REVIEW ARTICLE

THE RAUWOLFIA ALKALOIDS

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Over the past two or three decades much attention has been paid by clinicians to the treatment of hypertensive disease. This has resulted in the introduction and trial of a number of drugs, including, amongst others, thiocyanates, nitroprussides, azides, hydrazinophthalazine (hydrallazine), veratrum, the ganglion blocking agents, ion exchange resins, barbiturates and the various extracts and alkaloids of *Rauwolfia serpentina* Benth.

The introduction and use of *Rauwolfia serpentina* is of particular interest; not only has it intriguing pharmacological properties, but it represents something of a return to the use of vegetable drugs, in an age which is becoming increasingly devoted to the use of synthetic chemicals in medicine.

Although rauwolfia may ultimately not survive to take its place alongside the solanaceous drugs, the cardiac glycosides and opium, a study of its properties seems likely to lead to a clearer understanding of the complex mechanism underlying hypertensive disease in man. In this way it may establish itself firmly in the history of medicine. This may seem to be a jaundiced view to take of a new drug which is being widely used and widely studied. Many natural products have proved to be the starting points for further investigation and have yielded place to newer drugs with structures based upon the originals. A study of the literature indicates that this may be the fate of rauwolfia.

Historical

Rauwolfia serpentina under many names has been used for centuries in folk medicine in India. There are also reports of its use in Burma, Malaya and Java. It is variously called *sarpagandha* (Sanskrit)[1], *Chota-chand* (Hindi)[1,3,4], *chandra* or *chota-chand* (Bengali)[1], *dhan barua* or *dhan marua*, *pagla-ka-dawa* (Bihari)[1,2], *chota-chand*, *chandra*, *karavi*, *harkai* (Bombay)[1,2,4], *harkaya* (Marhatti)[1,2], *atalagandhi* or *patala garuda* (Telugu)[1,2], *Chuvana-avilpori* (Malay)[1,2], *dhannerna ordhan-barua* (Oriya)[2], *covannamiloori* (Tamil)[2], *chandrika* (Sanskrit)[2,3], *Tsjovanna Amelpodi* (Malealie, Malabar Coast), *chivan amelpodi* (Tamil), *Ratu-eka-weriya* (Cyng.), "*Ophioxylon of Serpents*", and *Ophioxylon serpentinum* (Lin.)[3], Trease and Evans[5] point out that the plant is mentioned in an ancient Hindu manuscript of 1000 B.C. In the second century A.D. it is to be found mentioned in Charaka's works[2] as *Sarpagandha*. Ainslie[3] describes the use of the root either as a powder or in the form of a decoction in snake bites and scorpion stings. He mentions also its use as a febrifuge, an anthelmintic, a stomachic and in obstetrics "to promote delivery in tedious cases". He seems to have been in some doubt about its utility

in snake bites, having "invariably trusted to the prompt use of Madeira wine and generally with success". Ainslie[3] gives references to earlier works. Dymock[4] indicates that the root was also used in dysentery: "In Bombay most of the labourers who come from the southern Concan keep a small supply of the root which they value as a remedy in dysentery and other painful affections of the intestines". The Pharmacopœia of India (1868)[6], and other publications[7–15], describe the use of the plant in folk medicine.

Distribution

Rauwolfia serpentina is found in India, Ceylon, Burma, Siam, Malaya and Java. Vakil[2] mentions its occurrence in the Himalayas, Assam, Pegu, Tennasserim, Bihar and the Deccan peninsula.

Botany and Pharmacognosy

The genus *Rauwolfia* belongs to the Apocynaceæ. The genus is named after Leonhard Rauwolf, a German doctor and botanist who travelled widely in Asia and Africa in search of medicinal plants which had been mentioned by the early Arab and Greek physicians. Rauwolf published the results of his studies in 1582. Years later a new genus of the Apocynaceæ was named *Rauwolfia* in his honour. The *Rauwolfias* are found all over the world in tropical and sub-tropical regions and there are about 130 species. These will be found listed in the *Index Kewensis*[16,17]. Botanical and pharmacognostical descriptions will be found in Dymock[4] and other works[5,7,8,11–25].

Rauwolfia canescens, *R. sellowii*, *R. perakensis*, *R. micrantha*, *R. hirsuta* (*R. heterophylla*), *R. semperflorens*, *R. vomitoria* and *R. densiflora* are other *Rauwolfia* species which have aroused interest. Wan[26], Youngken[13,27], Datta and Mukherji[24], Trease and Evans[5], and Martinez[28], deal with *R. perakensis*[13,26], *R. canescens*[5,13], *R. micrantha*[13,27], *R. hirsuta*[28], and *R. densiflora*[13]. Youngken points out that the roots and leaves of *R. canescens* were also used in Ayurvedic medicine[13]. He discusses some of the adulterants of *R. serpentina*[13], (*R. canescens*, *R. densiflora*, *R. micrantha* and *R. perakensis*). *Ophiorrhiza mungos* and white- and red-flowered *Clerodendron* species have also been described[22,23].

Chemistry

This is reviewed in detail by Phillips and Chadha[29], and by others[8,30–34]. The chemistry of the *Rauwolfia* species will therefore be dealt with briefly. A large number of alkaloids have been isolated; of a number of these the structure has been determined and physical constants are described (Fig. 1, p. 472; Table I, p. 476). The clinically important alkaloids (reserpine and rescinnamine) differ chemically from the others.

Alkaloids from Rauwolfia serpentina *Benth.*

Reserpine. Although the root contains numerous other substances which in extracts, concentrates or in powdered whole root modify its action, reserpine is the main active principle of *Rauwolfia serpentina.*

Its isolation from an oleoresin fraction was reported by Muller, Schlittler and Bein[35]. Reserpine was obtained as a relatively weakly basic alkaloid with m.pt. 262–3° C. and $[\alpha]_D^{23}$ −117 to −118° in chloroform. In 1932, van Itallie and Steenhauer[36,37] had reported the isolation of three alkaloids A, B and C from *R. serpentina*. In a more recent publication[38] alkaloid B (m.pt. 262° C.) has been claimed by Steenhauer to be identical with reserpine. Muller and his colleagues[35] obtained reserpine from the oleoresin fraction[39–41]. The extraction of reserpine is also described by Dorfman and his colleagues[39] and by others[40,41] Reserpine is the 3:4:5-trimethoxybenzoic acid ester of reserpic acid[44–46]. In 1953 Klohs and his colleagues[42] assigned to it the empirical formula $C_{35}H_{44}O_{10}N_2$ and made a preliminary study of its structure. Furlenmeier and his colleagues[43] proposed the formula $C_{33}H_{40}O_9N_2$. The structure of reserpine has now been established[47–50] (Fig. 1, I) and it has been synthesised[50a]. More recently, the stereochemistry has been investigated[51–54]. Reserpine is an indole derivative and is related to yohimbine.

Other Alkaloids

With few exceptions[56] these are all indolic (see Table I). As pure substances they have so far little or no clinical importance but some have been studied pharmacologically.

Rescinnamine (Fig. 1, III). Like reserpine, this is a tertiary indolic base related to yohimbine. It was isolated from *R. serpentina* and characterised as the 3:4:5-trimethoxycinnamic acid ester of methyl reserpate in 1954–55 by Klohs and his colleagues[57,58] and also in 1954 by Haack and his colleagues[59]. This alkaloid was called reserpinine by Haack[59]. However, this name has been used to describe another rauwolfia alkaloid of different structure (*q.v.*) and there is a possibility of confusion arising.

Deserpidine[60,64]. This alkaloid is closely related to reserpine, lacking only the methoxy group of ring A[60–64] (Fig. 1, X). Although it has been reported to be present in *R. serpentina*, its main source is *R. canescens*[60–64]; it is also known as *canescine*[60–64] and *recanescine*[63].

Serpine. This tertiary indolic alkaloid has recently been isolated from an alcoholic extract of the roots of the Cochin variety of *R. serpentina* by Chatterjee and Bose[65]. It is related to the tetra-hydro-β-carbolines, yohimbine and rauwolscine. A structural formula has been proposed (Fig. 1, V).

Sarpagine. This is identical with raupine and with serpagine. Raupine was isolated from *R. serpentina* in 1953–54[66–68]. Sarpagine was also isolated from the same source in 1953[69]. In 1954 the identity of raupine with sarpagine was established[70]. The constitution of sarpagine has been studied by Raymond-Hamet[50] and Thomas[71]. It is a weakly basic tertiary indolic alkaloid related to yohimbine. The physical constants[69] are given in Table I, but according to Bodendorf and his colleagues[66] the molecular formula is $C_{20}H_{26}O_3N_2$ ($C_{19}H_{22}N_2O_2 + CH_3OH$), m.pt. 325°, $[\alpha]_D^{20} = +63°$ in acetic acid.

Rauhimbine and iso*Rauhimbine*. Rauhimbine has been shown to be identical with corynanthine[72], an alkaloid previously isolated from

Pseudocinchona africana Chev.[34,73,74]. *iso*Rauhimbine and rauhimbine were isolated from *R. serpentina* by Hofmann[75]. The molecular formula $C_{21}H_{26}O_3N_2$, together with chemical and physical data, showed both compounds to be isomers of yohimbine and to be closely related to rauwolscine, an alkaloid isolated from *R. canescens*. Studies on the constitution, etc., of *iso*rauhimbine have been made by Le Hir and his colleagues[76] and by Chatterjee and Talpatra[77]. A partial structural formula has been suggested[76].

Yohimbine. The well-known pharmacologically active indolic alkaloid yohimbine[34] has been isolated from *R. serpentina* by Bader and his colleagues[78], and by Hofmann[72].

Other Yohimbine Isomers. These have been isolated by Bader and his colleagues[79] (alkaloid 3078); Weisenborn and his colleagues[80] (δ-yohimbine) (Fig. 1, VI); Hofmann[56] (δ-yohimbine, alkaloid C = an 11-methoxy-δ-yohimbine); Bader and his colleagues[81] (3-*epi*-α-yohimbine = 3-*epi*rauwolscine = alkaloid 3078)[79].

Reserpinine. This is another tertiary indolic base and is related to the alkalid tetrahydroalstonine. Reserpinine was isolated by Schlittler and his colleagues[82] in 1954. The m.pt. of the isolated compound was 238–9° C. (corr.) and a structural formula was proposed (Fig. 1, IV).' Weisenborn and his colleagues[80] also isolated reserpinine. The "alkaloid A" of Neuss and his colleagues[83] with the molecular formula $C_{22}H_{26}N_2O_4$ and related to tetrahydroalstonine is the "substance I" of Popelak and his colleagues[67], and is identical with the reserpinine of Schlittler and Weisenborn and their colleagues[80,82]. Recently, Janot and Le Men[84] have shown that *Vinca major* L. contains reserpinine. Raubasinine[59,67,85] is identical with reserpinine. Hofmann's "alkaloid C"[72] may also be reserpinine.

Reserpiline. Reserpiline[86] is closely related to reserpinine. It is an amorphous alkaloid possessing an additional methoxyl group (Fig. 1, XI).

Ajmaline (Fig. 1, IX). Ajmaline is from the chemist's point of view perhaps the most interesting and most studied of the many *Rauwolfia* alkaloids. It is a tertiary indoline base. Much work has been done upon the elucidation of its structure which is not settled. Robinson[97] has suggested a possible structure, referred to in some detail by Phillips and Chadha[29]. Ajmaline was first isolated in 1931 by S. and R. H. Siddiqui[87] who, in addition, obtained in crystalline condition the alkaloids ajmalinine, ajmalicine, serpentine and serpentinine. The molecular formula $C_{21}H_{26}O_2N_2$ was proposed[87] for ajmaline. Van Itallie and Steenhauer[37] isolated ajmaline at about the same time. Studies on the structure of ajmaline have been carried out by the Siddiquis[87–90] and others[91–97]. Notable amongst these has been the work of Robinson and his colleagues at Oxford[92,93,96,97]. It is considered identical with rauwolfine[29,36].

iso*Ajmaline.* This was isolated by Siddiqui[90] from a variety of *R. serpentina* known as "Dehra Dun". It is associated with *neo*ajmaline. Siddiqui[90] pointed out that the alkaloid content of "Dehra Dun" *R. serpentina* differed in yield and in character from that of the "Bihar" variety.

*iso*Ajmaline is formed when ajmaline or *neo*ajmaline are heated. *neo*-Ajmaline on heating melts at 205°; if heating is continued the melt re-solidifies forming *iso*ajmaline which melts at 264–266°. *iso*Ajmaline is an isomer of ajmaline[93,96].

Rauwolfinine[98–102]. This is an indoline alkaloid. Its structure is not completely known.

Serpentine[80,88,89,103–105]. This is a yellow quaternary indolic, anhydronium base. Siddiqui and Siddiqui suggested that its molecular formula was $C_{20}H_{20}O_3N_2 \cdot 1\frac{1}{2}\,H_2O$[87–89]. This has now been revised to $C_{21}H_{22}O_3N_2$[103]. It is a stereoisomer of alstonine[80] and very closely related to the alkaloid rauwolscine which occurs in *R. canescens*[106–111]. A structural formula has been proposed by Klohs and his colleagues[105] (Fig. 1, VII).

Serpentinine. Serpentinine isolated by the Siddiquis[87–89] and later by Schlittler and his colleagues[112,113] appears to be an indolic anhydronium base[112,113].

Ajmalicine. This is known also as π-tetrahydro serpentine. It was isolated from *R. serpentina* by Klohs and his colleagues[105], who proposed a structure for it (Fig. 1, VI). It is closely related to serpentine (Fig. 1, VII).

Minor alkaloids. The following appear in the literature: ajmalinine[87–89], serpinine[114], chandrine[115], methyl reserpate[56], R.S.51[116], thebaine[56], papaverine[56].

Alkaloids from other Rauwolfia *Species*

Rauwolfia canescens Linn. *R. canescens* contains reserpine[61,64,117], rauwolscine (α-yohimbine)[61,107,108,109,110,121,122], yohimbine[61], serpentine[61] canescine[61,62], pseudoyohimbine[61], recanescine[63] (probably the same as deserpidine[61,64] and 11-desmethoxyreserpine). Canescine and recanescine are probably identical[63]. β-Yohimbine has been isolated by Hofmann[118] from *R. canescens* roots. Stoll and his colleagues have isolated aricin, *iso*reserpinine, reserpiline and *iso*reserpiline from *R. canescens* leaves[119]. The alkaloids of *R. canescens* have at the moment more chemical than pharmacological interest. They are indolic and have a close relation to the *R. serpentina* alkaloids. The isolation of aricin (Fig. 1, VIII) is of interest. Aricin is a 10-methoxy derivative of ajmalicine; it is isomeric with reserpinine. Stoll and his colleagues[119] point out that there are biogenetic similarities between the rauwolfia and strychnos alkaloids.

Rauwolfia hirsuta Jacq. (= *R. heterophylla* Roem and Schult.) This species grows in South and Central America and in Mexico. Martinez[28] describes its occurrence in Mexico. Guatemalan *R. hirsuta* is known as "chalchupa"[123], the Colombian plant as "pinique-pinique"[124,125]. The following substances have been isolated from *R. hirsuta*: the chalchupins A and B[123], reserpine[126–129] and narcotine[126], rauwolscine[127,129] and alstonine[127,131] (Fig. 1, VII), serpentine[128–130], ajmaline[128], ajmalicine[129] yohimbine[129], heterophyllin (aricin = 5-methoxyajmalicine)[129], alkaloid A[131], rautensin (total alkaloid fraction)[131].

Rauwolfia micrantha Hook. Rao and Rao[132] have reported the isolation of bases A, B and C from an oleoresin fraction. Youngken[27] describes the microscopy of the roots and tests for alkaloids. It is also known as Malabar rauwolfia.

Rauwolfia sellowii. The alkaloid content of the various plant organs has been determined[133]. Seba and his colleagues indicate that *R. sellowii* contains ajmaline and serpentine[134].

Quantitative Estimation of Rauwolfia Alkaloids

Rauwolfia extracts, powdered root and reserpine are being widely used. They are available in various forms, including tablets, mixtures and solutions for injection. The reserpine content or hypotensive potency may be determined in a number of ways: Bakshi[135] pointed out that the *R. serpentina* alkaloids gave a blue fluorescence in ultra-violet light. This property was made use of in an assay method. Sheppard and his colleagues[136] exposed chloroform solutions of reserpine to a given intensity of ultra-violet light for a given period of time and measured the resulting fluorescence. They showed that the relationship concentration/fluorescence was linear over a range of 0·1 to 1·0 μg. of reserpine per ml.

McMullen and his colleagues[137] have studied the physical and chemical properties of the *R. serpentina* alkaloids with the objects of identifying and estimating pure reserpine, and reserpine in pharmaceutical preparations containing either the pure base or a mixture of alkaloids. They noted that the m.pt. was a good criterion of purity of reserpine, that the infra-red spectrum was an excellent method of identification and that the ultra-violet spectrum could, suitably modified, be used as a quantitative method of analysis. Three assay methods are described, together with a chromatographic method for separating reserpine from other rauwolfia alkaloids. Banes[138] pointed out that the ultra-violet absorption spectrum was not satisfactory for use in an assay procedure unless the reserpine had been isolated. In the absence of recanescine, identification of trimethoxybenzoic acid amongst the products after saponification of an alkaloid mixture indicated the presence of reserpine. Reserpine may be determined by use of a suitable method of extraction, treatment with vanillin and determination of the absorption at 532 mμ. In strongly acidic solution reserpine and reserpic acid give a red colour with vanillin. Another method of assay is described by Booth[139]. This is suitable for elixirs and depends upon the formation of a chloroform-soluble reserpine-bromophenol blue complex. The absorbance of the solution at 402 mμ is read. Sakal and Merrill[140] describe a spectrophotometric method for reserpine. Horhammer and his colleagues[141,142] describe methods suitable for extraction and determination of *R. serpentina* alkaloids from the plant.

In a drug so widely used and with such interesting properties as reserpine, it is inevitable that methods should be sought for its estimation in tissue and in body fluids, etc. These methods are generally based upon the fluorimetric, colorimetric and spectrophotometric characteristics. Fluorimetric and spectrophotometric methods are being used by Gillis and Lewis[143] to attempt to follow the distribution of reserpine in the rat's brain.

Fluorimetric methods for its estimation in blood and urine are described by Kelly[144] and others[145,146]. Sheppard and his colleagues[147] have labelled reserpine with ^{14}C in the 4-methoxy group of the trimethoxybenzoic acid moiety and have administered this compound to adult male rats with the object of studying the distribution of ^{14}C. The significance of their results will be discussed later.

PHARMACOLOGY

Although many careful studies have been made of the pharmacology of reserpine, it would be hazardous to assign to it a precise site or mode of action. In addition, pharmacological studies have been made upon extracts and concentrates of the whole root of *R. serpentina* and upon the now numerous purified alkaloids. Reserpine is without doubt the most studied and most important of these, and will be dealt with in some detail. The continued use and importance of extracts and of the powdered whole root makes it impossible to overlook the other alkaloids and preparations.

In 1931, Sen and Bose[148] observed that the dried root of *R. serpentina* contained 1 per cent. of total alkaloids which caused a slight fall in blood pressure with stimulation of respiration and relaxation of smooth muscle in the cat. They also observed a lowering of the blood pressure and sedation when the powdered drug was given to patients. In the same year Roy[149] noted that large doses of *R. serpentina* caused sleep, dulling of sensations, diminished reflexes and, if the dose was lethal, death from respiratory failure, the heart continuing to beat for some time. The work of Chopra and his colleagues[1] which followed the earlier investigations was both interesting and important. The alkaloid investigated was a dull brown-yellow substance and was used in 1 per cent. solution. It was toxic to *Paramœcium caudatum* and to white mice, cats and guinea-pigs. In chloralosed cats increased intestinal tonus and peristalsis was observed. Five to 10 mg. slowed the rate and decreased the depth of respiration. There was a fall in blood pressure partly due to cardiac slowing and partly to vasodilatation. There was stimulation of the virgin or pregnant cat uterus *in situ*. Chopra also noted sedation, hypnosis, impairment of sensory perceptions and of reflexes in frogs, and drowsiness and quietning in other species. The alkaloid of Chopra and his colleagues[1] was thought to be ajmaline, and was shown in 1940 by Raymond-Hamet[150,151] to be a sympatholytic.

Other work on the rauwolfia alkaloids followed from Calcutta[8,152–154]. The "Bihar" variety of *R. serpentina* was shown to be more hypnotic than that from Dehra Dun[152]. The pharmacology of serpentine, serpentinine and ajmaline were also studied. These acted as convulsant poisons in rats. Alcoholic extracts of *R. serpentina* were depressant. The total alkaloid hydrochlorides had a hypnotic effect. Alcoholic extracts and the total alkaloids antagonised picrotoxin, however the actions of ajmaline, serpentine and serpentinine were summated with those of the analeptic[152]. The total alkaloids and alcoholic extract were hypotensive, serpentine being

thought the chief hypotensive agent while ajmaline and serpentinine caused hypertension[153,154].

Studies were also made of the comparative sedative and hypotensive potencies of extracts and the purified total alkaloids from Bengal, Bihar

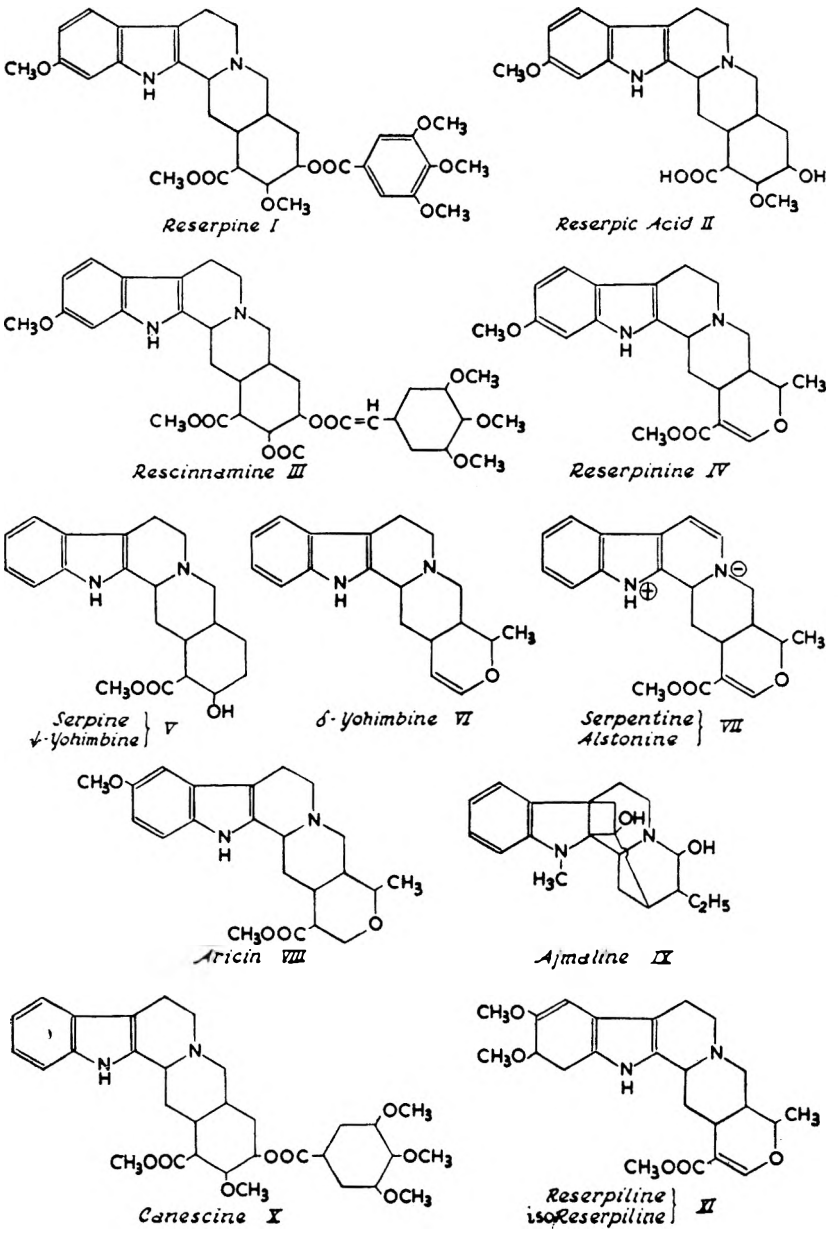

FIG. 1. The structure of some of the rauwolfia alkaloids.

and Dehra Dun varieties of *R. serpentina*[155–157]. An alcohol soluble, oil-free resin, free from alkaloids, was isolated. This caused sedation and hypnosis which were delayed in onset for 3 to 4 hours. The resin was synergistic with ether and chloralose and antagonistic to picrotoxin. Its site of action was thought to be the hypothalamus[155,156]. Gupta and his colleagues[158] investigated the hypnotic resin fraction further. It is interesting to note that Dymock[159] in 1891 isolated a crude resin fraction from *R. serpentina* (the root was shown to contain alkaloids at about the same time)[160,161]. Gupta and Kahali[157] noted that the *R. serpentina* alkaloids appeared to depress the vasomotor centre and also to act peripherally.

Before dealing with the pharmacology of reserpine, some of the later studies made upon extracts and concentrates and the whole root of *R. serpentina* will be considered.

Arnold[162] considered that *R. serpentina* acted as a sympatholytic agent with a marked central effect. Studies on dogs were made by Kramer and his colleagues[163] which showed that extracts lowered blood pressure and counteracted the effects of sympathomimetics.

A number of studies were made upon the actions of *R. serpentina* on vasomotor reflexes. Werner and his colleagues[164] elicited the carotid sinus reflex in cats by temporary occlusion of the common carotid arteries, and then, by artificially raising the intra-carotid pressure, produced a depressor response. Crude extracts of the roots of "Dehra Dun" *R. serpentina* reduced both pressor and depressor responses. Antagonism to the pressor effects of 100 m. units of pitressin was not shown, nor were cyanide-elicited reflexes antagonised. Werner and his colleagues[164–169] observed a non-competitive peripheral adrenergic blocking activity not primarily related to the vasomotor reflex blocking action. Work with the total purified alkaloids and with some of the purified bases, in which these were given intra-cisternally, pointed to a central action being responsible for the lowering of blood pressure and the blockade of vasomotor reflexes. Intracranial application of the *R. serpentina* alkaloids in animals decerebrated at the mid-collicular level did not influence the mean arterial blood pressure, nor did it abolish the pressor response to baroceptor stimulation. The extracts were assumed to interfere with the central control of vasomotor reflex activity and tone.

Vasodilatation in the kidney after injection of *R. serpentina* has also been observed in chloralosed cats. An alcoholic extract of *R. serpentina* (Dehra Dun) roots produced marked vasodilatation and blocked hæmorrhage-induced vasoconstriction in the renal vascular bed[169].

Other workers have investigated alkaloidal fractions from *R. serpentina*. Rubin and Burke[170] gave whole root powder to trained dogs for periods of 2 weeks or longer. The animals showed hypotension, bradycardia, sedation, miosis, relaxation of the nictitating membrane, ptosis, diarrhœa and tremor at dose levels of 80 and 320 mg. per kg. per day, the symptoms appearing within 48 hours. Bradycardia and miosis were blocked by atropine. At higher dose levels (320 mg. per kg. per day) there was

ultimately anorexia with emaciation and dehydration followed by death. Using a standardised alkaloidal extract of *R. serpentina* Gourzis and his colleagues[171] showed that hypotensive doses caused vasodepression and bradycardia. There was an increased pressor response to adrenaline and enhancement of the hypotensive and cardio-accelerator actions of isoprenaline. The carotid sinus reflex was reduced and the rise in blood pressure due to hypoxia was abolished. No change was seen in the responses to acetylcholine, histamine and efferent vagal stimulation but the hypertensive response which follows electrical stimulation of the afferent vagus was blocked.

Lim and his colleagues[173] have recently compared the responses to injections into the fourth cerebral ventricle and the carotid artery with intravenous administration of rauwolfia alkaloids, veratrum alkaloids and hydrallazine. Rauwolfia is considered to cause hypotension primarily by a central effect. Studies on the circulatory effects of *R. serpentina* have also been made by other workers[174,175]. Thuillier and Mouille[176] have shown that an extract of *R. serpentina* inhibits the action of acetylcholine on the isolated guinea-pig intestine. Kronheim and Koster[177] have noted a transient fall in adrenal ascorbic acid concentration after administration of either an alkaloid extract of *R. serpentina*, or of reserpine, serpentine, ajmaline or alkaloid F. A mixed alkaloid preparation of *R. serpentina* had no effect on the basal metabolic rate in rats[178].

Cronheim and Toekes[180] investigated the sedative actions of an alkaloidal extract of *R. serpentina*. Sedation was observed in dogs, cats, guinea-pigs and mice together with antagonism to the central excitant action of amphetamine in mice. Achelis and Kroneberg[181] compared the effects of the total alkaloids with those of reserpine on the dog's blood pressure. Hypotension due to the total alkaloids was not thought to result from the small amount of reserpine present since it was of speedy onset and long duration. Hypotension induced by reserpine on the other hand shows a slow onset coupled with bradycardia.

Gourzis[182,183] has shown that sedative doses of an alkaloidal extract of *R. serpentina*, unlike phenobarbitone, raise the emetic threshold to vomiting in veratrum-preparation treated dogs. A mouse ptosis bioassay of *R. serpentina* for reserpine-like activity has been described by Rubin and Burke[184].

Working mainly upon isolated tissue preparations, Banerjee and Lewis[185] showed that the alseroxylon fraction of the alkaloids of *R. serpentina* appeared to possess anticholinergic properties on skeletal, smooth and cardiac muscle. The effects were persistent and in some tissues there was a latent period before the maximum effect was shown. A delayed fall in body temperature in mice was observed.

The Pharmacology of Reserpine

In 1952 Muller, Schlittler and Bein[35] reported the isolation of reserpine —a weak base with a prolonged sedative action—from the oleoresin fraction of an extract of *R. serpentina*. The typical sedative effects of *R. serpentina* extracts were due after all to an alkaloid.

In 1953 Bein[186] showed that reserpine had hypnotic and hypotensive actions; it inhibited the pressor responses to electrical stimulation of the afferent vagi and sciatic nerves and also the carotid sinus pressor reflex. Pressor effects arising in response to electrical stimulation of the splanchnic nerves were not inhibited. The drug possessed no peripheral adrenergic- or ganglion-blocking activity. It potentiated the pressor action of adrenaline, noradrenaline and ephedrine. The hypotensive effect was not inhibited by atropinisation or vagotomy and there was no neuromuscular blocking activity. Peristalsis was stimulated. Bein considered that reserpine acted on the central nervous system. In the same year Bein and his colleagues[187,188] pointed out that reserpine not only caused sedation, hypnosis and hypotension, but was capable of inducing bradycardia and miosis, and that it also had laxative and body temperature-lowering effects. The Swiss investigators noted that reserpine was not a narcotic, and differed from the barbiturates and bromides. Reserpine-treated animals appeared tired and dazed, and slept peacefully but could always be wakened. It was felt that these long-lasting and delayed actions might be due to insolubility of the alkaloid or to its action being due to the need for prior formation of an active breakdown product. No direct peripheral vasodilator action was observed. Reserpine's action was not comparable with that of veratrine. A direct effect upon the sympathetic autonomic centres in the brain regulating the blood pressure was postulated and attention was drawn to the resemblance between the pharmacological effects of reserpine and the effects produced in 1947 by Hess[189] after stimulation of the mesencephalon. Hess's trophotropic-endophylactic system on stimulation gives rise to adynamia or sleep, miosis, relaxation of the nictitating membrane, hypotension, bradycardia, inhibition of respiration, hypothermia and defæcation. The analogy with reserpine is obvious.

