REVIEW ARTICLE

PERCUTANEOUS ABSORPTION

By J. W. Hadgraft, F.P.S., F.R.I.C., and
G. F. Somers, B.Sc., Ph.D., F.P.S.

From the Pharmaceutical Department, Royal Free Hospital and The Pharmacological Department, School of Pharmacy (University of London)

The application of preparations to the skin for cosmetic and medical purposes is as old as the history of medicine itself and references to the use of ointments, salves and pomades may be found in the early records of Babylonian and Egyptian medicine. In Roman times, drugs were sold by the "ungentarii" who were the compounders of ointments. Galen's formula for cold cream has persisted through the ages and, with some modification, it is still in use to-day.

Although the use of ointments goes back to antiquity, their use has, until recently, been largely empirical. It is only with the advances in dermatology that there has been an appreciation of the requirements of different ointment bases for different skin conditions. Before 1948, with the exception of hydrous ointment, official ointments were made with fatty materials such as soft paraffin, anhydrous wool fat, or beeswax or combinations of these substances. It was hardly appreciated that the therapeutic usefulness of an ointment depends as much on the kind of base used as on the active medicament. To-day, there are available many different synthetic substances allowing the formulation of a wide variety of preparations for application to the skin. The clinician has, therefore, a considerable choice of bases in the prescribing of dermatological preparations and the pharmacist needs to have an expert knowledge of the properties of the different preparations. Both should understand the main principles concerned in percutaneous absorption.

The percutaneous route has been used as a method of drug administration and, although of strictly limited value, may be useful in particular circumstances. With the introduction of toxic synthetic chemicals such as plasticisers in industry, and the use of highly potent insecticides in agriculture, hazards from the toxic effects after percutaneous absorption have become very real ones. The study of percutaneous absorption is of importance also in the elucidation of the normal functioning of the skin.

This review describes the main factors affecting percutaneous absorption, their assessment and application in preparations used in dermatology and drug administration.

Structure and Physical Properties of the Skin

Structure

The skin consists of an outer layer, the epidermis, and an inner layer, the dermis. The epidermis is a horny layer of keratinised epithelial cells, rich in lipoids and cholesterol. The thickness of this layer depends much on the position on the body and is largely determined by the amount

of wear and tear. Thus it is thickest on the palms of the hands and soles of the feet. The outer layers are being continuously shed and are renewed from the inner Malpighian layer, where transformation of protoplasm into keratin takes place. The layer of epidermis next to the Malpighian layer (or rete mucosum, prickle cell layer) is the stratum granulosum. Directly outside this is the stratum lucidum and the outermost layer is the stratum corneum. The cells of the Malpighian layer contain granules of the pigment, melanin, which are particularly abundant in the dark skinned races.

Beneath the epidermis is the cutis vera or true skin, a thick highly vascular layer consisting of connective and elastic tissue and containing many blood capillaries. The outer layer is folded into minute papillæ, over which the epidermis is moulded to form ridges. The papillæ may contain sensory nerve cells, or tactile corpuscles. The inner layer of the cutis vera passes into the sebaceous tissue.

The skin has a number of appendages—the hair follicles, sebaceous glands and the sweat glands. Each hair grows from a recess, or follicle, formed by the epidermal epithelium dipping into the subcutaneous tissue. The number of epithelial cells is much reduced in the follicle and at the base it thins out into a single layer of non-keratinised cells. The hairs arise from the base of the follicles and each hair consists of an external shaft and a root, called the hair bulb, implanted in the skin. The hair follicles are filled with sebum, a secretion from the sebaceous glands the ducts of which open into the upper portion of the hair follicle.

The sweat glands cover the whole of the human skin and each consists of a coiled tube in the dermis with a duct passing through the epidermis to the skin surface. The sweat glands are under vasomotor control and secrete sweat which helps to control the body temperature.

Physical Properties

The skin forms a relatively tough and impervious coating over the body. It has an important protective function and also plays an important part in the maintenance of a constant internal environment. It is not surprising that most substances penetrate the skin with difficulty and that the skin is highly selective in allowing substances to pass through it to the underlying tissues.

Externally, the skin is covered by a greasy secretion from the sebaceous glands consisting of waxes and cholesterol[1]. While this layer delays the penetration of water, prolonged immersion allows the waxes and cholesterol to be emulsified and water then penetrates into the horny epidermis. The keratin swells and the skin becomes white and wrinkled.

The main resistance to penetration of water and aqueous solutions through the epidermis is the presence of a polarised layer, or "electrophysiological barrier"[2], between the horny layer and the basal cell layer. This is situated between the acid stratum corneum (pH 5) and the slightly alkaline Malpighian layer, and represents an electrical double layer with positive H^+ ions on the one side and negative OH^- ions on the other. Substances applied to the skin penetrate mainly down the hair follicles[3–6].

Abnormal Skin

The normal condition of the skin can be altered by physical and pathological changes. These will affect percutaneous absorption. Removal of the epidermal layers by abrasion or cutaneous lesions allows medicaments to penetrate directly into the subcutaneous tissues and the circulation. With broken skin there is a definite danger of topically applied medicaments causing toxic effects when applied to large areas[7]. Degreasing the skin with solvents, such as benzene, chloroform, ether or acetone will remove the sebum from the skin and hair follicles, thus considerably facilitating percutaneous absorption[8]. Their main action is to dissolve the lipoid compounds in the living cells themselves[9]. Detergents also degrease the skin and are capable of bursting lipoid from the epidermal cells, thus creating conditions for percutaneous absorption. Astringents and caustic substances, for example, phenol, by precipitating cellular protein, may actually decrease absorption[10–12]. In lower concentrations, however, phenol may be absorbed with dangerous toxic effects.

In pathological conditions, functional changes alter the electrical behaviour and diminish skin resistance[9]. Changes in the skin membrane cause increased permeability and decreased polarisation currents, which can be demonstrated in inflammatory lesions. The normal acidity of the external skin may be changed to an alkaline reaction[13], as in seborrhœic dermatitis and eczema. Keratin is an amphoteric protein with an isoelectric point at pH 5·6. At the normal hydrogen ion concentration of the skin, there is a high resistance to hydrolytic agents, but in the presence of alkali the keratin becomes hydrated and behaves as a colloid gel[14]. At very alkaline values, hydrolysis occurs irreversibly with destructive effect. Increase in pH, therefore, will render the skin more permeable to external irritants.

Factors Affecting Percutaneous Absorption

The main factors affecting percutaneous absorption are (*a*) penetration and mode of absorption, (*b*) the temperature of the skin, (*c*) the properties of the medicament, (*d*) the influence of the vehicle and (*e*) the mode and duration of application.

Penetration and Mode of Absorption

Before a medicament can be absorbed it must first penetrate the skin. The main route of penetration is through the hair follicles[3–6] and sebaceous glands, but some penetration may occur through the sweat glands[16]. Except in special circumstances, absorption does not take place through the horny layer. The inuncted material passes through the follicle opening, along the hair and root sheaths and is absorbed through the sebaceous glands and the epidermal cells at the base of the hair follicles[9,15]. Here the epidermis thins out to a single layer of non-keratinised cells so that conditions are favourable for absorption, after which the medicament diffuses downwards into the highly vascular cutis vera, also horizontally and upwards into the epidermis[17].

Skin Temperature

It is well known that elevation of the skin temperature enhances percutaneous absorption. This may be due to a lowering of the viscosity of the sebum and facilitating its mixing with the inuncted preparation. A rise in skin temperature also increases the cutaneous circulation through vasomotor dilatation of the skin vessels. The skin temperature may be raised by the application of heat, irritant substances, or by covering with an air-tight dressing.

Properties of the Medicament

A major factor in skin penetration is lipoid solubility of the medicament[9,18]. It has been proved that lipoid soluble substances penetrate the skin, while lipoid insoluble substances do not. Salicylic acid, which is lipoid soluble, penetrates with ease; but sodium salicylate, which ionises in solution and is not lipoid soluble, does not penetrate the skin[9,19]. There are some substances, particularly the heavy metals, which are not lipoid soluble but undergo a chemical reaction with fatty acids in the sebum and are slowly absorbed[9]. The beneficial effects of ammoniated mercury have been stated to be due to the slow dissociation of mercuric ions under the influence of the acid reaction of the horny layer and sweat[20], which are then adsorbed on to the epidermal cell walls. Absorption into the general circulation does not take place[20–23].

Maximal percutaneous absorption occurs when the medicament combines lipoid solubility with a moderate solubility in water. These substances are soluble in the sebum, readily penetrate into the skin and then dissolve in the tissue fluids. The organophosphorus insecticides are particularly dangerous in this respect and it is difficult to oppose their absorption by the use of barrier creams. Progesterone has a similar chemical structure to œstradiol and the same lipoid solubility, but it is not so well absorbed because it is less soluble in water[24].

Influence of the Vehicle

The function of the vehicle in percutaneous absorption is to facilitate contact between the medicament and the absorbing cells in the sebaceous glands and at the base of the hair follicles. The vehicle itself cannot promote percutaneous absorption of a lipoid insoluble substance, nor can it transport the substance through the cell membrane. It can, however, retard penetration and absorption, for an ointment base which is not miscible with the sebum will hinder the penetration of the medicament into the hair follicle.

Absorption is best from vehicles which spread easily over the skin surface, readily mix with the sebum and so bring the medicament into contact with the absorption cell areas. It has long been known that absorption is better from animal and vegetable oils than from mineral oils, because they more readily penetrate the skin[3,5,25]. Organic solvents, like ether, chloroform, benzene and acetone penetrate the skin with ease[26], and enhance the percutaneous absorption of a medicament to such an extent that toxic effects can occur. Surface active agents, because of their

high wetting and detergent properties, should increase percutaneous absorption, but when they are incorporated in ointment bases there is little evidence that this occurs. It has been reported that deeper and more rapid penetration of ammoniated mercury is obtained by the addition of a wetting agent to a paraffin ointment base[27], but the addition of sodium laurylsulphate to a polyethylene glycol base fails to increase the absorption of phenolsulphonphthalein or potassium iodide[28]. In other experiments, the penetration of iron, bismuth and sulphonamides was promoted by the addition of alkylbenzene sulphonate to aqueous vehicles containing propylene glycol[15].

The addition of lipoids, such as cholesterol, increases the penetrating properties of liquid paraffin[5], but the application of cholesterol to the skin reduces permeability to water soluble salts by reinforcing the resistance of the fatty substances normally present in the skin[29].

The absorption of a medicament from an ointment will depend on its rate of liberation from the base, and this will depend upon the partition coefficient of the medicament between the base and the sebum. The inunction of a solution of phenol in oil will not have a toxic effect because the amount of phenol liberated is negligible, while aqueous solutions can have a caustic effect.

Incorporation of the medicament in an emulsified base affects absorption. It has been found that the absorption of diiodofluorescein ^{131}I is poor from an oil-in-water base, because the continuous aqueous phase probably retards penetration[30]. Absorption is better from water-in-oil emulsions, where the external phase is readily miscible with the sebum, and from cetomacrogol which possesses both lipophilic and hydrophilic properties.

Mode and Duration of Application

The mode of application affects percutaneous absorption. Friction and massage cause a local vasodilatation of the skin vessels, so increasing the skin temperature and promoting absorption. Mechanical pressure forces the ointment and the medicament into the hair follicles, mixes them with the sebum and displaces entrapped air[9,25].

The duration of application also affects absorption. With diiodofluorescein ^{131}I it has been shown that there is an increase in absorption with time[30]. After inunction, urinary excretion of mercury continues at a high level for at least a week after cessation of application. This is due to a store of accumulated mercury in the follicles[31].

Assessment of Percutaneous Absorption

The assessment of percutaneous absorption presents a number of fundamental problems. It is necessary to differentiate clearly between skin penetration and absorption.

The methods can be divided into two main groups (*a*) those which measure skin penetration and (*b*) those which measure systemic absorption. In all *in vivo* methods allowance must be made for an inherent variability between the test objects. Ideally, any scheme of quantitative

assessment should follow the general basic principles for biological assays defined by Dale[32] and enumerated by Emmens[33]. Simultaneous comparisons should always be made with a standard preparation under the same experimental conditions. However, strictly valid numerical assessments can rarely be made, for, in comparing absorption from different vehicles, the standard and test differ in composition and so do not comply with the biological assay principles. It is usually necessary to be content with generalised statements, such as "absorption is better from one vehicle than from another". Finally, the importance of species differences must be considered in transferring results obtained in experimental animals to man.

For the study of skin penetration medicaments are used which can be easily detected microscopically. Sections of the skin are prepared under carefully controlled conditions to prevent the movement of the medicament. The skin used may be animal or human skin removed in biopsies. Among the substances which have been employed in such investigations are oil soluble dyes[5], sulphonamides and iron and bismuth compounds[15]. Radioactive tracer materials may also be used where autoradiographs are prepared on photographic emulsions for comparison with normal sections[34]. A useful method for assessing skin penetration is to apply the medicament in solution to the skin under a bell jar. Changes in the concentration of the compound in the solution are measured at increasing time intervals[35,36]. This method is most suitable for solutions in volatile liquids and it gives valuable information on changes in penetration with both time and concentration.

For the study of skin absorption both *in vitro* and *in vivo* methods are used. *In vitro* methods are of a limited value only, for they bear little relation to the conditions occurring in normal skin. They do give useful information on the release of the drug from the base. The most commonly used methods are based on the diffusion of the medicament from cups in an inoculated agar plate. The zones of inhibition are measured, as in microbiological assay techniques[37,38]. Their main value is in determining the antiseptic activity of medicaments in ointment bases. Attempts have been made to simulate skin conditions by using natural and artificial membranes. Measurements have been made of the release of sodium chloride from various ointment bases through cellophane bags[39], and sodium iodide through sheep bladders[40].

For the assessment of skin absorption *in vivo*, the medicament is applied to the skin in the vehicle being tested. The amount absorbed is measured in the blood, urine, fæces or some particular organ by suitable chemical or physical methods. Substances used have included methyl salicylate[41] and iodine[42]. The availability of radioactive tracer substances has increased the sensitivity of such methods[30,43–45].

Alternatively, substances are applied to the skin which cause characteristic pharmacological actions when absorbed, such as local anæsthesia, inhibition of cholinesterase, growth of a particular organ or even death. Substances used have included the alkaloids[46,47], hormones[48,49], vitamins[50] and chemotherapeutic substances[51]. Some of these methods have been used for clinical assessment in man[41,52].

PERCUTANEOUS ABSORPTION

Radioactive tracer techniques provide new and sensitive methods for the assessment of percutaneous absorption, but they have some disadvantages when used in animal tests. Variations in absorption in different animals can be great, and it is difficult to use large numbers in a single test, because of increased radiation hazards and difficulties in the disposal of contaminated materials[30].

Dermatological Requirements

The determatological requirements of an ointment base differ according to the skin condition under treatment[53]. When the horny external layer of the skin has been damaged, or exfoliated, an ointment may be needed as a protective. Such ointments are usually inert, bland preparations, which are largely unabsorbed by the skin. They include the paraffins, which are particularly valuable in xerodermia and ichthyosis. They may include medicaments which are usually poorly absorbed and exert only a local action, e.g., ammoniated mercury, sulphur and zinc oxide.

Barrier creams are protective preparations, which are applied to the skin before exposure to irritant and other noxious substances. Their formulation will depend to a large extent on the type of substance which they are intended to guard against.

When it is desired to soften the skin an emollient ointment is used. Preparations of this type must penetrate the hair follicles. They usually contain an animal or vegetable oil, which is intended to replace a deficiency of the natural fats in the skin, as in xerodermia, ichthyosis and senile skin.

Ointments applied to the skin surface to convey a medicament to a localised area are used in the treatment of superficial lesions. In such preparations, it is important that the medicament should be released from the base but should not be absorbed systemically. Antiseptic ointments are best formulated in emulsified bases[54], such as hydrous emulsifying ointment, and it is important that the base chosen should have no adverse effect on the activity of the medicament.

In some conditions the medicament must penetrate through the skin to permeate the lower layers. In ringworm of the scalp, for example, fungal spores and mycelia become embedded deep in the hair follicles and are not reached by fungicides in the usual type of base. By the use of polyethylene glycols, ointments have been formulated, containing phenylmercuric nitrate or salicylanilide, which will destroy the mycelia and spores without the need for previous epilation[55,56]. Penetrating vehicles have also been used for the application of sulphur in acne vulgaris[57], and tyrothricin in the treatment of pyodermas[58].

Percutaneous Administration

The percutaneous route has only a limited sphere of usefulness in the administration of drugs. It has the advantage of enabling a high concentration of the drug to be built up in a certain region of the body for an intense localised action[59,60] with limited side effects. In addition, it offers an alternative route of administration for substances which are inactivated in, or irritate the gastro-intestinal tract, and would normally have to be given by parenteral injection. The main difficulty with percutaneous

administration is the control of doses. It is particularly useful when it is desired to build up the concentration of a drug slowly in the body and then to maintain it at a constant level. This was the basis of the use of mercurial ointments in the treatment of syphilis, where a depot of mercury was retained and slowly absorbed from the hair follicle[31].

A few of the more important drugs which have been administered percutaneously will now be discussed.

Vitamins and Hormones

The absorption of vitamins and hormones through the skin depends on their lipoid solubility. Oil soluble vitamins and hormones are well absorbed while, the water soluble ones are not.

Sex Hormones. The natural œstrogens are better absorbed through the skin than the synthetic ones, and the gradual and maintained concentration obtained in the body provides the optimal conditions for their action[61]. An intense local action is the basis of the application of œstradiol to promote development of the breast in the hypogonadal female[62]. Experience in the treatment of acne and various dermatoses, has shown that beneficial local effects can be obtained by the topical application of œstrogens in alcoholic solution, or in a vanishing cream base, without systemic effects[63].

The inclusion of œstrogens in cosmetic preparations for the improvement of senile skin has recently caused some controversy[64]. Provided they are used in moderation the amount of œstrogen absorbed does not appear to be significant. The indiscriminate use of œstrogens in cosmetic creams, however, is not to be encouraged, for systemic effects have been observed[65] and a possible carcinogenic action has not been disproved. Progesterone is not so well absorbed as the œstrogens[24,49,66] and the tissues are less sensitive to it. It has been used as a constituent of cosmetic creams.

Both testosterone and its propionate are absorbed through the skin, the free alcohol being more effectively absorbed than the ester[61,67]. Methyltestosterone is also absorbed, but to a less extent[68]. Percutaneous administration of testosterone propionate in sesame oil solution has been used in the treatment of dysmenorrhœa[69]. In other clinical conditions, both in the male and the female, percutaneous administration has been valuable for obtaining a localised effect[70,71].

Cortical Hormones. Cortisone and hydrocortisone can only be absorbed through the skin to a small extent. Systemic effects have not been observed, even when large doses have been applied over extensive areas of the skin. The application of a total dose of 750 mg. of hydrocortisone, over a 3 day period in a washable ointment, failed to increase the blood and urine concentrations of 17 : 21-dihydroxy-20-ketosteroids[72]. It has also been shown that the topical application of hydrocortisone does not increase the circulating eosinophils[73].

Deoxycortone is absorbed through the skin, and it has been reported that percutaneous absorption from a solution in eucalyptol is as effective as a subcutaneous injection in oil[25].

Vitamins. The fat soluble vitamins can be absorbed through the skin and vitamin A deficiency in animals has been cured by the percutaneous administration of the vitamin[74]. Absorption is better from organic solvents than from oily solutions or ointment bases[75]. Any beneficial effects from the application of vitamin A ointment in the treatment of ichthyosis, however, may be attributed to a non-specific effect on the epidermis, and even the administration of excessive doses does not significantly increase the total vitamin A plasma levels[76].

Vitamin K and its analogues are absorbed through the skin and percutaneous administration in light liquid paraffin and kerosene has been described as a convenient way of administering menaphthone to new-born infants[77].

Toxicity Hazards following Percutaneous Absorption

A brief mention must be made of the dangers following the absorption of toxic substances through the intact skin. Skin absorption is an industrial hazard in the use of some solvents, plasticisers, detergents and, more recently, in the handling of radioactive materials. The introduction of new and potent pesticides, particularly the organo-phosphorus compounds, raises a very definite occupational risk in agriculture. These compounds are found to penetrate the waxy cuticle of the insect so it is not surprising that they penetrate human skin with ease. Protection is afforded by special clothing and suitable barrier creams as a second line of defence.

In medicine there must always be a danger of toxic effects when potent medicaments are applied to large areas of broken skin. Attention has recently been drawn to an increasing number of instances of boric acid and borax poisoning in babies, many of which have been fatal[7]. The two most important factors involved are the concentration of the drug in the preparation and the area of broken skin to which it is applied.

References

1. Rothman, *Oppenheimer's Handbuch der Biochemie des Menschen und der Tiere*, 2nd Ed., 1934, Supp. Vol. 2, 157.
2. Rein, *Ztschr. f. Biol.*, 1927, **85**, 195.
3. Eller and Wolf, *Arch. Derm. Syph., N.Y.*, 1939, **40**, 900.
4. Miescher, *Dermatologica*, 1941, **83**, 50.
5. Harry, *Brit. J. Derm.*, 1941, **65**, 82.
6. Strakosch, *J. Pharmacol.*, 1943, **78**, 65.
7. Wilson, *Pharm. J.*, 1956, **176**, 199.
8. Starkenstein and Hendrych, *Arch. exp. Path. Pharmak.*, 1936, **182**, 664.
9. Rothman, *J. Lab. clin. Med.*, 1943, **28**, 1305.
10. Macht, *Arch. int. Pharmacodyn.*, 1938, **58**, 1.
11. Burgi, *Rev. med. la Suisse Romaine*, 1927, **57**, 461.
12. Meyenberg, *Dermat. Wschr.*, 1941, **112**, 31.
13. Anderson, *International Congress of Dermatology*, 1952.
14. Rothman and Flesch, *Ann. Rev. Phys.*, 1944, **6**, 205.
15. McKee, Sulzberger, Hermann and Baer, *J. Invest. Derm.*, 1945, **6**, 43.
16. Abramson and Engel, *Arch. Derm. Syph., N.Y.*, 1941, **44**, 190.
17. Herrmann, Sulzberger and Baer, *Science*, 1942, **96**, 451.
18. Jacobs, *Sect. III, Cowdry's General Cytology*, 1924, p. 99, *per* Rothman, *op. cit.*
19. Hopmann, *Der Balneologe*, 1939, **6**, 5.
20. Perutz, *Jadassohn's Handbuch der Haut und Geschlechshankheiten*, 1930, **5**, 2.
21. Current Comment, *J. Amer. med. Ass.*, 1936, **107**, 1722.

