimethylformamide before carrying out the titration. G. B.

Sulphonamides, Analysis of Mixtures of, by Paper Electrophoresis. S. Ljungberg. (*Svensk farm. Tidskr.*, 1957, **61**, 529.) Mixtures of sulphonamides were dissolved in 5 M acetic acid and separated by electrophoresis on paper. After drying, the position of the sulphonamides on the paper was detected in ultra-violet radiation. The appropriate areas were cut from the paper and digested for 1 hour with hydrochloric acid, and the quantity of sulphonamide in the resulting solution determined by the colorimetric method of Bratton and Marshall, or by measuring the ultra-violet absorption. The error of the method was about 5 per cent. Mixtures of sulphadiazine, sulphamerazine and sulphamethazine were separated in 20 hours' electrophoresis, but mixtures of sulphadiazine, sulphamerazine and sulphathiazole required 40 hours. G. B.

GLYCOSIDES

Methyl 4-*iso*thiocyanatobutyrate, a New Mustard Oil as a Glucoside (Glucoerypestrin) in *Erysium* Species. A. Kjaer and R. Gmelin. (*Acta chem. scand.*, 1957, **11**, 577.) Seed extracts of *Erysimum rupestre* DC yield a glassy glucoside fraction, which on acetylation gives crystalline glucoerypestrin tetra-acetate monohydrate. De-acetylation with methanolic ammonia furnished the alkali labile glucoerypestrin. Enzyme hydrolysis with myrosinase at pH 6·7 gave a mustard oil, which in turn yielded the thiourea $H_2N{\cdot}CS{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}COOMe$ on treatment with methanolic ammonia. Reaction of the *iso*thiocyanate with aniline and 1-naphthylamine in methanolic solutions afforded the crystalline phenylthiourea $C_6N_5NH{\cdot}CS{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}COOMe$ and the 1-naphthylthiourea $1\text{-}C_{10}H_7NH{\cdot}CS{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}COOMe$ respectively. The structure of the new mustard oil which follows as methyl 4-*iso*thiocyanatobutyrate was confirmed by synthesis from methyl 4-aminobutyrate hydrochloride

and thiocarbonyl chloride. Strong acid hydrolysis of glucoerypestrin yields hydroxylamine in accordance with the structure (I) for the glucoerypestrin anion.



(I)

J. B. S.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

8-Azapurines, Metabolism of. J. D. Smith and R. E. F. Matthews. (*Biochem. J.*, 1957, **66**, 323.) Growth of *E. coli* is inhibited by 8-azaguanine (I), 8-aza-adenine (II), 8-azahypoxanthine (III) and 5(4)-amino-1*H*-1 : 2 : 3-triazole-4(5)-carboxyamide (IV). (I) and 8-azaxanthine (V) inhibit the growth of *B. cereus*, whilst development of the tobacco leaf virus is inhibited by (I), (II) and (IV). A number of 8-azapurines and substituted triazoles were not inhibitory. In all cases those which are inhibitory are incorporated as 8-azaguanine in the organism's ribosenucleic acid. In *B. cereus* this occurs in amounts up to 40 per cent of the ribosenucleic acid guanine, the highest ratio being found in the terminal purine nucleoside 2′ : 3′-(monohydrogen phosphate) residues liberated by pancreatic ribonucleases. *B. cereus* ribosenucleic acid containing (I) has been fractionated and the shorter nucleic acid chains shown to contain more 8-azaguanine than the longer chains. They also contain a greater proportion of the cyclic-8-azaguanylic acid residues. Assimilation of (I) by *B. cereus* can be readily reversed by addition of guanine. Ethanol extracts of *B. cereus* grown in the presence of (I) have been shown to contain 8-azaguanosine and 8-azaxanthosine. Some (I) is incorporated into *B. cereus* dexoxyribonucleic acid, and 8-azaguanine desoxyribonucleoside has been tentatively identified in hydrolysates of the nucleic acid. J. B. S.