During 1953 a number of contributions to the study of reserpine were made in America. Trapold and others[190,191] showed that reserpine in dogs caused respiratory depression, increased intestinal mobility, miosis, persistent hypotension and bradycardia, the miosis and bradycardia being abolished by atropine. One mg. per kg. of reserpine did not significantly depress cardiac stroke volume, output and index, as measured by Fick's method, until maximum hypotension was established. Reserpine produced vasodilatation which was the initial cause of its hypotensive effects. Maintenance of hypotension was due to reduced cardiac output, but cardiovascular changes seemed to be secondary to depression of the central nervous system, the drug acting on higher centres such as those of the hypothalamus. Plummer and his colleagues[192] confirmed the effects of reserpine in the dog, rat and rabbit. Cronheim and his colleagues[193] pointed out that reserpine was the most potent single *Rauwolfia serpentina* alkaloid so far examined, and noted that other active alkaloids present (ajmaline, ajmalicine, serpentine, serpentinine and alkaloid G) had not shown hypotensive activity in doses of 1 to 3 mg./kg./day in dogs.

Further contributions came from Swiss workers in 1954[194–199]. Again, hypotension and sedation of long duration were noted. Sedation was

(continued on p. 480.)

TABLE I THE RAUWOLFIA ALKALOIDS

R.s. = *Rauwolfia serpentina* Benth. *R.h.* = *R. hirsuta* Jacq. *R.sl.* = *R. sellowii.*
R.c. = *R. canescens* L. *R.m.* = *R. micrantha* Hook. *R.sm.* = *R. semperflorens* Schlecht.
R.t. = *R. tetraphylla* L. *R.v.* = *R. vomitoria* Afz. *R.o.* = *R. obscura.*
R.cf. = *R. caffra* (Sonder)

Alkaloid	Plant source	Identical with	Molecular formula	Physical constants			References
				m.pt. °C.	$[\alpha]_D$	$[\alpha]_{5461}$	
Ajmalicine	*R.s. R.h.*	Alkaloid F π-Tetrahydro serpentine δ-Yohimbine	$C_{21}H_{24}O_3N_2$	250–2	$[\alpha]_D^{23}$ – 48·5° (C_5H_5N)	—	105
Ajmaline	*R.s. R.h. R.sl.*	Rauwolfine	$C_{20}H_{26}O_2N_2$	205	$[\alpha]_D^{33}$ + 128° ($CHCl_3$)	—	29, 97, 87–97
Ajmalinine	*R.s.*	*Alkaloid C	$C_{20}H_{26}O_2N_2$	180–1	$[\alpha]_D^{33}$ – 97° ($CHCl_3$)	—	87–89
Alstonine	*R.v.* *R.o.*	—	$C_{21}H_{24}O_3N_2$	225–6	*R.v.* $[\alpha]_D^{20}$ – 115 ± 4° ($CHCl_3$) *R.o.* $[\alpha]_D^{22}$ – 109 ± 6° ($CHCl_3$)	—	127, 131
Aricin	*R.c. R.h.*		$C_{32}H_{28}O_4N_2$	190		—	129
Alkaloid A	*R.s.*	Reserpinine alkaloid C	$C_{32}H_{26}O_4N_2$	240–1		—	131
*Alkaloid C	*R.s.*	Ajmalinine		177	$[\alpha]_D$ = – 76·4°	—	87–89
Alkaloid C	*R.s.*	Reserpinine, alkaloid A	$C_{32}H_{26}O_4N_2$	240–1	$[\alpha]_D^{20}$ = – 127° (C_5H_5N)	—	56
Alkaloid F	*R.s.*	Ajmalicine π-Tetrahydro serpentine	$C_{21}H_{24}O_3N_2$	253–4	$[\alpha]_D^{20}$ – 37 ± 6° (CH_3OH)	—	105
Alkaloid 3078	*R.s.*	3-*epi*-α-Yohimbine 3-*epi*Rauwolscine	$C_{21}H_{26}O_3N_2$	125–8 181–3	$[\alpha]_D^{26}$ – 96° (C_5H_5N)	—	79
Base A	*R.m.*	—	—	264–6	—	—	132
Base B..	*R.m.*	—	—	247–8	—	—	132
Base C	*R.m.*	—	—	157–9	—	—	132

TABLE I—*continued*

Alkaloid	Plant source	Identical with	Molecular formula	Physical constants: m.pt. °C.	Physical constants: $[\alpha]_D$	Physical constants: $[\alpha]_{5461}$	References
Canescine	*R.c.*	11-Desmethoxy reserpine	$C_{32}H_{38}O_8N_2$	230–4	$[\alpha]_D - 163 \pm 2°$	—	60–64
Chalchupine A	*R.h.*	Rauwolscine	—	230–2	—	—	123
Chalchupine B	*R.h.*	—	—	—	—	—	123
Chandrine	*R.s.*	—	$C_{35}H_{38}O_8N_2$	230–1	—	—	115
Corynanthine	*R.s.*	Rauhimbine	$C_{21}H_{26}O_3N_2$	218–25	$[\alpha]_D^{20} - 82°$ (C_5H_5N)	—	34, 72–74, 75
Deserpidine	*R.c.*	Recanescine, 11-Desmethoxy reserpine	$C_{32}H_{37}O_8N_2$	228–32	$[\alpha]_D^{24\cdot5} + 137°$ ($CHCl_3$)	—	60–64
11-Desmethoxy reserpine	*R.c.*	Deserpidine Recanescine	—	228–32	$[\alpha]_D^{24\cdot5} + 137°$ [$CHCl_3$]	—	60–64
Heterophyllin	*R.h.*	Aricin	$C_{22}H_{26}O_4N_2$	190	—	—	129,119
*iso*Ajmaline	*R.s.*	—	$C_{20}H_{26}O_2N_2$	264–6	$[\alpha]_D^{35} + 72\cdot8°$ (C_2H_5OH)	—	90, 93, 96
*iso*Reserpiline	*R.c.*	—	$C_{33}H_{38}O_9N_2$	211–2	—	—	119
*iso*Reserpinine	*R.c.*	—	$C_{33}H_{40}O_4N_2$	225–6	—	—	119
*iso*Rauhimbine	*R.s.*	—	$C_{21}H_{26}O_3N_2$	225–7	$[\alpha]_D^{20} - 104°$ (C_5H_5N)	$[\alpha]_{5461}^{20} - 129°$ (C_5H_5N)	75, 76, 77
Methyl reserpate	*R.s.*	—	$C_{23}H_{30}O_5N_2$	244–5	$[\alpha]_D^{20} - 106°$ (C_5H_5N)		56
Narcotine	*R.h.*	—	$C_{20}H_{23}O_7N_2$	205–7	—	—	126
*neo*Ajmaline	*R.s.*	—	$C_{20}H_{26}O_2N_2$	205–7	—	—	90, 250
Papaverine	*R.s.*	—	$C_{20}H_{21}O_4N$	147	—	—	56
Pseudoyohimbine	*R.c.*	—	—	265–78	—	—	61
Raubasine	*R.s.*	Substance II δ-Yohimbine Alkaloid F Ajmalicine π-Tetrahydro-serpentine	—	—	—	—	—

TABLE I—*continued*

Alkaloid	Plant source	Identical with	Molecular formula	Physical constants			References
				m.pt. °C.	$[\alpha]_D$	$[\alpha]_{5461}$	
Raubasinine	*R.s.*	Substance I Alkaloid C Reserpinine Alkaloid A	—	—	—	—	—
Rauhimbine	*R.s.*	—	$C_{21}H_{26}O_3N_2$	218–25	$[\alpha]_D^{20}$ – 82° (C_5H_5N)	$[\alpha]_{5461}^{20}$ – 94° (C_5H_5N)	72, 34, 73, 74, 75
Raupine	*R.s.*	Sarpagine + CH_3OH	$C_{20}H_{26}O_3N_2$	325	$[\alpha]_D^{20}$ + 63° (CH_3COOH)	—	50, 66–71
Rauwolfine	*R.s. R.h.*	Ajmaline	$C_{20}H_{26}O_2N_2$	205	$[\alpha]_D^{33}$ + 128° ($CHCl_3$)	—	29, 97, 87–97
Rauwolfinine	*R.s.*	—	$C_{19}H_{24}O_2N_2$	235–6	$[\alpha]_D^{32}$ – 34·7°	—	98–102
Rauwolscine	*R.h. R.c.*	α-Yohimbine	$C_{21}H_{26}O_3N_2$	—	—	—	61, 107–110, 121, 122
Rauwolfine	*R.cf. R.s.*	Ajmaline	$C_{21}H_{26}O_2N_2$	160	—	—	274
Recanescine	*R.c.*	11-Desmethoxy reserpine Deserpidine	$C_{33}H_{37}O_8N_2$	—	—	—	60–64
Rescinnamine	*R.s.*	—	$C_{36}H_{42}O_9N_2$	238–9	$[\alpha]_D^{24}$ – 97° ($CHCl_3$)	—	57–59
Reserpiline	*R.s. R.c.*	—	$C_{33}H_{38}O_8N_2$	—	$[\alpha]_D^{24}$ – 40° (C_2H_5OH)	—	86, 119
Reserpine	*R.s. R.c. R.h.*	—	$C_{33}H_{40}O_9N_2$	262–6 277–8 (corr.)	$[\alpha]_D^{23}$ – 117–8° ($CHCl_3$)	—	35–46, 51–54
Reserpinine	*R.c. R.s.*	Alkaloid A	$C_{32}H_{36}O_4N_2$	240–1	$[\alpha]_D^{23}$ – 117° ($CHCl_3$)	—	80, 82, 83, 67, 84
Sarpagine (Serpagine) ..	*R.s. R.h.*	Raupine – CH_3OH	$C_{19}H_{22}O_3N_2$	320	$[\alpha]_D^{20}$ + 54° (C_5H_6N)	—	50, 66–69, 71
Serpenine	*R.s.*	—	—	315	—	—	114
Serpentine	*R.h.* *R.c. R.sl.* *R.s.*	—	$C_{21}H_{20}O_5N_2$	157–8	$[\alpha]_D^{40}$ + 188° (H_2O)	—	106–111, 80, 87–89, 103–105

TABLE I—*continued*

Alkaloid	Plant source	Identical with	Molecular formula	Physical constants: m.pt. °C.	Physical constants: $[\alpha]_D$	Physical constants: $[\alpha]_{5461}$	References
Serpentinine	*R.s.*	—	$C_{20}H_{20}O_3N_2$	263–5	—	—	87–89, 112, 113
Serpine	*R.s.*	—	$C_{21}H_{26}O_3N_2$	213	$[\alpha]_D^{20}$ + 70·1° (C_5H_5N)	—	65
Substance I	*R.s.*	Alkaloid A Reserpinine	$C_{33}H_{40}O_4N_2$	228	$[\alpha]_D^{20}$ − 123° ($CHCl_3$)	—	—
Substance II	*R.s.*	Raubasine Alkaloid F Ajmalicine π-Tetrahydro-serpentine δ-Yohimbine	$C_{21}H_{24}O_3N_2$	247–8	$[\alpha]_D^{20}$ − 61° ($CHCl_3$)	—	—
π-Tetrahydroserpentine ..	*R.h. R.s.*	Ajmalicine	$C_{21}H_{24}O_3N_2$	250–2	$[\alpha]_D^{23}$ − 48·5° (C_5H_5N)	—	105
Tetraphyllicine	*R.t.*	—	$C_{20}H_{26}N_2$	320–2	$[\alpha]_D^{27}$ + 21° (C_5H_5N)	—	—
Tetraphyllin	*R.t.*	—	$C_{22}H_{26}O_4N_2$	220–3	$[\alpha]_D^{28}$ − 73°($CHCl_3$) − 35°(C_5H_5N)		
Thebaine	*R.s.*	—	$C_{19}H_{21}O_3N$	195	$[\alpha]_D^{20}$ − 279° (C_5H_5N)	—	56
R.S.51	*R.s.*	—	—	—	—	—	116
3-*epi*-α-Yohimbine	*R.s.*	Alkaloid 3078 3-*epi*-Rauwolscine	$C_{21}H_{26}O_3N_2$	125–8 181–3	$[\alpha]_D^{26}$ − 96° (C_5H_5N)	—	79
α-Yohimbine	*R.c.*	Rauwolscine	—	—	—	—	—
δ-Yohimbine	*R.h. R.s.*	Alkaloid F Ajmalicine π-Tetrahydro sepentine	$C_{21}H_{24}O_3N_2$	258–9	$[\alpha]_D^{20}$ − 45° (C_5H_5N)	—	80
β-Yohimbine	*R.c.*	—	$C_{21}H_{26}O_3N$	246–9	—	—	—
Yohimbine	*R.c. R.h. R.s.*	—	$C_{21}H_{26}O_3N_2$	235–7	$[\alpha]_D^{20}$ + 105° (C_5H_5N)	—	34, 72, 78

different from that produced by phenobarbitone or sodium bromide as shown by EEG studies. Reserpine 10^{-6} was shown to inhibit barium-chloride constriction of the isolated blood vessels of the rabbit's leg, but not to cause dilatation by itself. There was dilatation of the coronary vessels of the mammalian heart which was inhibited by tripelennamine, phentolamine, barium chloride and acetylcholine but not by atropine. Reserpine was shown to inhibit the constriction caused by barium chloride and pitressin but it does not antagonise the actions of histamine, adrenaline or noradrenaline. Similar results were obtained by Gillis and Lewis[143]. Reserpine antagonised the characteristic central effects of caffeine, cocaine, morphine and hyoscine, but not convulsions due to leptazol, nicotine or picrotoxin. In experiments on isolated organs[197] reserpine was used in a solution with ascorbic acid or it was dissolved in a mixture of propylene glycol, ethanol and distilled water. These solubilising agents may modify the drug's effects, and need careful control experiments[197].

A number of contributions from America appeared in 1954. Schneider and Earl[200] compared the sedative effects induced by reserpine and barbiturates in monkeys and showed that these differed from one another in the influence upon the EEG and behaviour. Plummer and his colleagues,[201], discussing their own and other investigators' findings, pointed out that reserpine induced quiet and sedation in monkey, dog, cat, rabbit, guinea-pig, rat and mouse. Dogs and guinea-pigs were very susceptible, the monkey much less so. The latency and prolongation of sedation was seen, but there was no tolerance to the drug. Reserpine was primarily a tranquilising and inactivating agent allowing but not inducing sleep. Augmentation of secretory and motor activity of the dog gastrointestinal tract was seen, with increase in volume and hydrochloric acid content of the gastric juice. Oxyphenonium (an anticholinergic agent) eliminated the increased secretion.

Plummer and his colleagues[201] noted that the acute toxicity of reserpine is low—monkeys tolerate 400 mg. per kg. per day by mouth, and rats 1 g. per kg. per day by mouth. There is profound sedation but the animals always recover. Monkeys tolerate 4 mg. per kg. intravenously and rats 8 mg. per kg. intravenously, larger doses cannot be given owing to the insolubility of the drug. The dosage in dogs was limited because of the complication of diarrhœa. Rats, dogs and monkeys have been given as much as 4, 35 and 3 mg. per kg. per day respectively by mouth for as long as six months without sign of undue toxicity.

Reserpine hypotension was thought[191,202] to be initiated by central nervous system depression, possibly hypothalamic in location, which caused vascular relaxation followed by a reduction in cardiac output. In dogs a peripheral action was not demonstrable[191,202].

Barrett and his colleagues[203] studied the actions of reserpine on gastric motility in the guinea-pig, cat, dog and rabbit. No peripheral cholinergic activity was found using the isolated ileum and colon. Anticholinergic activity was shown on the acetylcholine-stimulated ileum of guinea-pig, dog and rabbit but little effect was seen when cat tissues were used. There

was slight antagonism to acetylcholine activity on the isolated rabbit colon but no influence on the colon of the dog or cat. In the dog, reserpine caused gastric secretion inhibited by oxyphenonium but not by tripelennamine. Esterification was shown to be necessary for reserpine-activity by Plummer and his colleagues[204]. Reserpic acid and trimethoxy-benzoic acid both lacked sedative and hypnotic properties[204]. Schneider[205] showed that reserpine antagonised the effects of morphine in white mice subjected to a beam of heat focussed on the tip of the tail but itself did not change the pain threshold. Rauwolfia extracts and reserpine were found by Jenney[206] to lower the electroshock seizure threshold significantly in mice.

Chen and his colleagues[207,208] showed that reserpine exerted a facilitation reaction on the central nervous system in mice and potentiated the convulsive effects of leptazol and caffeine but not of strychnine. There was reduction of the electrically-induced convulsive seizure threshold. The anticonvulsant actions of phenytoin and other central nervous system depressants were opposed by reserpine. Moyer and others (1954)[209] studied changes in cardiovascular and renal hæmodynamics after reserpine administration in dogs. Renal hæmodynamics and electrolyte secretion were not significantly altered and no consistent effect could be observed on cardiac output.

A number of recent publications deal with the actions of reserpine on the nervous system. Longo and Napolitano[212] examined the effects of reserpine administration on the rabbit EEG and on the response to hypothalamic stimulation in the rabbit. The first phase after drug administration was one of excitation with decreased rest periods, prolongation of active periods and occasional outbursts of spike forms. This phase was followed by a blockade of the response usually elicited by sensory perceptions, and the motor and emotional responses caused by electrical stimulation of the hypothalamus were much reduced or abolished. Reserpine was thought to act on the diencephalic nuclei by depressing the ascending and descending pathways of the reticular formation. Gangloff and Monnier[213] stimulated the cortex, diencephalon, rhinencephalon and reticular formation of the rabbit's brain, and recorded and studied spontaneous brain activity and the electrically induced discharge in the cortex, the dorsal, medial and lateral thalamic nuclei, the rhinencephalon and the reticular formation of the brain stem. Reserpine was shown to depress the diencephalo-cortical system, to raise the after discharge threshold caused by thalamic and cortical stimulation and to increase the electrical ground activity of the rhinencephalon. A sleep pattern after reserpine was not seen. An action at the thalamic-cortical level is suggested. Rinaldi and Himwich[214] gave doses of up to 0·5 mg. per kg. of reserpine to unanæsthetised, curarised rabbits and could observe no change in the electrical activity of the brain while 1·5 to 2·0 mg. per kg. gave rise to an alertness pattern. Reserpine was thought to stimulate the meso-diencephalic activating system. Chusid and his colleagues[215] have studied the behavioural changes after reserpine administration in epileptic monkeys, and found that these were similar to those observed

in similarly treated human subjects. (A motion picture showing the effects on the behaviour of normal monkeys after reserpine administration has been made by Earl, Dibble and Wolf[216]).

Bein[217] pointed out that reserpine did not exert its influence upon respiration by acting on vagal respiratory reflexes or upon the medullary inspiratory and expiratory centres nor were motor pathways and neuromuscular transmission affected. Schneider and his colleagues[218] showed that 5 mg. per kg. of reserpine facilitated the knee jerk in spinal cats and increased the height of the monosynaptic spike as measured in the ventral root of the spinal cord of the decerebrate cat in which the dorsal spinal root was electrically stimulated. Chen [219] has shown that reserpine facilitates electrically induced hind-leg extensor seizures in mice. It antagonised the actions of phenytoin in a competitive fashion. Reserpine was thought to facilitate seizure spread in the central nervous system. Schneider[220] tested the activity and the responsiveness of diencephalic sympathetic centres in cats, before and after reserpine (0·5 mg. per kg. by intravenous injection or 10 mg. per kg. by mouth). Intact cats showed excitement followed by quiet and some signs of discomfort; there was miosis, salivation, diarrhœa and squinting on exposure to light. After the drug, cats in "sham rage" following hypothalamic transection became quiet and unresponsive and made no efforts to climb or to walk. After intravenous injection of 0·1 mg. per kg. reserpine, a pressor response could still be obtained from direct electrical stimulation of the hypothalamus, but the carotid sinus pressor response was markedly antagonised. Reserpine was considered to have an indirect damping effect on the central nervous system (diencephalic-sympathetic centre), blocking or inhibiting afferent stimuli centrally. In another publication Schneider and Earl[221] have shown that there are quantitative differences between the reactions to reserpine in monkeys and in dogs, cats, rabbits and rats. Jungle-born macaca monkeys became calm and relaxed and even playful under the drug's influence, the EEG being unchanged. The drug in monkeys was thought to act mainly on the brain stem. Recently Brodie and his colleagues[222–225] have given additional confirmation to observations that that lysergic acid diethylamide produces mental disturbances by suppression of some of the central actions of 5-hydroxytryptamine (5-HT). They have noted[222] that 5-HT acts like chlorpromazine and reserpine, in markedly potentiating the hypnotic action of hexobarbitone in mice by increasing the sensitivity of the central nervous system to the hypnotic drug. 5-HT and reserpine were shown to exert a common central potentiating action on hexobarbitone and ethanol in mice[223] which was antagonised by lysergic acid diethylamide. It was suggested that certain actions of reserpine might be due to 5-HT release as indicated by a marked increase in urinary excretion of 5-hydroxyindoleacetic acid in dogs treated with reserpine[223]. Reserpine also released 5-HT from the intestine[224] of rabbits receiving 5 mg. per kg. by intraperitoneal injection. The animals were later killed and the 5-HT content of a portion of the small intestine estimated, using the method of Udenfriend and others[226]. The intestinal 5-HT content fell

to 15 to 20 per cent. of the average normal amount, returning to normal values after 5 days.

It is the general view that the site of action of reserpine is in the brain. Evidence for a peripheral site of action has, however, been given by McQueen, Doyle and Smirk[210]. In the innervated but otherwise isolated hind limb of the rabbit[211] perfused with a blood-dextran medium at a constant rate, there was a rise in limb perfusion pressure (not a fall as might have been expected) after injection of reserpine into the systemic circulation. Direct injection of reserpine into the perfused limb caused a reduction in vasomotor tone. Reserpine had a depressant effect on the actions of vasopressor substances when these were injected into the rat hindquarters preparation and on the response of the isolated rat diaphragm to nervous stimuli. All these reserpine effects were prolonged, suggesting binding of the drug by the musculature. Somewhat similar conclusions have been drawn by Gillis and Lewis[143].

McQueen, Doyle and Smirk[227] have observed cutaneous vasodilatation in the ear vessels of rabbits. This was mainly mediated *via* the nervous system, since, when tested in rabbits which had undergone cervical sympathectomy on the left side together with removal of the superior cervical and stellate ganglia, there was usually dilatation after reserpine in the innervated ear, but no change or constriction in the denervated ear. Gillis and Lewis[143], in studies upon isolated organs, have shown that reserpine (0·1 μg. per ml.) in the isolated perfused kitten's heart caused an increase in outflow followed by a decrease in heart rate and amplitude. The effects were long lasting but usually reversible. One μg. per ml. of reserpine reduced vasoconstriction caused by 100 μg. per ml. barium chloride. Reserpine 1·0 or 0·1 μg. per ml. had little effect upon the response of the heart to adrenaline, noradrenaline or 5-HT. The alkaloid had no direct action upon the isolated guinea-pig ileum, but reduced the responses to acetylcholine, barium chloride, histamine and 5-HT. A delayed maximum inhibitory effect was noted but the responses were not quantitative. Antagonism to the effects of acetylcholine, 5-HT and potassium on the rat's uterus was shown by reserpine, once again with a delayed maximal effect. In the isolated perfused rat hind quarters no direct effect upon outflow was observed with reserpine but the constrictor response to adrenaline was inhibited. Neuromuscular transmission in the isolated sartorius muscle-ischiad nerve preparation of the frog was not modified by the alkaloid, and there was no direct effect upon the isolated frog's rectus abdominis muscle nor upon acetylcholine- or potassium-induced contractions in this tissue.* Reserpine is considered to inhibit some fundamental biochemical contractile process within or upon the surface of the muscle cells.

In the metabolic studies on rats, Sheppard and his colleagues[147] showed a concentration of the drug in fatty tissues which may account for its prolonged action. There is no specific accumulation of reserpine in the

* Since this article went to press a stimulant action on the frog rectus abdominis muscle has been shown [143, 143a].

brain and in view of the somewhat conflicting evidence on the precise site of action this is a significant finding.

A number of other studies have so far been made upon various aspects of the pharmacology of reserpine. For example[228], low doses cause vomiting in pigeons with a graded dose-response relationship within the range of 0·04 to 0·10 mg. per kg. body weight, the use of this response as a possible assay method has been suggested. Barrett and his colleagues[229] have shown that reserpine causes gastric secretion in dogs by stimulation of parasympathetic ganglia. Meier and his colleagues[230] have analysed the actions of a number of hypotensive substances including reserpine. Using anæsthetised rabbits the hypotensive agents were classified on the basis of (*a*) degree of maximal fall of blood pressure, (*b*) duration of the total fall of blood pressure, (*c*) duration of the maximal fall of blood pressure, and (*d*) the action integral which is the product of the intensity and duration of effect. The properties of reserpine were linked with those of hydrallazine since with both drugs the duration of the hypotension was approximately related to the dose.

So far no mention has been made of any influence of reserpine upon the endocrine glands. This subject was investigated in 1954 by Gaunt and his colleagues[231], who found that there was a reduction in fluid intake and urine volume when guinea-pigs were given 3 μg. per 100 g. per day and rats 10 μg. per 100 g. per day. On water-loaded rats, reserpine had an antidiuretic effect but sodium excretion was little changed. No changes in male gonadial function were noticed in the rat unless reserpine was given in doses causing loss of weight and inanition when there was a lowered secretion of androgens. In female rats reserpine (5 or 10 μg. per 100 g. per day) disturbed the normal vaginal œstrous cycle. Reserpine at the lower of these doses reduced the conception rate, number of live births and number of young per litter in rats. Mercier-Parot and Tuchmann-Duplessis[233] have recently shown that 50 μg. per day of reserpine cause disappearance of œstrus in the rat. No evidence was obtained for suppression of ACTH release. Reserpine caused adrenal hypertrophy, and thymic atrophy at a dose of 10 μg. per 100 g. per day. After adrenalectomy in the rat, reserpine reduced survival time suggesting that it increased the need for the cortical hormone and was an adrenal stimulant. Reserpine reduced the blood pressure in DOCA-salt—or cortisone induced hypertension. Sturtevant[232] in 1953 had also produced hypotension in rats with DOCA-salt induced hypertension by the addition of 5 per cent. of *R. serpentina* roots (4 g. of root per day) to their diet.

A thyroxine-induced increase in oxygen consumption was antagonised by reserpine in rats, although it had no influence upon that produced by 2:4-dinitrophenol[234]. Kuschke and Frantz[235] have shown that reserpine causes hyperglycæmia in rabbits without change in sugar tolerance. The hyperglycæmic and sedative effects of reserpine were inhibited by hydergine but bilateral splanchnicotomy did not prevent the hyperglycæmia. Turner and Carl[236] found that reserpine dilates melanophores and lipophores of fish causing display of colour. This effect was inhibited by cocaine and by ephedrine and partly by ergotamine,

but not by phenylephrine, pitocin or thyroid while adrenaline caused blanching.

Before discussing the pharmacology of the other rauwolfia alkaloids it is necessary to refer to the influence of reserpine upon normal human subjects. A very considerable amount of attention has been paid to the influence of reserpine upon hypertensive, psychiatric and other patients and this contrasts with the lack of data available about the normal individual. The study of the effects of reserpine upon the normal ambulant human subject is difficult, since small doses produce little demonstrable effect whilst larger doses of the order of 20 to 50 μg. per kg. may cause unpleasant side effects, including extreme lethargy, drowsiness, shivering, nasal congestion, conjunctival suffusion, weakness and even orthostatic fainting. These effects appear six to twenty-four hours after a single oral or intravenous dose and may persist for several days. There is a slight lowering of the blood pressure, particularly when standing and shortly after meals. Moderate bradycardia is usually present but miosis is not consistently observed. When miosis is present it appears in about twenty-four hours and may persist for twenty-four to forty-eight hours. Plethysmographic studies have shown an increase in hand blood flow which is not necessarily associated with a change in blood pressure[237].

The Pharmacology of Other Rauwolfia Alkaloids

Rescinnamine. The pharmacological properties of this alkaloid are similar to those of reserpine[57,238,239].

Reserpinine. According to Schlittler and his colleagues[82] reserpinine is not sedative or hypotensive. Kroneberg[240] showed that it was neither an adrenergic blocking agent nor spasmolytic, that it potentiated the effects of adrenaline and noradrenaline and caused a fall in blood pressure, but was quantitatively weaker than reserpine.

Serpine. Chatterjee and Bose[65] have described this as a hypotensive drug with a short-lived action. It is an adrenergic blocking agent rather like yohimbine[65]. Dasgupta and Werner[241] have also studied the properties of serpine. In rats, 5 mg. per kg. by intravenous injection cause quietening the LD50 being approximately 10 mg. per kg. Six mg. per kg. produced respiratory distress in monkeys and 7 mg. per kg. inco-ordination and occasional convulsions in rabbits. Serpine induced hypotension in cats and monkeys with suppression of the carotid sinus reflexes. Although an adrenergic blocking agent it did not cause adrenaline reversal. There was some antagonism to the pressor response seen after electrical stimulation of the splanchnic nerves. Serpine caused peripheral vasodilatation in perfused limbs of cats and monkeys, was a weak ganglion blocking agent and stimulated intestinal motility. No specific blockade of the pressor response to electrical stimulation of the medulla and hypothalamus was seen.

Sarpagine-Raupine. Raupine (and raubasin) have been shown by Kroneberg and Achelis[242] to exert adrenergic blocking actions on the blood pressure and nictitating membrane of the cat.

Ajmaline. Ajmaline was shown to have an inhibitory action on frog nerve fibres and ultimately prevented passage of the impulse[243]. Chopra and Chakravarti[245] showed that ajmaline raised the blood pressure of decerebrate cats but caused hypotension in spinal animals. Gupta[246] also observed that ajmaline caused hypertension but that in animals with experimental hypertension it caused a fall in blood pressure, findings confirmed by Chopra and his colleagues[247]. Dasgupta and Werner[248] showed that ajmaline caused a marked fall in blood pressure and inhibition of vasomotor reflexes after intracisternal injection into monkeys. There was reduction in the pressor response to sciatic nerve stimulation in chloralosed and decerebrate cats. Hypertension due to stimulation of the hypothalamus was not suppressed nor was "sham rage" abolished. One to 3 mg. per kg. per day did not cause hypotension in dogs[193].

Serpentine. This alkaloid produces hypotension and inhibits intestinal movements[244,249]. It is more toxic to mice than ajmaline or serpentinine and causes a rise in blood pressure in decerebrate cats, but a fall in spinal cats[245]. It lowers the blood pressure in experimental hypertension[246,247]. It is a more active substance than ajmaline[174]. Its hypotensive activity is probably due to acute vasodilatation[170].

neo*Ajmaline and* iso*ajmaline*[250]. These alkaloids stimulate and then depress the central nervous system in frogs, cats and guinea-pigs. The frog heart *in situ* and isolated rabbit and guinea-pig hearts are depressed. There is peripheral vasodilatation in perfused cat and frog preparations with hypotension in intact, decerebrate and spinal cats. In experimental hypertension *neo*ajmaline and *iso*ajmaline reduced the blood pressure. Respiratory depression is produced by large doses. Death is caused by respiratory failure.