22. Maloff, *Dtsch. med. Wschr.*, 1928, **54**, 1381.
23. Moncorps, *Arch. exp. Path. Pharmak.*, 1930, **155**, 51.
24. Isler and Mosimann, *Ann. Endocrinol.*, 1950, **11**, 69.
25. Vallette, *Pharm. J.*, 1953, **170**, 461.
26. Vallette, *J. Physiologie*, 1951, **43**, 41; *Therapie*, 1951, **6**, 443; *ibid.*, 1952, **7**, 139.
27. Duemling, *Arch. Derm. Syph., N.Y.*, 1941, **43**, 264.
28. Mayers, Nadkarnie and Zopf, *J, Amer. pharm. Ass. Sci. Ed.*, 1949, **38**, 231.
29. Matschak, *Arch. exp. Path. Pharmak.*, 1936, **182**, 688.
30. Hadgraft and Somers, *J. Pharm. Pharmacol.*, 1956 in the press.
31. Cole, Gammel, Rauschkolb, Schreiber and Sollman, *Arch. Derm. Syph., N.Y.*, 1926. **14**, 683.
32. Dale, *Analyst*, 1939, **64**, 554.
33. Emmens, *Hormones; A Survey of Their Properties and Uses*, The Pharmaceutical Press, London, 1951, 113.
34. Leblond and Gross, *Endocrinology*, 1948, **43**, 306.
35. Burgi, *Schweiz med. Wschr.*, 1937, **18**, 433.
36. Heidiger, *Klin. Wschr.*, 1928, **7**, 1553.
37. Reddish, *Proc. Amer. Drug. Manuf. Assoc.*, 1929, **16**, 116.
38. Reddish and Wales, *J. Amer. pharm. Ass., Sci. Ed.*, 1929, **18**, 576.
39. Rae, *Brit. J. Derm.*, 1944, **56**, 92.
40. Luff, *Pharm. J.*, 1891, **50**, 206.
41. Brown and Scott, *J. Pharmacol.*, 1934, **50**, 32.
42. Nyiri and Janitti, *ibid.*, 1932, **45**, 85.
43. Johnston and Lee, *J. Amer. pharm. Ass., Sci. Ed.*, 1943, **32**, 278.
44. Cyr, Skauen, Christian and Lee, *ibid.*, 1949, **38**, 615, 618.
45. Tronnier and Wagener, *Hautarzt*, 1953, **4**, 214.
46. Macht, *J. Amer. med. Ass.*, 1938, **110**, 409.
47. Hadgraft and Somers, *J. Pharm. Pharmacol.*, 1954, **6**, 944.
48. Zondek, *Klin. Wschr.*, 1929, **8**, 2229.
49. Zondek, *Lancet*, 1938, **234**, 1107.
50. Hume, Lucas and Smith, *Biochem. J.*, 1927, **21**, 362.
51. Zondek, *Nature, Lond.*, 1942, **149**, 334.
52. Moncorps, *Arch. exp. Path. Pharmak.*, 1929, **141**, 25.
53. Hadgraft and Brain, *Lancet*, 1949, **257**, 78.
54. Mumford, *Brit. J. Derm.*, 1938, **50**, 540.
55. Brain, Crow, Haber, McKenna and Hadgraft, *Brit. med. J.*, 1948, **1**, 723.
56. Haber, Brain and Hadgraft, *ibid.*, 1949, **2**, 626.
57. MacKee, Wachtel, Karp and Herrmann, *J. Invest. Derm.*, 1945, **5**, 309.
58. MacKee, Sulzberger, Herrmann and Karp, *ibid.*, 1946, **7**, 175.
59. Fussganger, *Med. Chim.*, 1934, **2**, 195.
60. Emmens, *J. Endocrin.*, 1941, **2**, 368.
61. Moore, Lamar and Beck, *J. Amer. med. Ass.*, 1938, **111**, 11.
62. MacBryde, *ibid.*, 1939, **112**, 1045.
63. Shapiro, *J. clin. Endocrin.*, 1952, **12**, 751.
64. Peck and Klarman, *Practitioner, Lond.*, 1954, **173**, 159.
65. Goldberg and Harris, *J. Amer. med. Ass.*, 1952, **150**, 790.
66. Leighty, Wrick and Jeffers, *Endocrinology*, 1941, **28**, 593.
67. Nelson, Greene and Wells, *ibid.*, 1940, **26**, 651.
68. Greene, Oppenheimer, Burrill and Nelson, *ibid.*, 1941, **29**, 979.
69. Abarbanel, *ibid.*, 1940, **26**, 765.
70. Foss, *Lancet*, 1938, **235**, 1284.
71. Spence, *ibid.*, 1939, **237**, 820.
72. Witten, Shapiro and Silber, *Proc. Soc. exp. Biol., N.Y.*, 1955, **88**, 419.
73. Smith, *Arch. Derm. Syph.*, 1953, **68**, 50.
74. Sobel, *J. Amer. med. Ass.*, 1955, **157**, 1537.
75. Montagna, *Proc. Soc. exp. Biol., N.Y.*, 1954, **86**, 668.
76. Flesch, *J. Invest. Derm.*, 1952, **19**, 353.
77. Vollmer, *Amer. J. Dis. Child.*, 1942, **64**, 462.

RESEARCH PAPERS

THE MICROBIOLOGICAL ASSAY OF VITAMIN B_{12} IN LIVER EXTRACTS

I. Experimental Design and Problems of Validity

By F. A. Robinson, M. E. H. Fitzgerald and J. J. Grimshaw

From the Research Division, Allen & Hanburys Ltd., Ware

Received March 26, 1956

Introduction

For many years these laboratories have been concerned with the production of liver extracts and with the estimation of their cobalamin contents. This paper deals with the experimental methods employed, with the development of the present experimental design, and with some of the problems of validity encountered. In a later publication it is hoped to discuss other aspects of the work and suggest possible explanations for some of the anomalous results.

Methods

The microbiological assay of the "*Lactobacillus lactis* Dorner factor" by a tube assay method was first described by Shorb[1], who related the response of the organism to the hæmatopoietic activities of liver extracts in U.S.P. units. Using the same organism, Foster, Lally and Woodruff[2] described a plate assay method.

Many types of microbiological assay have since been evolved. Skeggs, Nepple, Valentik, Huff and Wright[3] used a tube assay method with *L. leichmannii* 4797, and Emery, Lees and Toothill[4] described a similar method and presented a statistical analysis of a (3,3) assay design. Bessel, Harrison and Lees[5] described a cup-plate assay and Burkholder[6] a tube assay, both using the *B. coli* mutant described by Davis. In a later paper Harrison, Lees and Wood[7] described a modified plate assay medium, and reported results obtained with large plates (*cf.* Brownlee, Loraine and Stevens[8]) as well as with the more usual six-cup petri dish. Hutner, Provasoli, Stokstad, Hoffmann, Belt, Franklin and Jukes[9] described a method using *Euglena gracilis* and Ford[10] introduced the use of the protozoan *Ochromonas malhamensis.*

The work described in this paper has been confined to the use of *B. coli* N.C.T.C. 8134 in plate assays and *L. leichmannii* N.C.T.C. 7854 in both plate and tube assays.

B. coli *Plate Assays*

Harrison and others[7] recommended a (2,2) assay design since, for pure vitamin B_{12}, the log dose/response regression was rectilinear over the dose range 0·005 μg./ml. to 5·0 μg./ml. of vitamin B_{12}. Using five plate replicates they obtained standard errors of 0·14 to 0·23 mm. per zone. They found that the assay was valid for vitamins B_{12a}, B_{12b} and B_{12c} when

the solutions were treated with potassium cyanide, thus converting the compounds into cyanocobalamin before assay.

We have used the *B. coli* plate assay medium of Harrison and others[7] but prefer to use shredded agar which is autoclaved, filtered and added while still hot to the rest of the medium. The complete medium, except for glucose, is distributed in 125-ml. amounts in 150-ml. screw-capped bottles and autoclaved for 30 minutes at 10 lb. pressure. The autoclaved medium can be stored at room temperature and gives satisfactory assays for periods of storage of two months.

An 18-hour inoculum is prepared from a weekly slope culture and grown in 10 ml. of peptone broth. The inoculum density is read on an E.E.L. Colorimeter* and the volume of inoculum to be added is calculated from a chart showing the optimal amount of inoculum that must be used for a given inoculum density to produce the best growth zones.

The bottles of medium are steamed for 30 minutes immediately before use and the clear solution is decanted into a hot conical flask from the insoluble residue of phosphate. To the decanted medium is added 1 ml. of 20 per cent. sterile glucose solution, the flasks and contents are cooled in a water bath to 45–48° C., and the inoculum is then added and thoroughly mixed.

Nine plates are filled on levelling tables using a fast-running bulb pipette graduated at 12·5 ml., allowed to set and then refrigerated for two hours before cutting. Except that plates, pipettes and dilution tubes are dry-sterilised before use, aseptic precautions are unnecessary with this method of assay. Six holes to the plate are cut using a guide-frame to ensure vertical, evenly spaced holes. Careful attention to the cutter has been found to be necessary; silver-steel is preferable to stainless-steel and the cutters are ground and trued on a lathe at weekly intervals. The discs of agar are removed by suction and the plates are refrigerated for about three hours, water being removed from each set of three plates by suction immediately before filling. The holes in the plates are then filled with the appropriate test and standard solutions, the three plates in a set being filled with the same solutions. After incubation at 37° C. for 16 hours the diameters of the zones of exhibition are measured with Lee-Guiness spring-bow dividers to the nearest 0·1 mm.

L. leichmannii *Plate Assay*

The medium used is given in Table I, 230-ml. amounts being stored in screw-capped bottles. The storage life of this medium at room temperature is less than that of the *B. coli* medium and it is renewed monthly.

To each 230-ml. portion of medium cooled to 42–44° C. is added 10 ml. of a saline-washed 18-hour culture of *L. leichmannii* giving an E.E.L. reading at 37 to 40. Except that 25 ml. of medium is added to each plate using a sterilised measuring cylinder the technique of plate preparation is exactly as described for the *B. coli* plate assay.

Usually we carry out *B. coli* and *L. leichmannii* plate assays in parallel

* Evans Electroselenium Ltd., Harlow, Essex.

TABLE I

COMPOSITION OF MEDIUM USED IN *L. leichmannii* PLATE ASSAYS

Glucose	20 g.
Sodium acetate (hydrated)	20 g.
Sodium citrate	20 g.
Acid hydrolysed casein (A and H)	10 g.
*Salts A	60 ml.
*Salts B	40 ml.
Polysorbate 80	20 ml. of a 10 per cent. solution in neutralised absolute ethanol
L-Cystine	20 ml. of a solution containing 20 μg./ml.
DL-Asparagine	10 ml. " " " 20 μg./ml.
DL-Tryptophane	10 ml. " " " 20 μg./ml.
Xanthine	20 ml. " " " 1 μg./ml.
Adenine	10 ml. " " " 1 μg./ml.
Guanine	10 ml. " " " 1 μg./ml.
Uracil	10 ml. " " " 1 μg./ml.
p-Aminobenzoic acid	5 ml. " " " 1 μg./ml.
Nicotinic acid	2 ml. " " " 1 μg./ml.
Riboflavine	8 ml. " " " 200 μg./ml.
Pyridoxine hydrochloride	0·4 ml. " " " 1 μg./ml.
Calcium *d*-pantothenate	0·4 ml. " " " 1 μg./ml.
Aneurine chloride hydrochloride	0·4 ml. " " " 1 μg./ml.
Biotin	4 ml. " " " 1 μg./ml.
Folic acid	4 ml. " " " 1 μg./ml.
Thioglycolic acid	2 ml.
Sodium chloride	40 g.
Agar	35 g.
Water up to	2000 ml.

* *Salts A* 25 g. K_2HPO_4, 25 g. KH_2PO_4 } 250 ml. of solution.

* *Salts B* 35 g. $MgSO_4.7H_2O$, 7·5 g. $MnSO_4.7H_2O$, 2·1 g. $FeSO_4.4H_2O$ } 250 ml. of solution.

and it is convenient for each operator to prepare, fill and read three sets of plates with each organism.

The design of the assay, which is similar to that of the *B. coli* plate assay, will be discussed later.

L. leichmannii *Tube Assays*

The *L. leichmannii* tube assay of vitamin B_{12} has been very widely studied. Of particular interest are first the work of Loy, Haggerty and Kline[11,12], who dealt with the effect of reducing agents and with other aspects of the assay, and secondly, the long series of collaborative studies undertaken by the Association of Official Agricultural Chemists[13–15]. Using the medium of the U.S.P. XIV 4th Supplement, and the turbidimetric method of measuring growth the collaborators found on the whole, good inter- and intra-laboratory agreement and the method has now been made official.

Although we have now selected for use the U.S.P. medium (Medium 1) we prefer to measure the bacterial growth by titration of the lactic acid produced rather than by the turbidimetric method.

We have employed one variant of the U.S.P. medium (Medium 2) in which the ascorbic acid has been replaced by thiomalic acid at a concentration of 0·5 g. per litre of medium (double strength), and another variant (Medium 3) in which 6 mg. of potassium cyanide per litre of medium (double strength) has been added to the first variant. This medium is adjusted to pH 6·8 before sterilization, and the others to pH 6·0. All assays are run in triplicate at each dose level.

To each tube is added 5 ml. of medium (double strength) followed by the standard or test solution, and the volume is then adjusted to 10 ml. by the addition of distilled water. The tubes are then autoclaved for 10 minutes only at 10 lb. pressure, cooled, and inoculated with one drop of a dilute suspension of *L. leichmannii* that has been washed 5 times with sterile saline and then suspended in sterile saline to give a reading on the E.E.L. Colorimeter of about 15. The tubes are incubated for 65 hours at 37° C. and the growth is measured by titrating the acid produced to the initial pH value with 0·1N sodium hydroxide, using either bromothymol-blue indicator for Media 1 and 2, or bromocresol-purple for Medium 3.

Results

The results obtained graphically on 14 samples of liver extracts at various stages of production when assayed by five different methods, are given in Table II. Where the assay was valid the results obtained statistically are also given. Where the assay was invalid the reasons for the invalidity are recorded. To illustrate the way in which the results were calculated the values obtained in the *L. leichmannii* plate assay of Sample No. 11, together with the analysis of variance and estimation of potency and fiducial limits, are given in Table III.

TABLE II

COMPARISON OF VITAMIN B_{12} CONTENTS (μg./ml.) OF CRUDE LIVER EXTRACTS OBTAINED BY FIVE METHODS OF ASSAY

Sample	*B. coli* plate assay			*L. leichmannii* plate assay			*L. leichmannii* tube assay								
							Medium 1			Medium 2			Medium 3		
	Graph	Calc.	Valid	Graph	Calc.	Valid	Graph	Calc.	Valid	Graph	Calc.	Valid	Graph	Calc.	Valid
1	0·20	0·20	+	0·33	0·32	+	0·23	0·19	+	0·16	0·17	+	0·21	—	C.C.
2	0·30	0·30	+	0·48	0·50	+	0·24	0·23	+	0·23	0·28	+	0·14	—	P. & C.C.
3	2·2	2·2	+	3·0	3·0	+	1·4	—	O.C.	1·8	1·7	+	1·7	1·7	+
4	1·3	1·3	+	3·6	4·0	+	2·0	2·0	+	1·8	1·6	+	2·1	1·9	+
5	1·6	1·4	+	2·3	—	P. & O.C.	1·3	1·4	+	1·5	1·8	+	2·1	—	C.C.
6	2·6	2·8	+	completely invalid			2·6	2·7	+	3·4	3·4	+	2·4	2·2	+
7	2·4	2·3	+	3·6	3·6	+	3·1	3·1	+	2·8	2·6	+	2·9	2·9	+
8	2·1	2·0	+	4·2	4·4	+	1·7	1·8	+	1·2	—	O.C.	1·8	—	C.C.
9	1·8	1·8	+	3·3	3·6	+	1·7	1·7	+	1·8	1·7	+	3·4	3·4	+
10	1·8	1·9	+	3·0	3·2	+	1·8	1·8	+	2·3	—	O.C.	2·9	2·8	+
11	2·2	1·9	+	2·7	2·6	+	1·7	1·8	+	1·2	—	O.C. & P.	1·2	—	C.C.
12	4·0	4·0	+	7·1	6·8	+	3·4	3·6	+	3·6	3·7	+	2·9	—	C.C.
13	4·1	4·0	+	7·0	6·5	+	3·5	3·5	+	4·5	—	O.C. & P.	5·2	—	C.C.
14	2·9	2·6	+	4·2	4·1	+	2·9	2·8	+	3·3	3·1	+	1·3	1·5	+

Graph: Result estimated graphically, fitting regression lines by eye.
Calc.: Calculated result where assay is valid.
Valid: State of validity. If invalid: P = lack of parallelism; O.C. = opposed curvature; C.C. = combined curvature.

TABLE III

POTENCY OF SAMPLE NO. 11 (see Table II) BY THE *L. leichmannii* PLATE ASSAY

	Diameters of zones in 0·1 mm.						Coefficients of orthogonal contrast				
	Standard (1 μg./ml.) diluted			Test solution (1/3 dilution) diluted							
	1/20	1/10	1/5	1/20	1/10	1/5	Lp	L_1	L_1'	L_2	L_2'
Zone Size	113	130	149	111	128	143	−10	68	4	0	4
	111	130	149	108	124	141	−17	71	5	1	−1
	115	129	140	111	127	142	− 4	56	−6	−4	−2
Totals	339	389	438	330	379	426	−31	195	3	−3	1

Analysis of Variance

Adjustment for mean = 294144·5

Source of variation	S of S	d.f.	M.S.	F	P
Preparations	53·4	1	53·4	7·98	<0·05
Regression	3168·8	1	—	—	—
Parallelism	0·8	1	0·8	<1	—
Combined curvature	0·3	1	0·3	<1	—
Opposed curvature	0·0 (3)	1	0·0 (3)	<1	—
Between doses	3223·3	5	—	—	—
Between plates	12·3	2	6·15	<1	—
Error	66·9	10	6·69	—	—
Total	3302·5	17	—	—	—

In the analysis of variance the subdivision of the between doses sum of squares has been carried out by the method of detached coefficients or orthogonal contrast (Bliss and Marks[14]) Lp, L_1, L_1', L_2, L_2', have their usual significance and refer, in turn, to the five contrasts in the order shown in the above Table.

Estimation of Potency and Validity.

The contrast for preparations is significant at P = 0·05 but not at P = 0·01. None of the contrasts for parallelism, combined curvature or opposed curvature is significant, however, and the assay may be considered valid.

Using the accepted notation (see Finney[13], Chapter V).

Mean potency (R) = antilog $\frac{d\,(k^2-1)\,Lp}{6L_1}$

In all assays discussed in this paper the log dose interval (d) = 0·3010 and k (number of doses of standard and test material) = 3.

Then $\log R = \frac{4d\,Lp}{3L_1}$ and R = 0·86.

Since the test solution was initially diluted 3-fold and the standard contained 1 μg./ml. of cyanocobalamin, then:—

Potency of extract = 3 × 0·86 = 2·58 μg./ml.

The index of significance of the slope (g) = $\frac{N\,t^2\,s^2\,(k^2-1)}{12\,L_1^2}$ = 0·0104.

Since g is small the 95 per cent. fiducial limits are given by:—

$$\text{Log RL, RU} = \left[\frac{d\,(k^2-1)}{6L_1}\left\{Lp \pm t\sqrt{Ns^2\left[1+\frac{(k^2-1)\,Lp^2}{12L_1^2}\right]}\right\}\right]$$

$$= \frac{4d}{3L_1}\left\{Lp \pm t\sqrt{18\,s^2\,(1+2Lp^2/3L_1^2)}\right\}$$

From the error mean square (analysis of variance) s^2 = 6·69 and for 10 degrees of freedom t = 2·228 at P = 0·05.

Whence 95 per cent. fiducial limits are 2·31 — 2·91 μg./ml. (89·5–112·8 per cent.).

Table IV gives an analysis of the results obtained with Sample No. 11 by the *B. coli* plate assay, the calculations being carried out in a similar manner to those in Table III.

For the three *L. leichmannii* tube assays the analysis was carried out in a different way. The value of s^2 was obtained in the normal way

TABLE IV

POTENCY AND FIDUCIAL LIMITS OF SAMPLE NO. 11 (see Table II) BY THE *B. coli* PLATE ASSAY

The results given below were analysed by the method described for the *L. leichmannii* plate assay (see also under "Discussion").