Di*iso*propyl Phosphorofluoridate and other Anticholinesterases, Action of, on Amino Acids. R. F. Ashbolt and H. N. Rydon. (*Biochem. J.*, 1957, **66**, 237.) The action of di*iso*propyl phosphorofluoridate dyflos, di*iso*propyl phosphochloridate (DC1P) and other phosphorus-containing anticholinesterases on a number of amino acids has been studied under "physiological" conditions. Dyflos differs markedly from DC1P in that it reacts appreciably with the phenolic hydroxyl group of tyrosine under "physiological" conditions, causing *O*-di*iso*propylphosphorylation, the product being identified by comparison with a synthetic specimen. Tyrosine is able to compete with water in its reaction with dyflos at pH 7-8. Di*iso*propylphosphorylation of the α-amino group occurs to a small extent with tyrosine and phenylalanine. DC1P is even less reactive in this respect. Tetraethylpyrophospate (TEPP) is much less specific but more reactive than dyflos towards amino acids. It reacts equally readily with the phenolic and α-amino groups of tyrosine, with the α-amino group of phenylalanine, and the ε-amino group of lysine. It is slightly less reactive with the α-amino and β-hydroxy groups of serine. The significance of the results in relation to the mode of action of di*iso*propylphosphorofluoridate and other anticholinesterases is briefly discussed. J. B. S.

Poliomyelitis Vaccine: Safety and Antigenic Potency Testing. Report from the Biological Standards Control Laboratory, M.R.C. Laboratories. (*Brit. med. J.*, 1957, **2**, 124.) The British poliomyelitis vaccine is prepared by the formalin-inactivation method introduced by Salk (1953) but differs in its type I component from that produced in North America. The Mahoney strain has been replaced by the partially attenuated Brunenders strain. As in America, strains MEF-I and Saukett are used in preparing the type II and III components of the British vaccine. The general regulations for the testing of the British vaccine have been laid down in the T.S. Amendment Regulations No. 1131 (1956) and are based on the present American ones, but the introduction of an attenuated strain in this country has necessitated some modification of the American methods of testing. In Britain concurrent tests are made on each batch of trivalent vaccine by the control laboratory and the manufacturer, after the latter has shown that the tests for safety and potency on the individual type components are satisfactory. In the final tests the vaccine is examined for the presence of live virus by the inoculation of cell cultures and monkeys, and for its antigenic potency in monkeys. The three tests are described in detail. S. L. W.

BIOCHEMICAL ANALYSIS

Adrenaline, Estimation of, in Peripheral Blood. B. V. Franko, A. D. Bragg and D. T. Watts. (*Arch. int. pharmacodyn.*, 1957, **111**, 123.) This method is a modification of that used by Gaddum and Lembeck. It involves the use of the isolated uterus of the rat, suspended in a calcium and glucose deficient Ringer solution. An organ bath of 3 ml. capacity, of the overflow type was used. All apparatus was constructed in polyethylene to prevent haemolysis when whole blood was used for the assay. Lysergic acid diethylamide, 5 μg./l., was added to the Ringer solution in order to prevent stimulation of the tissue by the 5-hydroxytryptamine in the blood. These modifications permit the estimation of adrenaline in the peripheral arterial blood of the dog. Although insufficiently sensitive to estimate the adrenaline level under basal conditions, it could be used for this purpose under conditions of stress. The adrenaline could be estimated in whole blood provided that the assay was conducted within one minute of the blood being shed. The method is rapid and simple, and blood samples as small as 0·3 ml. can be used. At least 95 per cent of the adrenaline added could be recovered at the end of one minute. If the blood samples could not be assayed immediately the plasma was separated from the cells and used for assay. The method described here has been utilised for the determination of adrenaline in peripheral arterial blood of the dog under conditions such as haemorrhagic hypotension, anoxia and after the administration of such drugs as nicotine, histamine and acetylcholine. It was found that the maximum blood adrenaline level observed during haemorrhagic hypotension is approximately 100 times that of the basal level of less than 1 μg./l.