Serpentinine. Serpentinine diminishes the renal vasoconstrictor activity of adrenaline but does not alter adrenaline hypertension[251]. It has hypotensive activity[252].

Ajmalicine. Ajmalicine was shown not to have hypotensive activity in dogs by Cronheim, Stipp and Brown[193].

Ajmalinine. Ajmalinine causes hypotension with renal vasodilatation. It is a sympatholytic[253,254].

*R.S.*51[116]. This lowers blood pressure in normo-tensive and hypertensive animals. It is a peripheral vasodilator, an adrenergic blocking agent and a histamine liberator. Hypotension is not due to an action on brain centres, sympathetic ganglia, the myocardium or the splanchnic blood vessels.

Rauwolfinine[98]. This alkaloid has hypertensive properties.

Rauwolfine. This alkaloid may be identical with ajmaline but will be given separate, brief consideration. Rauwolfine was found to increase myocardial resistance to fibrillation in dogs and cats. De Boer[256,257] showed that it decreased the rate of the frog, cat and rabbit heart, and caused artificial changes in rhythm with depression of intraventricular conduction. It did not prevent auricular or ventricular fibrillation in cats or rabbits. Studies of the effects of rauwolfine on frog and mammalian hearts have also been made by Hartog[258].

THE ALKALOIDS OF OTHER *Rauwolfia* SPECIES

R. canescens[259]

Rauwolscine. This is a cardiovascular depressant with hypnotic activity and a relatively high toxicity[260]. It causes hypotension, blocks the effects of adrenaline on the cat blood pressure and antagonises the action of adrenaline on the mammalian and amphibian heart[261,262]. Mukherjee and Sen[263–265], have observed a reversible depressant effect upon the toad heart. There was an increase in coronary flow in the perfused guinea-pig and rabbit hearts when the perfusing fluid contained 10 μg. per ml. of rauwolscine; a solution of 1 mg. per ml. caused irreversible standstill. Rauwolscine caused hypotension in the normal cat with inhibition of cardiac and plain muscle, blocking of the vagus and antagonism to the pressor effects of adrenaline. Werner and his colleagues[266,267] gave rauwolscine by intra-cisternal injection to monkeys and showed that it caused hypotension with inhibition of the carotid sinus pressor reflexes. It is a vasodilator and has adrenergic blocking activity at high dose levels. Rauwolscine, when injected into the trunk of an animal, causes vasoconstriction of the innervated but otherwise isolated hind limb. In cats and monkeys there is a marked fall in peripheral resistance without alteration of cardiac output.

Canescine, Recanescine, Deserpidine[268–271]. These appear to be identical substances with properties very similar to those of reserpine and rescinnamine. The CH_3O group at the 11-position in reserpine does not seem to be essential for its pharmacological actions.

R. hirsuta (*R. heterophylla*)[131,272]

The *R. hirsuta* alkaloids are pharmacologically active and are described as "rautensin" by Mezey and Uribe[131,272]. This substance is probably a mixture of alkaloids. It causes in dogs and cats apathy, adynamia, hypotension, bradycardia, ECG changes and shortness of breath. Death is caused by respiratory failure.

R. sellowii[134]

Aqueous extracts are toxic to mice. The total alkaloids cause hypotension.

R. vomitoria[273]

Crude aqueous extracts prepared from the roots have been used. In the bilaterally vagotomised dog, these caused inhibition and reversal of the pressor response to adrenaline with less inhibition of the tone and movement of intestinal smooth muscle. The extracts themselves caused a rise in blood pressure and an increase in the tone and amplitude of movements of the small intestine.

SYNTHETIC AND NATURALLY OCCURRING COMPOUNDS RELATED TO THE RAUWOLFIA ALKALOIDS

Rauwolfine[274]. This is obtained from *Tabernæmontana ventricosa* Hocht (Apocynaceæ) which grows in South Africa. It has sympatholytic properties.

Esters of Yohimbine and Corynanthine. Le Hir[275] has studied the 3:4:5-trimethoxybenzoic acid esters of yohimbine and corynanthine and compared their properties with those of reserpine. In mice these esters were found to be less toxic than reserpine. They did not augment the hypnotic effects of amylobarbitone. The yohimbine derivative caused hypotension and adrenaline reversal, with a diminished response to carotid sinus occulsion. The corynanthine ester had somewhat similar properties. Huebner and his colleagues[276] have prepared the following yohimbé alkaloid derivatives and shown that they lack the characteristic sedative actions of reserpine:—

o-benzoyl, -anisoyl, -veratroyl and 3:4:5-trimethoxybenzoylyohimbine,
o-3:4:5-trimethoxybenzoylcorynanthine,
o-3:4:5-trimethoxybenzoyl-α-yohimbine,
o-acetylyohimbine, yohimbic acid amide and hydrazide, *N*-methyl yohimbine.

Discussion

Only the pharmacological properties and mode of action of reserpine will be considered. Reserpine is an intriguing drug. Its interest lies very much in its apparent lack of specificity and selectivity of action. If appropriate steps are taken, then a response to reserpine administration can be obtained from almost all systems, tissues and organs. This response is rarely a dramatic one, compared with the effects of say adrenaline or histamine, and it usually takes a little time to appear and then it is relatively persistent. In some cases, changes are not evident for up to one year as has been described in the onset of Parkinsonism. Opinion on the site of action of reserpine favours the brain and central nervous system as being the primary areas affected, and the rest of the organism being secondarily influenced. This may well be so, but there is good evidence for the direct action of the drug on other tissues. It is possible that the best explanation of the mode-of-action of reserpine is that it exerts a depressant effect upon some ubiquitous biochemical system in the cells, which is concerned with respiration or with some other aspect of the cell's economy. This might explain the delayed action since the effects of depression of metabolite synthesis are not always seen at once. In addition it is conceivable that this same, admittedly hypothetical, effect would, when exerted in nervous tissue, produce more profound and readily demonstrable effects than when exerted elsewhere.

It is interesting to speculate upon the part played by 5-HT in the central nervous actions of reserpine. The possibility that this substance is involved in mental activity has been discussed by Gaddum[277] and by Woolley and Shaw[278]. 5-HT has been found in the brain, and its distribution is both interesting and suggestive[279,280]. It has been postulated that the central effects of reserpine are mediated by liberation of 5-HT[222–226] since both depress mice and potentiate the actions of hexobarbitone in these animals. These potentiatory effects are antagonised by lysergic acid diethylamide. The antagonism between reserpine and lysergic acid diethylamide may point to an action of the former being mediated by

5-HT. Recently, however, Cerletti and Rothlin[281] have investigated the actions of 2-brom-(+)-lysergic acid (BOL 148) and have shown that this powerful peripheral antagonist to 5-HT does not produce the mental aberrations characteristic of lysergic acid diethylamide. This finding at once throws open the question about the part played by 5-HT in the brain. It would, however, be interesting to know how the distribution of 5-HT (and noradrenaline) in the brain compares with that of reserpine, in a well sedated animal.

Bein[217] in a recent paper has discussed the mode of action of reserpine with particular reference to its influence upon central reflex mechanisms. He has considered the behaviour of central autonomic efferent impulses after afferent nerve stimulation. Inhibition of the carotid sinus pressor reflex in the cat by reserpine has been shown to depend upon a connection being maintained between the medulla and the mid-brain. Bein has also discussed the influence of reserpine upon respiration. Spontaneous respiration is influenced by reserpine in a characteristic manner which is in some ways analogous to its action upon the carotid sinus pressor reflex. It is felt that reserpine may activate inhibiting centres in the brain which probably lie rostral to the caudal colliculi. It acts upon central regulatory mechanisms which integrate both somatic and autonomic functions[217].

Tripod and Meier[198] account for the direct and antagonistic actions of reserpine upon the isolated blood vessels of the rabbit hind quarters and upon the coronary vessels of the isolated mammalian heart by postulating the possible existence of "master receptors", which are different from those which subserve adrenergic, cholinergic and histaminergic functions. These determine the order of priority of the primary effects of drug upon cell, and also its antagonisms.

In conclusion it appears that we are still some way from knowing exactly at what point in the metabolism of the cell reserpine acts, nor do we know precisely its mode of action. It seems not improbable that the biochemist and not the pharmacologist will provide the final answer. It is possible that the action is upon the contractile substance of muscle cells, and it may be exerted upon some step in the synthetic, degradative or oxidative processes which go on in all cells. This may not, of course, explain the mental effects of the drug or its influence upon nervous tissues.

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RESEARCH PAPERS

CHARACTERISTICS OF CARBAMATE FORMATION BY ALKYLOXY-1-PHENYLETHYLAMINES IN RELATION TO DEVELOPMENT OF ANALGESIC ACTIVITY WITHIN THE SERIES

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STRONGLY basic primary amines absorb carbon dioxide to give bicarbonates and carbamates[1]. Addition of bicarbonate or carbon dioxide to solutions of primary arylalkylamines caused marked reduction in recoverable amine when the solution was basified and extracted with a solvent[2]. Apparently the retained base was held in solution as the carbamate ion. Previously it had been observed that the amount of carbon dioxide absorbed by 1-(*p-cyclo*hexyloxyphenyl)ethylamine base, unlike the corresponding *iso*propyl ether, was appreciably less than the theoretical requirement for formation of a bicarbonate or carbamate. This implied the formation of a distinct molecular species by the *cyclo*hexyl ether. These two ethers had distinct pharmacological properties[3] not accounted for by differences in their physical properties, e.g., they had almost identical pKa numbers, 8·67 and 8·75 at 37° C., and a similar gross distribution pattern in rat tissues[4]. They seemed to be metabolised to the same extent. Since carbon dioxide and bicarbonate are present in biological fluids, the properties of the carbamates formed by these two bases in solution have been investigated in detail while seeking differences between them sufficient to explain the pharmacological distinction.

EXPERIMENTAL

Carbamate formation by pure bases. Freshly distilled bases, from pure hydrochlorides, solidified and attained a constant weight increase after several days exposure to carbon dioxide in closed vessels. The amines chosen were non-volatile at room temperature and pressure. The products regenerated the bases when distilled under reduced pressure. Increases in weight were expressed as the number of moles of base absorbing one mole of carbon dioxide or its equivalent (bicarbonate formation requires absorption of water). Figure 1 shows that three types of amine were distinguishable by their weight increases within the series; those that combined in ratios greater than, equal to, or less than two moles of amine to one mole equivalent of carbon dioxide. This criterion also roughly classified them into those that had marked, doubtful and no analgesic activity respectively as assessed by the method of D'Amour and Smith[5] in rats. The ratio is less than two when bicarbonate is produced, and is equal to two for the substituted ammonium carbamate. A ratio greater

than two was explicable only by postulating partial enolisation of the carbamate to give a proportion of the species $RN = C(ORNH_3)_2$.

Attempts to analyse the products for total and non-carbamate amine were not wholly successful due to decomposition in aqueous-ethanolic solution. The product from 1-(*p-cyclo*hexyloxyphenyl)ethylamine gave

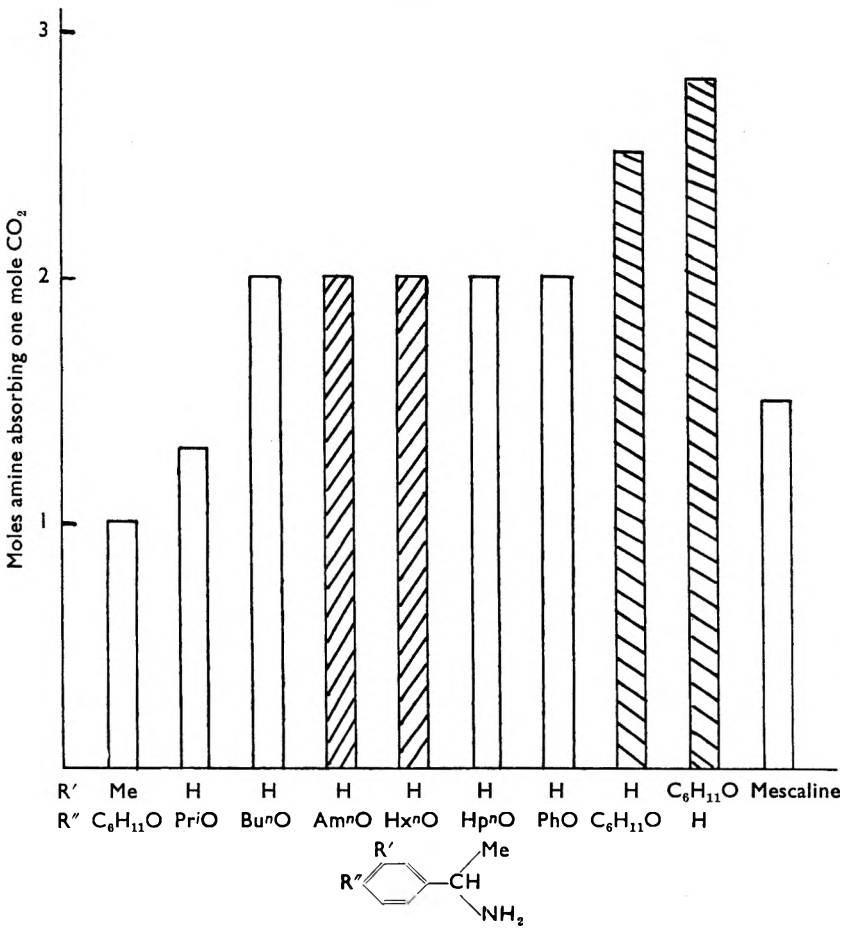

FIG. 1. Absorption of carbon dioxide by primary arylalkylamines. L–R upward hatching = slight analgesic activity; downward hatching = marked analgesic activity; □ = inactive.

92·5 per cent. total base after basifying a solution in acid ethanol, but only 64 per cent. after careful solution in dilute ethanolic sodium hydroxide.

Carbamate formation in solution. Little information exists on carbamate formation between amines and bicarbonate in solution. Reaction of free amino groups in hæmoglobin with carbon dioxide has been studied to explain certain features of carbon dioxide uptake and release by blood[6]. Formation of histamine carbamate has been investigated in relation to its action on isolated intestine[7].

Experiments were confined to the morphine-like 1-(*p-cyclo*hexyloxyphenyl)ethylamine and the corresponding but inactive *iso*propyl ether. Since the analgesic action followed tissue but not plasma concentration[4] there was little basis for using plasma as solvent and initial experiments have been made using simple aqueous solutions.

Solutions of the amine hydrochlorides (10^{-5} to 10^{-2}M) in sodium bicarbonate (0 to 0·6M) or phosphate buffer under different partial pressures of carbon dioxide were incubated at 37° C. to constant pH. Excess of 5N sodium hydroxide was added to retain carbamate and the liberated base was extracted with ether for estimation by the salicylaldehyde method[2]. In a few experiments the aqueous residues were acidified and carbon dioxide removed in a current of nitrogen. After basifying the liberated amine was extracted with ether. Total recovery was almost quantitative.

Results

In phosphate buffer under carbon dioxide without added sodium bicarbonate, the recovery of the *cyclo*hexyl ether decreased, that of the *iso*propyl ether increased, with falling pH. To explain this behaviour many hypothetical equilibria were analysed but only the following sequence could give a quantitative basis to this result and those with added bicarbonate.

$$RN^+H_3 + HCO_3^- \overset{k_1}{\rightleftharpoons} RNH_3HCO_3 \quad .. \quad .. \quad .. \quad .. \quad I$$

$$RN^+H_3 + HCO_3^- \overset{k_2}{\rightleftharpoons} RNHCOOH + H_2O \quad .. \quad .. \quad .. \quad II$$

$$\left.\begin{matrix} RN^+H_3 \\ RNHCOOH \end{matrix}\right\} \begin{matrix} + CO_2 \\ - H^+ \end{matrix} \overset{k_3}{\rightleftharpoons} RN^+H{=}C(OH)_2 \text{ or } RN^+H_2COOH \quad .. \quad III$$

$$RN^+H{=}C(OH)_2 - HCO_3^- \overset{k_4}{\rightleftharpoons} RNH{=}C(OH)_2{\cdot}HCO_3 \quad .. \quad .. \quad .. \quad IV$$

$$RNHCOOH \rightleftharpoons RNHCOO^- + H^+ \quad .. \quad .. \quad .. \quad V$$

Measurement of carbon dioxide evolution after tipping the solid hydrochlorides into equilibrated sodium bicarbonate solutions under carbon dioxide (100 per cent.) at 37° C. in closed constant pressure systems, gave the pKa of the carbamic acid; 8·38 and 8·75 respectively for the *cyclo*hexyl and *iso*propyl ethers. Within the range of pH used, the contribution of hydrogen ion from the substituted ammonium ion was negligible. At pH $< 7{\cdot}5$ it was therefore permissible to neglect reaction V and the sequence I to IV was summarised in the hyperbolic function VI

$$r = \frac{1}{A}\left(\frac{[H^+] + B}{C[H^+] + D}\right) \quad .. \quad .. \quad .. \quad VI$$

where r is the ratio of recovered to retained amine, A = $[CO_2]$; $B = k_1k_6[CO_2]$; $C = k_3(1 + k_4[HCO_3^-])$; $D = k_2k_6$; k_6 = the first dissociation constant of carbonic acid.

Calculation of the constants. The values of the equilibrium constants k_1 — k_4 given in Table I were calculated from data derived from the hyperbola relating r to $1/[H^+]$. Due to the enormous elongation of the abscissæ relative to r, this relation was approximately linear over the range of pH used and could be equated to the tangent at an intermediate point within the range (Fig. 2). There was good agreement between found and calculated values for recovered amines under a variety of conditions (Table II). The slope of the tangent to this hyperbola is negative or positive depending on whether B is less or greater than ADr when all constants are positive, as they must be in this example. This relation is solely dependent on the value of k_1 and k_2 and is independent of $[H^+]$. The apparent stabilising effect of hydrogen ion on the carbamate of the *cyclo*hexyl ether shown by the positive slope in Figure 2 is due solely to using $[H^+]$ as a convenient measure of the relative amounts of bicarbonate and carbon dioxide in solution. A less apparent difference in behaviour between the two carbamic acids was reflected in the nature of constant C. For the *iso*propyl ether this could be equated to k_3 under all conditions, i.e., $k_4 = 0$, whereas for the *cyclo*hexyl ether, C contained a $[HCO_3^-]$ dependent term, and a definite value could be ascribed to k_4. It was inferred that the carbamic acid derived from the *cyclo*hexyl ether formed a product capable of association with bicarbonate ion, as was indicated by the break in the curve between pH 6 and 7 (Fig. 2) for recovery of *cyclo*hexyl ether from phosphate buffer under carbon dioxide but without added bicarbonate. Presumably carbon dioxide formed little bicarbonate in phosphate buffer below pH 6. The evidence of Figure 1 where a combining ratio of about 2·5 to 1 implied partial formation of the species $RN = C(ORNH_3)_2$ suggested that the species $RN^+H = C(OH)_2$ formed part of the carbamic acid derived from the *cyclo*hexyl ether in solution (reaction III). The carbamic acid from the *iso*propyl ether, also associated with hydrogen ion, could

TABLE I

VALUES OF EQUILIBRIUM CONSTANTS FOR REACTIONS OF THE *cyclo*HEXYL AND *iso*PROPYL ETHERS OF 1-(*p*-HYDROXYPHENYL)-ETHYLAMINE WITH BICARBONATE AND CARBON DIOXIDE

	*cyclo*Hexyl ether	*iso*Propyl ether
k_1	$10^{2\cdot755}$	$10^{0\cdot599}$
k_2	$10^{2\cdot160}$	$10^{0\cdot839}$
k_3	$10^{2\cdot154}$	$10^{1\cdot470}$
k_4	$10^{2\cdot210}$	0

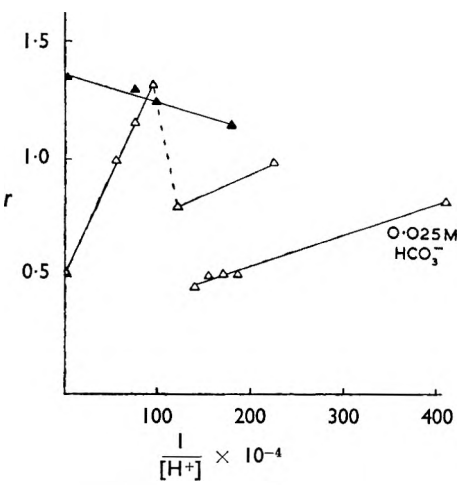

FIG. 2. Relation between ratio of recovered and retained amine, *r*, to $1/[H^+]$ in buffered solutions. △ = 1-(*p-cyclo*hexyloxyphenyl)ethylamine; ▲ = 1-(*p-iso*propyloxyphenyl)ethylamine. Phosphate buffer (except where bicarbonate is indicated) under 100 per cent. carbon dioxide.

TABLE II

RECOVERY OF ETHERS OF 1-(*p*-HYDROXYPHENYL)ETHYLAMINE FROM BICARBONATE—CARBON DIOXIDE SOLUTIONS IN EQUILIBRIUM AT 37° C.

Molar concentrations		Carbon dioxide per cent.	pH	Recovery per cent	
Amine	Bicarbonate			Found*	Calc.
		*cyclo*Hexyl ether			
10^{-4}	0	5	6·90	78	82
10^{-4}	0·013	5	7·30	81	77
10^{-4}	0·063	5	8·00	74	77
10^{-4}	0·125	5	8·31	73	77
8×10^{-3}	0·059	50	6·97	49	55
5×10^{-5}	0	100	5·98	60	58
2×10^{-4}	0·025	100	6·18	36	36
10^{-4}	0·025	0	8·66	89	81
		*iso*Propyl ether			
10^{-4}	0	50	4·41†	74	73
10^{-4}	0	50	5·98†	78	72
10^{-4}	0	50	6·19†	74	71
10^{-3}	0·100	5	7·82	81	79
10^{-3}	0·375	50	7·57	43	49
10^{-3}	0·147	50	7·40	57	53
10^{-3}	0·058	50	7·00	61	61

* Calculated to 5 per cent. (*cyclo*hexyl ether) or 10 per cent. (*iso*propyl ether) experimental loss.
† In phosphate buffer.

have the structure RN^+H_2COOH. A partial analogy may exist in the relation between urea and urethane, the former probably existing partly as $N^+H_2 = C\begin{matrix}O^- \\ \diagdown \\ \diagup \\ NH_2\end{matrix}$ and capable of salt formation with acids. Urethane behaves as an amide and does not form salts with acids.

DISCUSSION

There seems to be little doubt that the carbamic acids derived from the two amines have a distinct difference, best interpreted on the available evidence by the postulate that the *cyclo*hexyl ether gives a carbamic acid that can exist as its enolic form. This species seems to bear more resemblance to the "ortho" form of a ketone than to a carboxylic acid. Whether such a difference is related to the pharmacological distinction between the two can only be conjectural at this stage but the following observations support the conclusion. Among a long series of 1-(alkyloxyphenyl)ethylamines studied, only *m*- and *p-cyclo*hexyl ethers have so far shown marked analgesic activity. Others have often been strong depressants and slight activity could be due to this. The *p-cyclo*pentyl ether had no activity. Models showed that an axial hydrogen[8] of the *cyclo*hexyl ring in the "chair" form could form a hydrogen bond with carbonyl oxygen of the carbamic acid group. C——H——O bonds are poorly substantiated but the ease of elimination of axial hydrogen from a *cyclo*hexyl ring suggests that it may have sufficient degree of polar character to allow it to participate in hydrogen bond formation. This could be a driving force to induce enolisation. Such a bond is not possible for the *iso*propyl ether. Moreover, steric interference by alkyl substituents

ortho to the ether group prevent the bonding and 1-(3-methyl-4-*cyclo*-hexyloxyphenyl)ethylamine had no analgesic activity. Figure 1 indicates that this ether reacted with carbon dioxide in a similar manner to the *iso*propyl ether.

SUMMARY

1. Reaction of 1-(alkyloxyphenyl)ethylamines with carbon dioxide and bicarbonate or with bicarbonate alone, in solution can lead to formation of carbamic acids.

2. Some differences in the behaviour of two such acids in solution are discussed in relation to the analgesic activity of 1-(*p-cyclo*hexyloxyphenyl)-ethylamine.

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ESTIMATION OF DRUG ANTAGONISMS ON THE ISOLATED GUINEA-PIG VAS DEFERENS

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The use of the guinea-pig vas deferens was described by Waddell[1] for studies of pilocarpine and adrenaline. Apart from this the preparation appears to have received little attention; being well adapted for drug antagonism tests it should be more widely known.

Experimental

Large adult male guinea-pigs were killed by a blow and bled, the vasa deferentia carefully removed, without stretching, and placed in cold aerated Tyrode's solution. A single vas deferens was suspended in a 10 ml. bath filled with Tyrode's aerated to a constant temperature. Contractions were recorded by a lightly loaded lever with a magnification of eight. The time of drug contact was one minute, followed by two washings of fresh Tyrode's to relax the preparation. Temperature variations were found to be important; thus with adrenaline the greatest contractions were observed at 32–34° C., while with acetylcholine and histamine it was 36–38° C.

Results

(−)-Adrenaline, (−)-noradrenaline*, acetylcholine, and histamine, when applied to the preparation gave a contraction, and the result of increasing doses is illustrated in Figure 1. When the response of the preparation was plotted against the logarithm of the dose, a linear form is obtained over a wide range of dose, the slope of the curve is steep, and the preparation exhibits a well defined maximum.

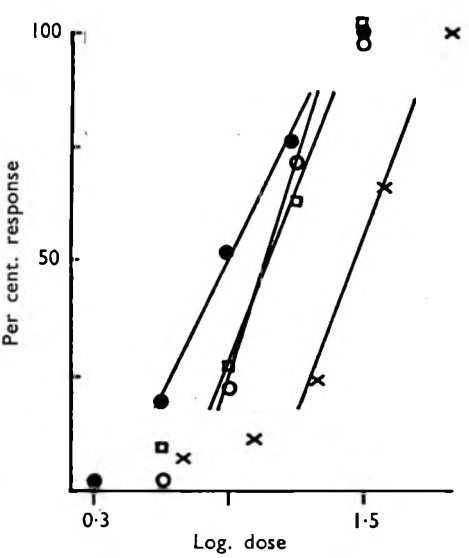

Fig. 1. Dose response curves for acetylcholine ●——●, noradrenaline ○——○, adrenaline □——□, and histamine ×——×, on the vas deferens preparation.

The regression coefficient *b* has been calculated for each of the dose response lines shown in Figure 1. For adrenaline *b* is 96·4, noradrenaline 104·7, acetylcholine 83·8, and for histamine 45·6.

* (−)-Adrenaline and (−)-noradrenaline were used throughout the work.

All four substances give a contraction and this is useful for a study of specific antagonists, the method being illustrated in Figure 2 with 883 F. (diethylaminomethylbenzodioxane, sympatol) and noradrenaline.

Drug antagonism is conveniently studied by a modification of the method described by Schild[2] for the estimation of pA values. The

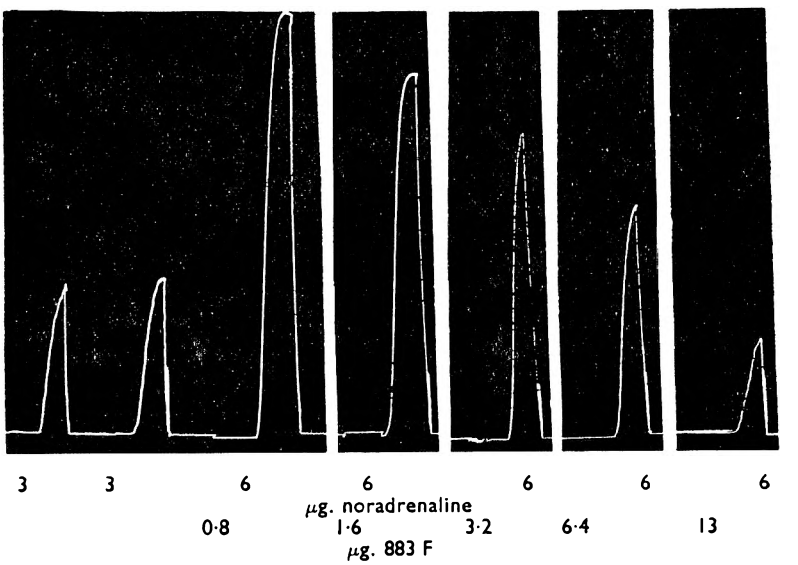

FIG. 2. Determination of antagonism of 883 F., to noradrenaline, showing decline of noradrenaline response as described in text.

preparation was first tested with a standard dose of, for example, 3 μg. of noradrenaline, to give a constant response. The antagonist was then added, e.g., 0·8 μg. of 883 F., and followed after five minutes with a double dose of the stimulating drug (6 μg. of noradrenaline). This procedure was repeated, the dose of the antagonist being doubled each time, until the contraction with the double dose of noradrenaline in the presence of the antagonist was approximately the same size as that obtained with the single dose of noradrenaline without the antagonist.

TABLE I

CONCENTRATION OF ANTAGONISTS REQUIRED TO REDUCE RESPONSES OF (−)-ADRENALINE, (−)-NORADRENALINE AND ACETYLCHOLINE

Antagonist	Concentration of antagonist in μg./ml. required to reduce contraction		
	(−)-adrenaline	(−)-noradrenaline	Acetylcholine
Atropine	75	120	2×10^{-4}
Mepyramine	375	300	0·6
Sympatol	0·8	0·9	
Piperoxan	0·02	0·08	3·2
Yohimbine	12	24	

Table I gives the concentration of the antagonist in μg./ml. required to depress the contractions caused by adrenaline, noradrenaline, and acetylcholine. Noradrenaline requires more antagonist to reduce the response than does adrenaline.

The Table also shows that the antagonists of acetylcholine seem to be more specific than those of the two sympathomimetic amines used, and also that despite the large doses of stimulating agents needed to contract the preparation, it is nevertheless sensitive to small amounts of antagonist.

Summary

1. The isolated vas deferens of the guinea-pig gives a contraction with (−)-adrenaline, (−)-noradrenaline, acetylcholine, and histamine, which is measured quantitatively.

2. The preparation is a convenient one for the study of drug antagonisms.

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A NEW NON-AQUEOUS METHOD OF ASSAY FOR THE BARBITURIC ACIDS AND SOME COMMERCIAL PRODUCTS

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Non-aqueous titrimetry has been applied to the assay of barbiturates by several groups of workers. Autian and Allen[1] developed a potentiometric method using a solvent system of chloroform and polyethylene glycol 400. Their studies were modified and extended by Swartz and Foss[2], but these workers also failed to find a suitable indicator for this solvent system. They expressed a desire to employ chloroform alone, which is an excellent solvent for most barbituric acids, but found that such a titration was not suitable as precipitation of the barbiturate salts obscured the indicator end-point. Because of its low dielectric constant, chloroform was also a poor solvent for potentiometric titrations. They circumvented this difficulty by adding 10 per cent. polyethylene glycol 400, but were then forced to use a potentiometric titration method. Roland[3] used 75 per cent. ethanol in water and titrated the salts potentiometrically with 0·1N hydrochloric acid. He deplored the use of indicators and stated that in his opinion they were not accurate. This, of course, is understandable for such weak acids in a system containing 25 per cent. water.