Contrasts and mean squares from analysis of variance

Contrast	Sum	Mean square	F	P
Preparations	−91	460·055	586·80	<0·01
Regression	185	2852·083	—	—
Parallelism	5	2·083	2·66	>0·1
Combined curvature ..	−11	3·361	4·29	⩾0·05
Opposed curvature ..	1	0·028	<1	—
Error		0·784		

The sums of the contrasts are the values of Lp, L_1, etc., used in the determinations of potency and fiducial limits.

Potency = 1·91 μg./ml.

95 per cent. fiducial limits 1·82–2·00 μg./ml. (95·3–104·7 per cent.).

TABLE V

POTENCY AND FIDUCIAL LIMITS OF SAMPLE NO. 11 (see Table II) BY THE *L. leichmannii* TUBE ASSAY

In all tube assays a standard solution containing 0·02 mμg./ml. of cyanocobalamin was employed and the test material was diluted accordingly (see text).

U.S.P. Assay (*Medium* 1)—

Contrasts and Standard Errors

Contrast	Sum	Standard error	Ratio: sum/standard error
Preparations	44	3·18	13·84
Regression	106	2·58	41·09
Parallelism	−2	2·58	0·78
Combined curvature ..	8	4·47	1·79
Opposed curvature ..	4	4·47	1·79

s^2 = 0·556 (from within dose groups sum of squares). For 12 degrees of freedom t = 2·18 and none of the contrasts for parallelism, combined curvature or opposed curvature is significant. Then potency = 1·76 μg./ml.

95 per cent. fiducial limits = 1·66 — 1·87 μg./ml. (94·3–106·3 per cent.).

Medium 2—

Contrasts and Standard Errors

Contrast	Sum	Standard error	Ratio: sum/standard error
Preparations	−96	4·38	21·92
Regression	195	3·55	54·93
Parallelism	9	3·55	2·54
Combined curvature ..	7	6·16	1·14
Opposed curvature ..	21	6·16	3·41

s^2 = 1·06 (from within dose groups sum of squares). t = 2·18 and the contrasts for parallelism and opposed curvature are significantly high. This assay was therefore invalid and no further calculations were carried out.

Medium 3—

Contrasts and Standard Errors

Contrast	Sum	Standard error	Ratio: sum/standard error
Preparations	−65	4·14	15·70
Regression	187	3·36	55·65
Parallelism	−3	3·36	0·89
Combined curvature ..	−45	5·83	7·72
Opposed curvature ..	−7	5·83	1·20

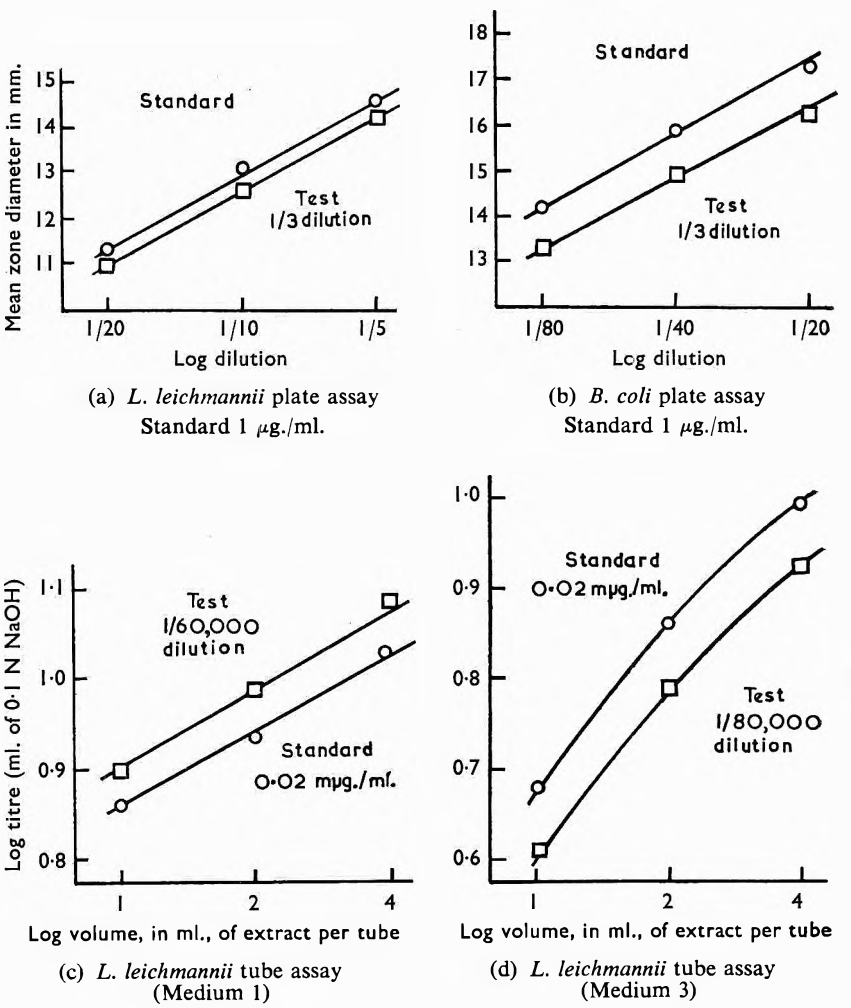
s^2 = 0·944 (from within dose groups sum of squares).

Potency = 1·15 μg./ml.

Fiducial limits not estimated (see Discussion).

from within dose groups and the significance of the five contrasts was then estimated by a "t" test by dividing each contrast by its standard error and comparing with $t = 2{\cdot}18$, as in this instance there are 12 degrees of freedom for the error term. The procedure is described elsewhere in detail[16,17]. We now use the same analysis of variance employed for the plate assay except that there is of course no term for difference between plates. The same formula as that used for the plate assays is used for the estimation of fiducial intervals. The results are given in Table V.

Figure 1 gives the graphical plots of the four valid assays of Sample No. 11. The plot of the thiomalic acid assay is not given, as this assay was invalid on two counts.



(a) *L. leichmannii* plate assay
Standard 1 μg./ml.

(b) *B. coli* plate assay
Standard 1 μg./ml.

(c) *L. leichmannii* tube assay
(Medium 1)

(d) *L. leichmannii* tube assay
(Medium 3)

FIG. 1. Graphical representation of results obtained in the assay of Sample No. 11 (See Table II) by four different methods.

Discussion

For the last nine years many workers have used cup-plate and tube assay methods for estimating vitamin B_{12}, with either *B. coli*, *L. lactis* Dorner or a strain of *L. leichmannii* as the test organism. The methods, developed originally for the assay of the pure vitamin, have been applied, often without modification, to the assay of natural products with the pure vitamin as standard. In particular the assay of liver extracts by this means has been widely practised and the anomalies encountered with this type of material have been mentioned in several papers[18–21].

Dawbarn and Hine[20] used a four point (2,2) plate assay at levels of 0·005 μg./ml. and 0·2 μg./ml. of vitamin B_{12} with *B. coli* as test organism as well as a tube assay method with *L. leichmannii*. They reported that sheep liver extracts almost invariably gave invalid assays shown by a lack of parallelism between the dose-response curves of the standard and test solutions, the latter tending to give a significantly lower slope. One assay is reported on rumen contents where they interpolated three doses between their usual 40-fold dilutions and found a curvilinear regression with a significantly higher slope through the three greatest concentrations. For liver extracts, agreement at the "1/5 level" between *B. coli* plate assays and *L. leichmannii* tube assays was reasonably satisfactory, the extracts being prepared by macerating 10 g. of liver, extracting with water and diluting to 250 ml.

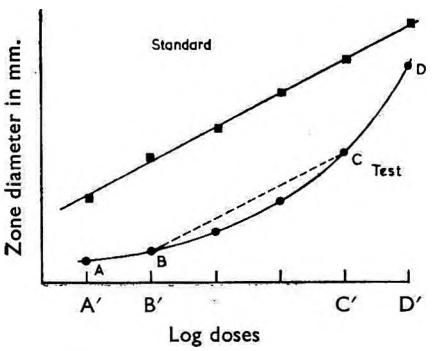


Fig. 2. Hypothetical curvilinear log dose response regression given by test material, showing apparent validity at B—C.

We consider these observations to be of considerable practical importance. A limitation of the (2,2) design is that it cannot show departure from linearity. Figure 2 illustrates a hypothetical case where a rectilinear dose-response for the standard is compared with a curvilinear dose-response for the test material. If it so happened that samples were usually diluted within the dose-range B′–C′ an apparently valid series of assays could be obtained and the occasional change in slope and hence non-parallelism such as arises in the range A–B, C–D, could be explained as random variation.

Figure 3 shows a pair of dose-response curves that have been obtained with a liver extract assayed over a wide range of dilutions by the *B. coli* plate method. A (2,2) design employing any pair of alternate points at a concentration of test material greater than 0·05 μg./ml. would have given an apparently linear non-parallel response (e.g. points A and B, as joined on the graph). This type of graph has been obtained for many extracts and thus, whereas for simple solutions the (2,2) design may be

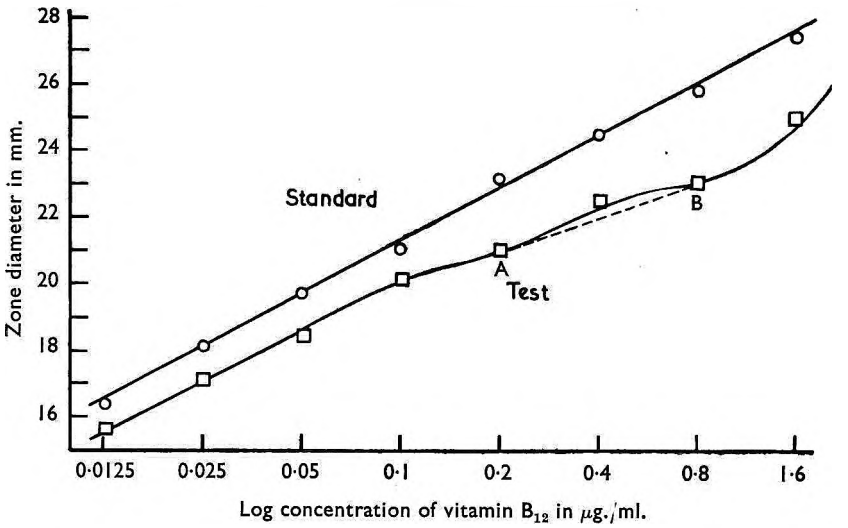


FIG. 3. Graphical representation of results obtained in the assay of a liver extract by the *B. coli* plate method over a wide range of vitamin B_{12} concentrations, showing curvilinear regression of test material and rectilinear regression[12] of standard (1 μg./ml.).

satisfactory provided a careful check is kept on parallelism (see "Antibiotics: Properties and Uses"[22], p. 82), the design is basically unsound for complex mixtures where the test dose-response regression may not be rectilinear over the whole range in which the standard gives a linear response.

Jerne and Wood[23] quote three assumptions that must be made if a body of data is to be regarded as yielding a fundamentally valid assay:

(1) The hypothesis of the validity of the experimental design.

(2) The hypothesis of the existence of a single-valued dose-response relationship.

(3) The hypothesis of similarity of the test and standard preparations. As has been shown, condition (3) cannot be assumed to hold for liver extracts, and similar considerations apply to assays of other complex materials for various ingredients. It would seem prudent to test any new preparation over a wide range of concentrations against a standard before assuming that a simple assay design is adequate.

Reference to Figure 3 shows that within a range of concentrations from 0·0125 μg./ml. to 0·05 μg./ml. of vitamin B_{12}, the standard and test materials gave parallel dose-response curves for the *B. coli* plate assay of liver extracts. Applying exactly the same considerations to the *L. leichmannii* plate assay the effective range was found to be 0·05 to 0·2 μg./ml. of vitamin B_{12}.

As a result of these findings a (3,3) assay design has been adopted. Thus for *B. coli* plate assays 3 doses of the standard at 0·0125, 0·025 and 0·05 μg./ml. of vitamin B_{12} are employed and 3 doses of the test solution at equivalent concentrations. For the *L. leichmannii* plate assays

the concentrations of vitamin B_{12} used are 0·05, 0·10 and 0·20 μg./ml. Fiducial limits (95 per cent.) of about $\pm$ 10 per cent. are usually obtained.

All assays are computed statistically and examined for departure from parallelism and combined and opposed quadratic curvature as described under "Results". As Table II shows, there is generally good agreement between the graphical and calculated results except where one of the contrasts L_1', L_2 or L_2' approaches significance. For example, in the *B. coli* plate assay (Table IV) the contrast for combined curvature is borderline, and in this instance the agreement between the two results is not so good as in the *L. leichmannii* plate assay.

Using *L. leichmannii* for tube assays very good results have been obtained with the U.S.P. medium over the range 0·02 to 0·10 mμg. of vitamin B_{12} per tube, only one out of the 14 samples included in Table II giving an invalid result; the assay could not be repeated in this instance owing to loss of material. A (3,3) design has been adopted with standard solutions containing 0·02, 0·04 and 0·08 mμg. of vitamin B_{12} per tube and corresponding dilutions of the test solution. The results generally agreed with those obtained in the *B. coli* plate assay.

Figure 1c and the corresponding analysis in Table V show the results obtained in the assay of Sample No. 11.

Medium 2 has given rather more trouble. Preliminary investigations suggested that the same range of dilutions should be used as for the U.S.P. medium. Table II shows, however, that invalid assays were quite often obtained with this medium, four out of the last seven assays being invalid. Where the assays were valid the results agreed fairly well with those obtained by the *B. coli* plate and U.S.P. tube assay methods. Invalidity showed as opposed curvature and as lack of parallelism, an observation recalling the work of Loy and others[11,12] who gave a graph of the results obtained with the U.S.P. medium to which thiomalic acid had been added; this graph would certainly not have been rectilinear had it been plotted on a log-log scale.

Medium 3 gave very variable results, although other workers have claimed that cyanide improves the validity and gives higher results. We have found that the response obtained was sometimes higher and sometimes lower, and that in many instances it was not possible to obtain a rectilinear dose-response curve for either test or standard solution. Figure 1d and the corresponding analysis in Table V for Sample No. 11 show a typical response of this type. A (2,2) assay for this material would have given an apparently valid result and in fact would give a good approximation of the potency. Even where the samples did give valid assays, the results varied widely from stage to stage of the extraction process and no attempt has been made to equate the mean quadratic regression, as checking pairs of quadratic regressions for validity is tedious.

By treating this type of assay as though it were valid and by using the same method of analysis as that applied to the linear regression, it can be shown that the contrast for opposed curvature is always without significance and that "lack of parallelism" is also nearly always absent. The latter point can be partially confirmed by treating the assay as two

(2,2) designs taking the three points in pairs and showing that the regressions so obtained are almost invariably parallel. It is admitted that this treatment is far from rigorous but it was not thought worthwhile to investigate the assay further. (For further discussion on this problem see Finney, "Statistical Method in Biological Assay"[17], p. 107 *et seq.*)

As with the plate assay methods, the potencies obtained by the tube assays are estimated graphically. This method checks any gross errors in computation and avoids the waste of time involved in working through the calculation of an obviously invalid assay. In the case of Sample No. 11 (Table II) for example, the computation of the contrasts and their standard errors would not normally have been carried out on the results obtained with Medium 2 as a graphical plot showed that the assay was clearly invalid.

We consider that the U.S.P. tube assay is the most reliable method of estimating the vitamin B_{12} content of liver extracts and gives results that are easily interpreted. It is not proposed to discuss in this paper the complications introduced by the use of different reducing agents in the medium. (See Loy and others[11,12], Denton and Kellog[24], Shenoy and Ramasarma[19], Dawbarn and Hine[20].) In view of our findings, however, that the *L. leichmannii* tube assay gives results in close agreement with the *B. coli* plate assay we believe that the latter may give results in closer agreement with the clinical activity than has generally been conceded.

The *L. leichmannii* plate assay presents a problem in that apparently valid assays on all counts were obtained on twelve out of the fourteen samples of Table II, and yet the results were invariably higher than those obtained by the *B. coli* plate assay, occasionally being twice and in one instance three times as high.

Use of Internal Standard

An obvious possibility, when faced with the problem of assaying a complex product, is to use as standard a preparation that closely resembles the preparation being assayed. Sometimes as in penicillin assays, it is recommended that the active ingredient be extracted from the mixture containing it and assayed against the pure standard. Alternatively, interfering substances can often be removed by solvent extraction, enzymatic action, adsorption or other means. Occasionally, however, neither of these methods is satisfactory because the active ingredient appears to exist in the form of a complex with some other constituent of the preparation, the complex then giving a response different from that given by the substance in the pure state. Such a complex of vitamin B_{12} may be present in liver extracts[21,25–27].

An internal standard has sometimes been used to overcome the difficulties introduced by the presence of interfering substances. Thus in the microbiological assay of aneurine by the method of Fitzgerald and Hughes[28], the vitamin is destroyed in an aliquot portion of the test solution by autoclaving with sodium sulphite, excess sulphite being removed by titration with hydrogen peroxide. Successive amounts of aneurine are

then added to portions of the aneurine-free preparation, giving a series of internal standards against which the untreated preparation is assayed.

Beck[29] described a method of this type for the assay of liver extracts in which treatment with acid was used to hydrolyse the vitamin B_{12}, thus rendering it microbiologically inactive. We investigated the use of internal standards obtained by hydrolysing liver extracts with sulphuric acid, sodium carbonate or sodium hydroxide followed by the addition of pure cyanocobalamin. Beck's original method, which was developed in conjunction with an *L. leichmannii* tube assay, was found to be very cumbersome, requiring a great deal of troublesome preparative work and the use of aseptic precautions throughout. Modified to suit the plate assay method, however, it has given encouraging results with both *B. coli* and *L. leichmannii* as test organisms.

Alkali-stable Factors

It is possible that the presence of alkali-stable factors in liver extracts may be partly responsible for some of the invalid results obtained[18,24]. Further discussion of this problem is reserved for a later paper.

Summary

1. Experimental methods are given for the assay of cobalamins in liver extracts using plate and tube assay methods with *B. coli* N.C.T.C. 8134 and *L. leichmannii* N.C.T.C. 7854 as test-organisms.

2. The use of a (2,2) design for complex materials is criticised and the adoption of a (3,3) design is advocated for liver extracts and similar materials.

3. Details of the experimental design and statistical analysis employed are related to the assay of fourteen samples of liver extract and the results are discussed.

We wish to thank Mrs. M. Davis, Miss D. Forder and Miss B. Legge for technical assistance.

References

1. Shorb, *J. biol. Chem.*, 1947, **167**, 455.
2. Foster, Lally and Woodruff, *Science*, 1949, **110**, 507.
3. Skeggs, Nepple, Valentik, Huff and Wright, *J. biol. Chem.*, 1950, **184**, 211.
4. Emery, Lees and Toothill, *Analyst*, 1951, **76**, 696.
5. Bessel, Harrison and Lees, *Chem. & Ind.*, 1950, 561.
6. Burkholder, *Science*, 1951, **114**, 459.
7. Harrison, Lees and Wood, *Analyst*, 1951, **76**, 696.
8. Brownlee, Loraine and Stevens, *J. gen. Microbiol.*, 1949, **3**, 347.
9. Hutner, Provasoli, Stokstad, Hoffmann, Belt, Franklin and Jukes, *Proc. Soc. exp. Biol., N.Y.*, 1949, **70**, 118.
10. Ford, *Brit. J. Nutr.*, 1953, **7**, 299.
11. Loy, Haggerty and Kline, *J. Assoc. off. agric. Chem., Wash.*, 1951, **34**, 225.
12. Loy, Haggerty and Kline, *ibid.*, 1952, **35**, 161, 169, 726.
13. Krieger, *ibid.*, 1953, **36**, 846.
14. Krieger, *ibid.*, 1954, **37**, 781.
15. Krieger, *ibid.*, 1955, **38**, 65.
16. Bliss and Marks, *Quart. J. Pharm. Pharmacol.*, 1939, **12**, 82, 182.
17. Finney, *Statistical Method in Biological Assay*, Charles Griffin & Co., London, 1952.
18. Robinson, Williams and Brown, *J. Pharm. Pharmacol.*, 1952, **4**, 27.

19. Shenoy and Ramasarma, *Arch. Biochem. Biophys.*, 1954, **51**, 371.
20. Dawbarn and Hine, *Austral. J. exp. Biol. med. Sci.*, 1951, **32**, 1.
21. Robinson, Fitzgerald, Fehr and Grimshaw, *Nature, Lond.*, 1954, **174**, 558.
22. Edwards, Kenwick, Robinson, Sykes and Thomas, *Antibiotics: Properties and Uses*, The Pharmaceutical Press, London, 1952.
23. Jerne and Wood, *Biometrics.*, 1949, **5**, 273.
24. Denton and Kellog, *Arch. Biochem. Biophys.*, 1953, **46**, 135.
25. Wijmenga, Lens and Geerts, *Acta Haematologica*, 1954, **11**, 372.
26. Wijmenga, Veer and Lens, *Biochim. Biophys. Acta*, 1950, **6**, 229.
27. Östling and Nyburg, *J. Pharm. Pharmacol.*, 1953, **5**, 46.
28. Fitzgerald and Hughes, *Analyst*, 1949, **74**, 340.
29. Beck, *Ann. pharm. franc.*, 1954, **12**, 132.