M. M.

Adrenaline, Noradrenaline and Hydroxytyramine, Separation of. N. Kirshner and McC. Goodall. (*J. biol. Chem.*, 1957, **226**, 207.) A procedure is described in which the weak cation exchange resin Amberlite IRC–50 is used to separate adrenaline, noradrenaline and hydroxytyramine in less time than that required by previous methods and in amounts which vary from 0·02 to 1·0 mg. Extracts of adrenal glands were prepared in 10 per cent trichloroacetic acid. After filtration the acid was removed by extraction with ether and the pH of the resultant solution was adjusted to 6·1. This solution was

then passed through the resin column and eluted with ammonium acetate buffer solution at pH 5·0. The amines were determined by their absorption at 279 mμ. In 3 experiments with pure solutions the per cent recovery of adrenaline was 85–95, noradrenaline 95–97 and hydroxytyramine 83–95. In 5 experiments with trichloroacetic acid extracts of adrenal glands the recovery of adrenaline, calculated from the optical density at 279 mμ, ranged from 88 to 103 per cent and the recovery of noradrenaline from 73 to 103 per cent. This separation of adrenaline, noradrenaline and hydroxytyramine is dependent upon the pH of the resin and the pH of the eluting fluid. Increasing the pH of the resin to 6·6 greatly enhanced the separation of adrenaline from noradrenaline but decreased the separation of noradrenaline from hydroxytyramine. Decreasing the pH of the resin below 6·0 increased the resolution of the hydroxytyramine and noradrenaline fractions but decreased the separation of adrenaline from noradrenaline. Changing the pH of the eluting buffer caused similar shifts in the resolving capacity of the resin. Increasing the ionic strength of the eluting buffer increased the rate at which the compounds migrated down the column. Increasing the molarity of the buffer beyond 0·4 M caused increased losses of adrenaline, noradrenaline and hydroxytyramine and decreased the resolution for all 3 compounds. M. M.

Dextran in Plasma, Estimation of. R. E. Semple. (*Canada J. Biochem. Physiol.*, 1957, **35**, 383.) When dextran is used to estimate plasma volume, concentrations of 60–150 mg./100 ml. of plasma have to be measured accurately and fairly rapidly. The methods that are already in use are accurate only for amounts greater than 300 mg./100 ml. The method described here is satisfactory for 50–150 mg. of dextran/100 ml. of plasma. First the plasma protein is removed with trichloroacetic acid. The supernatant fluid is then dialysed against water to remove the glucose. The carbohydrate concentration of the resulting aqueous extract is determined by a modified anthrone technique. Anthrone solution is added to the extract and the optical density of the solution is read in a spectrophotometer against the distilled water-anthrone blank at $\lambda = 625$ mμ. The results show that mean recoveries were between 99·8 and 100·1 per cent. When single plasma samples were analysed the standard deviations from the means varied, with the dextran concentration, from 1·7 to 2·5 per cent. These deviations were reduced to 1·4 to 1·7 per cent by the use of duplicate plasma samples. The whole procedure takes 3–4 hours.

M. M.

Noradrenaline and Adrenaline, Urinary Excretion of. N. T. Kärki. (*Acta physiol. scand.*, Supp. 132.) This paper gives a detailed account of the adrenaline and noradrenaline content of normal human urine. In order that the adrenaline and noradrenaline can be estimated quantitatively by a biological method the amines are first extracted from the unhydrolysed urine. This is done by adsorption on to aluminium oxide at pH 8·5. To minimise inactivation of the amines the pH is allowed to remain at this high value for not longer than 25 minutes. The amines were then eluted with 1 N sulphuric acid. The recovery of adrenaline and noradrenaline added to urine was approximately 73 per cent. The stability of the extracts was good: no reduction in biological activity being observed during storage at + 4° for 10 days. In the study of the stability of noradrenaline added to urine, no variation in the content occurred when the urine was stored in the cold (+ 4°) at either pH 4 or pH 6·5. The content did not change when the urine of pH 4 was stored for 2 days at room temperature, but a marked decrease occurred when the pH of the urine was 6·5 during the