Vespe and Fritz[4] titrated barbituric acids in dimethylformamide as solvent and their investigation was confirmed and expanded by Ryan, Yanowski and Pifer[5]. It was noted in this laboratory, however, that the end-point in such a system faded rapidly even when the titration flask was protected carefully from the atmosphere. It was noted, furthermore, that when using this solvent, there was a distinct tendency to overestimate the barbiturate content of certain coloured tablet preparations. In addition, dimethylformamide is unpleasant to work with and constitutes an actual danger to the respiratory system.

The main objection to the methods employed by other workers[1,2,3] was that the titration could be carried out only potentiometrically. It was decided, therefore, to seek a solvent system which did not possess the disadvantages that are inherent in dimethylformamide, but which yet permitted a visual titration of the barbiturates.

For the titration of weak acids in nonaqueous solvents, most workers have employed a methoxide titrant which is prepared with either metallic sodium, potassium or lithium. A recent publication by Crisafio and Chatten[6] has shown that a 0·1N solution of potassium hydroxide in anhydrous methanol was equally satisfactory for the titration of the bile acids and was much simpler to prepare than the conventional methoxides. Caldin and Long[7] proved that, in reality, a solution of sodium hydroxide in methanol did not differ in methoxide content from one prepared by using metallic sodium. It would appear reasonable to extend their findings on sodium hydroxide to include potassium hydroxide.

Experimental

Apparatus. 5 or 10 ml. burette, graduated in 0·02 ml., electromagnetic stirrer, 125 ml. suction flask and a small Büchner funnel.

Reagents. (1) chloroform, A.C.S. grade, (2) anhydrous methanol, A.C.S. grade, (3) benzoic acid, A.C.S. grade, (4) potassium hydroxide, A.C.S. grade, (5) dimethylformamide, Eastman white label, (6) potassium hydroxide 0·1N in anhydrous methanol, and (7) thymol blue indicator in 0·5 per cent. in anhydrous methanol.

Standardisation of titrate. Accurately weigh approximately 200 mg. of benzoic acid and dissolve in 50 ml. of chloroform, and 1 ml. of methanol and 4 drops of thymol blue indicator and titrate to a violet colour. A blank with the solvent system used here was approximately 0·10 ml. of titrant.

The contents of the beaker or flask can be conveniently protected from the atmosphere by using a piece of rubber dental dam or cardboard with a hole sufficiently large to permit the burette tip to pass through.

Procedures. (*a*) To assay bulk barbiturates, accurately weigh a sample of 40 to 50 mg. into a 150 ml. beaker, dissolve in 50 ml. of chloroform by stirring electromagnetically, add 1 ml. of anhydrous methanol, 4 drops of thymol blue indicator and cover the beaker. Titrate to a violet end-point with 0·1N potassium hydroxide in methanol.

(*b*) For commercial barbiturate samples, weigh and powder 20 tablets. Place an accurately weighed sample equivalent to 40 or 50 mg. of the drug in a 150 ml. beaker. Add 40 ml. of chloroform and stir electromagnetically for 10 minutes. Filter under suction into a 125 ml. flask using a Büchner funnel with Whatman No. 1 filter paper. Wash the beaker and funnel with 10 ml. of chloroform, add to the filtrate 1 ml. of anhydrous methanol and 4 drops of thymol blue solution. Cover the flask and titrate to a violet end-point with 0·1N potassium hydroxide solution in methanol.

Experimental Results and Discussion

The precision of the method for barbituric acids was tested by titrating 5 consecutive samples of U.S.P. grade phenobarbitone and calculating the standard deviation. These results are recorded in Table I. The study was extended to a number of other pure barbituric acids and triplicate assays for each are shown in Table II. The criterion of purity for these samples is shown by their melting points as well as by comparative duplicate assays utilising the dimethylformamide procedure[4]. Potentiometric titrations were performed on a Fisher titrator which was equipped with the conventional glass-calomel electrode system and a suitable arrangement to preclude the atmosphere. Figure 1 illustrates the excellent agreement that was obtained between the potentiometric and visual end-points for phenobarbitone in the chloroform-methanol system.

TABLE I

Titration of U.S.P. grade phenobarbitone using a chloroform-methanol solvent system

Mg. taken	Mg. recovered	Per cent. recovery
45·3	45·3	100·0
44·1	43·9	99·5
46·2	46·3	100·2
53·3	53·2	99·8
47·6	47·7	100·2

Mean—99·9 ± 0·3

TABLE II

COMPARATIVE RESULTS OF THE TITRATION OF PURE BARBITURIC ACIDS IN CHLOROFORM-METHANOL AND IN DIMETHYLFORMAMIDE

Barbituric acid	M.pt.° C.	In chloroform-methanol		Dimethylforformamide	
		mg. taken	mg. recovered	per cent.	per cent.
Amylobarbitone	156·3–156·9	48·0	47·9	99·8	100·4
		41·8	41·7	99·8	100·0
		44·7	44·8	100·2	
Barbitone	189·2–189·8	43·3	43·0	99·4	100·2
		41·1	40·8	99·3	99·2
		56·4	55·8	98·9	
Cyclobarbitone	171·3–172·8	41·7	41·4	99·3	99·2
		49·8	49·6	99·6	99·4
		46·0	45·8	99·6	
5-Allyl-5-phenylbarbituric acid	156–156·7	44·0	44·2	100·4	100·4
		45·8	45·9	100·2	100·2
		42·3	42·4	100·2	
Aprobarbital	140–142	46·7	46·7	100·0	100·0
		46·0	45·8	99·6	100·4
		42·7	42·4	99·3	
Propallylonal	180–181·2	51·7	51·0	98·6	99·8
		46·6	46·4	99·6	100·4
		47·7	47·5	99·6	
Hexethal	124·8–126·5	52·9	52·1	98·5	99·6
		49·0	48·6	99·2	99·3
		52·4	51·7	98·8	
Thiamylal	133·7–134·5	51·9	52·5	101·1	99·1
		45·8	46·3	101·1	99·4
		48·7	49·4	101·4	
5 - Methyl - 5 - phenylbarbituric acid	225·5–226·2	45·3	45·2	99·8	100·0
		49·4	49·4	100·0	100·0
		44·1	44·2	100·2	
5 - Ethyl - 5 - (1-*cyclo*hepten - 1 - yl) barbituric acid	170–172·2	43·0	42·7	99·3	100·4
		43·3	43·2	99·8	100·8
		44·1	44·0	99·8	
Pentobarbitone	128·2–129·4	50·6	49·9	98·6	98·8
		54·2	53·9	99·4	98·4
		49·9	49·4	99·0	

The precipitation of the barbiturate salts, when the acids were titrated in chloroform alone, as reported by Swartz and Foss[2] was confirmed in this laboratory. It was noted, however, that if 1 ml. of anhydrous methanol was added to the chloroform before commencing the titration precipitation did not occur. Since the titrant employed in this investigation was potassium hydroxide in anhydrous methanol, the addition of a further 2·5 ml., the amount required for most titrations, provided sufficient methanol to keep the barbiturate salts in solution. It was further noted that the precipitate which formed, when 1 ml. of methanol was not added, frequently redissolved before the titration was completed. This did not occur in every instance, however, and consequently it was deemed advisable to add the 1 ml. of methanol as a step in the standard procedure. It is the author's opinion that this resulted in a sharper visual end-point than that obtained in chloroform alone. It was also observed that the end-point was more permanent in either the chloroform or the chloroform-methanol system than in dimethylformamide.

TABLE III

STANDARDISATION OF POTASSIUM HYDROXIDE IN METHANOL

Mg. benzoic acid	Ml. titrant	Normality
202·2	21·74	0·0762
182·3	19·66	0·0760
180·3	19·37	0·0762
180·7	19·42	0·0762
193·6	20·80	0·0762

As outlined in the procedure, the titrant was standardised by dissolving approximately 200 mg. of benzoic acid, accurately weighed, in 50 ml. of chloroform, adding 1 ml. of anhydrous methanol and 4 drops of thymol blue solution. The 1 ml. of methanol, in this instance, serves no purpose except to keep the solvent system constant. The results of 5 consecutive titrations are given in Table III, which shows the reproducibility of the method.

It was reported earlier that the use of dimethylformamide as solvent resulted, in almost every instance, in the overestimation of the barbiturate content of coloured tablet preparations. As a result of this finding, a comparison was conducted between the two solvent systems. The results appear in Table IV. It is noted that the recoveries with dimethylformamide are not only higher than expected but are erratic. This may be accounted for by the fact that the extracts with this solvent were intensely coloured by the tablet dye, whereas the chloroform extracts were only faintly coloured or colourless. It had been hoped to perform comparative assays on these products by some other standard method, but owing to the other ingredients in the tablet, none were found to be suitable. In order to justify the results obtained with the two solvent systems, therefore, the tablet residues from the chloroform extracts were saved and dried. Known weights of phenobarbitone of U.S.P. grade were added to the residues and the phenobarbitone determined by the chloroform-methanol procedure. The recoveries were quantitative. The residues were again dried, further known weights of the phenobarbitone added and the assays carried out using dimethylformamide. In every instance, overestimation occurred with this latter solvent which was about the same as that found when the unknown tablets were assayed.

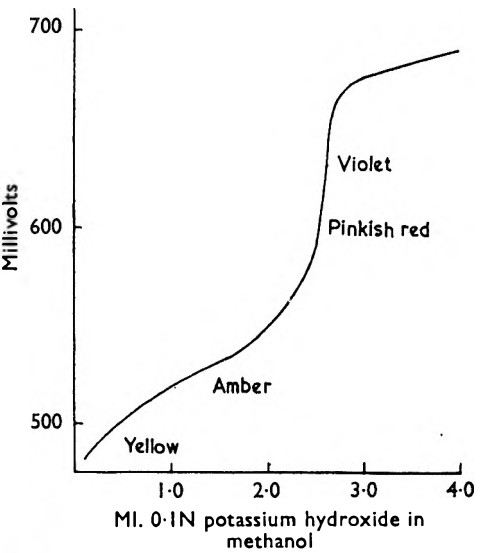

FIG. 1. Titration of phenobarbitone in chloroform-methanol system.

Vespe and Fritz[4] noted that when phenobarbitone was combined with aminophylline, the baribituric acid content could not be determined by extraction with dimethylformamide, due to interference by the theophylline. Although the same drug also interferes with direct chloroform extraction, the use of this latter solvent enables the analyst to apply a modification of David's[8] method to such mixtures. By treating the preparation with ammonia and silver nitrate as instructed by David, the procedure need be

carried only to the stage where the barbituric acid is extracted by shaking with 25 ml. portions of chloroform. When this has been accomplished, the drug can be determined by the direct titration previously outlined. It is necessary to use 1 ml. of methanol for each 50 ml.of chloroform extract. The results of such a modification, when applied to commercial products, are given in Table V along with the analyses of certain other phenobarbitone (uncoloured) preparations.

Comparative assays were performed, where possible, by the B.P. 1953 method for the phenobarbitone tablets. Agreement between the non-aqueous and the official procedure appears to be quite satisfactory in all but three instances. The preparations in question, which are a half grain tablet, a one grain and a grain and a half, are those of one manufacturer. For each the recovery by the official procedure was considerably higher than that which was obtained when using the non-aqueous technique. Titration of the phenobarbitone residues revealed that some additional substance was being weighed as phenobarbitone and that the actual amount of drug present in the residue agreed very closely with the non-aqueous values. The phenobarbitone residues of all other

TABLE IV

COMPARISON BETWEEN PER CENT. RECOVERIES IN THE CHLOROFORM-METHANOL SOLVENT SYSTEM AND DIMETHYLFORMAMIDE FOR COLOURED PRODUCTS

Product	Chloroform-methanol	Dimethyl-formamide
I	98·0	109·5
	98·2	102·5
II	100·0	107·8
	100·8	102·2
III	100·2	105·7
	100·0	105·8
IV	96·8	122·0
	95·6	113·4

TABLE V

RECOVERY OF PHENOBARBITONE FROM COMMERCIAL TABLETS

Product	Per cent recovery	
	Non-aqueous	B.P. 1953
Aminophylline and phenobarbitone ..	98·6	*
	96·8	
Aminophylline and phenobarbitone ..	92·1	*
	91·8	
Aminophylline and phenobarbitone ..	90·4	*
	90·5	
Aminophylline and Phenobarbitone ..	101·9	*
	101·4	
Phenobarbitone gr 1½	98·1	98·5
	98·3	
Phenobarbitone gr. 1¼	98·0	98·3
	98·2	
Phenobarbitone gr. 1¼	89·0	97·9†
	89·1	
Phenobarbitone gr. 1	91·3	90·5
	90·9	
Phenobarbitone gr. 1	94·5	97·8†
	94·4	
Phenobarbitone gr. ½	97·5	97·3
	97·7	
Phenobarbitone gr. ½	92·9	99·1†
	92·6	
Phenobarbitone gr. ½	94·6	95·0
	94·4	
Phenobarbitone gr. ¼	91·1	91·4
	91·5	
Phenobarbitone gr. ¼	96·1	97·6
	96·6	
Phenobarbitone with sodium pentobarbitone	87·2	*
	87·7	

* Not official in B.P. † Overestimated by B.P. procedure.

tablets, when also determined volumetrically, were found to be reasonably pure drug. These results indicate that the official procedure, which is much slower than the non-aqueous method, may lead to overestimation with certain products.

Sodium salts of barbituric acids, as bulk or in tablets or capsules, were extracted as the free acid by Swartz and Foss[2], who used the "Schmall apparatus". These workers acidified an aqueous solution of the salt and extracted the drug with chloroform. Evaporation of the solvent to 50 ml. was followed by the addition of 10 per cent., polyethlene glycol 400. The titration was performed potentiometrically. To use the procedure described in this report, it is necessary to add 1 ml. of anhydrous methanol after the chloroform extract has been evaporated to 50 ml. A visual titration can then be performed as previously described.

Summary

1. A new non-aqueous technique has been devised for the barbituric acids which is rapid, accurate and can be performed visually.

2. The procedure has been successfully applied to commercial samples of phenobarbitone tablets as well as those of phenobarbitone and aminophylline. The advantages over existing methods have been discussed.

3. The use of potassium hydroxide in methanol as a titrant in non-aqueous titrimetry has been extended to the barbiturates.

I would like to thank Dr. Leo Levi for many of the barbituric acid specimens which he carefully purified, and Miss M. McClure for her assistance in titrating some of the phenobarbitone tablets.

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BIOASSAY OF SODIUM-RETAINING ACTIVITY OF PURE CORTICOIDS AND OF EXTRACTS OF ADRENAL VEIN BLOOD

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The mineralocortical activity of the cortical hormones is a complex function affecting both renal and extra-renal factors. The different methods of assay, of necessity, measure the effect of the cortical steroids only on some of the factors involved. The interpretation of the results is thus rendered difficult as the response measured varies from one method to the other.

Some of the available methods have a reasonable degree of accuracy and sensitivity in the hands of their authors. Because of the complexity of the technique, however, they do not prove easily reproducible in the hands of other workers. Some achieve their accuracy at the cost of a time-consuming procedure and either have a limited range of sensitivity[1] or the necessity for large quantities of the active material[2].

The use of the $^{24}Na/^{42}K$ ratio[3] produced an accurate and sensitive method. The technical difficulties involved, however, are considerable. In addition to the preparation and measurement of the isotopes, a special physical absorption technique is needed to estimate the $^{24}Na/^{42}K$ ratio. The method does not differentiate qualitatively between the sodium-retaining and sodium excreting corticoids.

A reproducible simple procedure that can achieve this differentiation may have a place in routine investigations.

Methods

In all the following experiments on rats, adrenalectomy was performed under ether anæsthesia through a central dorsal skin incision. The adrenal glands with the surrounding fat as well as the upper third of the renal capsule were removed. The animals were maintained on a drinking solution of 1·0 per cent. sodium chloride and 5·0 per cent. glucose and on rat cubes. At the end of the experiment, the drinking solution was replaced by tap water, and if the animals did not die within eight days the results were discarded.

The temperature of the room was kept constant throughout the whole procedure at 22–24° C.

1. *Qualitative method for detecting mineralocortical activity on adult rats*

Male albino rats of 150–200 g. were adrenalectomised. On the third post-operative day the cubes were withdrawn at 5 p.m. and the drinking solution replaced by a 5 per cent. glucose solution. At 11 a.m. next morning the animals were weighed and given, by stomach tube, 5 ml. per 100 g. body weight of a solution of 14 per cent. ethanol containing 5 per

* In receipt of a scholarship from Cairo University.

cent. glucose. The rats were then kept in a quiet and darkened place at a constant temperature. They started to get drowsy in about ten minutes and were generally under light anæsthesia in thirty minutes. At 12 noon, 4·0 ml. of 0·9 per cent. sodium chloride solution was injected subcutaneously to each rat. A long fine hypodermic needle was used. The needle, introduced dorsally near the tail, was pushed under the skin to the shoulder region and the solution evenly distributed. Ten minutes later, 0·1 ml. of aqueous solution of deoxycortone glycoside, "Percorten" was injected intraperitoneally to the test animals while the control animals were given the corresponding amount of water.

Thirty minutes later, a catheter was introduced suprapubically, usually without the need for more anæsthetic. Occasionally, if the anæsthesia was light, a few whiffs of ether was used.

A suprapubic longitudinal incision 1·5 cm. long was made in the midline. The bladder was secured and, without pulling, and with the use of a pair of sharp fine scissors, a catheter was introduced through a small incision in the apex of the bladder and tied with a fine silk ligature. Kinking the bladder or damaging its wall were carefully avoided. The penis was ligated and a small amount of distilled water was then injected through the inner tube to test the patency of the catheter, minimum pressure being used. The catheter was a double walled polythene tube. The outer tube had an external diameter of 3·5 mm.; the inner tube of 1·5 mm. (Fig. 1); the thinner tube was connected to a blunt needle fitted in a syringe and fixed in a holder with a screw which could be rotated by hand so that 1–2 ml. was injected steadily in 30–45 seconds.

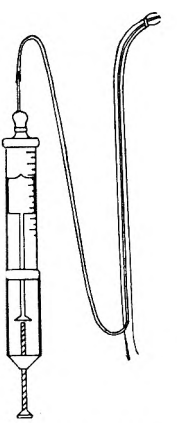

FIG. 1. Apparatus for washing the urinary bladder of the rat.

The rats were then put in a box which was kept warm by electric lamps and a thin thermometer was introduced into the rectum. The rectal temperature was kept as near to 37° C. as possible. Noises were avoided throughout the experiment.

Twelve noon, the time at which the sodium chloride had been injected, was considered zero hour. At 1.00 p.m. each bladder was washed out by injecting 2·0 ml. of distilled water through the inner tube. The washing was discarded. After this washing a graduated centrifuge tube was used to collect any fluid dripping from the catheter. Every 30 minutes the bladder was washed again by injection 2·0 ml. of distilled water. The washings were collected and the centrifuge tube changed. The collection was continued for at least one hour and a half.

The fluid in the centrifuge tubes was then made up to 10 ml. and the quantity of sodium estimated by the flame photometer.

2. *A quantitative method of assay on young rats*

Young female white rats 50–100 g. were used, with the same procedure for adrenalectomy and maintenance. Instead of single animals, about 40

rats generally in groups of ten were used. These were so arranged that animals with corresponding weights were evenly distributed.

On the fourth day after adrenalectomy, feeding was stopped at 11 p.m.; the saline-glucose drinking solution was available till 5 a.m. next morning, when it was replaced by 5 per cent. glucose only. At 10 a.m. the animals were injected with 4·0 ml. of Salt Load I (6·25 mg. sodium as chloride in 4 ml. 5 per cent. glucose).

Handling was kept to a minimum; for the injection, the animals were picked up by the tail and brought near a cone-shaped polythene container slit on its upper surface. They ran into the container which was then closed with a slit rubber stopper which allowed the tail to stay out. The thin needle (3 inches long) was introduced near the tail and pushed subcutaneously towards the shoulder region; then the injection was given slowly. One animal from each group was injected in turn.

At noon, each rat was caused to urinate by a gentle pull on the tail. One ml. of the Salt Load II (see below) and the corticoids were then administered to one rat from each group in turn, in the same order and taking the same precautions as for the first injection.

The Salt Load II contained 3·125 mg. sodium and 0·2 mg. potassium per ml. A tuberculin syringe with a fine needle was used to administer the corticoid in 0·1 ml. propylene glycol subcutaneously. The control group received the same amount of propylene glycol alone.

Once a rat was injected it was placed into a metabolism beaker. These were 1 litre pyrex glass beakers from the rim of which a tightly fitting wire mesh platform was suspended by wire hangers two-thirds of the way down the beaker. Before each platform was used it was waxed with hard paraffin wax and washed with cold distilled water. All the apparatus was kept thoroughly salt free. Each beaker had a perforated lid.

Six hours after the injection, the collection of urine was stopped from each rat successively in the same order as it had been started. The bladders were emptied by suprapubic massage after ether anæsthesia. Fæces lying on the wire mesh platform were removed with forceps, and the beaker and platform were washed four times with 20 ml. quantities of distilled water. The urine and washings were made up to 100 ml. The diluted urine was filtered through ashless acid-wash filter paper, and the amount of sodium estimated by the flame photometer.

Completeness of the operation was checked by keeping the rats on cubes and tap water only. They almost invariably died in eight days after the withdrawal of the saline drink.

Corticosterone (Compound B), 17-hydroxy-11-dehydrocorticosterone (cortisone, Compound E) and 17-hydroxy-corticosterone (hydrocortisone, Compound F) were dissolved in propylene glycol and given in 0·1 ml. of solution per rat. The effects were compared to those of deoxycortone acetate (DOCA) as a standard.

3. *Collection of adrenal venous blood*

(*a*) *Dog*

The operative technique described by Vogt[4] was used. Adult male dogs

of about 14 kg. body weight were anæsthetised with ether until a chloralose solution (70–80 mg. per kg. body weight) could be infused into the femoral vein.

Blood was collected, under heparin cover from the left adrenal gland into 50 ml. centrifuge tubes kept in an ice bath. The rate of flow was recorded. The blood was centrifuged and the plasma pipetted immediately after collection. Arterial blood from the carotid artery was collected before killing the dog and the left adrenal gland weighed. In dog C the splanchnic nerves were severed before blood collections started.

(*b*) *Rabbit*

Collection of adrenal plasma was carried out according to the method described by Vogt[4]. Rabbits of 2·5–3·0 kg. weight were anæsthetised with ether followed by chloralose infused into the jugular vein. Through a median abdominal incision the animal was eviscerated. Ligatures were then tied around all vessels draining into the inferior vena cava except the adrenal veins. Venules draining the fatty tissue surrounding the kidneys and those draining the vertebral column were tied.

A loose ligature was placed around the inferior vena cava above the entrance of the right adrenal veins. The aorta was ligated below the origin of the renal arteries and a cannula introduced into the cava below the level of the kidneys. Heparin was then administered through the jugular vein and a carotid cannula for blood pressure recording introduced. The ligature around the upper part of the vena cava was tied and the blood allowed to run into the cannula for the collection of samples. The blood pressure was maintained as constant as possible by infusing heparinized blood collected from another rabbit. The volume and time of each collection was recorded. At the end of the experiment the two adrenal glands were dissected out and weighed.

In the second experiment 10·0 units of ACTH dissolved in 40 ml. of saline were warmed to body temperature, and infused through the jugular cannula at the rate of 2·0 ml. per minute. A blood collection was carried out during the last phase of the infusion and for a short time afterwards.

4. *Chemical extraction and Assay*

(*a*) *Dog*

The method devised by Bush as reported by Bibile[5] was used. A mixture of ether and ethyl acetate 2 : 1 was prepared from freshly distilled stocks. Each volume of plasma was extracted by shaking it with its own volume of the mixture. After centrifuging, the non-aqueous phase was pipetted into a round-bottomed flask. The extraction process was repeated four times. The collected supernatants were evaporated *in vacuo*, at 40° C. until a thin film of fluid was left in the flask.

A volume of light petroleum corresponding to the initial volume of the plasma was added to the flask and its sides well scraped by a glass rod. A separating funnel was prepared containing 5/3 of the initial plasma volume of light petroleum, and the contents of the flask were added to the funnel. The evaporating flask was further washed and thoroughly

scraped with 70 per cent. redistilled ethanol in water, 1/7 of the initial plasma volume being used. The ethanolic washing was repeated, and the ethanol added to the funnel. The contents of the funnel were shaken thoroughly, and allowed to separate; the ethanol was then drained off. The whole process of ethanolic extraction was repeated twice. The combined ethanolic extracts were then evaporated *in vacuo* and the residue dissolved in propylene glycol.

When deoxycortone acetate was to be added to arterial plasma in recovery experiments, a concentrated solution of deoxycortone acetate in 40 per cent. ethanol was prepared and the solution added to the plasma before extraction started. The same volume of 40 per cent. ethanol was added to the control plasma.

In assaying adrenal samples, the control rats were injected with extracts of arterial plasma in propylene glycol. The volume of arterial plasma used was the same as the volume of adrenal plasma tested in the same experiment. Similarly, all standard doses of deoxycortone acetate were dissolved in extracts of the same volume of arterial plasma. In this way, the presence of active materials in the plasma not produced by the left adrenal and which might affect the assay in an unpredictable manner, was compensated.

Arterial plasma from control dogs was obtained and extracted in the same way. These extracts were assayed without the addition of any cortical steroids. The method of assay was used to test the mineralocortical activity of arterial plasma and adrenal venous plasma of dogs. 0·1 ml. of propylene glycol extract corresponded to between 2·0 and 2·25 ml. of adrenal plasma.

(*b*) *Rabbit*

It was not possible to collect sufficient arterial plasma from the animal supplying the adrenal blood to use its extract as a vehicle for the standard doses of deoxycortone acetate; these standard doses were thus given in 0·1 ml. propylene glycol alone. A sample of arterial plasma collected from two rabbits was extracted separately and assayed. Extracts of adrenal plasma were given to the rats in doses of 0·1 ml. propylene glycol per rat. Each 0·1 ml. corresponded to 1·7–3·5 ml. of the adrenal plasma.

Results

Preliminary experiments on mice, using the method of assay devised by Spencer[1], were abandoned because the day to day variations in the sodium excretion by the same mouse were large. In addition, repeated experiments on the same adrenalectomised mouse necessitated by the cross over technique of this method, were not well tolerated by the animals.

An attempt was made to study some of the variables involved in the use of the urinary excretion of sodium and potassium for the assay of mineralocortical activity. It was found that the urinary potassium excretion in adrenalectomised mice did not have a linear relation to the dose of deoxycortone acetate administered. Further, the urinary sodium excretion was

found to vary, for the same mouse, from day to day, over a considerable range, while the sodium excretion of groups of mice on the same post-operative day was reproducible.

Premedication with deoxycortone glycoside or with cortisone in the post operative period did not materially improve the results; neither did adrenaline-induced diuresis during the period of urine collection.

In the light of these observations the procedure described under the quantitative method of assay, using young adrenalectomised rats was followed. Another type of test used single anæsthetised rats and is in the nature of a screening test.

A qualitative biological method for detecting mineralocortical activity employing small groups of anæsthetised adult rats

As shown in Table I the amount of sodium excreted during 30 minutes, beginning 60 minutes after the injection of the sodium load, decreases with increases in the amount of "Percorten" administered.

TABLE I

EFFECT OF "PERCORTEN" ON SODIUM EXCRETION OF ADULT ANÆSTHETISED RATS

Experiment No.	Time of collection*	Na excretion in 30 minutes (μg. Na per minute)		
	Minute	Controls	3 μg. Percorten per rat	10 μg. Percorten per rat
1	90	3·51		2·21
	120	2·89		1·70
	150	3·50		1·50
2	90	5·00	1·70	0·56
	120	6·60	1·90	0·23
	150	3·70	3·10	0·20
3	90	7·80		2·90
	120	6·10		1·03
	150	8·30		0·90
4	90	3·13	3·93	2·27
	120	3·63	2·26	1·13
	150	2·36	2·90	1·13
5	90		2·36	2·63
	120		3·33	1·93
	150		2·53	0·90
Mean	90	4·89	2·66	2·12
	120	4·81	2·50	1·20
	150	4·24	2·84	0·93

* Zero time is taken as the time of the injection of the saline load.

Any damage to the bladder while introducing the catheter usually produced hæmaturia and partial or complete anuria, and these animals were discarded, as were also animals developing difficulty in breathing. Constant temperature and complete protection from environmental stimuli were found to be essential. The degree of anæsthesia was also found to play an important role. Animals with anæsthesia light enough to permit them to perform any degreee of spontaneous movements as well as animals with too deep a degree of anæsthesia were also discarded.

This method can be of some use as a screening test for mineralocortical activity. A group of 8 rats can be handled at one time and give a

result in a few hours. This method can also be of use in selecting the optimum dilution of the test material for quantitative estimation.

A quantitative method of assay employing larger groups of young conscious rats

Table II and Figure 2 show the relation between the dose of deoxycortone acetate and its effect on sodium retention. Table III contains the statistical analysis of the figures.

TABLE II

EFFECT OF DOCA ON SODIUM EXCRETION IN THE URINE OF YOUNG ADRENALECTOMISED RATS

Dose μg. DOCA	0·5	1·5	2·0	4·5	8·0
Number of rats	15	15	16	15	16
Mean Na excretion (per cent. of controls) ± S.D. of differences*.	88·1 ± 23	64·1 ± 20·7	51·3 ± 16·9	35·3 ± 17·4	25·0 ± 15

* Standard deviations of differences between test and control groups.

Tests of significance: Between control group and 2·0 μg., P 0·01
Between 1·5 μg. and 4·5 μg., P 0·05
Between 2·0 μg. and 8·0 μg., P 0·02

The effects on sodium excretion are expressed as per cent. of the mean control values. The deviations from linearity are small. The slope $b = 53{\cdot}3 \pm 10{\cdot}52$, giving a t value of 5·07 indicates that the response is dependent on the dose ($P < 0{\cdot}001$). The precision index however, is high:—

λ = (standard deviation of response)/slope = 0·714.

A dose of 2·0 μg. deoxycortone acetate gave a response significantly different from the control value ($P < 0{\cdot}01$).

TABLE III

ANALYSIS OF VARIANCE

Effects per cent. plotted against log dose of DOCA
Adjustment for means − 209,852·4

Nature of variance	Degrees of freedom	Sum of squares	Mean squares
Regression	1	37,268·3	37·263
Deviation from regression ..	3	418·7	139·57
Between doses	4	37,687·0	9421·7
Within doses	72	104,468·46	1450·95
Total	76	142,155·49	

Because of the flatness of the curve, the dose had to be increased by a relatively high factor to obtain responses differing significantly. In spite of attempts at standardising the conditions of the experiments, the variations between different groups of rats, necessitates the inclusion of a group of control animals in each assay and the expression of the responses of the test and standard animals as a percentage of the mean response of the control group. Because of variations in slope it is necessary to have two groups injected with known doses of the standard included in every assay.