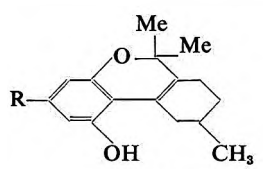
SYNTHESIS OF ALKYL SUBSTITUTED 1-(ALKYLOXYPHENYL)ETHYLAMINES

By A. McCoubrey

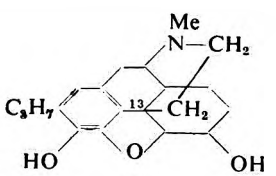
From the Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation), University of London, Maudsley Hospital, S.E.5

Received April 27, 1956

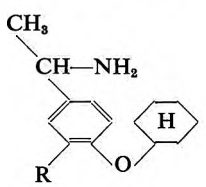
The ability of synthetic tetrahydrocannabinols (I) to induce ataxia in dogs was markedly influenced by the nature of the alkyl side chain R[1]. A vague resemblance both in structure and pharmacology between morphine and tetrahydrocannabinol suggested that suitable alkyl substituents might favourably influence the analgesic activity of 1-(*p-cyclo*hexyloxyphenyl)-ethylamine[2]. Methyl, ethyl and *n*-propyl derivatives (III; R = Me, Et, Pr, IV; R = Pr) were readily obtained but attempts to synthesise III (R = *n*-amyl or *n*-hexyl) by various routes were unsuccessful. Among various failures it was noteworthy that the bromine



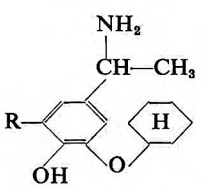
I



II



III

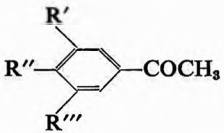


IV

of 4-bromo-2-*n*-hexylphenol was not replaceable by lithium, and its *cyclo*hexyl ether, unlike the 2-ethyl analogue, gave only traces of a Grignard reagent. *o-n*-Amylphenol, again unlike the ethyl analogue, could not be acetylated or chloracetylated in workable yield, apparently due to polymerisation.

III (R = Me, Et, or Pr) had increasing toxicity in mice as the alkyl chain was lengthened (LD50 approximately 80, 56 and 48 mg./kg. respectively) and while there was a noticeable increase in depressant activity, the analgesic action of the parent amine had disappeared. Conversely IV (R = Pr) had a short lived analgesic action at 3–5 mg./kg. intraperitoneally, a considerable increase in activity compared to the amine (IV; R = H). It was very toxic (LD50, 26 $\pm$ 2 mg./kg. intraperitoneally in mice). The corresponding *n*-hexyl ether was inactive. 2-*n*-propyldihydromorphine (II), like other morphine derivatives substituted in the benzene ring, was completely inactive.

TABLE I

OXIMES and 1-PHENYLETHYLAMINES DERIVED FROM SUBSTITUTED ACETOPHENONES 

			Oxime				1-Phenylethylamine hydrochloride								
									Found per cent.			Required per cent.			
R′	R″	R‴	Formula	M.pt. °C.	Found per cent. N	Required per cent. N	Formula	M.pt. °C.	C	H	N	C	H	N
OC_6H_{11}(i)	OH	C_3H_5(iii)	$C_{17}H_{23}O_2N$	107	4·7	4·8	$C_{17}H_{24}O_2NCl$(v)	175	65·1	8·4	4·4	65·1	8·9	4·5
OC_6H_{13}(ii)	OH	C_3H_5(iii)	$C_{17}H_{25}O_2N$	85	—(iv)	—	$C_{17}H_{26}O_2NCl$(v)	148	64·5	9·3	4·3	64·7	9·5	4·4
H	OC_6H_{11}(i)	Me	$C_{15}H_{21}O_2N$	100	5·7	5·7	$C_{15}H_{22}ONCl$(vi)	202	66·8	8·8	5·5	66·9	8·9	5·2
H	OC_6H_{11}(i)	Et	$C_{16}H_{23}O_2N$	121	5·6	5·4	$C_{16}H_{24}ONCl$(vii)	226	67·3	9·2	5·0	67·7	9·2	4·9
H	OC_6H_{11}(i)	Pr^n	$C_{17}H_{25}O_2N$	114	5·2	5·1	$C_{17}H_{28}ONCl$	169	69·1	9·5	4·5	68·6	9·4	4·7

(i) C_6H_{11} = *cyclo*hexyl. (ii) C_6H_{13} = *n*-hexyl. (iii) C_3H_5 = allyl. (iv) Found: C, 70·1; H, 8·6. Required, C, 70·1; H, 8·6 per cent. (v) R‴ = *n*-propyl.
(vi) B.pt. of base, 140–145° C./1 mm. (vii) B. pt. of base, 150–160° C./2 mm.

Experimental

Amines (Table I) were all prepared by reduction of the corresponding acetophenone oximes by sodium amalgam and acetic acid in methanol. Cyclohexylations were by the method previously described[3]. Hydrogenations were at room temperature and pressure. Boiling ranges refer to air bath temperatures.

2-n-*Propyldihydromorphine.*—Morphine hydrochloride was hydrogenated on palladised charcoal in methanol to dihydromorphine (89 per cent.). The *allyl ether* crystallised from light petroleum (b.pt. 60–80° C.) in prisms, m.pt. 77–78° C.(48 per cent). Found: C, 73·3; H, 7·5; N, 4·2. $C_{20}H_{25}O_3N$ requires C, 73·4; H, 7·6; N, 4·3 per cent. Rearrangement of this ether (1·1 g.) in boiling diethylaniline (45 minutes) gave an alkali soluble material (0·7 g.) which did not crystallise. It was hydrogenated on Raney nickel in methanol to 2-n-*propyldihydromorphine* (structure assigned from method of synthesis), prisms from light petroleum (b.pt. 60–80° C.) m.pt. 99° C. (95 per cent.). Found: C, 72·7; H, 8·3; N, 4·1. $C_{20}H_{27}O_3N$ requires C, 73·0; H, 8·2; N, 4·3 per cent.

4-Cyclo*hexyloxy*-3-*methylacetophenone.*—4-Hydroxy-3-methylacetophenone gave the *cyclo*hexyl ether, b.pt. 145–150° C./0·4 mm. (16 per cent.), characterised as the 2:4-*dinitrophenylhydrazone*, red needles from ethyl acetate-ethanol, m.pt. 181° C. Found: N, 13·4. $C_{21}H_{24}O_5N_4$ requires N, 13·6 per cent.

4-Cyclo*hexyloxy*-3-*ethylacetophenone.*—Reduction of 5-bromo-2-hydroxyacetophenone by the Huang-Minlon method[4] gave 4-bromo-2-ethylphenol, b.pt. 160–170° C./22 mm. (75 per cent.), characterised as the *α-naphthylurethane*, needles from benzene, m.pt. 140° C. (Found: C, 61·8; H, 4·4; N, 3·7. $C_{19}H_{16}O_2NBr$ requires C, 61·6; H, 4·3; N, 3·8 per cent.). The *cyclo*hexyl ether, b.pt. 210–212° C./16 mm. (3 per cent.). (Found: Br, 28·1. $C_{14}H_{19}OBr$ requires Br, 28·3 per cent.) was converted to the Grignard reagent and added to acetic anhydride at — 70° C. to give 4-*cyclo*hexyloxy-3-ethylacetophenone, b.pt. 190–192° C./3 mm. The 2:4-*dinitrophenylhydrazone* crystallised from ethyl acetate in red needles, m.pt. 152° C. Found: C, 61·6; H, 6·2; N, 13·2. $C_{22}H_{26}O_5N_4$ requires C, 62·0; H, 6·1; N, 13·2 per cent. The same ketone was obtained more conveniently by acetylation of *o*-ethylphenol. The product on fractional distillation gave 3-*ethyl*-4-*hydroxyacetophenone*, b.pt. 170–180° C./1 mm., m.pt. 95° C. (60 per cent., calc. on phenol reacting). Found: C, 73·1; H, 7·4. $C_{10}H_{12}O_2$ requires C, 73·2; H, 7·3 per cent. The *cyclo*hexyl ether gave the same 2:4-dinitrophenylhydrazone (mixed m.pt.) as the product from the first method.

4-Cyclo*hexyloxy*-3-n-*propylacetophenone.*—Hydrogenation of 3-allyl-4-hydroxyacetophenone[5] on palladised charcoal in ethanol gave 4-*hydroxy*-3-n-*propylacetophenone*, prisms from benzene-ethanol, m.pt. 89–90° C. (100 per cent.). Found: C, 73·8; H, 8·0. $C_{11}H_{14}O_2$ requires C, 74·2; H, 7·9 per cent. The *cyclo*hexyl ether, b.pt. 145–150° C./0·3 mm. (14 per cent.) was characterised as the 2:4-*dinitrophenylhydrazone*, red needles from ethyl acetate-ethanol, m.pt. 141° C. Found: C, 62·6; H, 6·1. $C_{23}H_{28}O_5N_4$ requires C, 62·7; H, 6·4 per cent.

3 - *Allyl*-5-cyclo*hexyloxy*-4-*hydroxyacetophenone*.—3-*cyclo*-Hexyloxy-4-hydroxyacetophenone[6] (2·3 g.) gave an allyl ether (not purified) which rearranged in boiling diethylaniline (1 hour) to the above *product*, b.pt. 170–180° C./0·6 mm. (1·85 g., 69 per cent.). It crystallised from light petroleum (b.pt. 80–100° C.) in needles m.pt. 58° C. Found: C, 74·3; H, 8·5. $C_{17}H_{22}O_3$ requires C, 74·4; H, 8·3 per cent.

3-*Allyl*-5-n-*hexyloxy*-4-*hydroxyacetophenone*.—4-Benzyloxy-3-hydroxyacetophenone[6] gave the n-*hexyl ether*, plates from light petroleum (b.pt. 60–80° C.) m.pt. 74° C. (77 per cent.). Found: C, 77·3; H, 8·1. $C_{21}H_{26}O_3$ requires C, 77·3; H, 8·0 per cent. Catalytic debenzylation on palladised charcoal gave 3-n-*hexyloxy*-4-*hydroxyacetophenone*, needles from light petroleum (b.pt. 60–80° C.), m.pt. 48° C. (89 per cent.). Found: C, 71·4; H, 8·5. $C_{14}H_{20}O_3$ requires C, 71·2; H, 8·5 per cent. The allyl ether (not purified) at 200° C. for 35 minutes gave the above *product*, b.pt. 175–180° C./0·9 mm. It crystallised from light petroleum (b.pt. 80–100° C.) and finally from ethanol, in needles, m.pt. 83° C. (47 per cent.). Found: C, 73·8; H, 8·8. $C_{17}H_{24}O_3$ requires C, 73·9; H, 8·7 per cent.

2-*Bromo*-4-n-*hexylphenyl* cyclo*hexyl ether*.—*p*-Bromophenyl caproate, b.pt. 125–130° C./2 mm., in 5 g. quantities was treated with an equal weight of aluminium chloride in tetrachlorethane and then heated at 120°C. for 30 minutes to give 4-*bromo*-2-caproylphenol, b.pt. 145–150° C./1 mm. (86 per cent.). It crystallised from light petroleum (b.pt. 60–80° C.) in plates, m.pt.58–59° C. Found: C, 53·3; H, 5·6; Br, 29·1. $C_{12}H_{15}O_2$ Br requires C, 53·2; H, 5·5; Br, 29·5 per cent. The 2:4-*dinitrophenylhydrazone* crystallised from ethyl acetate in orange needles, m.pt. 206° C. Found: N, 12·1; Br, 17·2. $C_{18}H_{19}O_5N_4Br$ requires N, 12·4; Br, 17·7 per cent. Reduction with hydrazine gave 4-*bromo*-2-n-*hexylphenol*, b.pt. 125–145° C./1 mm. (86 per cent.), crystallised from light petroleum (b.pt. 80–100° C.) in needles m.pt. 52° C. Found: C, 56·3; H, 6·7; Br. 31·6. $C_{12}H_{17}OBr$ requires C, 56·0; H, 6·6; Br, 31·2 per cent. Carbonation of the product of reaction with lithium butyl (3 equivalents) gave only valeric acid and unchanged material. The *cyclo*hexyl ether, b.pt. 195–200° C./0·5 mm. (16 per cent.). Found: C, 63·9; H, 7·8; Br. 23·6. $C_{18}H_{27}OBr$ requires C, 63·7; H, 8·0; Br, 23·6 per cent.) slowly reacted with magnesium in boiling ether (dibutyl ether or phenetole had no advantage) whence reaction with acetic anhydride at — 70° C. gave a trace of ketone, isolated as the 2:4-dinitrophenylhydrazone. It could not be purified.

2-n-*Amyl*-4-*bromophenol*.—*p*-Bromophenyl valerate by similar methods to the above gave 4-bromo-2-valeroylphenol, b.pt. 120–121° C./1 mm. (45 per cent.). Found: C, 51·6; H, 5·0; Br, 31·3. $C_{11}H_{13}O_2Br$ requires C, 51·4; H, 5·1; Br, 31·1 per cent. The 2:4-*dinitrophenylhydrazone* crystallised from ethyl acetate in red plates m.pt. 231° C. Found: N, 13·0; Br, 18·6. $C_{17}H_{17}O_5N_4Br$ requires N, 12·8; Br, 18·3 per cent. The above *product* crystallised from light petroleum (b.pt. 60–80° C.) in needles m.pt. 30° C. Found: C, 54·4; H, 6·0; Br, 32·7. $C_{11}H_{15}OBr$ requires C, 54·3; H, 6·2; Br, 32·9 per cent.

Summary

1. The synthesis of alkyl substituted 1-(alkyloxyphenyl)ethylamines is described.

2. Introduction of alkyl groups into the benzene ring of 1-(*p-cyclo*-hexyloxyphenyl)ethylamine or dihydromorphine abolished their analgesic activity.

3. 1-(3-*cyclo*Hexyloxy-4-hydroxy-5-*n*-propylphenyl)ethylamine had analgesic activity in rats but was very toxic.

Part of this work was done in 1949 during tenure of a Beit Memorial Fellowship for Medical Research at the University of Leeds. Facilities provided by Professor Challenger are gratefully acknowledged.

References

1. Adams, McKenzie and Loewe, *J. Amer. chem. Soc.*, 1948, **70**, 664.
2. McCoubrey, *Brit. J. Pharmacol.*, 1953, **8**, 22.
3. McCoubrey, *J. chem. Soc.*, 1951, 2931.
4. Huang—Minlon, *J. Amer. chem. Soc.*, 1946, **68**, 2487.
5. Arnold and McCool, *ibid.*, 1942, **64**, 1315.
6. McCoubrey and Iyengar, *J. chem. Soc.*, 1951, 3430.

A PHARMACOGNOSTICAL STUDY OF THE FRUIT OF *PASTINACA SATIVA* L. CULTIVATED IN EGYPT

By I. R. Fahmy, A. Hifny Saber and Ezz. A. E. Kadir

From the Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Egypt

Received September 23, 1955

Pastinaca sativa L. is a common dietetic plant, its roots being used as a vegetable. Nevertheless, it is mentioned by many authors as having photo-sensitising properties, causing dermatitis when applied locally. Inflammation had been noticed on the back of a student after being rubbed by a handful of *Pastinaca sativa* plant while sunbathing, and developing after a few hours into a large blistered surface[1]. Belringer[2] mentioned that wild parsnip (*P. sativa*) as well as cultivated parsnip appeared to be the plant most commonly associated with photo-sensitivity. He also found that the flowers, leaves and stalks when applied to the skin caused erythema in 24 hours and vesication in 48 to 72 hours, followed in a few days by desquamation and faint pigmentation. He mentioned that the active principle is found in both the aqueous and the ethanolic extracts of the plant. Kuske[3] investigated the photo-dynamic properties of a group of ketonic compounds known as furocumarins and found these substances when applied to the skin in sunlight produced erythema after 48 hours followed by prolonged pigmentation.

Van Urk[4] analysed the fruits of the plant and stated that they were free from any skin-irritating substance, contrary to Wittstein[5] who stated that they contained a volatile base and a substance which causes skin irritation. Abu-Shady claimed that they had similar dermatic irritation properties to those of *Ammi majus*[6] and advised the cultivation of the former plant in Egypt as a source of another drug for the treatment of leukodermia. All the studies mentioned in the literature deal mostly with the root[7–9], and with the exception of the cursory knowledge of the fruit nothing has been mentioned of any other organ of the plant, or about the nature of the substance causing the phyto-photo-dermatitis.

This work deals with the cultivation of the plant in Egypt, records detailed macro- and microscopy of the fruit, and refers to the principle or principles, causing the phyto-photo-determatitis.

Botanical Study and Cultivation of the Plant

Pastinaca sativa L. is a biennial small shrub, ½–1 metre to 1½ metres in height: it belongs to the family Umbelliferæ, Subfamily Apioidæ, Tribe Peucedenæ[10,11]. The plant has been grown successfully in Egypt by sowing the fruits in several localities in Cairo and Giza, the best time for sowing being in the late autumn for the fruit, and early spring for the vegetative crop (Fig. 1).

Macroscopical characters of the fruit (Fig. 2)

The Cremocarp is broadly elliptical, lenticular in shape, with thin wings on the lateral sides. It splits into two mericarps which are dorsiventrally

compressed, and measure from 5 to 8 mm. in length and 4 to 6 mm. in breadth; they are notched at apex and base and crowned by a small pyramidal stylopod, terminating with the remains of style and stigma. The basal portion of the fruit is thin and membranous, being an extension of the pericarp and continuous with the lateral wings. The dorsal side of the mericarp shows five prominent ridges, the two lateral ones extending outwards to form two thin lateral wings. The intercostal regions, separating the primary ridges, show brown bands indicating the position of vittae which run from the top, but do not reach the base of the fruit. The commisural side shows two broader similar bands but the vittae here run only to three-quarters the length of the fruit. The seed is albuminous and fills the whole loculus of the mericarp except for small cavities at the lateral sides and at the base. Odour is slight, becoming more distinct on crushing, the taste is slightly bitter but distinctly pungent.

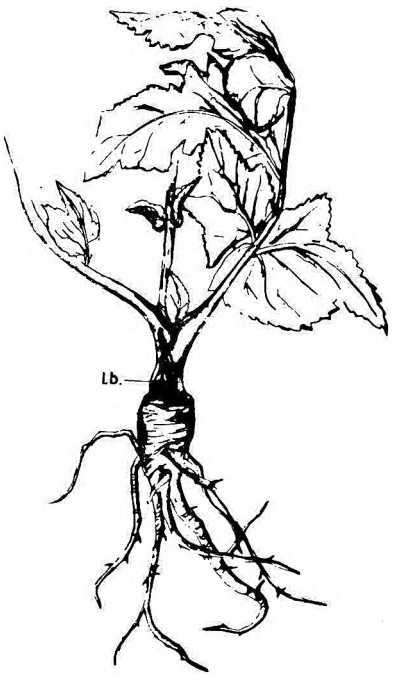


FIG. 1. Young plant of *Pastinaca sativa*, showing tuberous root and bases of radical leaves (l.b.) (× ¼).

Microscopical Characters of the Fruit

Epicarp (Fig. 3, A, B and C). There is a single layer of subrectangular, or tangentially elongated cells on the ribs, measuring from 36 to 80μ in length, 15 to 36μ in width and 11 to 29μ in height, with straight or slightly wavy anticlinical walls, containing small prismatic or cluster crystals of calcium oxalate measuring from 3 to 8μ in diameter. Stomata are present. These are mostly of cruciferous type. Trichomes are few, warty, conical, unicellular and non-glandular, measuring from 14 to 40μ in length and 15 to 22μ in width at the base. The cuticle is distinctly striated.

Mesocarp (Fig. 4, A, B and C). This consists of an outer zone of several layers of parenchyma cells, and an inner sclerenchyma zone of several layers of lignified fibre cells crossing each other. Some of these cells are elongated, with pointed ends and narrow lumen; others have no pointed ends, but with thick pitted walls and measuring from 43 to 75μ in length and 5 to 15μ in width (Fig. 5, A, B, C and D). Vittae are found traversing the mesocarp in the inner region of the outer parenchymatous zone and are usually abutting on, or even embedded in, the fibrous layer. Vascular bundles run through the ribs (Fig. 4, A) and

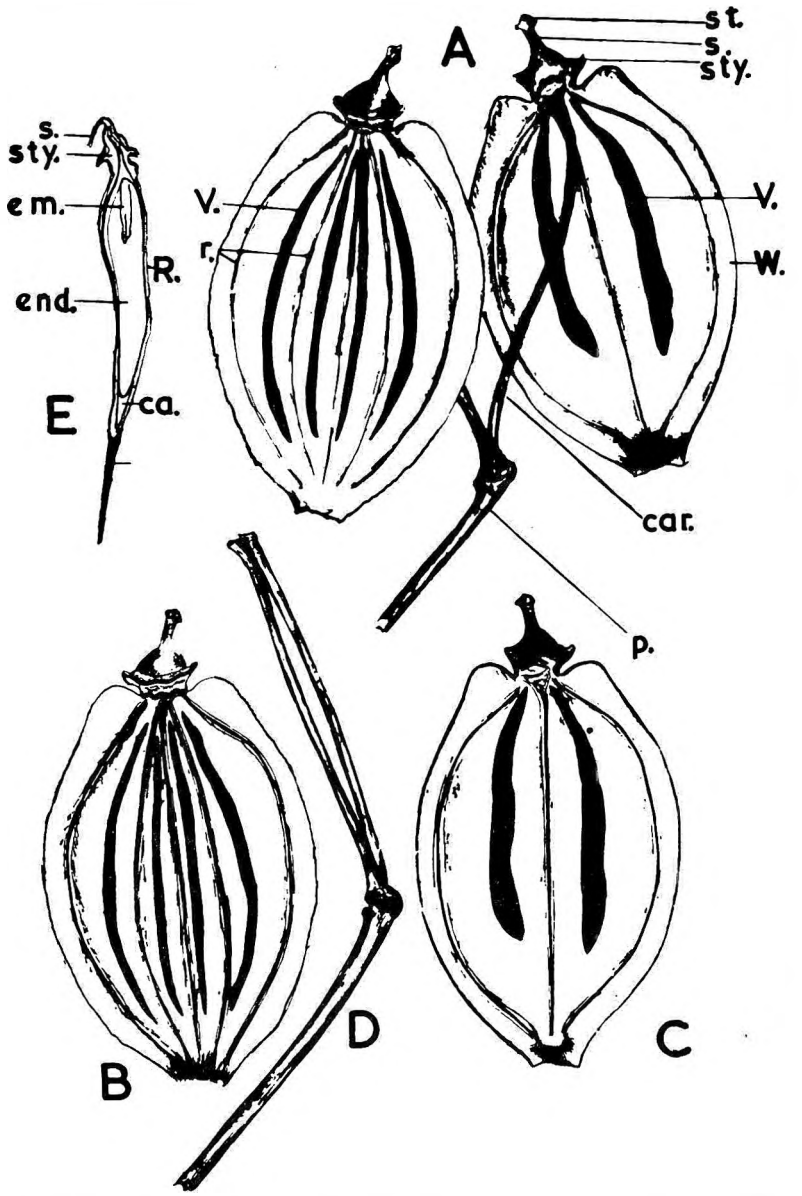


FIG. 2. Fruit of *Pastinaca sativa* L.: A., cremocarp splitting into 2 mericarps and attached to carpophore; B., mericarp from dorsal side; C., mericarp from commisural side; D., isolated carpophore attached to pedicel; E., M.L.S. in mericarp (all × 7); ca., cavity; car., carpophore; em., embryo; end., endosperm; p., pedicel; R., raphe; r., ridge; s., style; st., stigma; sty., stylopod; V., vitta; W., wing.