storage at room temperature. The biological activities of the extracts were determined using the blood pressure of the hexamethonium—treated cat or rat and the rectal caecum of the hen as test preparations. The adrenaline and noradrenaline content was then determined by the use of a suitable formula. To determine the normal excretion of noradrenaline in men, women and children 356 twenty-four urine samples from 291 subjects were used. To determine the adrenaline excretion 240 samples from 182 subjects were used. In the group of children from 1·5 to 6 years old, the mean 24-hour excretion of noradrenaline in the urine was 5·6 μg. and that of adrenaline 1·3 μg. In the children of 7–16 years the mean noradrenaline excretion was 14·5 μg./24 hours and that of adrenaline 2·8 μg. The excretion levels in this group were approximately twice those of the preceding group, and a highly significant regression was established between the amount of noradrenaline excreted and the weight of the child. In those subjects from 17 to 29 years the mean excretions were 24·5 μg. of noradrenaline and 5·1 μg. of adrenaline per 24 hours. In the group from 30 to 59 years the mean excretions were 25·2 μg. of noradrenaline and 5·4 μg. of adrenaline per 24 hours. In the group from 60 to 96 years the mean excretion of noradrenaline was 23·1 μg. and that of adrenaline 4·4μg./24 hours. When the amounts of noradrenaline were calculated per kilogram of body weight it was found that the mean noradrenaline output was highest for both sexes in the 7 to 16 year old group and that it decreased with increasing age. No differences in the 24 hour noradrenaline excretion were observed between the sexes when the mean excretions for the various age groups were compared. No statistically significant differences in the urinary excretion of adrenaline, calculated per kilogram of body weight were found between the different age groups and between the sexes. The urine volumes excreted by the children were large but the mean concentration was low, being only about half that of the urine of adults. The amounts of noradrenaline excreted by 85 subjects from 17 to 29 years were not found to bear any relationship to the urine volume. The excretion of the two amines was found to vary with the time of day. With both adrenaline and noradrenaline the amount excreted during the night was considerably lower than the amount excreted during the day. Muscular work was found to cause a very large increase in the amounts of both amines excreted. The greatest increase was 35 times the normal level. M. M.

CHEMOTHERAPY

Erythromycin Group of Antibiotics. L. P. Garrod. (*Brit. med. J.*, 1957, **2**, 57.) A series of *in vitro* experiments were undertaken to determine (1) the activity of oleandomycin, alone and in combination, with tetracycline, and (2) the closeness of the relationship between erythromycin, oleandomycin, and spiramycin from which might be deduced how far acquired resistance to each involves the others. The *in vitro* antibacterial activity of oleandomycin was found to be somewhat less than that of erythromycin, and the claims that a 2:1 mixture of tetracycline and oleandomycin exerts a synergic action *in vitro* were not confirmed. Spiramycin was shown to have a considerably lower activity *in vitro* than erythromycin. Studies of cross-resistance among the members of this group showed that, whereas complete cross-resistance develops in strains of staphylococci habituated *in vitro*, erythromycin-resistant strains isolated from patients may or may not be resistant to oleandomycin and spiramycin. To what extent the clinical use of these two antibiotics may produce bacterial resistance to erythromycin remains uncertain, but it would be safer to assume that cross-resistance may sometimes follow the use of any of the three. S. L. W.