The effect of pure cortical steroids on sodium excretion

As is shown in Table IV, the three cortical steroids with an oxygen at C(11) increase sodium urinary excretion under the conditions of the test. As a rule, sodium excretion increased with increasing dose of the steroid. Small doses of deoxycortone acetate (e.g., 2·0 and 4·0 μg. per rat) given to a group of animals concurrently, showed a marked sodium retention as usual.

When deoxycortone acetate was administered in conjunction with steroids causing increased sodium excretion, antagonism between the two steroids was observed in every case.

The potency of the steroids which increase the sodium excretion can be assessed roughly by their power to antagonise the sodium retaining effect of deoxycortone acetate. A dose of 10·0 μg. of each of these steroids was administered in conjunction with 4·0 μg. of deoxycortone acetate. The results obtained indicate that the potency of these compounds descends in the order; hydrocortisone, cortisone, corticosterone. To investigate further the quantitative nature of this antagonism several doses of deoxycortone acetate were administered in conjunction with two doses of cortisone. It was found that about 4·0 μg. deoxycortone acetate neutralised the effect of 10·0 μg. of cortisone in promoting sodium excretion. Eight μg. of deoxycortone acetate neutralised the effect of 31·25 μg. cortisone, while 2·0 μg. deoxycortone acetate produced no significant antagonism.

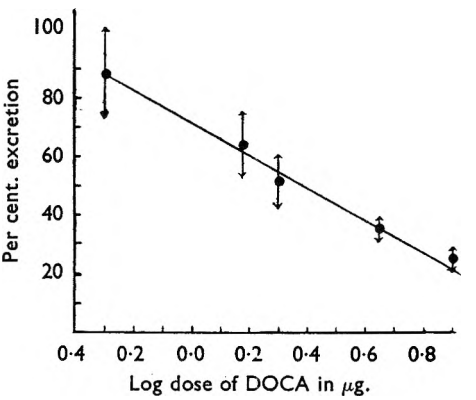

FIG. 2. The effect of deoxycortone acetate on urinary excretion of sodium by adrenalectomised rats (dose response curve). Ordinate: total sodium excretion expressed as percentage of the mean response of the control group. Abscissa: log dose of deoxycortone acetate per rat.

Corticosterone which is the weakest steroid of the group did on one occasion cause some degree of sodium retention when administered in a dose of 20·0 μg. per animal. In the other eight experiments carried out on this compound however, there was an increase in sodium excretion or an antagonism to the sodium retaining power of deoxycortone acetate.

Mineralocortical activity of adrenal venous blood of dog

Recovery. Extracts of arterial plasma did not cause any sodium retention (Table V). If anything, a tendency to enhance sodium excretion was observed, but this effect was not significant. When arterial plasma was extracted after the addition of 4·0 μg. deoxycortone acetate per 0·1 ml. of plasma this extract caused sodium retention to an extent slightly less

TABLE IV

PURE CORTICOIDS AND URINARY SODIUM EXCRETION

Steroid	Dose in μg.	DOCA μg.	No. of animals	Sodium excretion* per cent. ± S.E.
Hydrocortisone ..	5·0		15	219 ± 25
	20·0		14	474 ± 12
	10·0	+ 4·0	8	210 ± 31
Corticosterone ..	5·0		14	128 ± 24
	10·0†		7	135 ± 17
	20·0‡		13	240 ± 41
	40·0		7	189 ± 29
	10·0	+ 4·0	19	80 ± 13
Cortisone ..	31·25		8	187 ± 30
	125·0		7	203 ± 24
	500·0		8	290 ± 44
	10·0	+ 4·0	11	112 ± 14
	31·25§	+ 2·0	7	163 ± 21
	31·25	+ 8·0	6	103 ± 10

* Sodium excretion expressed as per cent. of mean excretion of control group.
† A group of 7 rats, run concurrently, each received 4·0 μg. DOCA. Their mean sodium excretion ± S.E. = 30 ± 9·7.
‡ A group of 8 rats, run concurrently, each received 4·0 μg. DOCA. Their mean sodium excretion ± S.E. = 34 ± 10·1.
§ A group of 6 rats, run concurrently, each received 2·0 μg. DOCA. Their mean sodium excretion ± S.E. = 25·8 ± 8·4.

TABLE V

DOG'S ARTERIAL PLASMA TESTED FOR MINERALOCORTICAL ACTIVITY

Experiment No.	No. of rats in each group	Mean sodium excretion of test group as per cent. of mean excretion of control group ± S.D. of differences		
		Extract of 0·75 ml. arterial plasma	4·0 μg. DOCA	Extract of 0·75 ml. arterial plasma containing 4·0 μg. DOCA†
1	7	103·0 ± 58*	61·02 ± 16	66·9 ± 16
2	7	115·0 ± 29·6	43·9 ± 20·9	64·0 ± 20·8

* Standard deviations of differences between test and control groups.
† The DOCA was added to the arterial plasma before extraction.

TABLE VI

MINERALOCORTICAL ACTIVITY OF ADRENAL VENOUS PLASMA IN THE DOG

Dog	A		B		C§
Assay	I	II	I	II	I
Volume of plasma per rat (ml.)	2·0*	2·0*	2·0†	2·0†	2·25‡
Mean systolic pressure during collection of sample (mm. Hg).	64		35		32
No. of rats per group	8	7	7	7	9
Mean Na excretion:					
Of test group as per cent.	78·98	64·2	102·4	96·3	75·06
Of mean excretion of control group ± S.D. of differences	± 20·14	± 21·93			± 22·32
DOCA equivalent μg./ml. plasma	0·68	0·66	0	0	0·33
DOCA equivalent μg. g./min.	0·95		0		0·44

* 2·0 ml. of the adrenal plasma collected over 1·98 min.
† 2·0 ml. of the adrenal plasma collected over 1·32 min.
‡ 2·25 ml. of the adrenal plasma collected over 3·06 min.
§ The splanchnic nerves were severed before the collection.

(differences non-significant) than that produced by 4·0 μg. deoxycortone acetate alone. In view of the fact that extracts of arterial blood usually increase sodium excretion, it is not quite clear whether recovery was incomplete or whether some of the effect of the added DOCA was masked.

Extracts of adrenal venous plasma. In order to obtain as high a concentration of corticoids in the adrenal effluent as possible, the blood obtained at higher blood pressures, and consequently at a more rapid rate of flow, was discarded. Only the later samples were used, when blood loss had diminished the pressure and blood flow. A sodium retaining activity of the samples examined could not be established with certainty (Table VI). The calculated activity (last column) being very near the threshold may not be real. Nevertheless, when the same sample was assayed a second time, the results agreed remarkably well (Dogs A and B, Table VI).

The results may either be interpreted as a sign of low release of sodium retaining hormones, or of high secretion of sodium diuretic hormones.

TABLE VII

MINERALOCORTICAL ACTIVITY OF RABBIT ADRENAL VENOUS PLASMA

Experiment No.		I	II		III
Sample assayed		Adrenal venous plasma	Adrenal venous plasma	Adrenal venous plasma after ACTH	Arterial plasma
No. of rats per group		11	16	16	8
Extract of plasma administered per rat	Volume (ml.)	3·0	1·7	3·5	3·0
	Collection time (min.)	1·62	2·85	3·05	
Mean sodium excretion of test and of standard groups as per cent. of mean of control group	Test group (plasma)	41·6	89·7	79·8	129·9
	Significance from control	$P < 0·001$	$P < 0·4$	$P < 0·2$	$P = 0·5$
	Standard group (2·0 μg. DOCA)	47·0	50·2	50·2	
	Significance from control	$P < 0·001$	$P < 0·001$	$P < 0·001$	
Estimated mineralocortical activity of plasma as μg. DOCA per ml. plasma		1·2	0·0	0·0	0·0

Mineralocortical activity of adrenal venous blood of the rabbit

Corticosterone being less active an antagonist of deoxycortone acetate than hydrocortisone, an animal species secreting predominantly corticosterone was also investigated. Adrenal venous plasma extract from one rabbit showed a significant sodium retaining power (Table VII), when extracts of 3 ml. plasma were injected into each rat of the test group. A second rabbit, however, showed no such activity. Arterial plasma on the other hand, had a slight sodium excreting effect.

DISCUSSION

The method reported here for the quantitative assay of mineralocortical activity offers certain advantages.

The procedure is simple. It has been the aim throughout this work to develop this method along lines which permit its adoption as a routine

method of investigation not requiring elaborate technical facilities or excessive labour.

It has a range of sensitivity. Significant sodium retention was achieved with 2·0 μg. doses of deoxycortone acetate in experiments where 10–15 rats per group were employed. In some experiments 1·0 and 1·5 μg. deoxycortone acetate gave significant sodium retention ($P < 0{\cdot}03$), but the results were not always reproducible. The highest dose used in the experiments reported here was 8·0 μg. deoxycortone acetate.

It is able to differentiate between mineralocorticoids and corticoids with an oxygen atom at C(11) (11-oxycorticoids).

The reported method, on the other hand, suffers from some serious handicaps. The index of precision is high — 0·714. As a result of this inaccuracy it is necessary to increase doses by a high factor (3–4) to achieve significant differences in response.

This method was used in a comparative study of the action of some of the known corticosteroids on electrolyte excretion. There was a demonstrable qualitative difference between deoxycortone acetate and the 11-oxycorticoids on the excretion of sodium by adrenalectomised animals. The 11-oxycorticoids caused an increase in sodium excretion instead of a decrease. Similar results were obtained by others[7–8].

Simpson and Tait's method, on the other hand, does not reveal such qualitative differences, the only difference being a quantitative one; the $\frac{Na}{K}$ ratio drops in all cases. An important factor which may account for this apparent discrepancy between the results obtained here and those obtained by Simpson and Tait is the duration of the experiment. Simpson and Tait found a decrease in the urinary $\frac{Na}{K}$ ratio after giving cortisone only when they used a two hours' collection period. Such a drop in the ratio was not obtainable with a six hours' collection.

Davis and Howell[9] pointed out that an important consideration which has often been neglected in studying water and electrolyte balances in response to cortisone is the role of glomerular filtration. This applied to dogs and rats. In normal dogs observed over two days, the authors found that cortisone and ACTH caused increased salt loss only in those dogs in which glomerular filtration rate was raised by the hormone. The rise in glomerular filtration and plasma flow was most profound with cortisone treatment and least with deoxycortone acetate treatment.

Roberts and Pitts[10], in their acute experiments on dogs demonstrating the sodium retaining power of cortisone, collected urine over a period of 2–3 hours only, after the administration of cortisone. The glomerular filtration rate and plasma flow were not significantly altered during this period. The changes in the subsequent period were not investigated.

Another factor which may be involved in a six hours' urinary collection and not in a shorter period has been indicated by Bloodworth[11]. In normal dogs, he found that administration of cortical extract for three to six hours caused a transfer of fluids from the intracellular to the

extra-cellular compartments. When deoxycortone acetate was used over the same period no such transfer was observed.

Deoxycortone acetate too, seems to have different actions when its effects are followed over a prolonged period. While its sodium retention effect is easily demonstrable a few hours after administration, its cold protection effect is demonstrable only twenty-four hours after administration[12]. The same holds for many of its effects on carbohydrate metabolism[13,14].

Spencer[1], in one experiment, estimated the sodium retaining activity of dog adrenal venous serum to be equivalent to 4·0 μg. deoxycortone acetate per ml. Simpson, Tait and Bush[15] demonstrated the presence of a highly active mineralocorticoid in the secretion of the adrenal gland of both dog and monkey. When the mineralocortical activities of all the fractions are added together the dog adrenal is estimated to secrete the equivalent of 20 μg. deoxycortone per minute.

The maximum effect of dog adrenal venous plasma in experiments recorded here, was equivalent to 0·95 μg. deoxycortone acetate per g. of gland per minute. This figure, however, was not significant (Table VI, Dog A). The reason why this figure is so low is, presumably, that the effect of the mineralocorticoids is masked by the action of 11-oxycorticoids in promoting sodium excretion.

Using the eosinophil test Bibile[5] estimated that the output from the dog's adrenal was equivalent to 11·3 μg. of cortisone or of hydrocortisone per g. per minute. The response utilised in this test is obtained with 11-oxycorticoids and not with deoxycortone. The results in Table IV show that the effect of 10 μg. hydrocortisone was not neutralized by 4 μg. deoxycortone acetate. The results therefore suggest that the mineralocorticoid activity of the dog's adrenal plasma was equivalent to more than 4 + 0·95 μg. per g. of gland per minute. This figure is compatible with the results of Simpson and others.

Bush[15] reported that in the rabbit the adrenal cortex secretes predominantly corticosterone. According to the work reported here, corticosterone is less effective than hydrocortisone in antagonising the sodium-retaining effect of deoxycortone acetate. From Bush's figures it also follows that the average concentration of corticosterone in the rabbit is low, namely 3·2 μg. per ml. of adrenal plasma. The rabbit thus seemed to be an animal where the neutralising effect of 11-oxycorticoids against the sodium-retaining action of DOCA-like substances, might cause less interference than in the dog. A significant mineralocortical activity of adrenal venous plasma was indeed found in one of two rabbits and was of the order of 2·7 μg. deoxycortone acetate equivalent per g. adrenal gland per minute.

Summary

1. A qualitative and a quantitative method of estimating mineralocortical activity are reported. Together, they supply a simple routine method of assay.

2. A comparative study of sodium-retaining activity of some pure

corticoids indicated the presence of an antagonism between 11-oxycorticoids and deoxycortone acetate. Quantitative data on this antagonism are reported.

3. Attempts to measure the mineralocortical activity of adrenal venous plasma in the dog and in the rabbit have been made.

It is a pleasure to acknowledge my indebtedness to Dr. M. Vogt for her invaluable and continuous help and guidance and to Professor J. H. Gaddum for his advice and help with the statistical analysis of the data reported.

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THE ASSAY OF HEPARIN

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For many years the potency of heparin has been assayed in these laboratories by a method which originated as a modification of that of Kuizenga, Nelson and Cartland[1] but which has since been so greatly modified that it is now similar to the method suggested by Foster[2,3] and adopted in the U.S.P. XIV.

As in the U.S.P. XIV, the assay consists of comparing by eye the clots produced when sheep plasma is recalcified in the presence of varying amounts of the standard heparin and the heparin under test. There are however, many technical differences between the two methods of assay.

In 1953 Blombäck, Blombäck, Corneliusson and Jorpes[4] published a paper on the reliability of the methods used in the assay of heparin and gave the anticoagulant activity of 20 samples of heparin sodium which had been assayed by four different methods—a fresh whole blood method, a thrombin method on plasma, and the methods of the U.S.P. XIV and the B.P. 1953. They found the U.S.P. XIV method to give 10 to 15 per cent. lower figures than the thrombin method for samples with 25 to 110 I.U. per mg., and sometimes lower figures for commercial samples.

In 1954 Jorpes, Blombäck and Blombäck[5], published the results of further research on the assay of heparin and included an *in vivo* method using sheep. When comparing the new and old Swedish standard heparins against the International Standard heparin, the U.S.P. XIV and the B.P. 1953 methods of assay again gave lower readings than the *in vivo* sheep, the fresh ox blood and the thrombin methods. They considered the U.S.P. XIV method the most difficult to handle and the least reliable.

In view of these statements it was thought necessary to check the reliability of our method and to show that on identical samples it did not give rise to similar discrepant results. Through the courtesy of Professor J. E. Jorpes of the Karolinska Institutet, Stockholm, samples of the two Swedish standard heparins were obtained and assayed against the International Standard heparin and the U.S.P. Heparin Sodium Reference Standard. Table I shows that the results obtained compared with those of Jorpes and others.

TABLE I

STRENGTH OF NEW SWEDISH STANDARD HEPARIN (I) AND THE OLD SWEDISH STANDARD HEPARIN (II) USING DIFFERENT METHODS OF ASSAY

International units per milligram of water-free substance

	In vivo sheep (Jorpes and others)	Fresh ox blood (Jorpes and others)	Thrombin method (Jorpes and others)	U.S.P. XIV (Jorpes and others)	B.P. 1953 (Jorpes and others)	Sheep plasma (Pritchard)
(I)	110	108	107	96	103	111
(II)	80	81	82	67	73	79

These results are in such close agreement with those given by the Swedish workers with their *in vivo* sheep, whole ox blood and thrombin methods that it was thought that a full description of our method of assay would be of value to others.

EXPERIMENTAL

Reagents

Sheep Plasma. Sheep are bled with aseptic precautions by cannula from the jugular vein, 225 ml. blood being taken into 25 ml. normal saline solution containing 3·8 per cent. sodium citrate B.P. The blood is stored at 4° C. for 5 days before the cells are separated by centrifuging for 45 minutes at 2800 r.p.m. and the plasma drawn off aseptically into a sterile bottle. Several sheep have been reserved for this purpose only and three or more are bled at a time as required and the plasma from these bulked. In order to maintain health and the quality of the plasma, the sheep are not bled more often than once every three weeks.

The bulked plasma is distributed into sterile 60 ml. bottles (filled only to the shoulder), capped with overstyle rubber caps held by rubber bands and stored in a cold room at — 10° C. until required. Stored in this manner, it may be used up to 4 or 5 months after the bleeding.

In the test, the bottle containing the solid frozen plasma is placed in a water bath at 37° C. and occasionally shaken until the plasma is completely liquid. This is then filtered through a small piece of cotton wool in a funnel to remove the small amount of coagulum which occasionally is deposited in some samples; those containing heavy deposits are not used. The thawed plasma may be used on the next day if kept overnight at 4° C. and may, if necessary, be diluted with normal saline to obtain a suitable clotting range.

Calcium Chloride Solution. A 2·5 per cent. solution of $CaCl_2 6H_2O$ (= 1·3 per cent. $CaCl_2$) in distilled water is used. The quantity per tube is kept constant at 0·25 ml. for all plasma.

Heparin Standard

The standard used, whether the International Standard, the U.S. Reference Standard or a subsidiary standard, is weighed rapidly and dissolved in sufficient sterilised normal saline solution (containing 0·13 per cent. chlorocresol B.P. as a preservative) to produce a solution containing exactly 10 International Units per ml. Sufficient may be made to last 4–8 weeks provided it is stored at 4° C. and not allowed to become contaminated by micro-organisms. The solution is not filtered or otherwise sterilised and it has been found useful to keep it in the graduated measure in which it is made, replacing the ground glass stopper with an overstyle rubber cap through which the solution is withdrawn by syringe.

Heparin Solutions for Assay

All solutions are diluted with normal saline, or powders dissolved in normal saline and further diluted, if necessary, to produce a solution

which may be expected to contain 10 units per ml. Preliminary tests may be made to determine the approximate potency but the final tests are made on a dilution which should be within 2–3 per cent. of the expected potency.

For samples of good quality a preliminary test using the Toluidine Blue method of MacIntosh[6] will give a reasonable estimate of the potency. Some samples of crude heparin, or heparin which has been degraded give less accurate but useful information by this test.

Apparatus

(1) Racks, wood or metal to hold 10 or more rows of 7 tubes.

(2) Test tubes, 10 mm. internal diameter, 65 mm. long, as uniform as possible.

After use, the tubes are rinsed and kept in a 1·5 per cent. Lissapol N solution overnight. They are then cleaned with a brush and soap, followed by thorough rinsing in tap water and distilled water and are dried in a hot-air dryer.

(3) Rubber stoppers for test tubes. Thoroughly washed in tap water and dried after use.

(4) Good quality accurate 1 ml. tuberculin syringes graduated in 0·01 ml. These are used instead of pipettes or burettes to measure the various solutions. There is no chance of "overshooting" the graduation marks as with a pipette or burette and errors due to variations in "draining time" when measuring such a viscous material as plasma are eliminated. It is essential, however, to test these syringes to ensure that the accuracy is within suitable limits (having an error of not more than 1·0 per cent. when tested at both 0·5 ml. and 1·0 ml.) and that there is no leak past the piston and between the nozzle and needle mount.

(5) Glass stoppered test tubes holding 10 ml. "Exelo" test tubes 100 mm. × 16 mm. with size C14 interchangeable ground glass stoppers are satisfactory.

Performance of Test

A heparin-plasma mixture is prepared for the standard and each of the unknown samples by placing 0·7 ml. of the standard heparin (10 units per ml.) and 0·7 ml. of each batch of heparin to be tested (diluted to contain an estimated 10 units per ml.) into a series of suitably labelled glass stoppered tubes. 6·3 ml. of plasma is added to each tube and the contents mixed thoroughly by inversion. This amount is sufficient for two identical rows of the standard and of each batch to be tested. If only preliminary tests are needed 0·4 ml. heparin and 3·6 ml. plasma is sufficient for one row. The following amounts of plasma are delivered into each row of the tubes in the rack: 0·76, 0·71, 0·65, 0·58, 0·50, 0·40 and 0·28 ml. The following amounts of the standard heparin-plasma mixture are placed in the first two rows of tubes: 0·24, 0·29, 0·35, 0·42, 0·50, 0·60 and 0·72 ml. The subsequent pairs of rows are filled in the same manner with similar doses of the heparin-plasma mixtures of each batch to be tested or in the case of preliminary tests a single row is used.

Not earlier than 20 minutes after the original heparin-plasma mixture is made 0·25 ml. calcium chloride solution is added to each tube which is then stoppered with a rubber stopper and the contents mixed by inverting 7–8 times making sure that the whole of the inside surface of the tube is wetted by the solution. The tubes are then allowed to stand overnight at room temperature.

It has been found that the most convenient order of adding the reagents to the tubes is to work along the columns from front to back with the plasma, along the rows from right to left with the heparin-plasma mixtures, and again from front to back with the calcium chloride solution.

Reading the results

The amount of clotting is estimated by eye using an arbitrary scale in terms of symbols which are later converted to numerical values and plotted as a curve. It has been found in practice (*cf.* Foster[2]) that it is easier to read the degree of clotting as trace, slight, ±, + or + + than to visualise it in figures such as 2, 3, 4, 6, and 10, or as a percentage. The examination of the tubes is best carried out by looking through the tubes which are held above eye level against a north light window. Tubes having clots of a value of "trace" (numerical value 2) or less can be shaken to detach the clot from the tube to make the comparison of the amount of clot easier.

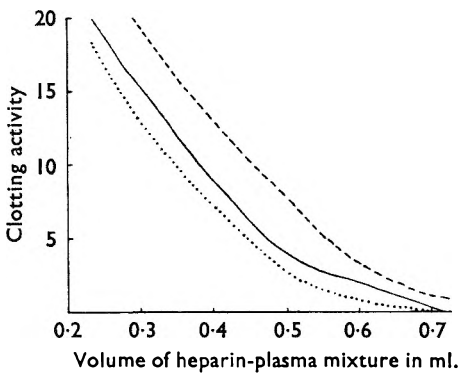

FIG. 1 Curves correlating clotting activity with heparin dosage.
—— International standard.
..... P6842. – – – P6853.

The first to be read are the standard tubes and having estimated the degree of clotting in each tube this is converted to its numerical value. The sum of the values of each pair of tubes containing the same dose of heparin-plasma mixture is used for the plotting of the graph, correlating the dose of heparin-plasma mixture and the clotting value, which is usually a smooth curve.

TABLE II

CLOTTING VALUES

Symbol	Numerical value	Appearance of clot
+ +	10	Clear, transparent, yellowish solid clot.
+ (+)	8	Transparent, yellowish solid clot with slightly granular appearance.
+	6	Opaque, granular, whitish solid clot.
±	4	Pale translucent clot filling whole tube. Slightly more opaque in upper portion.
S (slight)	3	Pale translucent clot extending to ¾ or over of tube. Nearly clear below.
tr (trace)	2	Thin but opaque clot extending ¼ to ½ of tube. Clear below.
?	1	Transparent ring of clot at surface of fluid in top quarter of tube.
—	0	No clot.

Having finally determined the degree of clotting in the standard tubes, the two rows of tubes for each product to be tested are examined and the degree of clotting in each tube directly compared with the clots in the standard tubes. In most instances the two rows of tubes for any one batch, standard or test, will be identical or very nearly so. Where tests are done on one row only, the numerical value of each tube is doubled.

Table II is intended as a guide to evaluate the degrees of clotting with the symbols and numerical values allotted to them. With practice it is easy to read intermediate values between those given in this Table.

Calculation of Results

From the plotted curve of the standard (see Fig. 1), the amount of heparin required to give clotting values of 12½, 10, 7½, 5 and 3 are obtained.

TABLE III

READINGS AND CALCULATIONS FOR A COMPLETE TEST

Row No.	Batch	Primary dilution in normal saline	Dilution in plasma	0·24 ml.	0·29 ml.	0·35 ml.	0·42 ml.	0·50 ml.	0·60 ml.	0·72 ml.
8	P6853	1/20	0·4/4	++	++	+(+)	+	±	?/tr.	−/?
7	P6842	1/21	0·4/4	+(+)/++	+/+(+)	±/+	S	?/tr.	−/?	−
6	P6019	1/11	0·7/7	++	+ (+)	+	±	tr.	?	−
5				++	+ (+)	+	±	?/tr.	?	−
4	P6018	1/8	0·7/7	++	+ (+)	+	±	>tr.	?	−
3				++	+ (+)	+	±	>tr.	?	−
2	I.S.	10 u./ml.	0·7/7	++	+ (+)	+	±	tr.	?	−
1				++	+ (+)	+	±	tr.	?	−
	Numerical values		P6853	20	20	16	12	8	3	1
			P6842	18	14	10	6	3	1	0
			P6019	20	16	12	8	3½	2	0
			P6018	20	16	12	8	4½	2½	0
			I.S.	20	16	12	8	4	2	0

Volumes of heparin-plasma mixtures required to produce similar clots at different clotting levels

Clotting level	International Standard	Batch P6018	Batch P6019	Batch P6842	Batch P6853
12·5	0·34	0·34	0·34	0·31	0·41
10·0	0·385	0·385	0·385	0·35	0·46
7·5	0·43	0·43	0·43	0·39	0·51
5·0	0·475	0·485	0·47	0·44	0·555
3·0	0·54	0·565	0·52	0·50	0·60

Potency as percentage activity of International Standard at different clotting levels

Clotting level	International Standard	Batch P6018	Batch P6019	Batch P6842	Batch P6853
12·5	100	100	100	109·5	83
10·0	100	100	100	110	83·5
7·5	100	100	100	110	84·5
5·0	100	98	101	108	85·5
3·0	100	95·5	104	108	90
Average ..		98·7	101	109·1	85·3

P6018. A 1 mg./ml. solution of the old Swedish Standard heparin
A 1/8 dilution contains 98·7 per cent. of 10 u. = 9·87 units.
P6018 contains 79 units/ml.

P6019. A 1 mg./ml. solution of the new Swedish Standard heparin
A 1/11 dilution contains 101 per cent. of 10 u. = 10·10 units.
P6019 contains 111 units/ml.

P6842. Production control sample
A 1/21 dilution contains 109·1 per cent. of 10 u. = 10·91 units
P6482 contains 229 units/ml.

P6853. Production control sample
A 1/20 dilution contains 85·3 per cent. of 10 u. = 8·53 units
P6853 contains 170 units/ml.

From the curves of each test sample the amount required to give clotting values over the same range are obtained and a percentage activity of the sample obtained for each value. The average of the five readings is taken as the percentage potency of the diluted sample compared to 10 units per ml., and the unitage of the undiluted sample calculated from this figure. The readings and calculations for a complete test are given in Table III.

Selection and Adjustment of Plasma

For most satisfactory results a clot with the value of $\pm$ (4), should be produced in the tube containing 0·42 ml. of the standard heparin-plasma mixture. This can be ensured by adjusting the amount of heparin contained in the 7 ml. heparin-plasma mixture, using proportionately more or less heparin according to the results given in a preliminary test. An alternative method, where the clots are very opaque and for ease in calculation, is to maintain the heparin-plasma mixture at 0·7 ml./7·0 ml. and to adjust the plasma by diluting with normal saline.

Discussion

It will be seen that the method of assay described above differs in many respects from that of the U.S.P. XIV. Whereas the latter requires the test to be read exactly one hour after recalcification of the plasma, in the above test, clotting is allowed to proceed for approximately 16 hours, after which time the amount of clot produced has nearly reached a maximum and thus does not change even if left standing for several hours longer. The degree of clotting in the tubes increases greatly between the 1 hour period and a period of approximately 16 hours, and it has been found that tests read at these two times do not give concordant results.

By allowing the reaction to proceed to near completion it is not necessary to select only those plasma which when recalcified form a solid clot within 5 minutes. In practice very few batches of plasma are found to be entirely unsuitable for use, and these are rejected not on the speed with which they clot but because the various grades of clot are not easily differentiated.

The use of plasma obtained by careful bleeding from the jugular vein minimises its contamination by tissue juices. Storage of the blood at 4° C. for 5 days before separating the plasma in the centrifuge has resulted in a very satisfactory plasma for this test, requiring slightly more heparin per unit volume to inhibit clotting than plasma separated immediately after bleeding.

Variations in the amount of calcium chloride used in the recalcification do tend to affect the degree of clotting produced in the tubes but complete tests carried out using amounts of calcium chloride 25 per cent. above and below the suggested amount gave results showing that the accuracy of the test is not dependent on the use of a critical amount of calcium chloride and that preliminary recalcification tests on the plasma are not necessary. Other differences in technique have been made with the object of reducing the time and labour involved in putting up the test without reducing its accuracy.

It is satisfactory to know that Professor Jorpes[7] is prepared to accept the results of this method of assay although he has on several occasions criticised the U.S.P. method.

The advantages of the method are that—

(1) It is reliable and accurate. With samples of commercial grade heparin the fiducial limits of error (P = 0·95) of a single assay are ± 1·7 per cent. and for the mean of a duplicate assay ± 1·2 per cent.

(2) It is easy to perform. After a very short period of training most technicians can carry out the assay and obtain results very close to those of more highly experienced workers.

(3) It allows a direct comparison between standard and sample at various clotting levels—essential for accuracy and an advantage in the detection of abnormal heparins.

(4) As the clotting is allowed to proceed to near completion there is no urgency in reading the results which can be done by several observers and their findings correlated.

(5) The plasma can be stored for several months and is thus immediately available when a test is required.

(6) The amount of calcium chloride solution necessary for recalcification of the plasma is not critical and need not be adjusted for individual batches of plasma.

Summary

1. A method is described for the assay of heparin consisting essentially in the comparison by eye of the clots produced in approximately 16 hours when sheep plasma is recalcified in the presence of varying amounts of Standard Heparin and the heparin under test.

2. The results obtained are believed to be accurate and reliable and the technique of the assay reasonably simple.

Thanks are due to Mr. R. A. Taggart for the statistical analysis of the results.

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THE ANTICOAGULANT ACTIVITY OF DEXTRAN SULPHATE

I. AN *in vitro* COMPARISON BETWEEN THE ACTIONS OF DEXTRAN SULPHATE AND HEPARIN ON THE VARIOUS STAGES OF BLOOD COAGULATION

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THE anticoagulant properties of synthetic polysaccharide sulphuric acid esters were first reported by Bergström[1,2]. The sodium salt of dextran sulphate was studied by Ricketts and Walton[3], who found that dextran sulphates of low molecular weight were no more toxic than heparin, and that the greatest anticoagulant effect was attained when the number of sulphate groups exceeded 1·3 per glucose unit. Ricketts[4] also found that a chain length of 5 to 20 glucose units was most suitable for therapeutic use.