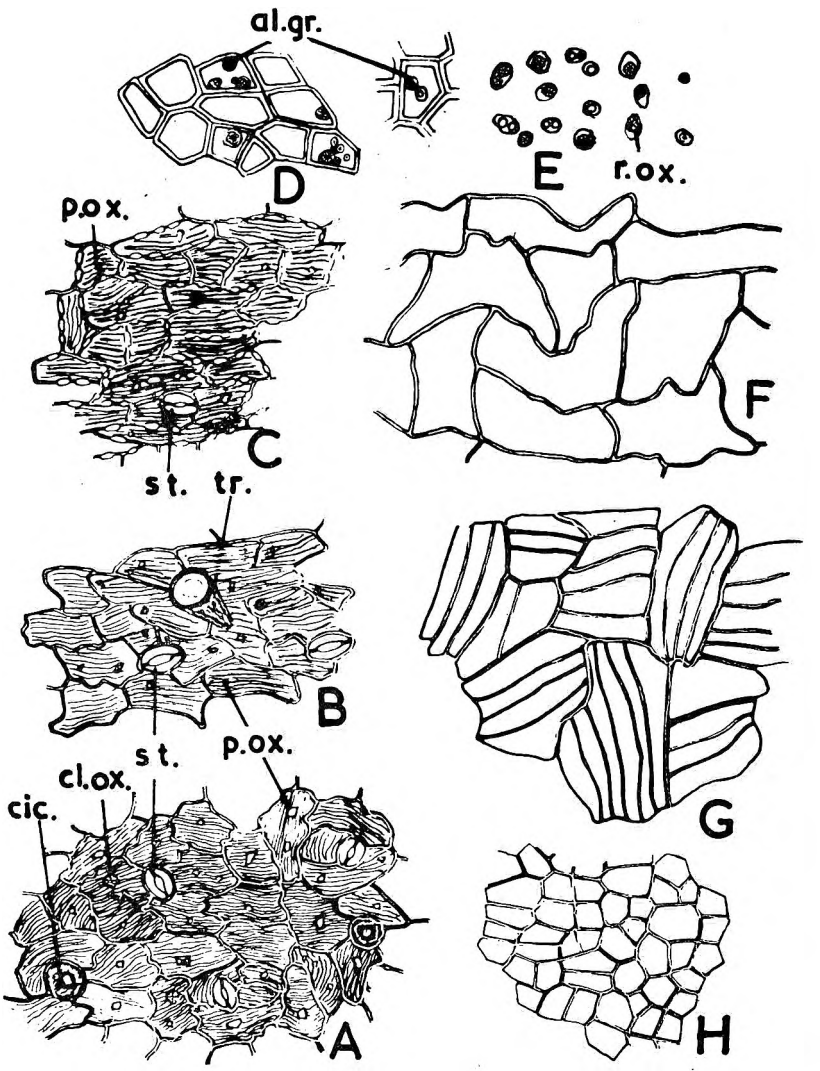


FIG. 3. A., Epicarp between the ridges; B. and C., epicarp on the ridges; D., endosperm cells; E., aleurone grains; F., endocarp; G., endocarp showing parquetry arrangement; H., seed-coat (all × 200); al.gr., aleurone grain; cic., cicatrix of hair; cl.ox., clusters of calcium oxalate; p.ox., prisms of calcium oxalate; r.ox., rosette of calcium oxalate; st., stoma; tr., trichome.

appear in transverse section as triangular or rounded structures on the outer side of the fibrous layer. Each bundle shows small phloem patches either on the outer side, or on the lateral sides of the xylem. This exhibits a few slender, annular and spiral vessels. The two vascular bundles of the wings show phloem patches on both the outer and inner sides. The fibrous layer from the dorsal and ventral sides of the fruit passes into each wing as a single band of fibrous cells, and joins there with the vascular

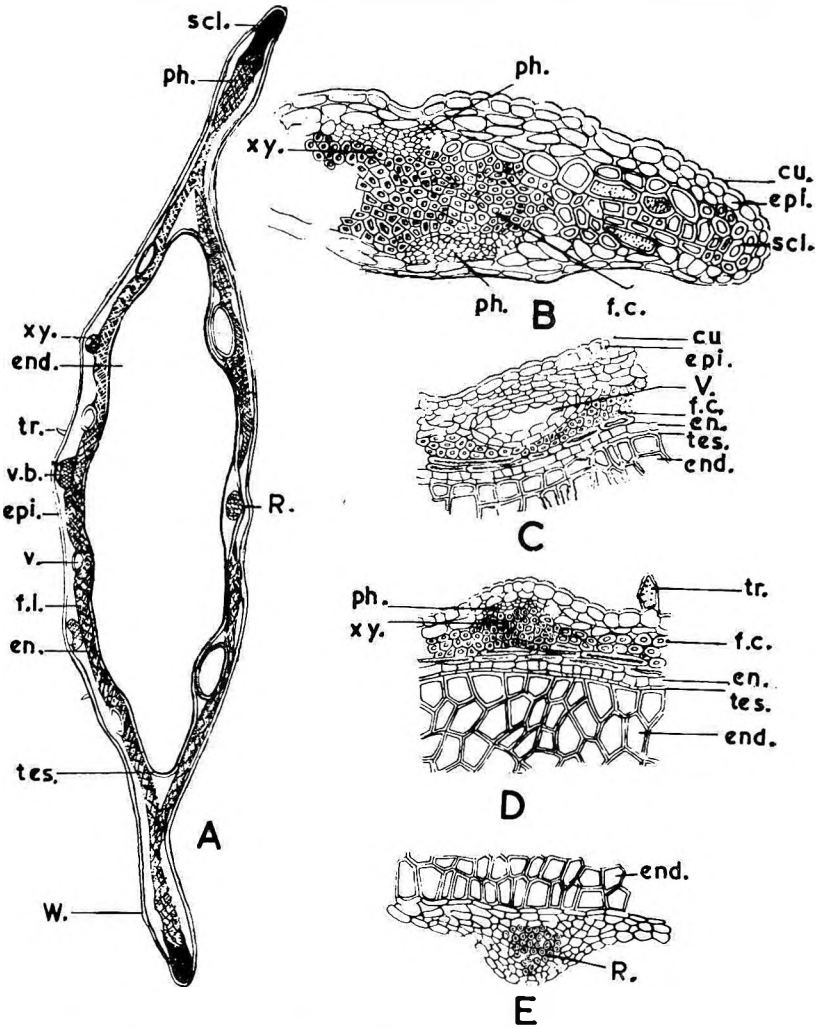


FIG. 4. A., Diagram of T.S. of mericarp (× 36); B., T.S. in wing of mericarp; C., same in region of dorsal vitta; D., same in one of ridges; E., in raphe (all × 120); cu., cuticle; en., endocarp; end., endosperm; epi., epicarp; f.c., fibrous cells; f.l., fibrous layer; ph., phloem; R., raphe; scl., sclereids; tes., testa; tr., trichome; V., vitta; v.b., vascular bundle; W., wing; xy., xylem.

bundle. The fibrous layer in the wing is thicker than in the other parts of fruit, and in addition to the fibrous cells, it shows sclereids and lignified, pitted, parenchymatous cells (Fig. 4, A and B).

Endocarp (Fig. 3, F and G). This is a single layer of polygonal, elongated cells with nearly straight or sinuate walls; measuring from 72 to 144μ in length, 36 to 80μ in width and 11 to 15μ in height; most of the cells are divided by parallel partitions, exhibiting parquetry arrangement. The endocarpal cells appear in transverse section as long narrow rectangular cells, showing occasional thin partitions.

Seed-Coat (Fig. 3, H). A single layer of small polygonal, isodiametric cells, with brownish walls, measuring from 11 to 36μ in length, 9 to 25μ in width and 4 to 7μ in height. In transverse section, it appears to be

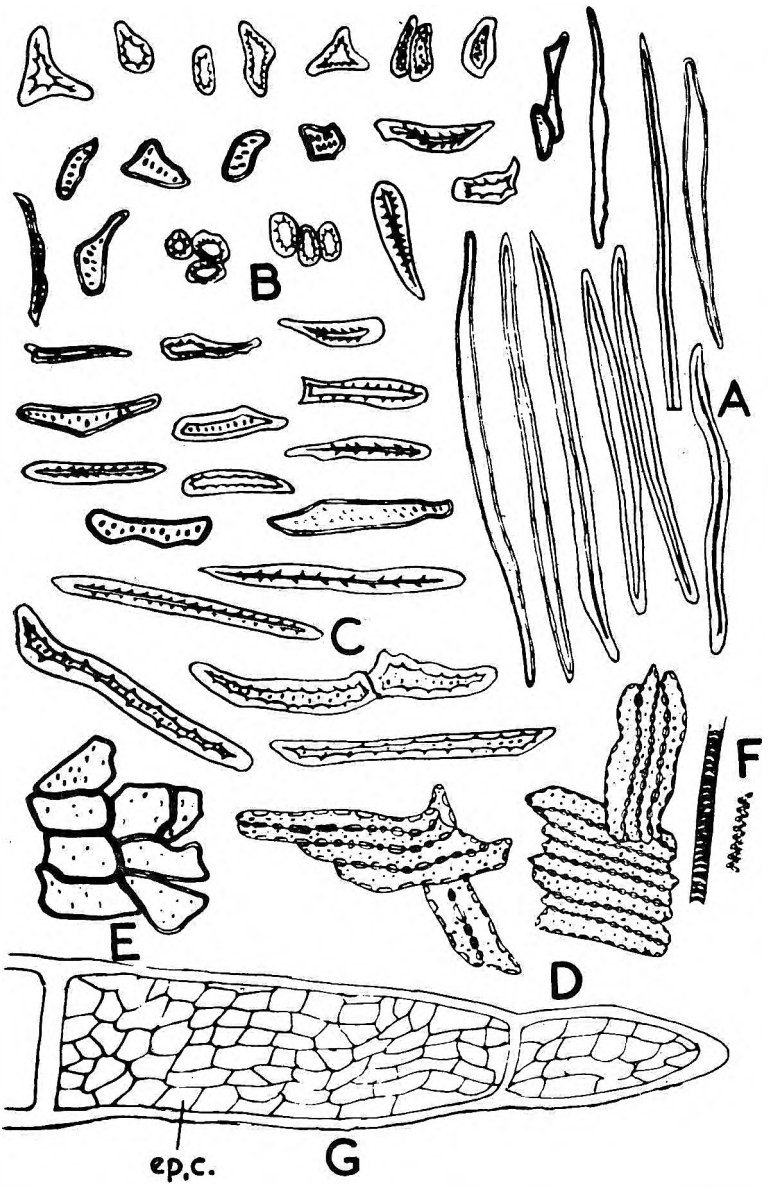


FIG. 5. Isolated elements from mesocarp of fruit; A., elongated fibrous cells with pointed ends; B., sclereids from wing; C., fibrous cells with non-pointed ends and thick pitted walls; D., fibrous cells crossing each other; E., lignified pitted parenchymatous cells from wing; F., vessels; G., part from the top of a vitta (all × 220); ep.c., epithelial cell.

formed of narrow rectangular cells, which are collapsed in many places; the seed-coat widens in the region of the raphe, where a slender vascular bundle is found.

Endosperm (Fig. 4, A and D). This is formed of polygonal, isodiametric cells, with thick cellulosic walls; measuring from 6 to 16μ in diameter, and containing fixed oil, and several oval or rounded aleurone grains. These contain distinct micro-rosette crystals of calcium oxalate (Fig. 3, E), 3 to 10μ in diameter. These are mostly larger than those found in other umbelliferous fruits.

Powder

The powder of Pastinaca fruit, is pale yellowish-brown, with distinct characteristic terebinthinate odour and a slightly bitter, very pungent taste. It is characterised microscopically by:

1. Fragments of epicarpal cells, with striated cuticle and showing occasional small calcium oxalate crystals, cruciferous stomata and non-glandular, warty, unicellular, conical trichomes or their scars.
2. Lignified, elongated, fibrous cells from the mesocarp (Fig. 5); some having pointed ends and thick walls, others with non-pointed ends and thick pitted walls, crossing each other.
3. Sclerenchymatous isodiametric cells in groups or isolated from the wing of the fruit.
4. Fragments of brown vittae, generally adhering to fibrous cells.
5. A few annular and spiral small vessels.
6. Endocarpal cells, mostly showing parquetry arrangement.
7. Endosperm cells with polygonal outlines and thick cellulosic walls; and containing aleurone grains with micro-rosette crystals of calcium oxalate.

Chemical Study

Both Klein[12] and Van Urk[4] have stated that the fruit contains an ethereal oil which contains butyric acid, acetyl ester and yields also methanol by steam distillation. Klein[12] attributed the skin irritation to a non-volatile alkaloid, pastinacin. But the present work shows that:

(i) The sublimate of the fruits of *Pastinaca sativa* L. is free of crystals, and gives a purplish-brown colour with concentrated sulphuric acid;

(ii) The fruit yields 12 per cent. ash and 0·9 per cent. acid-insoluble ash;

(iii) The decoction is clear when hot, but it becomes turbid on cooling and gives positive tests for reducing sugar, unsaturated compounds, and catechol tannin;

(iv) The fruits, when successively extracted with light petroleum, ether, chloroform and ethanol, yield 12·68; 1·78; 13·61 and 7·36 per cent. of dried extracts respectively calculated with reference to the air dried fruits;

(v) Three crystalline constituents were isolated, and were found in the following percentages: xanthotoxin 0·1 per cent., imperatorin 0·17 per cent. and bergapten 0·38 per cent.; the fruits containing in addition about 9·9 per cent. of fixed oil, as well as resinous substance, colouring

matter and chlorophyll. Light petroleum is the best solvent for the extraction of these principles from the fruit because their isolation from the ethanolic extract is much more tedious and lengthy with more extractive and colouring matter.

The crystalline constituents when investigated reacted similarly to those listed by Fahmy and Abu-Shady[6] who also isolated xanthotoxin (ammoidin), imperatorin (ammidin) and bergapten (majudin) from the fruits of *Ammi majus* in the following percentages: 0·5, 0·3 and 0·02 respectively.

These constituents are used in the treatment of leucodermia[13]. As the fruits of *Pastinaca sativa* were found to contain the same constituents but in different percentages, viz. 0·1, 0·17 and 0·38 respectively, and the leaves were found to contain only xanthotoxin[14] in the percentage of 0·08 (*cf.* those of *A. majus* which are totally free of these crystalline constituents[15]) the fruits and leaves of *P. sativa* can be used as a substitute for *A. majus*. They could be used also as a source for the preparation of these active constituents; particularly bergapten, of which the fruits contain about twenty times the quantity found in *A. majus*.

Summary

1. The macro- and microscopical characters of the fruit of *Pastinaca sativa* L. are described.

2. The fruit was found to contain xanthotoxin 0·1 per cent., imperatorin 0·17 per cent., bergapten 0·38 per cent., about 10 per cent. of fixed oil, a resinous substance, colouring matter and chlorophyll.

References

1. *Pharm. J.*, 1930, 125, 219.
2. Bellringer, *Brit. med. J.*, 1949, **1**, 984.
3. Kuské, *Arch. Derm. Syph.*, 1938, **54**, 193.
4. Van Urk, *Pharm. Weekbl.*, 1919, **56**, 1390.
5. Wittstein, through 8.
6. Fahmy and Abu-Shady, *Quart. J. Pharm. Pharmacol.*, 1948, **21**, 499.
7. Ibn-El-Bitar, *Mophradat Ibn El-Bitar*, 3, 4, 46.
8. Phil. Gustav Hegi, *Illustrierte Flora Von Mittel-Europa*, 1906, vol. V2, 1404.
9. Perrot, *Matières Premières Usuelles Du Règne Vegetal*, 1943–1944, 1682.
10. Rendle, *The Classification of Flowering Plants*, Vol. II, 1942, 411.
11. Bentham and Hooker, *ex. Ind. Kew*, 1894.
12. Klein, *Handbuch Der Pflanzenanlyse*, 1932, Vol. II, 218, 235, 247, 249, 295, 500, 502, 506, 507, 519; Vol. III, V.I, 959, V.II, 932.
13. Abdel-Moneim El-Mofty, *J. Royal Egypt. Med. Ass.*, Jan. 1950, I.
14. Kadir, 1954, Thesis presented for the degree of D.Pharm., Faculty of Medicine, Kasr-El-Ainy, Cairo University, Cairo, Egypt.
15. Hifny Saber and Hashim, *Proc. of Pharm. Soc. Egypt*, 1952, Vol. XXXIV, No. 12, 131.

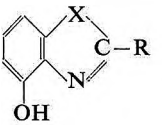
4-HYDROXYBENZAZOLES: PREPARATION AND ANTIBACTERIAL ACTIVITIES

By A. H. Beckett and K. A. Kerridge

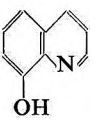
From The School of Pharmacy, Chelsea Polytechnic, London, S.W.3.

Received April 4, 1956

In a previous communication, the preparations and investigation of metal-chelating molecules as potential antibacterial agents were described[1]. As a continuation of these studies, a number of 4-hydroxybenzazoles (I; X = N or O, R = alkyl; X = S; R = NH_2) which may be regarded as analogues of the active, metal-reagent oxine (II), have been prepared and their antibacterial activity examined*.



(I)



(II)

It is known that variation in the alkyl substituent at the 2 position of benziminazole causes only slight variation of the basic strength of the derivatives[2]. Hence a study of the 2-alkyl-4-hydroxybenziminazoles seemed to offer the opportunity of modifying the lipoid solubility of the compounds by altering the size of the alkyl group without an attendant change in the electron donating capacity of the basic nitrogen atom. Such a study is of interest, because a relation between lipoid solubility and activity independent of the degree of metal-chelation is indicated in the "aza-quinolines"[3]. It also seemed reasonable to separately alter the electron availability of the basic nitrogen atom by replacement of the N (1) atom of the 4-hydroxybenziminazoles by O and S atoms by preparing suitably substituted benzoxazoles and benzthiazoles.

Preparation of the compounds. The 2-alkyl derivatives of 4-methoxybenziminazole were prepared by heating the appropriate aliphatic acid with 2:3-diaminoanisole in the presence of 4N hydrochloric acid, according to the general procedure of Phillips[4]. An 85 per cent. yield of the 2-benzyl derivative was obtained by heating together equimolecular proportions of *diamine* and phenylacetic acid, in the presence of 10 per cent. hydrochloric acid in a sealed tube for 40 minutes at 180 to 185° C.[5]

Since iminazole is not reduced in the presence of constant boiling hydriodic acid and red phosphorus[6], this reagent was used to demethylate the 2-alkyl-4-methoxybenziminazoles, by heating at 130 to 140° C. for one hour. The corresponding hydroiodides of the hydroxy derivatives (I; X = NH, R = alkyl) were obtained in yields of 85 to 95 per cent. by this process.

2-Alkyl-4-hydroxybenzoxazoles (I; X = O, R = H, Me, Et, or CH_2Ph)

*During the course of this work, Erlenmeyer and colleagues, *Helv. chim. Acta*, 1952, **35**, 1736, examined 4-hydroxybenziminazole, benzthiazole, and benzoxazole for antibacterial activity.

were prepared by condensing 2-aminoresorcinol hydrochloride with the appropriate acid, amide, or cyanide.

In attempting to prepare 2-amino-4-methoxybenzthiazole by ring closure of *o*-methoxyphenylthiourea with sulphuryl chloride, 2-amino-7-chloro-4-methoxybenzthiazole was obtained.

Experimental

Chemical

Microanalyses were made by Mr. G. S. Crouch, School of Pharmacy, University of London. Equivalent weights, except those of the benzoxazoles, were determined by titration with 0·2 N perchloric acid in acetic acid. It was found that the titration of benzoxazoles and picrates of benziminazoles with perchloric acid was not quantitative under the conditions employed. Details of new compounds are given in Table I.

Preparation of 2-alkyl-4-methoxybenziminazoles. 2:3-Diaminoanisole (5·52 g., 0·04 mole), dissolved in 4 N hydrochloric acid (40 ml.), was refluxed with the appropriate aliphatic acid (0·2 mole) under an atmosphere of nitrogen for 40 minutes. The cooled, neutralised (10 per cent. solution of ammonia) solution was extracted with ethyl acetate. 2-Alkyl-4-methoxybenziminazole derivatives were obtained from the ethyl acetate extract by evaporation of the solvent under reduced pressure. Ether was added to those residues which were not crystalline, and the side of the flask scratched with a glass rod to effect crystallisation.