Glyoxals, Antiviral Activity of. C. A. de Bock, J. Brug and J. N. Walop, (*Nature, Lond.*, 1957, **179**, 706.) The antiviral activity of some compounds with an $\alpha\beta$-dicarbonyl structure, and closely related substances was studied. Influenza virus A-USA-47 (A′ strain) was used and was cultured in eleven-day-old chick embryos. The eggs were inoculated with the seed virus and incubated at 36° for 48 hours. After this time the haemagglutination titre was tested. For estimation of antiviral activity the compounds were injected into the allantoic fluid of the eggs one hour before inoculation and the titre measured in the usual way. A compound was considered active if the difference between the logarithm of the haemagglutination titre of the eggs inoculated with virus only and those treated with the drugs was greater than + 0·6. A number of α-keto-aldehydes appeared to be active in the concentration used (0·1M solution or suspension in saline). *In vitro* investigations on some of the more active compounds showed a direct action on the virus. The virus particles lost their infective power when incubated with concentrations as low as 0·002M for 5 hours at 37°. Somewhat higher concentrations destroyed the enzymic activity of the virus against urinary mucin as substrate. Haemagglutinating power of the virus was destroyed by still higher concentrations or by prolonged incubation with one of the active compounds in a concentration of 0·004M at 37°. The virucidal action *in vitro* is strong enough to explain the activity in the allantoic test. G. P.

PHARMACOLOGY AND THERAPEUTICS

Hypoglycaemic Agent, Clinical Report of. J. Pomeranze, H. Fujiy and G. T. Mouratoff. (*Proc. Soc. exp. Biol. N.Y.*, 1957, **95**, 193.) A new synthetic oral hypoglycaemic drug, *N*-β-phenylethylformamidyliminourea (DBI) causes in doses of 100 mg. a significant decrease in the blood sugar concentration in normal adults and in diabetics. The configuration of the glucose tolerance curve was altered, unlike the sulphonylurea drugs which lower the fasting blood sugar but do not alter the glucose tolerance curve. DBI adequately replaced 40 units of the 70 required units in a young severe labile diabetic patient and totally replaced insulin in a less severe diabetic patient under 40 years of age and in a 68 year old patient whose diabetes had been present for 28 years. G. F. S.

Indole Carboxamidines and Aminomethylindoles as Antimetabolites of Serotonin. E. Shaw and D. W. Woolley (*J. Amer. chem. Soc.*, 1957, **79**, 3561.) A number of 6-aminomethyl-1 : 2 : 3 : 4-tetrahydrocarbazoles and 1 : 2 : 3 : 4-tetrahydrocarbazole-6-carboxamidines have been synthesised as potential antimetabolites of serotonin. The antiserotonin action of 1 : 2 : 3 : 4-tetrahydrocarbazole-6-carboxamidine and -6-*N*-phenylcarboxamidine, 6-aminomethyl-, 6-*NN*-dimethylaminomethyl-, and 9-benzyl-6-*NN*-dimethylaminomethyl-1 : 2 : 3 : 4-tetracarbazoles has been measured on carotid artery segments, on isolated rat uterus, and against the pressor action of serotonin in dogs. 1 : 2 : 3 : 4-Tetrahydrocarbazole-6-*N*-phenyl carboxamidine caused a sharp fall in the blood pressures of anaesthetised dogs when administered intravenously in doses of 2–7 mg./kg. 9-Benzyl-6-dimethylaminomethyl-1 : 2 : 3 : 4-tetrahydrocarbazole in daily doses of 25 mg./kg. administered orally to dogs caused refusal of food and behavioural changes after 3–4 days treatment. J. B. S.