A clinical trial with two samples of dextran sulphate having these characteristics has been reported by Ricketts, Walton, van Leuven, Birkbeck, Brown, Kennedy and Burt[5]. The dosage was based on that of heparin, the anticoagulant activity of the dextran sulphate having been assayed against heparin by a method employing the clotting time of recalcified, citrated sheep plasma[6]. This assumed, of course, that the anticoagulant actions of dextran sulphate and heparin were fundamentally similar, but a comparison of results from various centres[7] revealed interesting differences between the relative anticoagulant potencies of the two drugs when assayed by various methods. A comparative study of the anticoagulant properties of heparin and dextran sulphate was therefore undertaken.

EXPERIMENTAL

Materials

Dextran Sulphate. An Injection of dextran sulphate prepared for the clinical trial[5] (manufacturer's number, 52DSO39) and the British Standard dextran sulphate (Author's Preparation I_4[4]) were both tested. The preparations behaved similarly; the Injection was assayed against the British Standard by the one-stage prothrombin time test and subsequent results are expressed in absolute concentrations of the latter; in each experiment an indication is given of which preparation was used.

Based on overall anticoagulant activity, numerous assays against International Standard heparin by various methods in 11 laboratories yielded a mean estimate of heparin potency for the British Standard of 25 u./mg.[7] which is about one-fifth of the potency of International Standard heparin (130 u./mg.[8]). Thus, in the following charts, a comparison between the two drugs on the basis of overall anticoagulant activity (clinical unitage) may be obtained by imagining the position of

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the dextran sulphate curves to be moved to the left on the drug concentration axis through a five-fold interval.

Heparin. The majority of the experiments were made with International Standard heparin, although in some, another powder heparin or a commercial, clinical solution were used. The three samples behaved similarly in the tests in which they were compared, and the results are presented as absolute concentrations of the International Standard, by using conversion factors obtained from an assay of the "powder" (one-stage prothrombin time test) and from the labelled potency of the commercial preparation. In each case it is stated which sample was used.

Thrombin and Fibrinogen. Lyophyllised human thrombin and fibrinogen were obtained from the Lister Institute and dissolved in 0·85 per cent. (w/v) sodium chloride solution ("saline") as directed on the ampoules. The potencies of the thrombin solutions were calculated from the labelled unitages. Veronal buffer[9] was added to the fibrinogen solutions because this was found to prevent precipitation when the solutions were repeatedly frozen and thawed.

Blood and Plasma. Human blood was obtained from healthy adult subjects or, in a few cases, from hospital patients not acutely ill. Nine volumes of blood were mixed with one volume of 3·8 per cent. (w/v) trisodium citrate (anhyd.) to obtain *citrated whole blood*: *citrated plasma* was obtained from this by centrifugation sufficient, unless otherwise stated, to remove the majority of the platelets.

Methods

Experiments were designed to compare the anticoagulant activities of heparin and dextran sulphate on various phases of the coagulation mechanism, and techniques were selected for testing limited portions of the clotting sequence. In presenting the results, it is convenient to consider the processes of clotting in the reverse order to that in which they naturally occur, because this allows the simpler tests to be considered first.

In the one-stage tests, replicate readings (usually 2 or 4) were made in a symmetrical sequence to eliminate bias due to progressive changes in any of the reagents[10]. With the two-stage tests, this plan could not always be followed but the control runs were repeated towards the end of those experiments in which changes in reagents might have produced the observed effects in test runs, and the important runs were repeated on another occasion before the results were accepted.

The reagents were kept in an ice bath until immediately before each test, and, with one exception to be noted, the clotting times were measured in a water bath at *ca.* 37° C. The anticoagulants, usually in saline, or occasionally in calcium chloride solution (when this was required in the system), were added to the reactions immediately beforehand.

Part 1: *The Reaction between Thrombin and Fibrinogen*

(i) *The thrombin clotting time of citrated plasma*[11]

Figure 1 shows the responses obtained with thrombin over a range of concentrations of the two drugs in citrated plasma. The observable

range of responses was passed through in rather less than a ten-fold range of heparin concentrations, but the same change in response occupied approximately a ten-thousand-fold range of dextran sulphate concentrations, only a part of which is shown. (Figure 1 suggests that at lower concentrations the response curve would cross, but in another experiment which tested the minimal interfering concentrations of both drugs, this was not observed: the dextran sulphate curve reached the control clotting time at an absolute concentration of about five times that required for the minimal effect of heparin.) The difference between the slopes of the response curves was somewhat diminished if the thrombin concentration was reduced but was clearly observable throughout the useful working range.

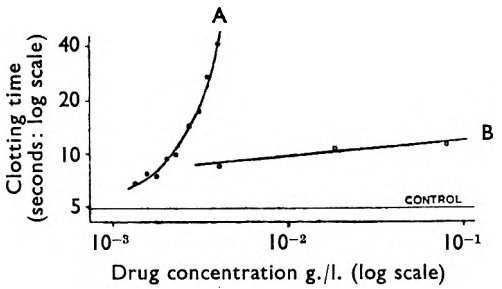

FIG. 1. *Part* 1: *The reaction between thrombin and fibrinogen.* The thrombin clotting time of citrated plasma. Final concentration of thrombin *ca.* 12 u./ml.; mean saline control clotting time, 4·9 sec. "Powder" heparin: British Standard dextran sulphate.

A. heparin; B. dextran sulphate. Each point is the mean of 4 readings.

(ii) *Interaction with a co-factor*

In a number of experiments, increasing concentrations of plasma (or serum) were added to clotting systems containing fixed concentrations of purified thrombin and fibrinogen and various concentrations of dextran sulphate or heparin.

Three differences between heparin and dextran sulphate were found in these experiments. First, the drugs differed in the minimal concentration of plasma required to prolong the clotting time of the purified system; with dextran sulphate, the clotting time did not lengthen until the concentration of plasma was raised to two to four times that required by a concentration of heparin giving a comparable clotting time in concentrated plasma. Occasionally, the higher plasma concentrations produced a negligible increase of clotting time with dextran sulphate. Second, in the effective (upper) range of plasma concentrations, to obtain a given increase in anticoagulant effect, a far greater increase (in Figure 2, approximately 12-fold) in concentration was required with dextran sulphate than with heparin: this clearly reflects the finding of the previous experiment. These two effects are shown in the data plotted in Figure 2.

In addition, dextran sulphate inconstantly lengthened the clotting times obtained with the lower plasma concentrations (peak at about 1 per cent. of plasma). This effect was not seen with heparin.

Additive Effect of Dextran Sulphate and Heparin. Figure 3 shows the results of testing both separately and together similar absolute concentrations of the two drugs. While this concentration of dextran sulphate

alone appeared not to interact with the co-factor, it clearly potentiated the interaction between heparin and the co-factor.

These experiments suggested that dextran sulphate reacted weakly with a co-factor similar to that required for heparin and did not block the reaction between heparin and its co-factor.

(iii) *Interaction with progressive antithrombin*

(*cf.* Antithrombin III in the classification of Seegers[12].)

Citrated plasma was defibrinated by the addition of a small quantity of thrombin. Observations were then made of the rate of decay of a larger quantity of thrombin, subsequently added[13] in the presence of various concentrations of heparin or dextran sulphate. The lowest concentration of heparin causing a definite increase in the decay rate appeared to be about 4×10^{-4} g./l., whereas with dextran sulphate about 8×10^{-3} g./l. appeared to be required to give a like effect. (These tests were made at 15° C. to obtain a slower reaction.)

These results suggest that heparin delays the clotting of plasma by thrombin partly by interfering directly with the thrombin-fibrinogen reaction, and partly by accelerating the destruction of thrombin by progressive antithrombin. The actions of dextran sulphate appear to be similar but very much weaker.

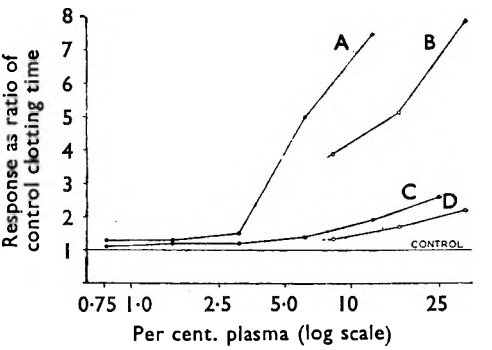

FIG. 2. *Part* 1: *Interaction with a co-factor.* The clotting times of mixtures of purified thrombin and fibrinogen with varying concentrations of plasma, and of heparin or dextran sulphate. For each drug, the thrombin concentration was constant. Control clotting times: for heparin, 6·6–8·7 sec.; for dextran sulphate, 6·3–6·8 sec. (there was some variation in control clotting times at different plasma concentrations, and the response ratios were calculated from the control clotting times obtained at the corresponding plasma concentrations). International Standard heparin: Injection of dextran sulphate. Each point is the mean of 2 readings.

A. Heparin $3{\cdot}0 \times 10^{-3}$ g./l.
B. Dextran sulphate 2·2 g./l.
C. Heparin $1{\cdot}5 \times 10^{-3}$ g./l.
D. Dextran sulphate $9{\cdot}0 \times 10^{-2}$ g./l.

Part 2: *The Conversion of Prothrombin to Thrombin*

(i) *In spontaneous coagulation*

Using the Thrombin Generation Test of Macfarlane and Biggs[14], curves were obtained showing the liberation and decay of thrombin after the recalcification of citrated, whole blood, with and without various concentrations of the anticoagulants. Blood samples from 22 subjects were each tested with both drugs and the results obtained are shown in Figures 4 and 5, with the controls obtained on the same samples. The drug curves have been corrected for the effects of the anticoagulants upon the fibrinogen used as indicator of thrombin concentration, by repeating the

tests on further portions of the same samples with corresponding concentrations of the drugs in the fibrinogen instead of in the clotting blood. For each drug concentration, the mean difference between the clotting times of the control curve (no drug) and of the curve obtained with the drug in the indicator, was then added to each reading obtained in the corresponding drug curve. For each sample of citrated blood the five runs were obtained in a random order. Each citrated sample was kept in a chilled, silicone-coated container until required for each run (longest storage, about 4 hours).

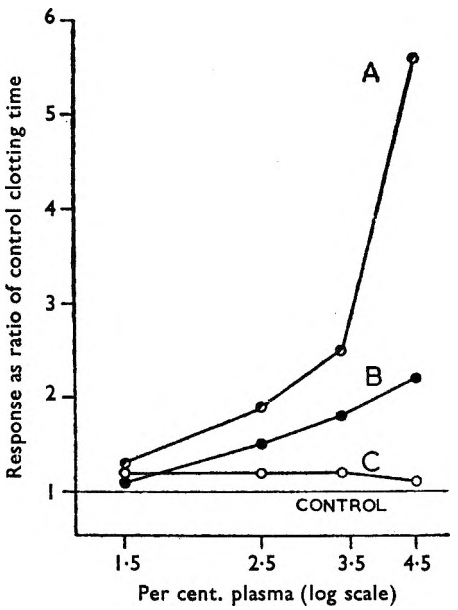

FIG. 3. *Part* 1 : *Interaction with a co-factor.* The clotting times of mixtures similar to those of Figure 2, with the same thrombin concentration throughout. Control clotting times 14·7–15·4 sec. International Standard heparin; British Standard dextran sulphate. Each point is the mean of 4 readings.

A. Heparin + dextran sulphate
B. Heparin $4{\cdot}6 \times 10^{-4}$ g./l.
C. Dextran sulphate $5{\cdot}7 \times 10^{-4}$ g./l.

The series of curves with heparin was observed up to the point of extinction because a concentration of the drug sufficient to delay the liberation of thrombin beyond about 30 minutes proved also sufficient to depress the quantity liberated below the limit of observation. With dextran sulphate, on the other hand, a delay as great as about 45 minutes was readily observed, because even under these conditions there was practically no diminution in the quantity of thrombin formed. Indeed, the series only stops at this point because the stock batch of fibrinogen was then exhausted and subsequent samples differed in their reaction to thrombin and would therefore not have given comparable results. In any event it is clear that the quantity of thrombin appearing in the system is far less affected by dextran sulphate than by heparin.

(ii) *In the two-stage prothrombin time test*

Figure 6 shows the results of a two-stage prothrombin time test with the highest concentrations of heparin and dextran sulphate which gave workable clotting times in the thrombin indicator. In each case, these concentrations were sufficient definitely to prolong the one-stage prothrombin time, although the heparin effect was the greater. In calibrating the indicator with known thrombin concentrations, allowance was made for the inhibitory effects of the anticoagulants.

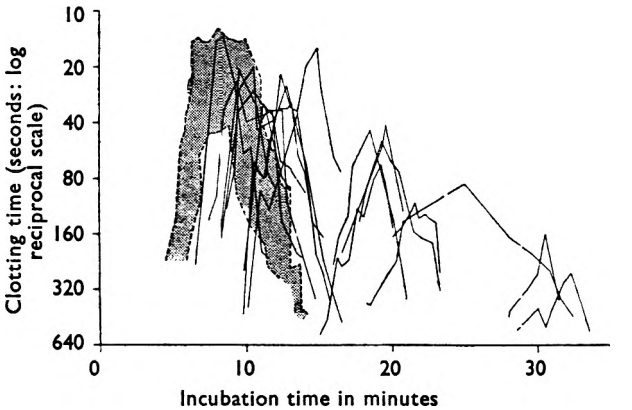

FIG. 4. *Part* 2: *Thrombin generation tests with various concentrations of heparin* (the concentrations are given in Figure 8): citrated whole blood. Time-scale from recalcification. The reciprocal clotting time-scale is proportional to thrombin concentration: this is plotted logarithmically to simplify the shape of the curves. The control curves obtained from the same blood samples fell within the hatched area. International Standard heparin. Each curve shows a single series of readings.

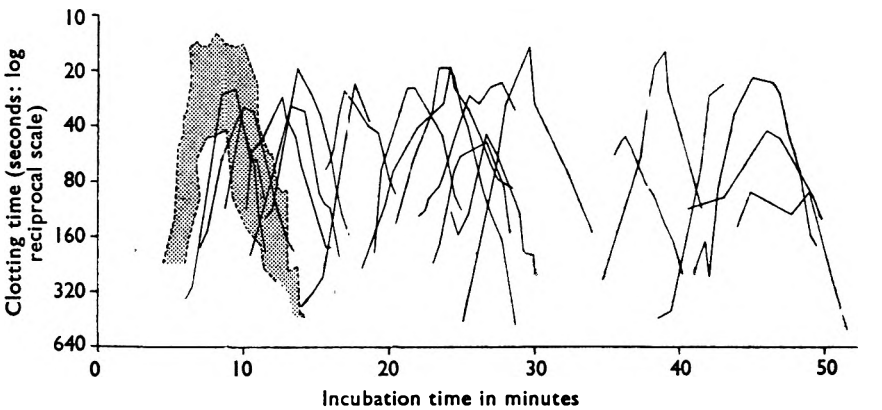

FIG. 5. *Part* 2: *Thrombin generation tests with various concentrations of dextran sulphate* (the concentrations are given in Figure 8). Plotted as Figure 4. Injection of dextran sulphate.

With dextran sulphate, the point of extinction was reached in the indicator reaction at lower concentrations than in the main reaction, but interference with thrombin liberation was not observed up to this point. As Figure 6 shows, the results with heparin followed closely the family of curves published by Biggs and Douglas[15]. In further tests with a different batch of brain extract, the quantity of thrombin liberated passed from the control level to extinction within a heparin range of approximately 8×10^{-5} to 8×10^{-4} g./l.; with dextran sulphate, a delay in the appearance of thrombin was observed in an experiment testing $3{\cdot}2 \times 10^{-3}$ g./l., but even in this case there was little reduction in the quantity of thrombin which appeared.

These results were thus very similar to those obtained with the thrombin generation test, and supported the finding that dextran sulphate did not interfere with prothrombin conversion over the tested range.

Part 3: *The Development of Blood Thromboplastin*

Biggs, Douglas and Macfarlane[16] showed that a powerful thromboplastin developed in clotting blood, and that it could be prepared from a reaction mixture composed of platelets and serum together with plasma which had been freed of prothrombin by treatment with alumina. The active constituents of this mixture were shown to be antihæmophilic globulin and factor V (supplied by the treated plasma), Christmas factor and factor VII (present in the serum), platelet factor(s), and calcium. The effect of heparin upon the development of blood thromboplastin was also studied, and guided by this work[17], the effects of heparin and dextran sulphate were compared in the following experiments.

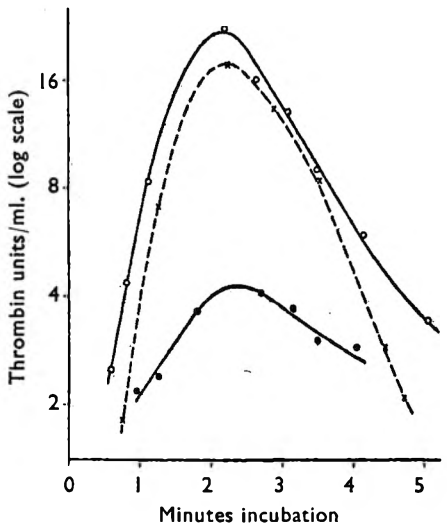

FIG. 6. *Part 2: "Two-stage" prothrombin time test*, with highest workable concentrations of heparin (2×10^{-3} g./l.: clinical solution, solid circles) and dextran sulphate (8×10^{-3} g./l.: Injection, open circles) compared with saline control (broken line).

(i) *Relative overall effects of the two drugs*

(*a*) *In spontaneous coagulation.* Accepting the proposition of its originators[14] that the thrombin generation test may broadly be interpreted in terms of the generation of blood thromboplastin, information on the early stages of coagulation may be obtained from Figures 4 and 5. The data for both drugs have been summarised in two ways in Figures 7 and 8.

In Figure 7, the shortest observed clotting time (corrected, as explained above) in each of the thrombin generation tests have been plotted against the times at which they occurred. This presentation therefore shows in one diagram the main information to be obtained from Figures 4 and 5, namely that heparin reduces the quantity of thrombin liberated in proportion as the appearance of thrombin is delayed, whereas dextran sulphate merely delays the appearance of thrombin over the range of drug concentrations that were tested.

In Figure 8, the times of observing the shortest clotting times are plotted against the corresponding drug concentrations. The points from the two drugs appear to fall on broadly parallel regressions separated by an approximately four-fold dose interval. This agrees well with the

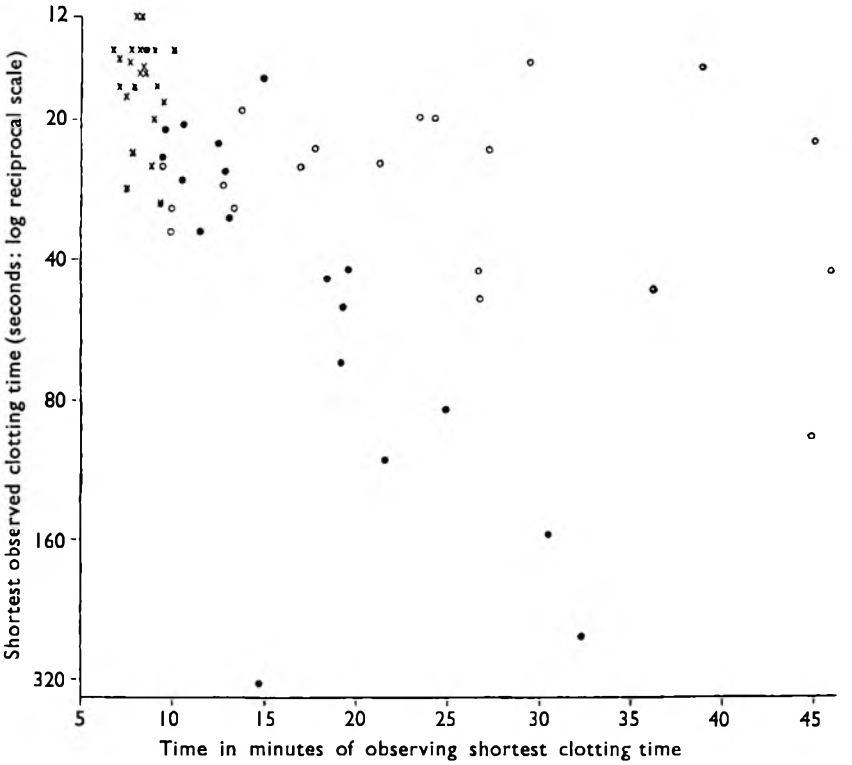

FIG. 7. *Part* 3: *Development of blood thromboplastin.* The data of Figures 4 and 5 replotted as indicated on the co-ordinates. Solid circles represent the heparin curves, open circles the dextran sulphate curves, and crosses the saline control curves.

mean potency ratio of approximately five-fold derived from the assays previously mentioned[7]. Figures 1, 7 and 8, taken together, strongly suggest that the anticoagulant effect of dextran sulphate on spontaneous coagulation chiefly depends on interfering with the development of blood thromboplastin.

(*b*) *In the generation of blood thromboplastin from active constituents.* Figure 9 shows the results of an experiment in which thromboplastin generation tests were carried out in the presence of various concentrations of the two drugs. The measurement of thromboplastin production is derived from the area under the graph by which the results of this test are ordinarily studied: this method of summarising the results gives expression to variations both in the rate of thromboplastin generation and in the final titre of activity which is developed[18]. As in the other two-stage tests, the effects of the anticoagulants were allowed for when the calibration curves for these experiments were prepared.

The figure shows a linear relationship between thromboplastin production and the logarithmic concentrations of both drugs. The difference between the positions of the curves approximately corresponds to the five-fold difference in overall anticoagulant activity previously mentioned,

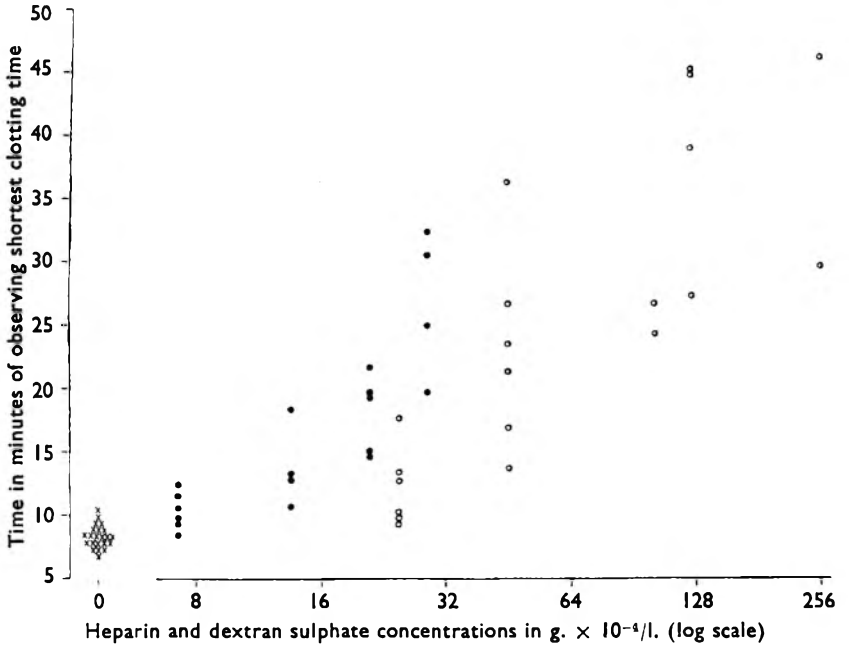

FIG. 8. *Part* 3: *Development of blood thromboplastin.* The data of Figures 4 and 5 replotted as indicated on the co-ordinates. Solid circles represent the heparin curves, open circles the dextran sulphate curves, and crosses the saline control curves.

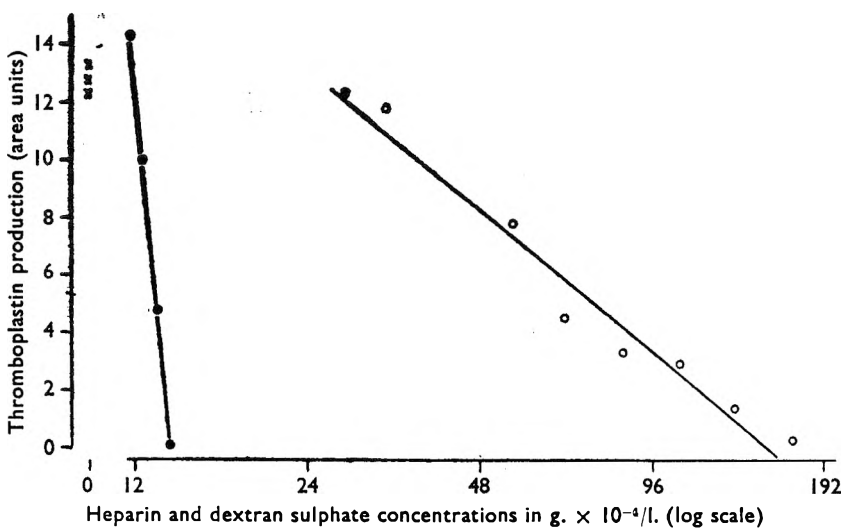

FIG. 9. *Part* 3: *Thromboplastin Generation Tests*, summarised as described in the text. Alumina-treated plasma[16], 4 per cent.; serum (not previously diluted), 2·5 per cent., buffered with amino*tris*(hydroxymethyl)methane 0·02 M, pH 7·3[26]. Heparin, International Standard, solid circles; dextran sulphate, British Standard, open circles; three control curves are represented by crosses.

but this adjustment does not, of course, eliminate the difference between the slopes of the curves, which contrasts strongly with the similarity between the corresponding slopes of Figure 8. However, reference to Figures 4 and 5 shows that with one exception there is little widening of the thrombin curves as either series passes to the right, which suggests that over the tested range there was no demonstrable alteration in the activity of the thromboplastin which was formed, but only a delay in its generation (*cf.* Figures 5c and 8 of Biggs and Douglas[15]; and Figure 1B of Ingram[19]). Thus, from Figure 8 it may be inferred that in blood clotting spontaneously, these drugs have a similar activity in delaying the generation of blood thromboplastin (at any rate in the low concentrations tested); but Figure 9 suggests that the quantity of thromboplastin formed is less affected by dextran sulphate than by heparin.

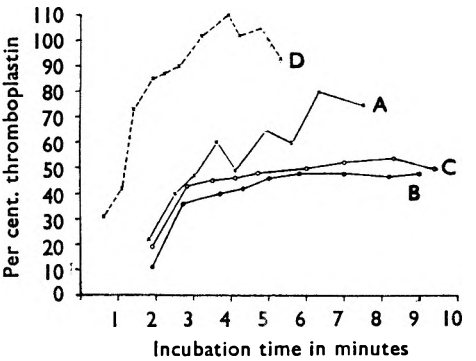

FIG. 10. *Part* 3: *Thromboplastin generation tests*, with platelets, alumina-treated plasma[16], serum and calcium chloride solution, buffered with amino*tris*(hydroxymethyl)methane 0·02 M, pH 7·3[25].

A. control curve
B. with $1{\cdot}06 \times 10^{-3}$ g./l. International Standard heparin.
C. with $1{\cdot}80 \times 10^{-3}$ g /l. British Standard dextran sulphate.
D. alumina-plasma, serum and calcium chloride preincubated for 5 min. before adding the platelets (at 0 min.).

(ii) *Relative effects on Reactions not involving Platelets*

When studying preliminary reactions leading to the generation of thromboplastin, Biggs, Douglas and Macfarlane[20] developed the technique of preincubating calcified mixtures of some of the reagents to which the remainder were added after an interval. The curve of the thromboplastin that was then generated was compared with the curve obtained when all the reagents were mixed at the same time, and it was found that preincubation of certain mixtures lead to a more rapid liberation of thromboplastin when the remaining reagents were added. In these cases it was supposed that preliminary, time-consuming reactions had taken place between the components which were preincubated together, so that when the thromboplastin system was completed, the usual latent interval was reduced. This technique was used in some of the following experiments.

In Figure 10 is shown the curve of thromboplastin generation obtained in the ordinary way from a mixture of alumina-treated plasma[16] (4 per cent.), platelets, serum (2·5 per cent., not previously diluted) and calcium chloride solution (the control curve). Concentrations of the two drugs were chosen which produced a similar and relatively small inhibition: these curves fall together somewhat below the control curve. The Figure

also includes the curve obtained on adding the platelets to a mixture of the other components which had been preincubated (without drugs) for 5 minutes. These four curves are each to be regarded as controls of different sorts for those of the following figure. Figure 11 shows four curves which were all obtained in the same way as the preincubation curve of Figure 10 except that the chosen concentration of each drug was added either at the beginning of the preincubation or with the platelets.

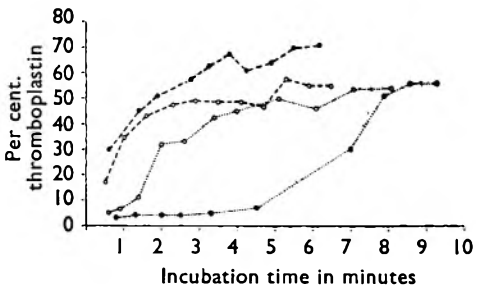

FIG. 11. *Part* 3: *Thromboplastin generation tests*, with alumina-plasma, serum and calcium chloride preincubated for 5 min. before adding platelets, and buffered with amino*tris*(hydroxymethyl)methane, 0·022 M, pH 7·3[25].

●·····● Heparin, $1{\cdot}16 \times 10^{-3}$ g./l. in the preincubated mixture.

○·····○ Dextran sulphate, $1{\cdot}97 \times 10^{-3}$ g./l. in the preincubated mixture.

The addition of platelets reduced these drug concentrations to 1·06 and $1{\cdot}80 \times 10^{-3}$ g./l. respectively, and the buffer concentration to 0·02 M (see Fig. 10).

●----● Heparin added with the platelets to a concentration of $1{\cdot}06 \times 10^{-3}$ g./l.

○----○ Dextran sulphate added with the platelets to a concentration of $1{\cdot}80 \times 10^{-3}$ g./l.

Taking, in Figure 11, the pair of curves shown in stipple (which represent the interference of the drugs with the reaction(s) of the preincubated mixture) the results suggested that, in the selected concentrations, heparin had by far the greater activity against reaction(s) not involving platelets. The other pair of curves show a much smaller difference.

As a simple rider to this experiment, clotting times were measured after recalcifying various mixtures of high spun and low spun citrated plasma (i.e. to obtain various platelet concentrations) containing different concentrations of the two drugs. One series of results with two platelet concentrations is illustrated in Figure 12. Each pair of points spans the same logarithmic interval of drug concentration, but it was necessary to stagger the pairs of heparin concentrations in order to keep the clotting times within the useful working range. For each drug, the curves from the different platelet concentrations are approximately parallel, but the heparin curves are separated by a greater vertical interval (about 0·035 reciprocal units, on the right hand scale) than those obtained with dextran sulphate (about 0·02 reciprocal units). In other words, a given reduction in the platelet count produces a greater prolongation of the clotting time in the presence of heparin than dextran sulphate. Now, the clotting time of recalcified, citrated plasma alone is relatively little affected by variations in the platelet count, provided that the plasma is allowed adequate contact with a "foreign" surface[21]. From this it may be inferred that reactions between factors other than platelets can to some extent replace platelet reactions in spontaneous clotting. The findings of Figure 12

thus suggest that dextran sulphate interferes less than heparin with reactions between non-platelet factors which confirms the inference drawn from Figures 10 and 11. Heparin has been shown to interfere more strongly with the thromboplastin generation test when fewer platelets are added to the reaction.[22].