Demethylation of 2-alkyl-4-methoxybenziminazoles. The methoxy derivative (2 g.) was heated with hydriodic acid (10 ml.) sp. gr. 1·7 and red phosphorus (0·2 g.) for 1 hour at 130 to 140° C. After cooling, the crystallised hydriodide of the corresponding hydroxybenziminazole was filtered off and the free base precipitated with ammonia solution.

Preparation of 2-ethyl- and 2-benzyl-4-hydroxybenzoxazoles. (I; X = O; R = Et or CH_2Ph respectively). 2-Aminoresorcinol hydrochloride (0·02 mole) was heated with either propio- or phenylaceto-nitrile (0·02 mole) in a sealed tube for 1 hour at 180 to 185° C. After cooling, the residues were recrystallised from 50 per cent. aqueous ethanol to give the respective 2-alkyl-4-hydroxybenzoxazoles.

4-Hydroxybenzoxazole. (I; X = O, R = H) was prepared by refluxing 2-aminoresorcinol hydrochloride (3·24 g.) with formic acid (10 ml.) for one hour. After distilling off the excess acid, the residue was sublimed, 200 to 215° C./1·5 mm., and the sublimate recrystallised from 30 per cent. aqueous ethanol.

4-Hydroxy-2-methylbenzoxazole. (I; X = O, R = Me) sublimed on heating a mixture of 2-aminoresorcinol hydrochloride (3·24 g.) and acetamide (1·3 g.) at 120° C./1 mm.

2-Amino-7-chloro-4-methoxybenzthiazole was formed on slowly adding sulphuryl chloride (10·0 g.; 0·075 mole) to a solution of *o*-methoxyphenylthiourea (9·1 g., 0·05 mole) in chlorobenzene (40 ml.), the temperature of which was maintained between 40 to 50° C. The evolution of hydrogen chloride was complete within five minutes, after which time the reaction mixture was cooled and the chlorobenzene decanted

TABLE I

4-HYDROXY- AND 4-METHOXYBENZAZOLES

Compound	M.pt. °C.	Physical form	Found			Formula	Required			Yield per cent.	Equivalent	
			C	H	N		C	H	N		Found	Required
12. 4-Methoxy-2-methylbenziminazole	163	grey-white prisms (*a*)	66·0	6·0	17·3	$C_9H_{10}N_2O$	66·0	6·2	17·3	60	163	162
13. 2-Ethyl-4-methoxybenziminazole	132	grey prisms (*a*)	68·05	6·9	15·9	$C_{10}H_{12}N_2O$	68·2	6·8	15·9	53	176	176
14. 4-Methoxy-2-propylbenziminazole	128	brown prisms (*a*)	68·9	7·2	14·7	$C_{11}H_{14}N_2O$	69·5	7·4	14·8	48	192	190
15. 2-Benzyl-4-methoxybenziminazole	173	grey prisms (*a*)	76·0	5·8	11·8	$C_{15}H_{14}N_2O$	75·6	5·9	11·8	85	239	238
1. 4-Hydroxybenziminazole	194	white prisms (*a*)	62·5	4·50	20·7	$C_7H_6N_2O$	62·75	4·5	20·9	87	135	134
2. 4-Hydroxy-2-methylbenziminazole	211 (decomp.)	white prisms (*a*)	64·2	5·4	19·0	$C_8H_8N_2O$	64·9	5·4	18·9	88	149	148
3. 2-Ethyl-4-hydroxybenziminazole	156	white prisms (*a*)	66·2	5·9	17·2	$C_9H_{10}N_2O$	66·7	6·2	17·3	89	163	162
4. 4-Hydroxy-2-propylbenziminazole	140	grey prisms (*a*)	68·5	6·8	15·9	$C_{10}H_{12}N_2O$	68·2	6·8	15·9	85	174	176
5. 2-Benzyl-4-hydroxybenziminazole	191	silver prisms (*a*)	74·9	5·45	12·55	$C_{14}H_{12}N_2O$	75·0	5·35	12·5	93	222	224
6. 4-Hydroxybenzoxazole	190 (*b*)	silver plates (*c*)	62·7	3·9	10·5	$C_7H_5NO_2$	62·2	3·7	10·4	58		
7. 4-Hydroxy-2-methylbenzoxazole	147·5	white plates (*c*)	64·65	4·8	9·3	$C_8H_7NO_2$	64·45	4·7	9·4	73		
8. 2-Ethyl-4-hydroxybenoxazole	87·5	white plates (*c*)	66·8	5·6	8·7	$C_9H_9NO_2$	66·2	5·5	8·6	65		
9. 2-Benzyl-4-hydroxybenzoxazole	189	grey plates (*c*)	75·0	4·85	6·1	$C_{14}H_{11}NO_2$	74·7	4·9	6·2	75		
10. 2-Amino-7-chloro-4-hydroxybenzthiazole	227	needles (*d*)	41·6	2·6	13·9	$C_7H_5N_2ClOS$	41·9	2·5	13·95	50	201	200·6

(*a*) Solvent, ethyl acetate. (*b*) Lit. m. pt. 181° C[7]. (*c*) Solvent, aqueous ethanol. (*d*) Solvent, ethanol.

All the hydroxy compounds in Table I showed a positive reaction with cupric and ferric ions at a concentration of 0·5 per cent. in 70 per cent. aqueous ethanol. 2-Ethyl-and 2-methyl-4-hydroxybenzoxazoles gave precipitates with 0·05 N silver nitrate in 0·05 N nitric acid solution.

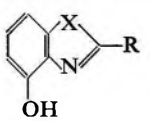
from the viscous residue of benzthiazole hydrochloride. Ether was added and the *hydrochloride* separated by filtration. 2-Amino-7-chloro-4-methoxybenzthiazole, m.pt. 202 to 203° C. (C, 44·8; H, 3·4; N, 12·9. Calc. for $C_8H_8N_2ClOS$: C, 44·8; H, 3·3; N, 13·05 per cent.) and not the expected 2-amino-4-methoxybenzthiazole was obtained on neutralising the hydrochloride with ammonia and recrystallising the base obtained from ethanol. The identity of the isolated compound was proved by a mixed m.pt. and a comparison of its infra-red spectrum with that of a sample of 2-amino-7-chloro-4-methoxybenzthiazole kindly supplied by Professor H. Erlenmeyer[7].

The corresponding hydroxy compound was obtained by demethylating with hydriodic acid as described under hydroxybenziminazoles.

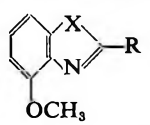
Other materials. 2:3-Diaminoanisole was prepared according to Lane and Williams[8]. 2-Aminoresorcinol, m.pt. 160·5° C. (decomp.), lit. m.pt. 159 to 160° C.[9], was obtained on hydrogenating 2-nitroresorcinol at 5 atm. in the presence of Raney nickel. *o-Methoxyphenylthiourea*, m.pt. 154·5° C., lit. m.pt. 156° C.[10], was prepared by a method similar to the one described for the preparation of *p*-tolylthiourea[11].

TABLE II

ANTIBACTERIAL ACTIVITIES OF 4-HYDROXY- AND 4-METHOXYBENZAZOLES



(III)



(IV)

Cpd. No.	Derivative (III)	M.I.C. in reciprocal molar concentrations against*					
		Staph. aureus	*B. subtilis*	*Bact. coli*	*Proteus vulgaris*	*Sh. sonnei*	*Myco. phlei*
1	X = N; R = H	1600	—	—	6400	—	—
2	X = N; R = Me	—	—	—	1600	—	—
3	X = N; R = Et	—	—	—	3200	—	—
4	X = N; R = Pr^n	—	—	—	3200	—	1600
5	X = N; R = CH_2Ph	—	3200	1600	12,800	1600	6400
6	X = O; R = H	—	—	—	1600	—	
7	X = O; R = Me	—	—	—	—	—	
8	X = O; R = Et	—	—	—	1600	—	
9	X = O; R = CH_2Ph	—	—	—	—	—	
10	X = S; R = NH_2 (7-Chlorine atom)	—	1600	1600	3200	1600	
	(IV)						
11	X = N; R = H	—	—	—	—	—	
12	X = N; R = Me	—	—	—	1600	—	
13	X = N; R = Et	—	—	—	1600	—	
14	X = N; R = Pr^n	—	—	—	1600	—	
15	X = N; R = CH_2Ph	—	—	—	1600	—	
16	X = S; R = NH_2 (7-Chlorine atom)	—	—	1600	3200	—	

— Signifies growth at M/1600.

* All these compounds are inactive at M/1600 concentration against *Str. pyogenes*.

Bacteriology

Minimum inhibitory concentrations of the compounds described were determined in a similar manner to that used previously[1]. The results are summarised in Table II.

Despite the metal chelating properties of the compounds, the low order of their antibacterial properties precludes any attempted correlation of chelation and antibacterial action in the present series.

Summary

1. 2-Alkyl-4-hydroxybenzazoles have been prepared as potential antibacterial agents acting through the chelation of metal ions.

2. All the 4-hydroxybenzazoles listed show chelation phenomena with cupric and ferric ions: 2-ethyl- and 2-methyl-4-hydroxybenzoxazole precipitate silver ions in dilute acid solution.

3. None of the compounds prepared possessed significant antibacterial activity.

We thank Dr. W. G. Smith and Mrs. P. M. Clark of Bradford Technical College for the bacteriological results, Imperial Chemical Industries for a gift of 2-nitroresorcinol, and one of us (K. A. K.) thanks the Pharmaceutical Society for the award of a scholarship.

References

1. Beckett, Clarke, Kerridge and Smith, *J. Pharm. Pharmacol.*, 1955, 7, 717.
2. Davies, Mamalis, Petrow and Sturgeon, *ibid.*, 1951, 3, 420.
3. Albert, Hampton, Selbie and Simon, *Brit. J. exp. Path.*, 1954, **35**, 75.
4. Phillips, *J. chem. Soc.*, 1928, 2393.
5. Porai-Koshits, Ginzburg and Efross, *J. Gen. Chem.* (*U.S.S.R.*) 1949, **19**, 1945.
6. Wyss, *Ber.*, 1897, **10**, 1364.
7. Erlenmeyer and Uberwasser, *Helv. Chim. Acta*, 1942, **25**, 515.
8. Lane and Williams, *J. chem. Soc.*, 1954, 2977.
9. Likhosherstov and Zhabotinokaya, *J. Gen. Chem.* (*U.S.S.R.*), 1932, **2**, 761.
10. *Chem. Abstr.*, 1941, **35**, 453[5].
11. *Organic Syntheses*, Vol. 22, Wiley & Sons Inc., p. 16.

ARYLOXYPROPANE DERIVATIVES

Part II. The Synthesis of Some Aryloxypropanolamines for Study as Local Anæsthetics

By V. Petrow, O. Stephenson and A. J. Thomas

From the Chemical Research Laboratories, The British Drug Houses Ltd., London, N.1

Received April 12, 1956

Following studies[1–3] on 1-aryloxypropane-2:3-diols such as mephenesin, attention was turned in these laboratories to the derived 3-aryloxy-2-hydroxypropylamines (IV; see p. 668)[4]. Compounds of this type had previously been prepared by Boyd[5,6] in 1909 and by Fourneau[7,8] in 1910, the French worker drawing attention to their antipyretic and analgesic properties. Some years later Pyman[9] discovered the local anæsthetic activity of 3-diethylamino-1-phenoxypropan-2-ol (IV; Ar = Ph; R = R′ = Et), thereby extending the range of biological properties shown by this versatile group of compounds. More recently Ing and Ormerod[10] have described 16 propylamines based on (IV), some of which were more active than procaine in the guinea-pig weal test.

Work outlined below was directed to the preparation of 3 main types (i) simple aryloxyhydroxypropylamines (IV); these were synthesised and examined prior to the independent studies of Ing and Ormerod[10], (ii) 3-(2′:6′-xylyloxy)-2-hydroxypropylamines (IV; Ar = 2:6-xylyl), which bear a formal resemblance to the lignocaine group of local anæsthetics[11,12] and (iii) the hitherto unknown 3-diphenylmethoxy-2-hydroxypropylamines (IV; Ar = Ph_2CH).

The simpler aryloxyhydroxypropylamines listed in Table I were readily prepared by condensing the 3-aryloxy-1:2-epoxypropanes (I) with the appropriate amines (II) (route a). Their biological study, kindly undertaken by Dr. S. W. F. Underhill and his staff showed that most of them had appreciable local anæsthetic activity when tested on the rabbit's cornea. Maximum potency was shown by 1-propylamino-(IV; Ar = *o*-tolyl; R = Pr; R′ = H) and 1-*iso*propylamino-2-hydroxy-3-*o*-toloxypropane (IV; Ar = *o*-tolyl; R = *iso*Pr; R′ = H). The *iso*propyl-derivative was examined further when it was found to be superior to procaine in local anæsthetic activity using the guinea-pig weal method. Unfortunately it proved to be somewhat more toxic than procaine and its development was not proceeded with.

Following the discovery of lignocaine [2-diethylamino-acetamido-*m*-xylene (hydrochloride monohydrate)] by Löfgren[11,12] we turned to the synthesis of the analogous 3-(2′:6′-xylyloxy)-2-hydroxypropylamines (IV; Ar = 2:6-xylyl). These (see Table II) were prepared not only by reaction (*a*) (above), but also by route (*b*) employing the chlorohydrin (III; Ar = 2:6-xylyl). Their biological study, for which we are indebted to Dr. A. David and Mr. B. G. Cross, B.Sc., F.P.S., revealed a high order of potency, 1-diethylamino-2-hydroxy-3:2′:6′-xylyloxy) propane

TABLE I

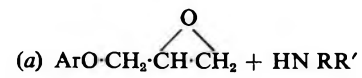
ARYLOXYPROPANOLAMINE DERIVATIVES

$ArOCH_2CHOHCH_2{\cdot}NRR'$

Ar	R	R′	Base (B) Hydrochloride (H)	M.pt. ° C. B.pt. ° C.	Formula	Found per cent. C	H	N	Cl	Required per cent. C	H	N	Cl
PHENYL	H	H	H*	—	$C_9H_{14}O_2NCl$	—	—	—	—	—	—	—	—
	H	Et	H*	126	$C_{11}H_{18}O_2NCl$	56·9	7·7	5·9	—	57·0	7·8	6·1	—
	H	*n*-Pr	H	141–2	$C_{12}H_{20}O_2NCl$	58·1	7·4	5·8	14·5	58·6	8·2	5·7	14·5
	H	*i*-Pr	H	115	$C_{12}H_{20}O_2NCl$	58·3	8·0	5·6	—	58·6	8·2	5·7	—
	H	*n*-Bu	H	140	$C_{13}H_{22}O_2NCl$	60·4	8·4	5·2	13·6	60·1	8·5	5·4	13·7
	H	Bz	H	185	$C_{16}H_{20}O_2NCl$	—	—	—	12·0	—	—	—	12·1
	Et	Et	B	128/1 mm.	$C_{13}H_{21}O_2N$	—	—	6·3	—	—	—	6·3	—
o-TOLYL	H	Et	H*	115	$C_{12}H_{20}O_2NCl$	58·9	8·3	—	—	58·6	8·2	—	—
	H	*n*-Pr	H	116	$C_{13}H_{22}O_2NCl$	60·0	8·3	5·2	13·7	60·1	8·5	5·4	13·7
	H	*i*-Pr	H	135	$C_{13}H_{22}O_2NCl$	60·1	8·4	—	13·6	60·1	8·5	5·4	13·7
	H	*n*-Bu	H	101	$C_{14}H_{24}O_2NCl$	61·3	8·7	—	12·9	61·4	8·8	—	13·0
	Me	Me	B	132/1·5 mm.	$C_{12}H_{19}O_2N$	69·1	8·7	—	—	68·9	9·2	—	—
	Et	Et	H*	115	$C_{14}H_{24}O_2NCl$	61·5	8·7	5·3	—	61·4	8·8	5·1	—
	n-Bu	*n*-Bu	B	170/1 mm.	$C_{17}H_{29}O_2N$	73·9	10·5	4·7	—	73·1	10·5	5·0	—
p-TOLYL	H	Et	B	76	$C_{12}H_{19}O_2N$	69·1	8·8	6·7	—	68·9	9·2	6·7	—
	H	Et	H*	170	$C_{12}H_{20}O_2NCl$	59·1	8·0	—	—	58·6	8·2	—	—
	H	*n*-Pr	H	170	$C_{13}H_{22}O_2NCl$	60·0	8·5	5·3	—	60·1	8·5	5·4	—
	H	*n*-Bu	H*	174	$C_{14}H_{24}O_2NCl$	61·8	8·8	—	—	61·4	8·8	—	—
o-CHLOROPHENYL ..	Et	Et	B	106–110/0·1 mm.	$C_{13}H_{20}O_2NCl$	60·9	7·6	—	—	60·5	7·8	—	—
p-CHLOROPHENYL ..	Et	Et	B	120/0·1 mm.	$C_{13}H_{20}O_2NCl$	60·3	8·1	5·3	13·8	60·5	7·8	5·4	13·8
	n-Bu	*n*-Bu	B*	140/0·05 mm.	$C_{17}H_{28}O_2NCl$	65·3	8·9	4·4	11·6	65·0	9·0	4·5	11·3
p-METHOXYCARBONYL PHENYL	H	*n*-Pr	H	180	$C_{14}H_{20}O_4NCl$	55·5	6·7	4·8	11·8	55·3	7·3	4·6	11·7
	H	*n*-Bu	H	185	$C_{16}H_{24}O_4NCl$	56·9	7·6	—	—	56·7	7·6	—	—
p-ETHOXYCARBONYL-PHENYL	H	*n*-Pr	H	153	$C_{15}H_{24}O_4NCl$	56·7	7·6	4·2	11·3	56·7	7·6	4·4	11·2
	H	*n*-Bu	H	157	$C_{16}H_{26}O_4NCl$	57·8	7·8	4·5	10·7	57·9	7·9	4·2	10·7
p - BUTOXYCARBONYL-PHENYL	H	*n*-Pr	H	114	$C_{17}H_{28}O_4NCl$	59·6	7·8	—	10·3	59·0	8·2	—	10·3
1-NAPHTHYL	H	*n*-Pr	B	104	$C_{16}H_{21}O_2N$	—	—	5·4	—	—	—	5·4	—
	H	*n*-Pr	H*	138	$C_{16}H_{22}O_2NCl$	—	—	4·7	12·0	—	—	4·7	12·0
	H	*n*-Bu	B	82	$C_{17}H_{25}O_2N$	74·9	8·7	—	—	74·7	8·5	—	—
	H	*n*-Bu	H	161–2	$C_{17}H_{26}O_2NCl$	—	—	4·4	11·5	—	—	4·5	11·5

*Inactive.

(IV; Ar = 2:6-xylyl; R = R′ = Et) being 3 times more active than lignocaine using the guinea-pig corneal reflex method. All members of this series, however, produced some evidence of local necrosis on subcutaneous injection, thus rendering them unsuitable for clinical trial (*cf.* also Fosdick and Carbon)[13]. Their high biological potency led us to examine their deoxy-analogues (V) (see Table II), which were readily obtained by condensing trimethylene dibromide with sodium 2:6-xylenoxide to give ω-2:6-xylenoxypropyl bromide, followed by reaction with (II) in the usual way. This structural modification, however, failed to eliminate the irritant properties shown by the parent group (IV; Ar = 2:6-xylyl).