Iron-Dextran; Treatment of Iron-deficiency Anaemia in Children. R. O. Wallerstein and M. S. Hoag. (*J. Amer. med. Ass.*, 1957, **164**, 962.) Iron-deficiency anaemia in 24 infants from 5 to 36 months was treated by intramuscular injection of solutions of an iron-dextran complex (Imferon). Dosage

was at the rate of 50 to 100 mg. of Fe daily (1 to 2 ml. of Imferon), and the total dosage varied according to age: under 6 months, 100 mg.; 6 to 12 months, 200 mg.; 12 to 24 months, 300 mg.; over 24 months, 400 mg. Haemoglobin values rose to about 11 g./100 ml. in 3 weeks. Rises of 4 per cent/day were seen in several infants with severe anaemia. Reticulocyte response occurred early and correlated fairly well with the degree of anaemia in patients with severe anaemia, though it was inconstant in patients with moderate anaemia. The injections caused no local tenderness, redness or swelling, but slight brownish discoloration of the subcutaneous tissues, lasting for several weeks, occasionally occurred. There were no systemic reactions. There is a wide margin of safety, and a total dosage of at least 1 g. may be given to infants. It is emphasised, however, that failure to respond to the proper intramuscular dose of iron is never an indication for giving more iron. S. L. W.

Iron, Intravenous, Toxic Reactions to. I. P. Ross. (*Lancet*, 1957, **2**, 77.) Patients given 100-mg. doses of saccharated iron oxide preparation (Ferrivenin) intravenously, following initial doses of 25–50 mg. showed toxic reactions in about 7·5 per cent of the cases, about half of these being serious enough to require the treatment to be discontinued. Massive doses (500–600 mg. by intravenous infusion) gave rise to a much larger proportion of toxic reactions. Reactions were least common in pure iron-deficiency anaemias and more frequent in the presence of fever, toxaemia or metabolic disturbance. Great care appears to be necessary with patients having disorders of the pulmonary capillary bed, who may be given doses of 25 to 50 mg. very slowly. As, even with careful selection of cases, the incidence of toxic reactions to 100-mg. doses is about 5 per cent, the development of less toxic preparations is desirable.

G. B.

Peganone; A Clinical Evaluation. C. H. Carter and M. C. Maley. (*Amer. J. med. Sci.*, 1957, **234**, 74.) After a control period of one year on established therapy, 38 chronic, refractory epileptics, suffering from mixed grand and petit mal epilepsy, were treated with peganone (3-ethyl-5-phenylhydantoin). Of the total of 38, 23 were children and 15 adults. In 9 cases peganone was used alone, and in the remainder it was combined with the previous medication. No incompatibilities were found with other anti-epileptic drugs. Dosage was commenced at 0·5 g. daily and gradually increased to a total of 3 to 4 g. daily in divided doses; in a few adults dosage was increased to a maximum of 5 or 6 g. daily. Thirteen of the patients were studied for more than one year, 10 for from 6 to 9 months and the remainder for short periods. In the group as a whole there was an overall improvement of 62 per cent in the number of seizures; 71 per cent of the cases showed a reduction of more than 50 per cent in the number of seizures. Routine blood studies, liver function tests and urinalyses were carried out on all patients. There were no deviations from normal and no evidence of other toxic effects such as skin rash or gum hyperplasia. The only side-effect noted was drowsiness in a few patients daily. The authors consider peganone to be a valuable addition to the available anti-epileptics because of its effectiveness and lack of toxicity. S. L. W.

Proclorperazine; Antiemetic Properties. D. G. Friend and G. A. McLemore. (*Arch. intern. Med.*, 1957, **99**, 732.) Proclorperazine, a new chlorpromazine congener, gave excellent or good results in 23 out of 25 cases of nausea and vomiting of various etiologies. It was given in a dose of 10 mg. by mouth or intramuscularly every 4 to 6 hours or as a rectal suppository of

50 mg. once daily. Several of the cases of nausea and vomiting had previously failed to respond to chlorpromazine therapy. In 2 cases with Ménière's syndrome with severe vertigo it effectively relieved the dizziness. Two cases of hiccough were also relieved. Slight drowsiness was a common but usually desirable side-effect. On long-continued therapy mild gastric irritation may occasionally occur, and in doses higher than 60 mg. a day it may cause confusion, dizziness or fainting. No jaundice, agranulocytosis or other serious toxic effects were observed. S. L. W.