(iii) *Effect of Dextran Sulphate on the reaction between Christmas Factor and Calcium*

Bergsagel[23] found that decalcified serum was less efficient in the thromboplastin generation test than untreated serum, and concluded that in serum, Christmas factor was combined with calcium; it appeared that a reaction between Christmas factor and calcium occurred at a preliminary stage in thromboplastin generation.

The effect of dextran sulphate upon this reaction was investigated as follows:—

(1) A concentration of dextran sulphate was determined which was just insufficient to inhibit the thromboplastin generation system with untreated serum (alumina-treated plasma, 4 per cent.; serum, not previously diluted, 2·5 per cent.; British Standard dextran sulphate, $4{\cdot}0 \times 10^{-4}$ g./l.). When the tests were repeated with serum which had been decalcified by dialysis, this concentration of dextran sulphate produced a definite inhibition of thromboplastin generation.

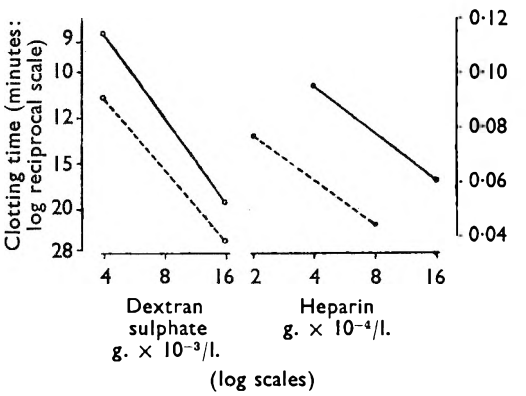

FIG. 12. *Part* 3: *Clotting times of recalcified citrated plasma.* Unbroken lines, platelets 170,000 per mm³. Broken lines, platelets 500,000 per mm³. International Standard heparin; British Standard dextran sulphate.

(2) Dialysed serum, diluted overnight with saline, was incubated with calcium chloride solution and successive thromboplastin generation tests

TABLE I

REACTION BETWEEN CHRISTMAS FACTOR AND CALCIUM

Rates of activation of decalcified serum while being incubated with calcium chloride solution.

Dextran sulphate g./l.		Rate of Activation, per cent. per min. (replicates)		D-S-rate as per cent. of C-rate	
		Control (C)	Dextran sulphate (D-S)	Replicates	Mean
$4{\cdot}0 \times 10^{-4}$	1	4·1	1·9	46	61
	2	4·3	3·2	74	
	3	6·9	4·3	62	
$2{\cdot}0 \times 10^{-4}$		1·8	1·6, 2·5	89, 139	114

The method of computing the rate of activation is described in the text.

were made with subsamples of the incubating mixture. Thromboplastin generation became progressively more rapid until after about 40 minutes the results approximated to those obtained with previously diluted but undialysed serum. The time was noted at which each thromboplastin curve reached an indicator clotting time of 25 seconds, and as the serum was incubated, this clotting time was reached progressively more quickly. The log. of the time taken to reach 25 seconds was then plotted against the length of incubation of the serum, and the slope of this regression was expressed as per cent. increase in activity per minute incubation. Rates were also obtained with two concentrations of dextran sulphate and the results (Table I) suggested that the higher concentration ($4{\cdot}0 \times 10^{-4}$ g./l.) diminished the rate of reaction between Christmas factor and calcium. At half that concentration there was no apparent effect.

As the magnitudes of the effects were small, a comparison with heparin was not attempted.

These results suggest that the reaction between Christmas factor and calcium is rather more sensitive to dextran sulphate than is the generation of thromboplastin from active constituents. This goes some way towards explaining the discrepancy between the findings of Figures 8 (thromboplastin generation in whole blood) and 9 (thromboplastin generation from active constituents). Figure 9 showed a much greater difference between the minimal interfering concentrations of the two drugs. It may be that the activity of the two drugs is most similar upon the preliminary reaction(s) leading to thromboplastin generation, but that dextran sulphate has a lower activity upon all subsequent reaction(s).

Discussion

Dextran sulphate shows very different activities on the different phases of the clotting mechanism.

Tested upon the spontaneous coagulation of whole blood, as in the U.S.P. heparin assay[6], dextran sulphate was found to have approximately one-fifth of the activity of heparin[7].

The clotting time of whole blood largely depends upon the length of time taken in the earliest stages of coagulation[24]. It is therefore likely that when tests involving whole blood are used to assay an anticoagulant of the heparin type, the potency ratio will reflect the activity of the drug upon early reactions, and the present work confirms this hypothesis. The evidence further suggests that the actions of dextran sulphate differ so greatly from those of heparin that a true comparison between the anticoagulant activities of the two drugs cannot be made.

Summary

1. The anticoagulant activity of dextran sulphate has been compared with that of heparin. Compared with heparin, dextran sulphate did not greatly affect the thrombin-fibrinogen reaction, and reacted only slightly with the progressive antithrombin and the heparin co-factor, over the concentrations tested; an effect upon prothrombin conversion was not observed.

2. The relative effect of dextran sulphate upon thromboplastin generation was similar to the potency ratio derived from whole-blood clotting time assays.

We are indebted to Dr. C. R. Ricketts for his advice; to the Director, the Department of Biological Standards, Medical Research Council, for samples of International Standard heparin and British Standard dextran sulphate, and for putting at our disposal the report, *The Collaborative Study of Dextran Sulphate*; and to Dextran, Ltd., for the Injection of Dextran Sulphate.

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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Analgesics, Separation of. G. Wagner. (*Arch. Pharm. Berl.*, 1956, **289**, 8.) Salicylic acid, salicylamide and salicyl-*iso*propylamide can be separated by paper chromatography, using paper buffered at a pH of 10 or above. Under these conditions acetylsalicylic acid is completely saponified. Separation may also be carried out by ionophoresis. In the case of salicylic acid and acetylsalicylic acid the separation is carried out at pH 4 to 5. Amidopyrine, 4-aminophenazone, 4-methyl aminophenazone and pyramidon may be separated on acid buffered paper using *n*-butanol saturated with water. Phenylbutazone, phenacetin and acetanilide may be separated from caffeine and codeine at a pH of 3 or 4. In ionophoresis, phenylbutazone wanders as an anion, while pyramidon is only transported to a slight extent to the cathode. For development of the pyrazolone derivatives Dragendorff's reagent is used, while aminophenazone may be detected after diazotisation by coupling with alkaline β-naphthol. The caffeine may be detected by an acid iodine-potassium iodide solution. G. M.

Antihistaminic Agents, Identification of. A. Osol and C. N. Sideri. (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 761.) About 25 mg. of the substance under test was dissolved in 5 ml. of sulphuric acid. The colour was observed for 2 minutes. The solution was then diluted to 20 ml. with water and observed for a further 2 minutes. Where no response was obtained the experiment was repeated using nitric acid.

Substance	Colour with sulphuric acid	Colour on dilution
Antazoline hydrochloride	Colourless	Colourless[1]
Chlorcyclizine hydrochloride	Brilliant yellow	Colourless
Chloropyrilene citrate	Dark red	Brown precipitate
Chlorpheniramine maleate	Colourless	Colourless [2]
Diphenhydramine hydrochloride	Deep orange-red	White turbidity
Doxylamine succinate	Light yellow	Colourless
Methapyrilene hydrochloride	Orange-brown	Greenish-yellow
Phenindamine tartrate	Orange-brown	Colourless
Pyrathiazine hydrochloride	Pink, then brownish	Brownish
Mepyramine maleate	Cherry red	Turbid; white or cream precipitate separates
Thenyldiamine hydrochloride	Pink, then vivid orange-red	Colourless
Thonzylamine hydrochloride	Red	Turbid; white or cream precipitate separates
Tripelennamine hydrochloride	Yellow (turbid)	Colourless (turbid)

[1] Deep red with nitric acid. [2] Colourless with nitric acid.

G. B.

Œstrogen Preparations, Analysis of. P. M. Sanders, D. Banes and J. Carol. (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 727.) Samples of equine œstrogen sulphates were hydrolysed in acid alcohol, aqueous acid and dioxan-trichloroacetic acid. Acid alcohol appeared to be the most reliable method and gave results 3 to 16 per cent. higher than those obtained by the other methods. Œstrogen sulphates were also hydrolysed by converting them to acetates with acetic anhydride in hot pyridine, followed by hydrolysis

of the acetates with sodium carbonate. Good recoveries were obtained in the assay of conjugated ketosteroids, but in the case of conjugated diols the 17-hydroxyl group appeared to be esterified to some extent and to resist conversion to the acetate, making the analytical results less reliable. G. B.

Podophyllum Resin, Quantitative Estimation of Active Substances in. H. Potěšilová. (*Československ. Farm.*, 1955, 4, 454.) Podophyllotoxin, α- and β-pelatins (series A and B) and picropodophyllin react quantitatively with alkali to give salts which result from the opening of a lactone ring; these compounds can be estimated by determining the amount of alkali required to open the ring, or the amount of acid required to close it after it has been opened. The method can be used to estimate the total amount of lactone-containing compounds, calculated as podophyllotoxin, in podophyllum resin. A 1-g. sample of resin is extracted with 10 ml. of chloroform; the chloroform is removed from the filtered extract and the residue is dried under reduced pressure at 100° C. to constant weight. A weighed sample (0·1 g.) of the residue is dissolved in 2 ml. of ethanol and the solution is immediately titrated with 0·1N sodium hydroxide, with phenolphthalein as indicator (to neutralise organic acids and free phenol groups). The neutral mixture is refluxed for 15 minutes on a water bath with 100 ml. of 0·1N sodium hydroxide, and the cooled solution is titrated with 0·1N hydrochloric acid. A further 10 ml. of 0·1N hydrochloric acid is added and the solution is heated in the same way, cooled and titrated with 0·1N sodium hydroxide. The ring-closing titration is preferred for determinations on the resin. Tests on commercial samples show that the chloroform-soluble fraction of the resin contains 79 to 97 per cent. of "podophyllotoxin." E. H.

Papaverine and its Salts, Colorimetric Determination of. O. N. Soboleva (*Aptechnoe Delo*, 1955, 4, No. 4, 37.) Papaverine reacts with formaldehyde to form methylene-dipapaverine (Freund and Fleischer, *Ber.*, 1915, **48**, 406), and when this is treated with bromine water and ammonia a greyish violet precipitate is formed; the precipitate dissolves in ethanol to give a violet-red or blue-violet colour. The coloured compound can be extracted from dilute ethanol by chloroform, but not by ether. None of the other opium alkaloids react in the same way. For the determination of papaverine, a solution containing 0·5 to 1·5 mg. of the hydrochloride is evaporated to dryness and the residue is stirred for 30 minutes with 2 drops of 35 per cent. formaldehyde solution and 0·2 ml. of 80 per cent. sulphuric acid. The product is transferred to a 25-ml. glass-stoppered calibrated tube, the dish being washed with 0·5-ml. quantities of water. The contents of the tube are shaken for 4 minutes with 0·5 ml. of bromine water; 5 ml. of ethanol and 1 ml. of 25 per cent. ammonia solution are added, and the volume is made up to 25 ml. The colour is measured in an absorptiometer fitted with a blue filter. The error is ±5 per cent. E. H.

Protoveratrine, Determination of. J. Levine and H. Fischbach. (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 713.) A mixture of protoveratrines A and B may be separated by partition chromatography, a column consisting of 1 g. of Celite with 1 ml. of the immobile phase (2 parts of buffer solution, pH 3·5 with 1 part of ethylene glycol) being sufficient for the separation of a mixture of approximately 1 mg. of each alkaloid. 200 ml. of benzene is passed through the column to remove protoveratrine A, followed by 150 ml. of ethylene chloride to remove protoveratrine B. Protoveratrine may be

assayed by passing a chloroform solution through a Celite column incorporating buffer solution pH 3·5 and chlorophenol red (3 : 3′-dichlorophenol sulphonphthalein). The quantity of dye removed is proportional to the quantity of alkaloid present in the solution under examination. G. B.

***Rauwolfia serpentina*, Differentiation of.** D. Banes and J. Carol. (*J. Assoc. off. agric. Chem., Wash.*, 1955, **38**, 866.) Spectrophotometric analysis of the aromatic acids obtained after hydrolysing the weak alkaloids of rauwolfia root has been employed for differentiating *Rauwolfia serpentina* from other species. Six samples of whole roots of *R. serpentina* from India were analysed for derived aromatic acids; the ultra-violet absorption spectra of the isolated acids resembled those of trimethoxycinnamic trimethoxybenzoic acid mixtures. The spectra of acids derived in the same manner from *R. heterophylla*, *R. micrantha*, *R. hirsuta*, *R. canescens*, and *R. sellowi*, either resembled the curve given by trimethoxybenzoic acid, or indicated a negligibly low concentration of acyloxy alkaloids. On the basis of these observations, spectrophotometric criteria were established for the chemical identification of ground rauwolfia roots: (1) Absorbance at 303 mμ greater than that at 288 mμ ($A_{303}/A_{288} > 1$) and absorbance ratio A_{303}/A_{273} greater than 0·8 indicate the presence of *R. serpentina* (although mixtures containing *R. serpentina* and large proportions of a species poor in acyloxy alkaloids, like *R. sellowi* or *R. micrantha*, would yield a similar spectrum): (2) Absorbance ratio $A_{303}/A_{288} < 0{\cdot}6$, and $A_{303}/A_{273} < 0{\cdot}9$ indicate a species of *Rauwolfia* other than *serpentina* with other species. In the examination of a number of commercial samples the spectrophotometric procedure yielded results which were consistent with those obtained by microscopic methods. Paper chromatography could be used on the mixed acids if desired. R. E. S.

Reserpine Preparations, Chromatographic Analysis of. D. Banes, J. Carol and J. Wolff. (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 640.) Serpentine, ajmaline, ajmalicine, reserpine, recinnamine and deserpidine may be separated from each other and identified by ascending paper chromatography, using a mobile solvent consisting of *iso*octane, benzene, formamide and *cyclo*hexane, and formamide (30 per cent.) in acetone as the immobile phase. After development, the paper is dried at 90° C. and exposed to hydrochloric acid fumes to increase subsequent fluorescence of deserpidine. The spots due to the alkaloids are examined in ultra-violet radiation, and the alkaloids identified by comparison with spots due to pure samples of known alkaloids run simultaneously with the samples under test. The authors also describe a method employing a column of diatomaceous silica (Celite 545), using citric acid and ethanol as the immobile solvent and a mixture of chloroform, *iso*-octane, water and ethanol as the eluent. By this method quantities of the order of milligrams of reserpine may be separated quantitatively, and determined by ultra-violet spectrophotometric analysis. G. B.

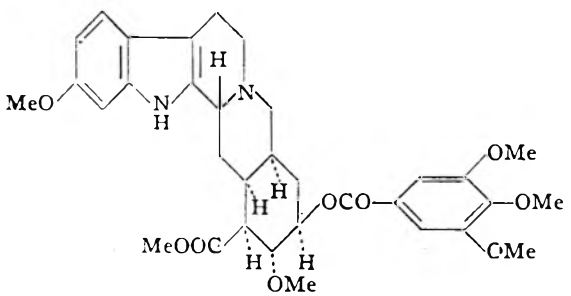
Reserpine in Pharmaceutical Products, Determination of. W. F. Bartelt and E. E. Hamlow. (*J. Amer pharm. Ass., Sci. Ed.*, 1955, **44**, 660.) Solutions of the material under test were placed on a column prepared from equal quantities of Solka-floc and Celite 545. The column was washed with water, followed by ethanol (27 per cent.) and again with water to remove impurities. A quantity of 5N acetic acid was then added to the column to elute the reserpine. A pair of platinum wires placed at the outlet of the column and connected to a voltmeter and a battery was used to show when the acetic acid (containing reserpine)

had reached the outlet. This solution was collected and assayed by determination of the ultra-violet absorption at 267 mμ. The method was found to be simple to use, and suitable for the routine assay of liquid preparations and tablets. G. B.

Sodium Carboxymethylcellulose, Non-aqueous Titration Assay For. C. N. Sideri and A. Osol. (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 759.) Sodium carboxymethylcellulose was assayed for sodium by heating with glacial acetic acid, cooling to room temperature and titrating with 0·1 N perchloric acid in dioxan. Slightly higher results were obtained by ignition of the sample before titration. These results were somewhat lower than those obtained by precipitating carboxymethylcellulose as a copper derivative and determining the copper content of it. Results based on sulphated ash determinations were higher, possibly because of the presence of iron and other substances in addition to sodium sulphate in the residue. G. B.

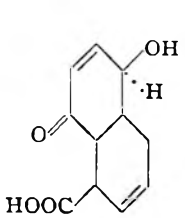
ORGANIC CHEMISTRY

Reserpine, Total Synthesis of. R. B. Woodward, F. E. Bader, H. Bickel, A. J. Frey and R. W. Kierstead. (*J. Amer. chem. Soc.*, 1956, **78**, 2023.) The total synthesis of reserpine (I) is briefly outlined.

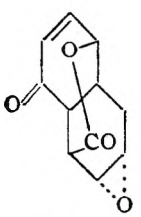


(I)

The adduct from *p*-benzoquinone and vinylacrylic acid was reduced by sodium borohydride to the alcohol (II) which was converted to an oxide with



(II)



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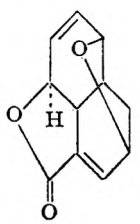

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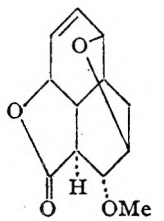
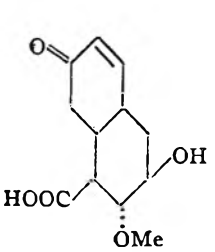


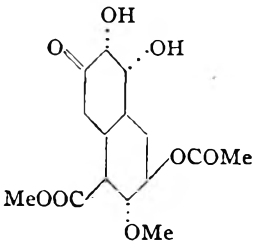
(V)

perbenzoic acid. Action of acetic anhydride and sodium acetate in benzene on the oxide gave the lactone (III) which was transformed by aluminium *iso*propoxide in hot *iso*propyl alcohol into the ether (IV) and thence by the action of sodium methoxide in methanol to the methoxy-ether (V). The bromohydrin obtained by the action of *N*-bromosuccinimide on (V) in warm aqueous solution

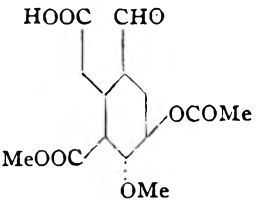
in the presence of sulphuric acid, was oxidised by chromium trioxide in acetic acid to a bromo-ketone which gave the hydroxy acid (VI) upon short treatment



(VI)

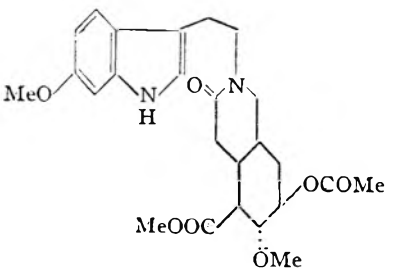


(VII)

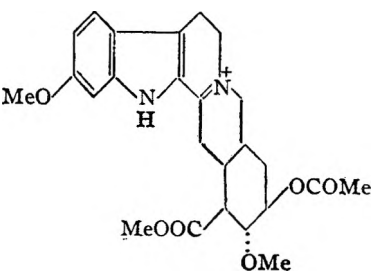


(VIII)

with zinc in cold glacial acetic acid. The methyl ester of (VI) was converted to the acetate by acetic acid in warm pyridine and thence to the diol (VII) by treatment with aqueous osmium tetroxide followed by potassium chlorate. The diol (VII) was transformed without isolation of the labile intermediates, e.g. (VIII) etc. to the lactam (IX), through successive treatments with aqueous periodic acid in ethereal diazomethane, condensation with 6-methoxytryptamine in benzene, and reduction with sodium borohydride in methanol. Boiling phosphorus oxychloride converted the lactam (IX) into the quaternary cation (X)



(IX)



(X)

which was reduced directly with aqueous methanolic sodium borohydride to (±)-methyl *O*-acetyl*iso*reserpate; resolution yielded (−)-methyl *O*-acetyl*iso*-reserpate. Hydrolysis with methanolic potash followed by treatment with hydrochloric acid to yield *iso*reserpic acid hydrochloride and then warming with *NN'*-di*cyclo*hexyl-carbodiimide yielded *iso*reserpic acid lactone. Isomerisation of the lactone with pivalic acid in boiling xylene gave reserpic acid lactone. Upon methanolysis this lactone yields methyl reserpate which can be transformed by 3:4:5-trimethoxybenzoyl chloride in pyridine to reserpine (I).

A. H. B.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

5-Hydroxytryptamine in Serum. D. F. Sharman and F. M. Sullivan. (*Nature, Lond.*, 1956, **177**, 332.) It is well known that 5-hydroxtryptamine (5-HT) is liberated from platelets during the clotting of blood and that the amount found in the serum varies according to the way in which the material is prepared.

The purpose of this paper was to investigate this variation. Human blood withdrawn from a vein in the arm by means of a silicone-treated syringe was used. The blood was allowed to clot either by placing it in a glass test-tube and letting it stand without agitation for 30 minutes or by placing it in a test-tube containing a glass marble and tipping the tube through $\pm 20°$ from horizontal at a rate of 15 cycles/minute for 30 minutes. In each case the clotted blood was centrifuged at 3000 rev./minute for 30 minutes, by which time the serum was free of platelets. Acetone extracts of the serum were assayed on the isolated uterus of the rat. The results indicated that agitation during the clotting results in a decreased amount of 5-HT in the serum. In another experiment it was shown that the clot does not adsorb 5-HT and that mechanical disturbance after clotting, even in the presence of the clot, does not destroy it. M. M.

Oxytocin and Vasopressin, Separation of. R. Hausmann. (*Arch. Pharm. Berl.*, 1956, **289**, 15.) By the action of pepsin on the protein hormone of pituitary posterior lobe, increasing amounts of the hormone peptide investigated by du Vigneaud are obtained. Glacial acetic acid also produces quantitative splitting. The resulting solutions may be separated by chromatography on paper, the position of the active principles being determined by biological tests on individual strips of the paper. Since vasopressin has an isoelectric point of 10·85, while that of oxytocin is 7·7, separation by high voltage ionophoresis is possible and indeed preferable to chromatography, since it is more rapid and there is less destruction of the activity. This is however only possible after a preliminary purification, e.g., by precipitation with ammonium sulphate. After separation by ionophoresis further purification can be achieved by paper chromatography. G. M.

Sarin, Enzymatic Hydrolysis of. F. C. G. Hoskin. (*Canada. J. Biochem. Physiol.*, 1956, **34**, 75.) The high toxicity of the organophosphorus cholinesterase inhibitors makes it difficult to study their metabolism and excretion in the intact animal. An investigation of the metabolism of *iso*propyl methylphosphonofluoridate (sarin) by rat serum enzyme has shown it to be hydrolysed to the less toxic *iso*propyl methylphosphonic acid. When this compound, labelled with ^{32}P, was given to rats it was excreted unchanged in the urine. It is suggested that the metabolism of sarin by the rat would lead almost exclusively to *iso*propyl methylphosphonic acid. G. F. S.

BIOCHEMICAL ANALYSIS

5-Hydroxyindoleacetic Acid and 5-Hydroxytryptamine in Urine, Test for. G. Curzon. (*Lancet*, 1955, **269**, 1361.) A simple and rapid test is described for the detection of 5-hydroxytryptamine (5-HT) and its metabolite 5-hydroxyindoleacetic acid using paper chromatography. Fifty μl. of urine is applied at a point 5 cm. from the edge of a rectangular piece of Whatman no. 4 paper. The urine is applied in 10 μl. portions and dried by a warm current of air. Five μl. of a 1 mg. per ml. ethanolic solution of 5-hydroxyindoleacetic acid or of a 0·2 mg. per ml. aqueous solution of 5-HT may be applied in parallel with the urine spot and also a normal urine for comparison. The sheet is fastened in cylindrical form and stood for 45 minutes in about 100 ml. of solvent in a wide-necked jar. The solvent for 5-HT is 8 g. of sodium chloride dissolved in 100 ml. of water, and 1 ml. of glacial acetic acid is added for the detection of 5-hydroxyindoleacetic acid. The paper is dried in a

warm oven, sprayed with colour reagent (2 g. dimethylaminobenzaldehyde dissolved in 5 ml. of concentrated hydrochloric acid and 95 ml. water), and heated at 55–60° C. for 15 minutes. Normal urines show a strong yellow urea spot and weak spots in the indoxyl-sulphate, tryptophan, and indoleacetic acid positions. Urines containing 20 mg. or more per litre of 5-hydroxyindoleacetic acid show a further blue-grey spot. Urines containing at least 2 mg. per litre of 5-hydroxytryptamine show a blue spot. The method may be useful in the diagnosis of metastasing carcinoid. G. F. S.

Insulin, Paper Chromatography of. G. Grodsky and H. Tarver. (*Nature, Lond.*, 1956, **177**, 223.) A paper chromatographic technique for the isolation and determination of insulin in small amounts of tissue has been devised. The proteins were precipitated from the hashed tissue by the addition of trichloroacetic acid, and the precipitate extracted with a mixture of ethanol-water and concentrated hydrochloric acid. After adjusting the pH to 8·5 to 9·0, and filtering out any precipitate, the insulin was precipitated by the addition of a mixture of ethanol and ether (3 : 5). The precipitate, in aqueous-ethanolic hydrochloric acid, was applied as a streak of droplets to Whatman 3 MM paper and chromatographed using *n*-butanol, water, acetic acid (12 : 5 : 2). Under these conditions insulin moves with R_F 0·21. After drying, the paper was immersed for 10 minutes in a solution of bromophenol blue (0·05 per cent.) and mercuric chloride (1 per cent.) in 2 per cent. acetic acid. Excess dye was washed out with dilute acetic acid, the insulin streak cut out, reduced to shreds, extracted with aqueous-ethanolic hydrochloric acid, and the intensity of the colour at 470 mμ measured. The amount of insulin was determined by comparison with known standards similarly treated, linear relationships being obtained with 0·1 to 0·2 mg. Chromatographic examination of liver protein, ribonuclease, and serum proteins showed that none of these behaved as did insulin, though glucagon appears to have properties similar to insulin. The production of insulin in fœtal beef pancreatic slices in the presence and absence of oxygen was also examined. Glucagon appeared to have a direct effect on insulin production. J. B. S.

Insulin, Paper Chromatography of. A. Light and M. V. Simpson. (*Nature, Lond.*, 1956, **177**, 225.) R_F values have been determined for crystalline insulin on Whatman No. 1 paper in various solvent systems. 2-Butanol/1 per cent. acetic acid (1 : 1) gave the most discreet spots, 20 μg. of insulin being easily detectable. The R_F value in this system varied slightly with the amount of insulin applied to the paper and with the distance travelled by the solvent front. The technique was used to detect insulin in various crude fractions, obtained by the procedure of Romans *et al.*, with the proviso that salt is removed by dialysis prior to chromatography, and also that excessive amounts of crude protein are not applied to the paper. The amount of insulin was estimated approximately by comparison with known insulin spots. Ribonuclease, serum album, or pancreatic fractions depleted of insulin showed no movement, but crystalline glucagon moved at the same R_F as insulin. Its presence in insulin samples may be detected by paper electrophoresis at pH 7·5. For preparative processes the use of Munktell No. 20 electrophoresis paper permitted insulin to move at its normal R_F even when the paper was heavily loaded with crude material. In a large-scale experiment 32 mg. of labelled insulin were isolated from 80 g. of calf pancreas slices which had been incubated in the presence of DL-leucine-1-^{14}C. J. B. S.

Isoniazid, Microdetermination of. J. Wagner, P. Kraus and B. Večerek (*Československ. Farm.*, 1955, **4**, 389.) A method for the determination of isoniazid in solutions, tablets or blood serum is based on its reaction with potassium mercuric iodide in alkaline solution to form a yellowish green turbidity which changes to orange-green on acidification. To 0·5 ml. of test solution (containing 4 to 20 μg. of isoniazid) 0·5 ml. of a 5 per cent. aqueous solution of potassium mercuric iodide and 1 ml. of N sodium hydroxide solution are added; after 1 minute the solution is acidified with 2 ml. of 2N acetic acid (to eliminate interference due to ammonium salts) and, after a further 5 minutes, the density of the turbid solution is measured in a 5-cm. cuvette in a Pulfrich photometer fitted with a S47 filter. The concentration is determined from a calibration graph constructed with solutions containing 8 to 40 μg. per ml. of isoniazid; over this range the optical density is proportional to the concentration. Determinations can be carried out directly on blood serum, previously deproteinised with barium hydroxide and zinc sulphate, but internal standards must be used in making the calibration graph. The error is $\pm$ 5 to 8 per cent. Hydrazine salts, phenylhydrazine and 2:4-dinitrophenylhydrazine react in the same way as isoniazid. E. H.

Phenobarbitone, Metabolite of, in Human Urine. E. J. Algeri and A. J. McBay. (*Science*, 1956, **123**, 183.) The *p*-hydroxy-derivative of phenobarbitone has been found in the urine of two patients who died after overdoses of phenobarbitone. The urines were extracted with ether at pH 7, the ether extracts washed with 0·2 N hydrochloric acid, dried with anhydrous sodium sulphate and shaken with 0·05 N sodium hydroxide until all the barbiturate was extracted. The ultra-violet absorbencies were measured at pH 9·5 and 2. The barbiturates were then re-extracted with ether from acid solution, the volume reduced and the ether solution submitted to paper chromatography. Two barbiturates were found; one located at R_F 0·5 was phenobarbitone, and the other at R_F 0·29 *p*-hydroxyphenobarbitone. In the first case the concentration of *p*-hydroxyphenobarbitone was 9·2 mg./100 ml. of hydrolysed urine and the barbiturate concentration of the blood 7·2 mg./100 ml. Forty-six per cent. of the *p*-hydroxyphenobarbitone was in the conjugated form. Similar results were obtained in the second case. G. F. S.

Staphylococcus Enterotoxin, Detection of. L. Levi, B. H. Matheson and F. S. Thatcher. (*Science*, 1956, **123**, 64.) The results are described of an infra-red spectrophotometric examination of boiled and lyophilised preparations obtained from cultures of enterotoxigenic and non-enterotoxigenic staphylococci (*Micrococcus pyogenes var. aureus*) in accordance with the "cold-ethanol" method developed by Thatcher and Matheson (*Can. J. Microbiol.*, 1955, **1**, 40). The specimens were finely powdered and 5 mg. was mixed intimately with 995 mg. of potassium bromide; 200 mg. of the mixture was then subjected in a vacuum to a pressure of 10,000 lb/sq. in. for about 5 minutes. The absorbancy of the clear disc thus produced, over the frequency range extending from 4000 to 650 cm.$^{-1}$, showed strong N–H and characteristic C–H stretching vibrations at 3400 cm.$^{-1}$ and 2900 cm.$^{-1}$, respectively. Marked absorptions noted at 1650 and 2540 cm.$^{-1}$ were indicative of the presence of polypeptide bonds, while the characteristic band occurring at 1065 cm.$^{-1}$ could be considered to be associated with ester linkages such as –C–O or C–O–P. The 1100 to 1000 cm.$^{-1}$ region proved to be the most informative, for the intensity of the absorption bands at 1065 cm.$^{-1}$ was always found to be higher for preparations showing enterotoxigenic activity than it was for preparations that were biologically inactive. R. E. S.