(*a*) $ArO{\cdot}CH_2{\cdot}\underset{\diagdown\,O\,\diagup}{CH}{\cdot}CH_2 + HN\,RR'$

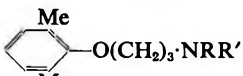
(I) (II)

$ArO{\cdot}CH_2{\cdot}CHOH{\cdot}CH_2NRR'$

(IV)

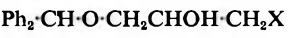
(*b*) $ArO{\cdot}CH_2{\cdot}CHOH{\cdot}CH_2{\cdot}Hal + (II)$

(III)



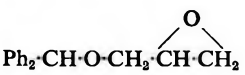
Me, $O(CH_2)_3{\cdot}NRR'$, Me

(V)



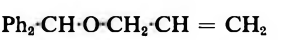
$Ph_2{\cdot}CH{\cdot}O{\cdot}CH_2CHOH{\cdot}CH_2X$

(VI)



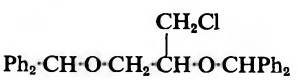
$Ph_2{\cdot}CH{\cdot}O{\cdot}CH_2{\cdot}\underset{\diagdown\,O\,\diagup}{CH}{\cdot}CH_2$

(VII)



$Ph_2{\cdot}CH{\cdot}O{\cdot}CH_2{\cdot}CH = CH_2$

(VIII)



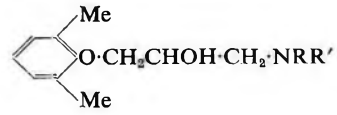
$Ph_2{\cdot}CH{\cdot}O{\cdot}CH_2{\cdot}\underset{|}{\overset{CH_2Cl}{CH}}{\cdot}O{\cdot}CHPh_2$

(IX)

Extension of the work to the hitherto unknown 3-diphenylmethoxy-2-hydroxypropylamine series (VI; X = NRR′) offered initial difficulty. 1:2-Epoxy-3-diphenylmethoxypropane (VII), required as an intermediate in route (*a*) was not obtained by heating diphenylcarbinol with epichlorohydrin in the presence of basic catalysts such as pyridine. The required epoxide (VII) was ultimately obtained in 40 per cent. yield by condensing sodium diphenyl methoxide with epichlorohydrin in benzene solution. For small scale work, however, epoxidation of diphenylmethyl allyl ether (VIII) proved a more convenient route to (VII). The allyl ether (VIII) had previously been described by D'yakonov[14], who obtained it in 32 per cent. yield by condensing diphenyldiazomethane with allyl alcohol. We now find that (VIII) is readily prepared in high yield by (i) heating diphenylmethyl bromide with excess allyl alcohol in the presence of powdered potassium hydroxide and (ii) by heating diphenylcarbinol with allyl alcohol in benzene solution under reflux in the presence of toluene *p*-sulphonic acid as catalyst and with continuous removal of the water formed during the reaction. Its epoxidation with perbenzoic acid

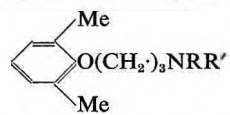
TABLE II

1-Amino-2-hydroxy-3(2′:6′-xylyloxy) propane derivatives



R	R′	Base/Salt	M.pt. ° C. B.pt. ° C.	Formula	Found per cent. C	H	N	Cl	Required per cent. C	H	N	Cl
H	Et	B	76	$C_{13}H_{21}O_2N$	70·3	9·5	6·0	—	69·9	9·5	6·3	—
H	Et	H	145–7	$C_{13}H_{22}O_2NCl$	60·5	8·8	5·3	—	60·1	8·5	5·4	13·7
H	*n*-Pr	B	79	$C_{14}H_{23}O_2N$	70·8	9·9	6·0	—	70·8	9·8	5·9	—
H	*n*-Pr	H	152–3	$C_{14}H_{24}O_2NCl$	61·1	9·0	5·2	12·8	61·4	8·8	5·1	13·0
H	*i*-Pr	B	75–7	$C_{14}H_{23}O_2N$	71·0	9·6	—	—	70·8	9·8	5·9	—
H	*i*-Pr	H	141–2	$C_{14}H_{24}O_2NCl$	61·5	8·7	4·5	—	61·4	8·8	5·1	—
Et	Et	B	121/0·2 mm.	$C_{15}H_{25}O_2N$	71·3	10·3	5·4	—	71·6	10·1	5·6	—
Et	Et	H	114–5	$C_{15}H_{26}O_2NCl$	62·4	9·2	4·7	12·5	62·5	9·1	4·9	12·3
Piperidine		H	164–6	$C_{15}H_{26}O_2NCl$	63·8	8·9	4·8	11·7	64·1	8·7	4·7	11·9

1-Amino-3(2′:6′-xylyloxy) propane derivatives



H	*n*-Pr	H	162–3	$C_{14}H_{24}ONCl$	65·3	9·8	5·3	13·4	65·2	9·3	5·4	13·8
Morpholine		H	170–1	$C_{13}H_{24}O_2NCl$	—	—	5·1	—	—	—	4·9	—
Δ^3-Piperideine		H	177–8	$C_{14}H_{24}ONCl$	67·8	8·5	4·4	13·0	68·2	8·5	5·0	12·6

in benzene solution furnished (VII) in yields greater than 90 per cent. Reaction of (VII) with (II) gave the propanolamines listed in Table III.

Attempts to prepare (VI; X = NRR′) by route (*b*) were only partly successful. Reaction of diphenylcarbinol with epichlorohydrin in the presence of sulphuric acid as catalyst[7,8] led to the formation of *sym*-tetraphenyl methyl ether in place of the expected (VI; X = Cl).

The required intermediate was ultimately obtained by making use of an earlier observation that diphenylmethylcarbinol reacts smoothly with ethylene chlorohydrin or cyanohydrin in the presence of toluene *p*-sulphonic acid as catalyst [*cf.* the preparation of (VIII) by method (ii) (above)] to give diphenylmethoxyethyl chloride and diphenylmethoxyethyl cyanide, respectively. Extending this reaction to diphenylcarbinol and α-monochlorohydrin in equimolar ratio led only to the formation of the bimolecular condensation product (IX). By using 3 molar proportions of α-monochlorohydrin, however, a 25 per cent. yield of 1-chloro-2-hydroxy-3-diphenylmethoxypropane (VI; X = Cl) was obtained, together with larger quantities of (IX). Reaction of (VI; X = Cl) with (II) gave (VI; X = NRR′) in the usual way.

Biological study of the diphenylmethoxypropanolamines listed in Table III was kindly undertaken by Dr. A. David and Mr. B. G. Cross, B.Sc., F.P.S. The compounds, in general, showed marked local anæsthetic activity by the guinea-pig corneal reflex method, and on application to an exposed human nerve ending stimulated by the Newton Victor electronic nerve stimulator. In common with the lignocaine analogues, however, they produced necrosis at the site of injection.

Experimental

M.pts. are uncorrected.

The following example illustrates the method used for the preparation of the simpler 3-aryloxy-2-hydroxypropylamines listed in Table I.

Preparation of 1-iso*Propylamino*-2-*hydroxy*-3-o-*toloxypropane* (IV; Ar = *o*-tolyl; R = *iso*Pr; R′ = H). Glycide *o*-tolyl ether (23 g.) and *iso*propylamine (30 g.) were slightly warmed until reaction commenced and the mixture then water-cooled to moderate the exothermic condensation. Reaction was then completed by heating on the steam bath for 1 hour. After distilling off excess amine, the residual solids were dissolved in benzene and the solution treated with hydrogen chloride. The precipitated *hydrochloride* was collected and purified by crystallisation from methanol/ether.

The following examples indicate the methods used for the preparation of the 3-(2′:6′-xylyloxy)-2-hydroxypropylamines listed in Table II.

Preparation of 1-*Diethylamino*-2-*hydroxy*-3-(2′:6′-*xylyloxy*)-*propane* (IV, Ar = 2:6-xylyl; R = R′ = Et). (*a*) Glycide 2:6-xylyl-ether was obtained in 70 per cent. yield by condensation of 2:6-xylenol with epichlorohydrin[15]. It had b.pt. 84° C. at 0·5 mm. Found: C, 73·5; H, 8·0. $C_{11}H_{14}O_2$ requires C, 74·1; H, 7·9 per cent.

The glycide ether (44·5 g.) was heated with diethylamine (21·9 g.) under reflux for 5 hours. Excess diethylamine was removed by distillation and

TABLE III

1-AMINO-2-HYDROXY-3-DIPHENYLMETHOXYPROPANE DERIVATIVES

R	R′	Base or Salt	M.pt. ° C. B.pt. ° C.	Formula	Found per cent. C	H	N	Cl	Required per cent. C	H	N	Cl
H	Me	B	83	$C_{17}H_{21}O_2N$	75·0	8·1	5·2	—	75·2	7·8	5·2	—
H	Me	H	135–6	$C_{17}H_{22}O_2NCl$	66·0	6·8	4·6	11·8	66·3	7·2	4·6	11·5
H	Et	B	90–1	$C_{18}H_{23}O_2N$	76·3	8·2	4·9	—	75·8	8·1	4·7	—
H	Et	H	117–9	$C_{18}H_{24}O_2NCl$	—	—	4·6	11·4	—	—	4·4	11·0
H	*n*-Pr	B	61–3	$C_{19}H_{25}O_2N$	76·2	8·5	4·7	—	76·2	8·4	4·7	—
H	*n*-Pr	H	106–9	$C_{19}H_{26}O_2NCl$	—	—	4·2	11·3	—	—	4·2	10·6
H	*i*-Pr	B	55–60	$C_{19}H_{25}O_2N$	76·0	8·2	4·8	—	76·2	8·4	4·7	—
H	*i*-Pr	H	199–201	$C_{19}H_{26}O_2NCl$	67·8	7·8	4·1	10·6	68·0	7·8	4·2	10·6
Me	Me	B	154/0·3 mm.	$C_{18}H_{23}O_2N$	75·7	7·9	4·7	—	75·8	8·1	4·9	—
Et	Et	B	156/0·4 mm.	$C_{20}H_{27}O_2N$	76·7	8·9	4·1	—	76·6	8·7	4·5	—
PIPERIDINE		B	57–9	$C_{21}H_{27}O_2N$	77·7	7·9	4·4	—	77·5	8·4	4·2	—
		Salicylate	136 8	$C_{28}H_{33}O_5N$	72·5	6·5	3·1	—	72·5	7·2	3·0	—
N-BENZYL PIPERAZINE		H	218–220	$C_{27}H_{34}O_2N_2Cl_2$	—	—	6·0	—	—	—	5·7	—
PIPERAZINE (BIS)		B	116–8	$C_{36}H_{42}O_4N_2$	76·2	7·6	4·9	—	76·3	7·5	5·0	—
PIPERAZINE (BIS)		H	216–8 (d)	$C_{36}H_{44}O_4N_2Cl_2$	—	—	4·4	11·5	—	—	4·4	11·1

the product fractionally distilled at 0·2 mm. to give a nearly quantitative yield of the required *base* as an oil.

(*b*) 1-*Chloro*-2-*hydroxy*-3-(2′ : 6′-*xylyloxy*)-*propane* was prepared by the method described earlier[16] and obtained in high yield as an oil, b.pt. 91° C. at 0·1 mm. Found: C, 61·3; H, 7·2; Cl, 16·7. $C_{11}H_{15}O_2Cl$ requires, C, 61·5; H, 7·0; Cl, 16·5 per cent.

The chlorohydrin (21·5 g.) in benzene (40 ml.) was treated with diethylamine (15 g.) and the mixture heated on the steam bath for 5 hours. After washing with water to remove diethylamine hydrochloride, the benzene solution was dried and treated with hydrogen chloride. The precipitated 1-*diethylamino*-2-*hydroxy*-3-(2′ : 6′-*xylyloxy*)*propane hydrochloride* was purified by crystallisation from ethyl acetate containing a trace of methanol.

Preparation of 1-*Bromo*-3-(2′ : 6′-*xylyloxy*)*propane.* Sodium (34·5 g.) dissolved in ethanol (750 ml.) was added to 2 : 6-xylen-1-ol (183 g.). 1 : 3-Dibromopropane (909 g.) was then added and the mixture heated under reflux on the steam bath for 4 hours. Excess alcohol was removed on the steam bath and the residue treated with water and thoroughly extracted with chloroform. The chloroform extracts were washed with water, dried and the solvent removed. The residue was purified by distillation under reduced pressure to give 1-*bromo*-3-(2′ : 6′-*xylyloxypropane* as an oil, b.pt. 80° C. at 0·5 mm. Found: C, 54·5; H, 6·51; Br, 32·6. $C_{11}H_{15}OBr$ requires C, 54·3; H, 6·2; Br, 32·9 per cent.

Reaction of the foregoing compound with (II) is illustrated by the following example.

Preparation of 1-n-*Propylamino*-3-(2′ : 6′-*xylyloxy*)*propane.* 1-Bromo-3(2′ : 6′-xylyloxy)propane (11·7 g.) was treated with *n*-propylamine (6·0 g.) when an exothermic reaction occurred. The mixture was heated under reflux for 2 hours. After allowing to stand overnight, the separated propylamine hydrobromide was collected and washed with ether, which was subsequently used to isolate the product in the usual way. The base so obtained was purified as the hydrochloride.

Preparation of diphenylmethyl allyl ether (VIII). (*a*) Diphenylcarbinol (87 g.) and allyl alcohol (32 g.) in benzene (250 ml.) were treated with toluene *p*-sulphonic acid (200 mg.) and the mixture refluxed for 20 hours in a Dean-Stark apparatus. The cooled solution was washed with sodium carbonate solution and then with water and the solvent removed. Fractionation under reduced pressure gave (VIII), b.pt. 120° C. at 0·2 mm. Found: C, 85·8; H, 6·9. Calc. for $C_{16}H_{16}O$: C, 85·7; H, 7·2 per cent.

(*b*) Diphenylmethyl bromide (160 g.) in allyl alcohol (200 g.) was heated under reflux on the steam bath and finely powdered potassium hydroxide (40 g.) added in 5 g. portions at 10 minute intervals with occasional shaking. Heating was continued for a further 8 hours, when the mixture was poured into water and the product isolated with chloroform. Fractionation under reduced pressure furnished (VIII), b.pt. 101° C. at 0·2 mm. Found: C, 85·7; H, 7·2. Calc. for $C_{16}H_{16}O$: C, 85·7; H, 7·2 per cent.

1:2-*Epoxy*-3-*diphenylmethoxypropane* (VIII). (*a*) The foregoing compound (26·4 g.) was added to a cold solution of perbenzoic acid (18·32 g.) in benzene (490 ml.) and the mixture left at 0° to 5° C. for 4 days. The benzene solution was then washed consecutively with dilute aqueous solutions of sodium hydroxide, sodium iodide and sodium thiosulphate and then with water, when the benzene was removed by distillation. Fractionation under reduced pressure gave 1:2-*epoxy*-3-*diphenylmethoxypropane*, b.pt. 114° C. at 0·1 mm. Found: C, 80·0; H, 6·5. $C_{16}H_{16}O_2$ requires C, 80·0; H, 6·7 per cent. The product solidified on standing.

(*b*) Diphenylcarbinol (92 g.) in methanol (100 ml.) was added to sodium methoxide (11·6 g. sodium in 160 ml. methanol) and the mixture taken to dryness under reduced pressure at 100° C. The resulting sodium salt was suspended in dry benzene (400 ml.), epichlorohydrin (92·5 g.) added and the mixture heated under reflux for 16 hours. After acidification with acetic acid, filtration and water washing, the benzene was removed by distillation and the residue purified by fractionation to yield (VII), b.pt. 130° to 134° C. at 0·4 mm.

(*c*) Experiment (*b*) was repeated with the addition of sodium iodide (2 g.). After filtration, the benzene layer was treated with hydrogen chloride to convert any (VII) present into (VI; X = Cl). The benzene solution was then washed with sodium thiosulphate solution and with water and the solvent removed. Distillation of the complex mixture under reduced pressure gave a fraction (24 g.), b.pt. 210° C. at 0·4 mm. which solidified and yielded $\alpha\alpha\beta\beta$-tetraphenylethane[17], m.pt. 207° to 209° C. on crystallisation from acetone. Found: C, 93·1; H, 6·8. Calc. for $C_{26}H_{22}$: C, 93·4; H, 6·6 per cent.

The condensation of 1:2-epoxy-3-diphenylmethoxypropane with (II) to give the propanolamines listed in Table (III) is typified by the following example.

Preparation of 1-*diethylamino*-2-*hydroxy*-3-*diphenylmethoxypropane* (VI; X = NEt_2). The epoxide (VII) (crude, 15 g.) in benzene (30 ml.) was treated with anhydrous diethylamine (7·9 g.) and the mixture heated under reflux for 6 hours. The product was isolated with chloroform and purified by fractionation under reduced pressure, a small fore-run being rejected. The main fraction (b.pt. 150° C. at 0·4 mm.) was identified as the required propanolamine, which was characterised as indicated in Table III.

sym-*Tetraphenylmethyl ether*. Diphenylcarbinol (23 g.) in epichlorohydrin (23·2 g.) was treated with conc. sulphuric acid (0·3 ml.) with swirling, when a slight exothermic reaction occurred. The mixture was heated on the steam bath for 24 hours, cooled and the product extracted with chloroform, which was washed with aqueous sodium bicarbonate and then with water. Removal of the chloroform followed by fractionation under reduced pressure yielded (i) unchanged diphenylcarbinol, b.pt. 110° to 114° C. at 0·5 mm., (ii) a fraction (11 g.), b.pt. 200° C. at 0·5 mm., which was crystallised from light petroleum (b.pt. 60 to 80° C.) to yield *sym*-tetraphenylmethyl ether[18], needles, m.pt. 109° C. Found: C, 89·4; H, 6·0. Calc. for $C_{26}H_{22}O$: C, 89·1; H, 6·3 per cent.

2-*Diphenylmethoxyethyl chloride.* Diphenylcarbinol (61·3 g.) ethylene chlorohydrin (54 g.), toluene *p*-sulphonic acid (500 mg.) and toluene (250 ml.) was heated under reflux in the Dean-Stark apparatus for 30 minutes. After washing with sodium bicarbonate solution and with water the solvent was removed and the residue fractionated under reduced pressure to give 2-diphenylmethoxyethyl chloride[19], b.pt. 122° C. at 0·3 mm. Found: C, 73·3; H, 6·2; Cl, 14·7. Calc. for $C_{15}H_{15}OCl$: C, 73·0; H, 6·1; Cl, 14·4 per cent.

By employing ethylene cyanohydrin (28·5 g.), 2-*diphenylmethoxyethyl cyanide* was obtained, b.pt. 158° C. at 0·3 mm., m.pt. 58° C. (after crystallisation from light petroleum). Found: C, 80·6; H, 6·2; N, 6·0. $C_{16}H_{15}ON$, requires C, 81·0; H, 6·4; N, 5·9 per cent.

Preparation of 1-*chloro*-2-*hydroxy*-3-*diphenylmethoxypropane* (VI, X = Cl). (*a*) A solution of 1:2-epoxy-3-diphenylmethoxypropane in benzene was saturated with hydrogen chloride. After standing 24 hours at room temperature the solution was washed free from acid, the solvent removed and the residue fractionated under reduced pressure to give 1-*chloro*-2-*hydroxy*-3-*diphenylmethoxypropane*, b.pt. 140° C. at 0·05 mm. Found: C, 69·7; H, 6·3; Cl, 12·6. $C_{16}H_{17}O_2Cl$ requires C, 69·4; H, 6·2; Cl, 12·8 per cent.

(*b*) Diphenylcarbinol (61·3 g.) α-monochlorhydrin (46·3 g.) and toluene *p*-sulphonic acid (500 mg.) in toluene (250 ml.) were heated under reflux in the Dean-Stark apparatus for 2½ hours when water (6 ml. 100 per cent. theory) had collected. The toluene solution was washed with aqueous sodium carbonate and with water and the solvent removed. Fractionation of the residue under reduced pressure gave a small fore-run (2·7 g.), b.pt. 130° to 190° at 0·3 mm. and a main fraction (58·3 g.), b.pt. 230° C. at 0·4 mm. On crystallisation from light petroleum it yielded 1-*chloro*-2:3-bis-(*diphenylmethoxy*)*propane* (IX), crystals, m.pt. 85° to 87° C. Found: C, 78·0; H, 6·1; Cl, 8·4. $C_{29}H_{27}O_2Cl$, requires C, 78·6; H, 6·2; Cl, 8·0 per cent.

When the reaction was repeated using three molar equivalents of α-monochlorohydrin (110·5 g.) condensation took place within 1 hour. Fractionation of the product yielded the required chlorohydrin (19 g.), b.pt. 140° at 0·05 mm. and (IX) (40 g.) m.pt. 85° to 87° C.

Preparation of 1-(4′-*Benzylpiperazino*)-2-*hydroxy*-3-*diphenylmethoxypropane* (VI; X = NC_2H_4NBz). The following example illustrates the method used for condensing (VI; X = Cl) with (II): (VI; X = Cl] (2·8 g.) in ethanol was treated with sodium hydroxide (400 mg.) dissolved in the minimum volume of water and benzylpiperazine (1·8 g.) added. After 1 hour at room temperature the mixture was heated on the steam bath for 3 hours, after which it was diluted with water and the product isolated with chloroform. Conversion to the hydrochloride and crystallisation from ethanol containing a little water furnished 1-(4′-benzylpiperazino)-2-hydroxy-3-diphenylmethoxypropane dihydrochloride.

Summary

1. Propane derivatives of types $ArO{\cdot}CH_2{\cdot}CHOH{\cdot}CH_2NRR'$,  $O(CH_2)_3{\cdot}NNR'$ and $Ph_2{\cdot}CH{\cdot}O{\cdot}CH_2CHOHCH_2NRR'$ have been prepared for examination as local anæsthetic agents.
2. Though active, the compounds proved irritant.

References

1. Berger and Bradley, *Lancet*, 1947, **252**, 97.
2. Berger and Bradley, *Nature, Lond.*, 1947, **159**, 813.
3. Hartley, *Quart. J. Pharm. Pharmacol.*, 1947, **20**, 388.
4. Petrow and Stephenson, *J. Pharm. Pharmacol*, 1953, **5**, 359.
5. Boyd, *J. chem. Soc.*, 1909, **95**, 1802.
6. Boyd, *ibid.*, 1910, **97**, 1791.
7. Fourneau, *J. Pharm. Chim.*, 1910, vii, 1, 99.
8. Fourneau, *Chem. Zent.*, 1910, **11**, 1790.
9. Pyman, *J. chem. Soc.*, 1917, **111**, 167.
10. Ing and Ormerod, *J. Pharm. Pharmacol*, 1952, **4**, 21.
11. Löfgren, *Arkiv. Kemi Mineral, Geol.*, 1946, 22A, No. 18.
12. Löfgren and Ekstrand, *Acta chem. scand.*, 1952, **6**, 1016.
13. Fosdick and Carbon, *J. Amer. chem. Soc.*, 1954, **76**, 1296.
14. D'yakonov, *J. Gen. Chem. U.S.S.R.*, 1951, **21**, 1986.
15. Petrow and Stephenson, *J. Pharm. Pharmacol.*, 1953, **5**, 362.
16. Stephenson, *J. chem. Soc.*, 1954, 1573.
17. Engler, *Ber.*, 1878, **11**, 927.
18. Stobbe, *Ber.*, 1901, **34**, 1967.
19. Protiva, Sustr and Borovicka, *Chem. Listy.*, 1951, **45**, 43.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ALKALOIDS

Alkaloids of Two Brazilian Apocynaceæ. W. B. Mors, P. Zaltzman, J. J. Beereboom, S. C. Pakrashi and C. Djerassi. (*Chem. Ind.*, 1956, 173.) Reserpine was isolated in nearly 0·08 per cent. yield from the root bark of *Rauwolfia grandiflora* Mart. Chromatography of the "reserpine" fraction obtained from the whole plant of *Lochnera* (*Vinca*) *Rosea* (L.) Reichb., var. *Alba* (Sweet) Hubbd. yielded ajmalicine in 0·036 per cent. yield. Counter current distribution of the strongly basic fraction using 18 transfers and a chloroform-buffer (citrate-phosphate buffer of pH 7·4) system gave bright yellow crystals of serpentine in *ca.* 0·02 per cent. yield. Counter-current distribution (19 transfers) of the weak bases between chloroform and pH 6 buffer followed by crystallisation of fractions 7 to 15 from methanol furnished 0·009 per cent. of colourless crystals m.pt. 170–190° C. Repeated recrystallisation from methanol gave an analytical sample with the following constants: m.pt. 200–201° C. $[\alpha]_D^{21}$ + 50° (ethanol). (Found: C, 73·98; H, 7·70; N, 8·19; N–CH_3, 4·14; OCH_3, 9·00 which corresponds to $C_{20-21}H_{26-28}N_2O_2$ with one *N*-methyl and one *O*-methyl group. The alkaloid exhibited a typical indole ultra-violet absorption spectrum; it appears to be a new alkaloid and the name "lochnerine" is proposed. A. H. B.