Rauwolfia, the Total Alkaloids of, and Reserpine, Sites of Action of, on the Sleep Centre. E. Frommel and P. Gold. (*Acta pharm. tox. Kbh.*, 1957, **13**, 345.) A comparison is made of the action of an extract containing all the active principles of rauwolfia with that of a specific member of the group, namely reserpine, on the sleeping time of various animals. It has previously been suggested that the action of reserpine involves a sleep centre which is different from that affected by phenobarbitone. This has been confirmed by showing that reserpine does not potentiate significantly barbiturate-induced sleep in guinea pigs. In contrast, the total alkaloids of *Rauwolfia serpentina* appear to overlap the site of action of phenobarbitone since these alkaloids potentiate its sleep-producing effect. Neither the total alkaloids of rauwolfia nor reserpine modified the hypnosis produced by sodium thiopentone in rabbits. Atropine was found to significantly enhance the sleep-inducing effect of thiopentone in rabbits, previously treated with the total alkaloids of rauwolfia. This action was less marked in those animals which had received reserpine. Although the administration of phenobarbitone significantly augmented the duration of thiopentone-induced sleep in rabbits treated with the total alkaloids, this effect was much smaller in animals given reserpine. The administration of atropine and phenobarbitone to rabbits treated with the total alkaloids produced the longest duration of thiopentone-induced sleep. There was less effect in the reserpine treated animals. These results indicate that the action of the total alkaloids of rauwolfia differ from reserpine on the sleep centres affected by phenobarbitone and thiopentone. However this distinction does not apply to the centres of nikethamide excitation because here both the total alkaloids and reserpine neutralised the agitation produced by nikethamide in guinea pigs and enhanced the sedative effect of phenobarbitone against nikethamide. M. M.

Ro 2-7113, Analgesic Activity and Toxicity of. W. M. Benson, D. J. Cunningham, D. L. Hane and S. Van Winkle. (*Arch. int. Pharmacodyn*, 1957, **109**, 171.) By intravenous or subcutaneous injection Ro 2–7113 [(±)-1-methyl-3-allyl-4-phenyl-4-propionoxy-piperidine hydrochloride] had the same order of toxicity in mice as alphaprodine (the corresponding 3-methyl derivative) and levorphanol, but was considerably more active as an analgesic. Orally the ratio of toxic dose to analgesic dose LD50/AD50 for all three drugs was much less, but was still more favourable in the case of Ro 2–7113. In rats Ro 2–7113 was twice as toxic by intravenous injection as alphaprodine and levorphanol, but more than twelve and seven times as active, respectively. Duration of activity of Ro 2–7113 by this route, like that of alphaprodine, was less than that of levorphanol. Subcutaneously in rats the ratio of LD50/AD50 was twelve times more favourable for Ro 2–7113 than for alphaprodine, and twice that of levorphanol. By the oral route the ratio for Ro 2–7113 was twice that for the other two. Similar results were obtained for rabbits and dogs. In dogs, sedation and respiratory and cardiac depression was seen with sufficiently large

doses. Peripheral cholinergic effects were also noted. The increased potency of the new analgesic is interesting since altering the position of the allyl group to the nitrogen gives a compound with much diminished, if not antagonistic, action. G. P.