CHEMOTHERAPY

2-Amino-1 : 3 : 4-thiadiazoles, the Carcinostatic Activity of. J. J. Oleson, A. Sloboda, W. P. Troy, S. L. Halliday, M. J. Landes, R. B. Angier, J. Semb, K. Cyr and J. H. Williams. (*J. Amer. chem. Soc.*, 1955, **77**, 6713.) The carcinostatic activity of several 2-R-amino-1 : 3 : 4-thiadiazoles (where R = H, Me, Et, Allyl, Phenyl, Acetyl groups) against several transplanted animal tumours in mice is reported. The activity of 2-amino-5-R-1 : 3 : 4-thiadiazoles (R = OH, SH, Cl) and 2-R-amino 5-methyl-1 : 3 : 4-thiadiazoles (R = Me and Allyl) is also recorded. The parent compound, 2-amino-1 : 3 : 4-thiadiazole was the most active; the lower 2-alkylamino and 2-acetylamino derivatives were also active and less toxic than the parent compound, while the 2-phenylamino derivative was inactive. In most cases, substitution in the 5-position reduced the activity of the 2-amino derivative. A. H. B.

***iso*Nicotinyl Hydrazones from D-Mannuronolactone and D-Mannuronic Acid.** C. H. Brown, H. E. Bond, S. A. Peoples and P. P. T. Sah. (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 591.) Alginic acid was hydrolysed by heating under reflux with formic acid for 20 hours. Formic acid was removed by distillation, and the resulting D-mannuronolactone recrystallised from warm water. D-Mannuronic acid *iso*nicotinyl hydrazone was obtained by the reaction of D-mannuronolactone with isoniazid in hot water, and crystallised by allowing to stand overnight in a refrigerator. D-Mannuronolactone *iso*nicotinyl hydrazone was made similarly, but absolute ethanol or methanol was used as the solvent, so as to prevent hydrolysis of the product. The LD50 dose, determined by oral administration to mice was 1100 mg./kg. for the D-mannuronolactone derivative and 1000 mg./kg. for the D-mannuronic acid compound, compared with 160 mg./kg. for isoniazid. In *in vivo* tests in mice infected with *Mycobacterium tuberculosis* H37Rv, these derivatives appeared to be at least as effective as isoniazid. Results were very similar to those obtained with the corresponding D-glucuronolactone and D-galacturonic acid derivatives. G. B.

Spiramycin: Clinical and Laboratory Studies. D. G. Hudson, G. M. Yoshihara and W. M. M. Kirby. (*Arch. intern. Med.*, 1956, **97**, 57.) Laboratory and clinical studies of a new antibiotic, spiramycin, are presented. *In vitro* spiramycin effectiveness was determined by the inhibition of the growth of streptococci and pneumococci in tryptose phosphate broth containing three per cent. human blood, and of staphylococci in broth without added blood. Results were compared with those obtained simultaneously using penicillin and erythromycin at the same concentration. Antibiotic dilutions ranged from 0·01 μg. to 50 μg./ml. Spiramycin was the least active antibiotic in inhibiting the growth of pneumococci and β-hæmolytic streptococci, and generally so against staphylococci. With non-hæmolytic streptococci tested, spiramycin was less active than erythromycin but more effective than penicillin. Generally, the order of increasing sensitivity to spiramycin was: staphylococci; streptococci; pneumococci. Using 117 strains of freshly isolated streptococci, there appeared little evidence of cross resistance between spiramycin and either penicillin or erythromycin. *In vivo* studies involved 29 adult patients with bacterial pneumonia: 2·0 g. of spiramycin was given initially, followed by 1·0 g. every six hours. These oral doses were well tolerated and well absorbed, rapidly producing serum concentrations of 1·0 to 7·0 μg./ml., which are at least 100 times as great as those required to inhibit pneumococci *in vitro*. The clinical effectiveness of spiramycin against bacterial pneumonia appeared

comparable with results previously obtained at the same hospital using erythromycin and penicillin. No patients with staphylococcal infections were treated, but the low level of cross resistance suggests that spiramycin may be useful in the treatment of infections caused by penicillin- and erythromycin-resistant strains, although the relationship between inhibitory concentration *in vitro* and blood levels obtained, is less favourable than with erythromycin and penicillin.

G. P.

PHARMACY

NOTES AND FORMULÆ

Ergometrine Decomposition of Unstabilised Solutions of. J. Reichelt and L. Šafařík (*Československ. Farm.*, 1955, **4**, 404.) The effect of heat, atmospheric oxygen and ultra-violet light on unstabilised 0·1 per cent. solutions of ergometrine maleate at pH 3·5 to 4·0 is studied. The decomposition products are separated by chromatography on Whatman No. 1 paper; the paper is first impregnated with a solution of formamide in ethanol (4:6 or 3:7) and the chromatogram is developed with chloroform, saturated with formamide. Chromatography of solutions which had been aerated for 12 hours in the dark at room temperature shows that, in addition to ergometrinine, two decomposition products are produced; the first of these (X) showed a dark red fluorescence in ultra-violet light, and the second (Y) a yellowish green fluorescence. Neither of the substances reacts with *p*-dimethylaminobenzaldehyde. Ergometrinine and (+)-lysergic acid are the only decomposition products detected in solutions which had been heated to 100° C. in the absence of air for 15 hours. E. H.

Oxytetracycline and Tetracycline, Parenteral. M. Katz, O. Klioze and S. Y. P'an. (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 751.) Dry powders intended for the preparation of injections of oxytetracycline and tetracycline were made according to several formulæ, and the powders and solutions prepared from them were examined for stability. Potentiometric titrations and *in vivo* tests for absorption and irritation were carried out to assess the suitability of the preparations for injection. A mixture of oxytetracycline(terramycin)hydrochloride 1, sodium glycinate 0·9 was suitable for the preparations of solutions for intravenous or intramuscular injection. With a mixture of oxytetracycline hydrochloride 1, and magnesium chloride 1 it was possible to prepare solutions of concentrations up to 50 mg./ml., less irritating and better absorbed by intramuscular injection. A mixture of oxytetracycline hydrochloride 1, ascorbic acid 4 was suitable for preparing intravenous solutions more stable than those containing sodium glycinate. A solution prepared from a mixture of tetracycline hydrochloride 1, sodium glycinate 0·9 was suitable for intravenous but not for intramuscular injection. A solution for intramuscular injection was prepared from tetracycline hydrochloride 1, magnesium chloride 1, ascorbic acid 2·5, while an intravenous solution was prepared from a mixture of tetracycline 1 and ascorbic acid 3. The dry powders were stable when stored for 6 weeks at 50° C., and solutions retained most of their potency when kept for periods up to 72 hours at 25° C., although solutions of tetracycline were generally somewhat less stable than those of oxytetracycline. Intravenous solutions prepared with ascorbic acid were compatible with most transfusion fluids, including dextrose, sodium chloride and Ringer's solutions.

G. B.

PHARMACOLOGY AND THERAPEUTICS

Atropine in the Treatment of Anticholinesterase Intoxication. A. S. Gordon and C. W. Frye. (*J. Amer. med. Ass.*, 1955, **159**, 1181.) This paper re-emphasises the necessity for large doses of atropine in the treatment of intoxication resulting from antichloinesterase agents such as organic phosphate compounds used as insecticides. These compounds also have a potential use as chemical warfare agents, and military recommendations for treatment are as follows. In mild or moderate symptoms of poisoning 2 mg. of atropine sulphate should be injected intramuscularly. In severe cases 4 to 6 mg. should be injected intravenously or intramuscularly. After the initial dose 2 mg. should be given at hourly intervals or oftener until signs of atropinisation appear or as long as muscarinic effects are present. A review of reported cases of poisoning from these anticholinesterase agents reveals a direct relationship between survival, the amount of atropine given and the speed of administration. The consequences of inadequate treatment are grave, whereas the effects of excessive administration of atropine, though uncomfortable and occasionally temporarily incapacitating, are not serious. It is important to note that there is a marked tolerance for atropine in the presence of anticholinesterase poisoning, and the failure of appearance of atropinisation after a 2 mg. dose offers further presumptive evidence of anticholinesterase intoxication. An over-all survey of the literature includes almost 1000 people who have received atropine in excess of the usual therapeutic maximum of 1 mg.; most have received 2 mg. or more and about one-third more than 10 mg. Of this number only 11 persons have died. Details are given in the paper of 25 cases of anticholinesterase poisoning treated with atropine. Other measures necessary in the treatment of this type of intoxication are also outlined. S. L. W.

4-*n*-Butoxy β-(1-piperidyl) Propiophenone Hydrochloride and β-Diethylaminoethyl *p*-*n*-Hexyloxybenzilate Hydrochloride, Local Anaesthetic and Pharmacological Properties of. R. B. Arora and V. N. Sharma. (*J. Pharmacol.*, 1955, **115**, 413.) Dyclonine, [4-*n*-butoxy β-(1-piperidyl) propiophenone hydrochloride] and β-diethylaminoethyl *p*-*n*-hexyloxybenzilate hydrochloride were both effective topical local anæsthetics in a series of ophthalmological surgical procedures in guinea-pigs. The benzilic acid ester was the more potent, but was slower in onset and more irritant. There was no parasympathomimetic or anticholinergic activity with either substance. On intravenous injection into anæsthetised dogs small doses of both drugs stimulated respiration and caused a fall in arterial pressure; with increase in dose (3 to 5 mg./kg.) respiration was depressed. The reduction in blood pressure was due both to a decrease in cardiac output and to a direct arteriolar dilatation; both compounds increased flow through the vessels of the isolated hind limbs of dogs. Intestinal motility was reduced in anæsthetised dogs with the benzilate, but not with dyclonine. In man, dyclonine, in a concentration of 1 per cent., and the benzilate in a concentration of 0·25 per cent., produced analgesia in ophthalmic operations, equivalent to that of a 4 per cent. solution of cocaine hydrochloride. G. P.

Calcium Methionate, Pharmacological Study of. G. V. Rossi, T. S. Miya and L. D. Edwards. (*J. Amer. pharm. Ass., Sci. Ed.*, 1956, **45**, 47.) Calcium chloride, gluconate and methionate were submitted to comparative trial in rabbits, rats and mice. Equimolecular concentrations produced similar effects on the blood pressure, smooth muscle (ileum and uterus) and diaphragm, the

effects being those characteristic of calcium compounds, and independent of the anion present. Calcium methionate was shown to be slightly more toxic than calcium gluconate, but produced the same effects on gross appearance, growth rate, blood picture, bone composition and tissue morphology in rats. Calcium methionate was effective in raising the blood calcium level in rats, and was slightly more rapidly absorbed and removed from the blood than calcium gluconate. It has the advantage of greater solubility, and solutions of high calcium content may be prepared without stabilisers. Solutions are suitable for oral or intravenous administration, but are too irritant for injection by the subcutaneous and intramuscular routes. G. B.

Chlorpromazine, Antagonism of 5-Hydroxytryptamine by. E. P. Benditt and D. A. Rowley. (*Science*, 1956, **123**, 24.) 5-Hydroxytryptamine (5-HT) or a related substance was found to be associated with mast cells in rat tissue and was liberated along with histamine by histamine-liberators such as ovomucoid, compound 48/80 and dextran. 5-HT produced hyperæmia and œdema after subcutaneous injection into the dorsum of the rat's paw; the extent of the response was indicated by azovan blue dye, injected into the tail vein immediately before administration of the 5-HT. Dibenamine antagonised these actions. Chlorpromazine (1·0 to 1·5 mg./kg. i/v) also antagonised the actions of 5-HT in this preparation and in addition antagonised similar actions of histamine. On the isolated rat's colon chlorpromazine in a concentration of about 10^{-6} antagonised the stimulant actions of both acetylcholine and 5-HT; recovery from this inhibitory effect was complete only after 30 minutes. G. P.

Chlorpromazine in the Treatment of Tetanus Convulsions. R. E. Kelly and D. R. Laurence. (*Lancet*, 1956, **270**, 118.) The object of this study was to discover a drug or combination of drugs which will abolish the muscle spasm of tetanus without affecting respiration or abolishing consciousness. Of a number of drugs tried on experimental tetanus the only ones of any promise were chlorpromazine and promethazine. Chlorpromazine was shown to be effective against rabbit tetanus in a dose of 1 mg./kg. body weight. This dose abolished "spontaneous" tetanus and reflex tetanus for an average of 77 minutes; if no afferent stimulus was applied the tetanus was abolished for periods up to twice as long. The animals were quiescent after this dose, but normal rabbits would hop about if encouraged. Respiration was unaffected. Promethazine also abolished tetanus but only in high dosage and for a shorter time. A child, aged 2½ years, with severe tetanus convulsions, was successfully treated by means of chlorpromazine alone given by intravenous infusion. Chlorpromazine was given for 16 days, the total daily dose, in mg./day, being 75, 180, 220, 200, 155, 225, 285, 275, 330, 250, 215, 300, 240, 115, 50, 30. The degree of control varied, but opisthotonic spasms occurred no more than three or four times a day, and minor spasms lasting less than 15 seconds ten to fifteen times a day. At no time was consciousness lost as a result of administering the drug. The child was always easily roused from sleep. Respiration was unaffected. Considerable intravenous thrombosis occurred and 10 mg. of heparin was added to each bottle of infusion. An intravenous drip was kept going throughout the period of chlorpromazine administration, the solutions used being at first 5 per cent. dextrose and then compound sodium lactate injection E.P., of which about 500 ml. was given each day. Large doses of tetanus antitoxin were given at the start, and penicillin to prevent respiratory complications. The child had a slow but uneventful convalescence. S. L. W.

Ergot Drugs, Effect of, on *Betta splendens*. L. T. Evans, L. H. Geronimus, C. Kornetsky and H. A. Abramson. (*Science*, 1956, **123**, 26.) The hallucinogenic drug, (+)-lysergic acid diethylamide, (LSD-25), induced a quiescent state in the Siamese fighting fish, *Betta splendens*; changes in the behavioral, vegetative and motor characteristics of the fish were seen when the drug was added to the aquarium water in a concentration of 5×10^{-7} M. Eight other ergot derivatives were also tested for these effects; only two, a monobromo derivative (BOL-148), and (+)-lysergic acid ethylamide (LAE-32), had activity approaching that of LSD-25. Among those with negligible activity were ergotamine, dihydroergotamine, (−)-LSD-25 and (+)-*iso*-LSD-25. Mescaline and pethidine were also tested and also had negligible depressant activity. G. P.

Ethinamate, The Pharmacology and Toxicology of. E. E. Swanson, R. C. Anderson and W. R. Gibson. (*J. Amer. pharm. Ass., Sci. Ed.*, 1956, **45**, 40.) Ethinamate (ethynyl*cyclo*hexyl carbamate, Valmid, Valamine) was tested for hypnotic action and toxicity in mice, rats and dogs. For oral administration an emulsion with 5 per cent. of acacia was used. A solution in polyethylene glycol 200 was given by intravenous injection. Electroencephalographic studies indicated that the mode of action of ethinamate is similar to that of barbiturates. Duration of hypnotic effect in rats and dogs was about half that of quinalbarbitone sodium. The ratio of hypnotic and toxic doses was approximately the same as for quinalbarbitone sodium. Ethinamate showed some protective action against electric shock and leptazol and some local anæsthetic effect. It had no antipyretic, analgesic or diuretic action. Liver appeared to play a role in the degradation of the drug, since rats with liver damage showed a prolonged sleeping time whereas nephrectomised rats did not. Intravenous injection of ethinamate into dogs previously anæsthetised with the same substance lowered the blood pressure and slowed the heart rate and respiration, eventually causing death from respiratory failure unless this was prevented by artificial respiration or administration of picrotoxin. G. B.

Glycyrrhizinic Acid, Preparation of, and Effects in Man. L. H. Louis and J. W. Conn. (*J. Lab. clin. Med.*, 1956, **47**, 20.) A method is described for the preparation of ammonium glycyrrhizinate, the sodium retaining principle from liquorice. When administered orally to ten normal subjects it had no effect upon protein or carbohydrate metabolism, nor was there any effect upon the renal excretion of uric acid and creatinine, or upon the level of circulating eosinophils. There were very significant effects upon electrolyte metabolism, the most intense being upon the retention of sodium and chloride. There was only a mild increase in the urinary potassium. A patient with Cushing's syndrome reacted similarly, but there was no effect upon the electrolyte metabolism in two cases of congenital hyperplasia. Ammonium glycyrrhizinate depressed the excretion of 17-ketosteroids, particularly in normal subjects with high levels of 17-ketosteroid excretion and in the patients with congenital adrenal hyperplasia. It also decreased the concentrations of sodium and chloride in thermal sweat, and it is capable of inhibiting the pituitary release of the melanophore stimulating hormone; the effect of 4 g. per day orally being roughly equivalent to 37·5 mg. of cortisone, 20 mg. of hydrocortisone and 5 mg. of 9-α-fluorohydrocortisone orally. The results suggest that ammonium glycyrrhizinate acts in the body very similarly to an adrenal steroid. Its peripheral effects are exclusively upon electrolyte and water metabolism, but it can depress both production of the adrenocorticotrophic and melanophore stimulating hormones. G. F. S.

Isoniazid, Streptomycin and PAS; Combined Use in Tuberculous Meningitis. R. Maggi, C. J. G. Diaz and F. C. Pfister. (*Antibiotic Med.*, 1956, **2**, 21.) Twenty children, with tuberculous meningitis, whose ages ranged from 1 to 11 years, were treated with combined therapy as follows. Isoniazid by mouth, 15 to 20 mg./kg./day was administered in three divided doses. Streptomycin, 30 mg./kg./day, was given by intramuscular injection every 12 hours. PAS in a 3 per cent. solution in triple distilled water was given by venoclysis in a dose of 0·5 to 0·6 g./kg./day in severe cases, and in other cases optionally by mouth every 6 hours with daily doses of 0·4 g./kg. body weight. This treatment was continued for 2 to 3 months, and was followed by maintenance treatment which lasted about 4 months and comprised the simultaneous use of two drugs with the basic and permanent treatment of streptomycin intramuscularly, given at intervals of 48 hours (later, 72 hours), and isoniazid by mouth 8 to 10 mg./kg./day in 2 divided doses, alternating every 4 weeks with PAS by mouth 0·4 g./kg./day in 4 divided doses. Of the 20 patients, 11 received extrathecal treatment exclusively, while the rest were treated in addition with intralumbar streptomycin injections in doses of 1 to 2 mg./kg./day. Comparison of the results between the two groups of children appeared to indicate distinct advantages for the extrathecal method of treatment. The mortality of the intrathecal group was 3 out of 9, whereas on the extrathecal group only 1 out of the 11 patients died. The clinical and humoral response of the latter group was also more prompt, and the frequency of blocks was considerably lower. The authors conclude that exclusive extrathecal therapy is not only possible but appears to be the method of choice. The prolonged use of isoniazid in this series did not cause any disagreeable side reactions or toxic symptoms.

S. L. W.

Mercaptomerin, Systemic Reactions to. W. C. Smallwood and H. L. Matthews. (*Lancet*, 1956, **270**, 121.) Four patients had systemic toxic reactions to subcutaneous injections of mercaptomerin. In three of the patients the reactions appear to have been allergic (the symptoms including fever, rigor, malaise, cyanosis, dyspnœa, and erythematous rash), while the fourth patient developed hæmorrhagic colitis, probably due to excretion of metallic mercury through the wall of the gut. These 4 cases were collected within a period of 2 years from a hospital with 120 medical beds, which suggests that systemic reactions to mercaptomerin may not be rare. The drug was given as directed by the makers. The 4 cases were under treatment when different consignments of the drug were in use in the hospital. Other patients treated with the same batches of mercaptomerin were not affected. In their time-relationship to the giving of the injection and in the prominence of cyanosis and circulatory collapse the reactions in these patients were very similar to those described following the use of other mercurials. Certain characteristics of these reactions suggest an allergic mechanism; they rarely follow the initial dose of the drug and their severity increases with each successive dose and is unrelated to the size of the dose. It appears that the effect of the first few doses is to sensitise the patient and that later doses induce an allergic response. In most cases and initially the response is mild, but if the mercurial is continued even in small doses violent and dangerous reactions may follow, as in two of the cases reported. The authors conclude that mercaptomerin can cause generalised systemic reactions which do not appear to differ from those caused by other organic mercurial diuretics given by other routes.

S. L. W.

Mustine in the Treatment of Malignant Effusions. A. S. Weisberger, B. Levine and J. P. Storaasli. (*J. Amer. med. Ass.*, 1955, **159**, 1704.) Forty-

three patients with pleural, pericardial or peritoneal effusions due to metastatic malignant disease were treated with mustine. Immediately prior to mustine therapy the patients were given 0·2 g. of quinalbarbitone and 50 mg. of chlorpromazine. In the patients with pleural and peritoneal effusions the mustine was administered in a single injection of 0·4 mg./kg. body weight. In 2 patients with pericardial effusion doses of 10 mg. and 22 mg. of mustine were used respectively. The mustine was prepared immediately preceding injection by adding 10 ml. of isotonic sodium chloride to each 10 mg. ampoule. In the treatment of peritoneal effusion a paracentesis was done and half the fluid removed. While there was still a free flow of fluid the mixture was administered either through a catheter inserted into the trocar or through a needle introduced at another site. A similar procedure was followed for the intrapleural and intrapericardial administration, except that the mustine was introduced directly through the needle used to remove the fluid. After administration of the mixture the patient was changed to a new position every 5 or 10 minutes for a period of an hour to ensure more uniform distribution of the material throughout the serous cavity. On the following day paracentesis was repeated and as much fluid removed as possible. The patients were followed closely with X-rays and physical examinations for evidence of reaccumulation of fluid. Of the 43 patients, 28 (65 per cent.) were significantly improved. In 20 of the 28 there was no reaccumulation of fluid; in 8 of the patients there was a marked reduction in the amount of fluid reaccumulating. Twenty of the 28 patients were still living at the time of the report, with improvement lasting from 6 to 24 months in 10 patients and 2 to 5 months in 10 patients. The best responses were obtained in patients with carcinomas of the breast or ovary. In this series the leukopenia following mustine therapy was very mild and much less than that following intravenous therapy with mustine. Nausea and vomiting were also minimal. This suggests that larger and more effective doses of mustine could be used without adverse side-effects. The results compare favourably with those obtained with radioactive colloidal gold and all patients with effusions due to metastatic malignancies should be given a trial of mustine therapy. S. L. W.

Neomycin in Urinary Tract Infections. R. J. Roantree and L. A. Rantz. (*Antibiotic Med.*, 1956, **2**, 103.) *In vitro* studies showed neomycin to be a particularly effective antibacterial agent against the *Escherichia coli*, paracolon, *Proteus*, and *Aerobacter* groups of bacilli; it was found ineffective against *Pseudomonas*. Neomycin was administered intramuscularly to 20 patients having urinary tract infections resistant to most of the other antibiotics. Except in 2 cases the dosage did not exceed 1 g./day and the duration of treatment was 5 days or less. The clinical result was gratifying in most cases. In those cases in which the urinary tract infection was the chief disease the fall in temperature and relief of symptoms was usually prompt. 15 of the 20 patients had sterile urine cultures immediately after treatment, though there were a number of reinfections later; the 5 other patients had *Pseudomonas* in their urine following treatment. No permanent damage to the kidney or to the eighth cranial nerve was noted in this series. This confirms previous observations that neomycin given in a dosage of 1 g. daily for 5 days or less does not result in toxic residual effects if the patient has normal renal function. If there is intrinsic renal disease resulting in nitrogen retention it is probably wise not to use neomycin. Neomycin is a useful agent for the treatment of urinary tract infections if it is reserved for those cases in which the organisms are resistant to less toxic drugs. S. L. W.

Phenoxymethylpenicillin: Plasma Penicillin Levels. R. L. Nichols, W. F. Jones and M. Finland. (*Proc. Soc. exp. Biol., N.Y.*, 1955, **90**, 688.) This paper presents comparisons of penicillin levels in plasma after administration of phenoxymethylpenicillin and benzylpenicillin. The subjects were 8 normal young men and 24 hospital patients, with ages ranging from 15 to 86, all of whom had normal renal function. Three dosage forms of penicillin were used, namely: (1) the free acid of phenoxymethylpenicillin in 200,000 unit tablets, (2) buffered potassium benzylpenicillin in 200,000 unit tablets, and (3) aqueous potassium benzylpenicillin intramuscularly. These were given at 3 dosage levels, namely, 200,000 units, 400,000 units and 1,000,000 units; individual doses were separated by at least 48 hours. All subjects received both the oral penicillins at least at one and the same dosage level and many also received an intramuscular injection of the same number of units of benzylpenicillin. Three of the young men received all 3 dosage forms at all 3 levels, and 5 subjects received all 3 dosage forms at each of the 3 levels. The results of the assays showed that oral phenoxymethylpenicillin gave higher and better sustained levels of penicillin activity in the plasma than oral buffered potassium benzylpenicillin at each of the 3 dosage levels. Intramuscular potassium benzylpenicillin yielded higher and better sustained levels than oral phenoxymethylpenicillin in equivalent doses of 400,000 or 1,000,000 units. An intramuscular dose of 200,000 units of potassium benzylpenicillin produced higher peak levels and these occured earlier, but were less well sustained, than with this amount of oral phenylmethoxypenicillin; the total amount of penicillin absorbed from this amount of penicillin was not significantly different for these 2 dosage forms. Persons over 60 attained peak levels later and, in general, had higher and better sustained levels of penicillin in the plasma from any given dose than did younger individuals. S. L. W.

Propoxyphene, Bioassay of Analgesic Activity of. C. M. Gruber, E. P. King, M. M. Best, J. F. Schieve, F. Elkus and E. J. Zmolek. (*Arch. int. Pharmacodyn.*, 1955, **104**, 156.) A controlled clinical study is reported of the analgesic activity of propoxyphene (α-(±)-2-propionoxy-4-dimethylamino-1:2-diphenyl-3-methyl-butane hydrochloride), compared with codeine phosphate, acetylsalicylic acid and a placebo, in patients with chronic pain. The drugs were given orally in identical capsules, containing approximately equiactive doses at two dose levels, one or two capsules every four hours, and in a randomised order. A complete "series" was one day on each drug and one day on placebo. At the end of each twenty-four hour period, when the drugs were changed, the patients were asked to estimate the number of hours of pain at each of four intensities (severe, moderate, slight or none), which were scored for evaluation. A statistical analysis showed the following to be significant (1) the variation among patients, (2) the variation among the drugs (entirely due to placebo) and (3) between the two doses of the placebo. Significant differences were not found between the analgesics or surprisingly between the doses of the analgesics. Since pain is subjective, the difference between the estimates of pain with the placebo and with the drugs may be used to determine the relief of pain according to the formula:—

$$\text{Per cent. pain relief} = \text{Placebo} - \text{Drug} \times 100 \div \text{Placebo}.$$

The slopes of the lines were almost parallel and there was no important difference in the pain relief between the drugs at the following dose levels, which are concluded to have the same analgesic activity—propoxyphene 50 mg., codeine phosphate 32·5 mg., and acetylsalicylic acid 325 mg. There was also no significant difference in side effects. G. F. S.

Radioactive Digitoxin, Metabolic Fate of, in Human Subjects. G. T. Okita, P. J. Talso, J. H. Curry, F. D. Smith and E. M. K. Geiling. (*J. Pharmacol.*, 1955, **115**, 371.) Biosynthetically-labelled ^{14}C-digitoxin was given intravenously, in multiple doses, to three patients whose prognoses were poor. Doses were between 0·1 and 0·5 mg.; the last dose was administered between 16 hours and 36 hours before death. Autopsies were performed within two hours of death and tissue samples assayed for both unchanged digitoxin and its metabolic products. Heart muscle showed no particular affinity for the glycoside, in comparison with other organs. The kidney and the contents of the gall bladder, jejunum, ileum and colon had the highest concentrations of unchanged digitoxin. The gall bladder contents, jejunum contents and the spleen were associated with high concentrations of the metabolic products. The liver had the greatest total amount of both the glycoside and its metabolites. It was also the main site of detoxification of the drug: most of the administered glycoside was metabolised in the body. The probable fate of injected digitoxin is as follows: after intravenous injection there is an initial rapid removal of the drug from the bloodstream; the drug is metabolised, during and after this time, in the liver and passes into the gastrointestinal tract *via* the biliary tract; it is to a large degree reabsorbed from the small intestine and enters the enterohepatic cycle; the kidney meanwhile excretes small quantities of the metabolites and lesser amounts of the unchanged drug. The kidney is the major excretory site for the drug and its metabolites. G. P.

Thiuram Disulphides and Related Compounds, Acute Toxicity and Disulfiram-like Activity. B. A. Barnes and L. E. Fox. (*J. Amer. pharm. Ass., Sci. Ed.* 1955, **44**, 756.) Several thiuram disulphides and related compounds were tested for toxicity in mice and for ability to induce acetaldehydæmia in rabbits. Di*cyclo*hexylthiocarbamoyl diethylthiocarbamoylsulphide,bis(dibutylthiocarbamoyl)sulphide and bis(di*iso*butylthiocarbamoyl)disulphide had approximately the same activity as disulfiram, while being less toxic. The presence of two amino groups and double-bonded sulphur appeared to be essential for disulfiram-like activity. A possible explanation is that the $\rangle C = S$ group inhibits acetaldehyde metabolism by linking to the enzyme in competition with the substrate, but that in order to have a sufficiently high activity a substituted amino group must be present. Ascorbic acid did not have any effect on the metabolism of ethanol nor on the ability of disulfiram to inhibit the metabolism of acetaldehyde. G. B.

3:5:3′-Tribromo-DL-Thyronine in Myxœdema. N. Compston and R. Pitt-Rivers. (*Lancet*, 1956, **270**, 22.) This study was undertaken not in anticipation of any therapeutic advantage of tribromothyronine over other thyroxine analogues but because the demonstration of high thyroxine-like activity of non-iodine containing analogues of thyroxine in man must cause a reorientation of views on thyroid physiology. Two patients with primary myxœdema were treated with tribromothyronine and both responded fully to a daily dose of 1 mg. of this compound given by intramuscular injection. In both cases normality was maintained, after treatment with tribromothyronine had been stopped, with 4 gr. of thyroid daily. From this it would appear that 1 mg. of DL-tribromothyronine is equivalent to 0·4 mg. of L-thyroxine. It may be assumed from previously published evidence that the D-isomer in DL-tribromothyronine is virtually inactive in human myxœdema. It therefore appears that tribromothyronine has only slightly less activity than thyroxine itself since 1 mg. of the DL-compound (containing 500 μg. of the L-isomer) had activity equivalent to 4 gr. of desiccated thyroid. S. L. W.