Senna, Standardized, as a Laxative in the Puerperium, a Clinical Assessment. A. S. Duncan. (*Brit. med. J.*, 1957, **1**, 439.) Senna has been regarded for many years as a safe laxative for pregnant and puerperal women, but its use has lapsed because of variability of the potency of preparations. The introduction of a stabilised consistent preparation, Senokot, has led to a revaluation of its use, and in the present trial the preparation was compared for laxative effect with cascara. As a preliminary step spontaneous bowel movements in 100 consecutive puerperal women were recorded; of these only 26 occurred by the third day, and in 23 an enema was required after the eighth day. Two further groups received either 0·4 g. of tab. extract cascara B.P. or two teaspoonsful of Senokot granules (equivalent to 0·7 g. of senna pod) on the morning of the third puerperal day. With the doses used the senna preparation was the more effective of the two, bowel movement occurring in about 85 per cent of the patients within 24 hours, whereas with cascara the figure was about 55 per cent. Also the proportion of normal stools was higher with the senna. No untoward side effects were noted with the laxatives, apart from gripes, which were more frequently observed with the senna preparation. A few patients complaining of severe morning sickness improved dramatically after Senokot, but this requires further investigation. Of the 290 women in the trial, 66 had been taking laxatives regularly before they became pregnant and 43 had taken liquid paraffin regularly during pregnancy. G. P.

Tolbutamide; Clinical and Biochemical Studies. M. F. Crowley, F. W. Wolff and A. Bloom. (*Brit. med. J.*, 1957, **2**, 327.) Tolbutamide was used in the treatment of 42 patients on standard diets whose diabetes was neither so mild as to be controlled by diet alone nor so severe as to need insulin immediately. Patients were maintained for a total of 6 weeks on tolbutamide and then for a further 6 weeks on dummy tablets unless relapse occurred, in which case tolbutamide was re-introduced. The dosage was 3 g. on the first day and 2 g. daily thereafter. Of the 42 patients treated 30 responded well, with a return to normoglycaemia, and a complete or almost complete disappearance of glycosuria. Twelve responded poorly or not at all. The majority who responded were middle-aged or elderly. Seven of the 30 who responded relapsed when the drug was stopped, six within the first 2 months; six of the 7 responded well when the drug was restarted and remained satisfactorily controlled on 2 g. daily. The remaining 23 remained well controlled on dietary regime alone. No toxic effects were recorded in any of the patients. The majority showed no weight change while on tolbutamide, but when it was discontinued there was a mean reduction of 3 lb. in weight in patients observed over the next 2 months. When tolbutamide 1 g. was given twice daily the blood concentration differed from that following a single dose of 2 g., but the effect on the blood and urine sugars was the same. This suggests that the level of blood tolbutamide is not the determining factor in the fall in blood and urine sugar. The fact that the plasma inorganic phosphorus did not fall during tolbutamide therapy and that the drug had no effect on the amino acid nitrogen levels indicates that tolbutamide does not act in the same way as insulin. S. L. W.

BOOK REVIEW

METHODEN DER ORGANISCHEN CHEMIE (Houben-Weyl). Fourth Edition. Edited by Eugen Müller. Volume XI, Part I. Stickstoffverbindungen II. Herstellung von Aminen. Pp. lviii + 1178 (including Index). Georg Thieme Verlag, Stuttgart, 1957. Moleskin DM. 208.00.

The present volume, the eighth to be published in the fourth edition of Houben-Weyl, and one of two to be devoted to the chemistry of nitrogenous compounds, is concerned entirely with the preparation of amines. Some idea of the scope and breadth of the information contained within this one volume can be gathered from the fact that it extends over nearly 1200 pages. The various preparative methods are classified under the following headings: direct amination, amination by substitution, by addition to olefinic substances, by reduction, by the use of organometallic compounds, by rearrangement, and by cleavage. The emphasis, throughout, is on practical methods, ofwhich numerous examples are given, in sufficient detail for each experiment to be carried out without further reference to the original literature. Factors affecting the course, rate and yields of the various reactions are discussed in detail, as for example the influence of concentration, solvent, temperature and metal salts on the reaction between alkyl halides and ammonia or amines. As with the previous volumes in this series, much other useful information has been condensed into the many tables throughout the book, which briefly summarise large numbers of additional examples. It is this type of information which is particularly valuable to the practical organic chemist. Literature references up to 1956 are given throughout. Although price places this and the other volumes of this series outside the range of the average pocket, they will form valuable additions to any research library, helping to fill the ever important need for the classification of reaction methods.

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