ANALYTICAL

Antihistamine Combination, Spectrophotometric Assay of. S. M. Blaug and L. C. Zopf. (*Drug Standards*, 1955, **23**, 147.) A mixture of pyrrobutamine diphosphate and methapyrilene hydrochloride was assayed by preparing a solution in ethanol (95 per cent.) containing 0·002 per cent. of methapyrilene hydrochloride and 0·0012 per cent. of pyrrobutamine diphosphate. The optical density was measured at 225 mμ (maximum for pyrrobutamine diphosphate) and 241 mμ (maximum for methapyrilene hydrochloride) using a blank to compensate for the absorption due to the ethanol. The absorption spectra of the two compounds are very similar, but it was found possible to calculate the quantity of each substance present by the use of simultaneous equations containing additional terms to correct for the interference of one component at the absorption maximum of the other. G. B.

Belladonna Alkaloids, Colorimetric Assay for. W. Saski. (*Drug Standards*, 1955, **23**, 149.) The suggested method of assay depends upon the formation of a stable blue colour when a benzene solution of belladonna alkaloids is treated with oleic acid and cupric nitrate. The full intensity of the colour is developed within 24 hours at room temperature. Although the absorption is a maximum at 675 mμ, it is preferable to make measurements at 710 mμ so as to reduce the interference due to chlorophyll. Samples of belladonna herb are extracted, and volatile bases removed from the alkaloidal extractive as described in the U.S. Pharmacopeia XIV. The residue is dissolved in benzene and an aliquot quantity of the solution treated with oleic acid and cupric nitrate and allowed to stand for development of the colour. A further quantity of the benzene solution is treated with oleic acid and used as a control. The content

of alkaloids is calculated from the light absorption at 710 mμ by reference to a standard curve prepared by similar treatment of standard solutions of atropine in benzene. The results by this colorimetric method are in agreement with those of the U.S.P. XIV assay. G. B.

Heroin and Quinine, Spectrophotometric Determination of. M. J. Pro, W. P. Butler, and A. P. Mathers (*J. Assoc. off. agric. Chem., Wash.*, 1955, **38**, 849.) Absorption data for heroin, quinine, and mixtures of these alkaloids in 0·1N sodium hydroxide are given. Preliminary studies demonstrated that heroin was rapidly hydrolysed to morphine in 0·1N sodium hydroxide and that at 297·5 mμ, the point of maximum absorption of morphine, equal molar concentrations of quinine and morphine have almost the same absorption, while at 330 mμ, the quinine maximum, morphine does not absorb. Absorbance curves prepared in 0·1N hydrochloric acid show that the quinine absorbance is much greater than that of heroin at the quinine maximum, 250·5 mμ while at the 318 and 347·5 mμ maxima, even large concentrations of heroin do not absorb. At 285 mμ, the heroin maximum, equal weight concentrations of quinine absorb appreciably. From observed values at different wavelengths the two alkaloids can therefore be determined simultaneously in this acid medium. The separation of diluents insoluble in anhydrous methanol is advantageous because samples found in practice may contain many inert substances.

R. E. S.

Marcoumar, Colorimetric Determination of. J. Bednář. (*Československ. Farm.*, 1956, **5**, 26.) Marcoumar (3-(1′-phenylpropyl)-4-hydroxycoumarin) can be determined by allowing it to react with diazotised *p*-nitraniline, extracting the acidified reaction mixture with benzene and measuring the colour of the extract. One tablet, containing about 0·3 mg. of pure Marcoumar, is heated on a water bath for 10 minutes with about 12 ml. of 10 per cent. sodium acetate solution in a 25-ml. calibrated flask. After being cooled, the contents of the flask are made up to 25 ml. with 10 per cent. sodium acetate solution. The solution is filtered, and 2 ml. of freshly prepared diazotised *p*-nitraniline solution are added to 5 ml. of the filtrate; the mixture is set aside for 20 minutes at room temperature. It is then acidified with 1 ml. of concentrated hydrochloric acid and extracted with 10 ml. of benzene. The benzene solution is dried over sodium sulphate and its optical density is measured in a Pulfrich photometer with a S47 filter; a solution prepared without the sample is used as a blank. The amount of Marcoumar present is read from a calibration curve, constructed from readings obtained with known amounts (0·25 to 2·5 mg.) of pure Marcoumar. To prepare the diazotised *p*-nitraniline, 2 ml. of 1 per cent. *p*-nitraniline in N hydrochloric acid are mixed with 2 ml. of 5 per cent. sodium nitrite solution; after being set aside for two minutes at room temperature, the solution is treated with 2 ml. of 5 per cent. urea solution; after a further five minutes it is made up to 20 ml. E. H.

Nux Vomica, Colorimetric Assay of. M. Karmazin and L. Böswart. (*Pharm. Zentralh.*, 1956, **95**, 10.) 1·5 g. of coarsely powdered nux vomica is shaken vigorously with a mixture of 20 g. of ether and 10 g. of chloroform, then treated with 3 g. of 50 per cent. sodium carbonate solution. After shaking for 30 minutes, 3·5 g. of water is added and the mixture shaken again for 2 minutes. The layers are separated and the ether-chloroform solution is filtered through cotton wool: 20 g. of the filtrate is evaporated to dryness and the residue is rubbed down twice with 2 × 10 ml. of hot 1 per cent. sulphuric acid,

the liquid being filtered into a measuring flask. After cooling, the volume is made up to 25 ml. with the dilute acid. To 10 ml. of this solution in an ice bath is added 3 ml. of saturated solution of ammonium reineckate and the mixture is allowed to stand for 1 hour in the ice bath. The precipitate is filtered off on a 3G4 filter, washed with 0·2 per cent. ammonium reineckate solution, and freed from liquid by vacuum. After drying the filter tube with filter paper, the residue is dissolved in 2 ml. of acetone and washed with acetone until the runnings are colourless. The filtrate is made up to 10 ml. and the extinction is measured, using a green filter of 525 mμ. The method is standardised against a mixture of equal parts of strychnine and brucine. G. M.

Quaternary Ammonium Compounds, Determination of. R. Reiss. (*Arzneimit.-Forsch.*, 1956, **6**, 77.) The method is based on the extraction of the iodides of the quaternary compound with chloroform. About 10 ml. of a solution containing 1 to 2 mg. of the compound is treated with 1 ml. of 10 per cent. sulphuric acid and 2 ml. of freshly prepared 10 per cent. solution of potassium iodide, and extracted with 10 ml., then with 2 × 5 ml. of chloroform. The chloroform solution is filtered and made up to 25 ml. To 20 ml. of this solution is added 20 ml. of water, 1 ml. of sulphuric acid and 5 ml. of 1 per cent. solution of sodium nitrite. After shaking for 2 minutes, the chloroform layer is filtered into a 2 cm. cell and the colour is determined, using a S49 filter. The method is standardised against known weights of the compound. The method is not applicable in presence of certain alkaloids, or when the chloroform extract is itself coloured. In some cases extraction from an alkaline medium may remove the latter difficulty. The method has been tested with cetyltriethylammonium bromide, dodecylbenzyldimethylammonium bromide, di*cyclo*hexyldibenzylammonium bromide and di*cyclo*hexylcetylbenzylammonium bromide, but cannot be used with tetraethylammonium bromide. G. M.

Sulphafurazole, Colorimetric Determination of. J. Blažek and Z. Stejskal. (*Československ. Farm.*, 1956, **5**, 27.) Sulphafurazole (Gantrisin) is determined by titration with silver nitrate in the presence of sodium borate. For injections (containing 400 mg. per ml.), 50 ml. of 55 per cent. ethanol and 0·3 g. of sodium tetraborate are added to 1 ml. of solution in a 100-ml. calibrated flask; 40 ml. of 0·1N silver nitrate solution are added from a burette, the flask being thoroughly shaken during the addition. The solution is made up to 100 ml. and filtered through a dry folded filter, the first 20 ml. of filtrate being discarded. Concentrated nitric acid (3 ml.) and ferric ammonium sulphate solution (3 ml.) are added to a 25 ml. aliquot of the clear filtrate, and the excess of $AgNO_3$ is titrated against 0·1N ammonium thiocyanate. Tablets can be determined in the same way; a quantity of powdered tablets corresponding to 400 mg. of sulphafurazole is treated directly in a 100-ml. calibrated flask. The precision is $\pm$ 1·6 per cent. E. H.

Tetracycline Antibiotics, Separation of. P. P. Minieri and A. G. Mistretta. (*Science*, 1955, **122**, 1234.) The countercurrent distribution system of McIlvaine's phosphate-citrate buffer at pH 4·5 versus chloroform has been used for the analytical separation of mixtures of the known tetracycline antibiotics. In a 50-tube distribution with this system, the peak tubes observed were as follows: chlortetracycline 26, tetracycline 39, and oxytetracycline 44; these values corresponded to K aqueous/solvent values of 1·13, 3·90 and 8·80 respectively. After the theoretical curves for each component have been calculated, the percentage composition of a mixture such as one containing tetracycline and chlortetracycline can be calculated from the peak heights, determined

spectrophotometrically. Crude samples containing 300 to 500 μg./mg. gave satisfactory results in the identification of major components with 3 to 5 mg. placed in the first tube of a 50-tube apparatus containing 10 ml. of each phase. Pigments with strong absorption in the ultra-violet, such as anhydrotetracycline were usually found in the low-numbered tubes because of their solubility in chloroform; their presence did not, therefore, interfere with the ultra-violet determination of the peak tubes, which were carried out on the upper phase at a wavelength of 265 mμ after suitable dilution. R. E. S.

Vitamin K_3, Estimation of. V. Sathe, J. B. Dave and C. V. Ramakrishnan. (*Nature, Lond.*, 1956, **177**, 276.) A modification of the method of Reddy and Srinivasan (*Curr. Sci.*, 1948, **17**, 22) is described for the spectrophotometric estimation of micro quantities of Vitamin K. Vitamin K_3 solution (2 to 30 μg. 2-methyl-1:4-naphthoquinone) is shaken with 0·5 ml. of ethanol and 0·1 ml. of a saturated solution of 2:4-dinitrophenolhydrazine in 2 N hydrochloric acid. After 10 minutes 0·25 ml. of 20 per cent. sodium carbonate is added and shaken well until the green colour appears. Three ml. of amyl alcohol, 1 ml. of ethanol and 1 ml. of water are added, shaken and kept for 5 minutes. The amyl alcohol layer is taken and the density read at 635 mμ in a spectrophotometer. The colour is stable for 10 hours. G. F. S.

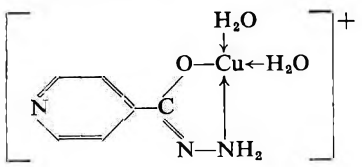
Water in Drugs, Determination of. G. J. Mulder and J. A. C. van Pinxteren. (*Pharm. Weekbl.*, 1956, **91**, 33.) A comparison of the Karl Fischer method with other methods for the determination of water in a number of pharmaceutical materials gave results in favour of the former. With the opium alkaloids, Karl Fischer results agreed generally with those obtained by drying at 70° C. *in vacuo*, but were in most cases appreciably higher than the loss on drying at 105° C. With dry extracts results were in all cases higher than those by ordinary drying methods; with thick extracts (belladonna, gentian, liquorice) higher results were generally obtained, but extract of valerian gave a much lower one since there was no interference by volatile products. For starches and pectin, results were in general slightly higher than those of ordinary drying. The method recommended for thick extracts is to rub the material (0·5 g.) down with sand (5 g.), take up with anhydrous methanol and make up to 25 ml.; an aliquot is then taken for the titration. G. M.

CHEMOTHERAPY

Isoniazid, Mode of Action of. A. Albert. (*Nature, Lond.*, 1956, **177**, 525.) Isoniazid has been shown to have an affinity for heavy metals, only slightly less than those of the common amino-acids. The suggestion has been made that chelation with a metal plays a part in the therapeutic action of the drug, since 1-*iso*nicotinyl-1-methylhydrazine, which cannot give an anion and consequently does not form chelates, has very little antituberculous activity. The affinity of other hydrazides for heavy metals was determined; of two isomers of isoniazid, nicotinic hydrazide (which had no antituberculous activity) had the same order of affinity for metals as isoniazid; picolinic hydrazide (which has about 1/8th the activity of isoniazid) had 10^3 to 10^5 times the affinity for metals of isoniazid. So that the property of being able to form chelation compounds with metals is not the only factor concerned in the action of isoniazid. Nor does the replacement of nicotinamide in diphosphopyridine nucleotide by isoniazid explain its

antituberculous action, since cyanacetic hydrazide, which appears to act as an antitubercular in the same way as isoniazid, is not a structural analogue of nicotinamide. G. P.

***iso*Nicotinyl Hydrazide, Mode of Action of.** S. D. Rubbo, J. Cymerman-Craig, J. Edgar, G. N. Vaughan and D. Willis. (*Nature, Lond.*, 1956, **177**, 480.) The relatively insoluble complex of isoniazid formed by chelation with copper appeared to be as active as isoniazid or verazide, both in anti-



X′ X = a univalent anion

tuberculous activity *in vitro* and by the healing ulcer technique in guinea-pigs. With the healing ulcer method two injections of the complex effected 85 per cent. cure in three weeks and with three injections 99 per cent. cure was obtained in four weeks. However the compound had a much higher local and general toxicity in mice and guinea-pigs than had isoniazid. The suggestion was made that isoniazid is probably inactive in the absence of a chelating metal, antituberculous activity occurring after the formation of a chelate metal complex.

G. P.

Pertussis Vaccines: Effect of Added Toxoids on Antigenicity. J. Ungar. (*Brit. med. J.*, 1956, **1**, 841.) This is the report of an investigation to determine whether or not the presence of either toxoid, diphtheria or tetanus, or of both, reduces the immunity response of mice to *H. pertussis* vaccines. Combined antigens that had been used for the immunisation of children, and having compositions based on earlier experiments on animals, were chosen. Two series of prophylactics were prepared by adding toxoids to each of two *H. pertussis* vaccines A and B, prepared on different occasions but otherwise similar. The following prophylactics were used: (1) Vaccine A 20,000 $\times$ 10^6 cells/ml. (2) Vaccine A plus diphtheria toxoid 25 Lf/ml. (3) Vaccine A plus diphtheria toxoid 25 Lf/ml., plus tetanus toxoid 2·5 Lf/ml. (4) Vaccine B 20,000 $\times$ 10^6 cells/ml. (5) Vaccine B plus diphtheria toxoid 25 Lf/ml. (6) Vaccine B plus diphtheria toxoid 25 Lf/ml., plus tetanus toxoid 2·5 Lf/ml. The pertussis vaccines were prepared from freshly isolated strains grown on Bordet-Gengou medium, killed with formalin and preserved in thiomersal. The added diphtheria and tetanus components were plain toxoids, purified by ammonium sulphate precipitation. The six products were tested three times concurrently for their ability to protect mice against intracerebral challenge. Similar mouse protection tests were carried out on a series of 12 plain *H. pertussis* vaccines, prepared from organisms grown for 48 hours on Bordet-Gengou medium, killed with formalin and standardised to 20,000 $\times$ 10^6 cells/ml. The results of these tests were compared with those on 16 different batches of a diphtheria-pertussis-tetanus prophylactic, prepared by the addition of both toxoids to *H. pertussis* vaccine suspensions similar to the 12 previously mentioned. Several of these tests on the two prophylactics were carried out concurrently, the animals being challenged at the same time with the same suspensions of virulent organisms. The results of the tests (which are given in detail in the paper) indicate that the antigenic response of mice to the two different *H. pertussis* vaccines investigated was not reduced by the addition of diphtheria toxoid or of

diphtheria and tetatus toxoids combined. This finding indicates that combined prophylactics will induce in children the same degree of protection against whooping cough as will a plain suspended vaccine. S. L. W.

Tetracyclines, Avidity of, for the Cations of Metals. A. Albert and C. W. Rees. (*Nature, Lond.*, 1956, **177**, 433.) Tetracycline, like chlortetracycline (aureomycin) and oxytetracycline (terramycin) showed high affinity for the cations of heavy metals. It is not known whether the antibacterial properties of the tetracyclines are related to their metal-binding properties as is the case with 8-hydroxyquinoline. However the binding properties are important since experiments have shown that alumina, given with the tetracyclines to prevent gastric irritation, completely inactivates them. G. P.

Tubercle Bacilli, Antituberculous Immunity Induced by Methanol Extracts of. D. W. Weiss and R. J. Dubos. (*J. exp. Med.*, 1956, **103**, 73.) Further confirmation was given of the increased resistance of mice to experimental tuberculosis (highly virulent bovine culture MV intravenously) by prior vaccination (intraperitoneally) with methanol extract of killed tubercle bacilli. Immunity was evident whether the challenge infective dose was large or small, causing a disease of short and long duration respectively. Two strains (avirulent H37Ra, and the attenuated strain BCG, substrain BCG-P) of tubercle bacilli were surface cultured, killed with phenol, washed with acetone, and then methanol extracted. Weight for weight, methanol extract possessed much less protective activity than the whole phenol-killed bacilli, but its primary toxicity was lower. The level of immunity elicited by whole cells and methanol extract was of the same order as that observed with living BCG. The duration of the methanol extract protective effect was greater (still present 62 days after vaccination) than that of living BCG-T, which gives only short duration immunity in mice. Also, the authors considered that the protective action of both whole phenol-killed bacterial bodies and their methanol extract, could be enhanced and prolonged by certain adjuvants; in particular, by an oil-arlacel mixture, and by highly purified typhoid somatic polysaccharide. G. P.

PHARMACOLOGY AND THERAPEUTICS

Acetazolamide in the Treatment of Epilepsy. B. Ansell and E. Clarke. (*Brit. med. J.*, 1956, **1**, 650.) Twenty-six epileptic patients were treated with acetazolamide; of these 23 were of the idiopathic type. There were 6 patients suffering from major epilepsy, 5 from minimal epilepsy, 4 from minor epilepsy, and 8 from mixed idiopathic epilepsy. The remaining 3 had symptomatic epilepsy of varying aetiologies and had resisted all therapy. 15 of the patients had acetazolamide alone over periods ranging from 4 weeks to 18 months. The majority were studied as out-patients. A dose of 125 mg. of acetazolamide twice a day was added to the previous medications in the first few cases. If improvement occurred and was sustained the other drugs were gradually eliminated. In most of those deriving no benefit the dose of acetazolamide was progressively increased and adapted to the individual until it was approximately 10 mg./kg. Excellent results (attacks completely dispelled) were obtained in 3 major, 2 minimal and 3 mixed epileptics; good results (attacks markedly decreased in frequency) were obtained in 1 of each of the major, minimal, minor and symptomatic groups, and in 2 of the mixed epileptics; slight results were obtained in 8 of the patients, and in 4 the treatment was of no value. Five of the patients complained of paræsthesia of the hands and feet, which usually persisted for about a week and recurred with an increase of dose. Excessive

drowsiness occurred in 4 patients (all had doses greater than 8 mg./kg.). One patient became depressed and irritable, but, on the other hand, behaviour difficulties decreased in 3 children. The authors conclude that acetazolamide may be of value as the sole therapeutic agent in the treatment of major epilepsy of moderate severity, in some cases of minimal epilepsy, and as adjuvant therapy in mixed idiopathic epilepsy. Repeated increase of dose is often necessary but as the drug is relatively non-toxic it can be used in doses of up to 20 mg./kg. The mode of action is unknown. It does not appear to depend on the production of a systemic acidosis. It is suggested that it may act specifically on the carbonic anhydrase in the epileptic brain. S. L. W.

Afroside B, Pharmacology of. M. J. Rand and A. Stafford. (*Austral. J. exp. Biol. med. Sci.*, 1956, **33**, 527.) Afroside B is a new crystalline cardiac glycoside obtained from *Gomphocarpus fruticosus*, growing in Queensland. In isolated perfused guinea-pig hearts a concentration of 1 in 7·5 million produced a positive inotropic response. A concentration of 1 in 10 million increased the strength of the isometric contraction of the cat papillary muscle. Toxicity experiments showed that by slow intravenous infusion in cats, anæsthetised with pentobarbitone, the mean lethal dose was 444 $\pm$ 5·6 μg./kg. Changes in the ECG were typical of those seen with other cardiac glycosides. In the guinea-pig, anæsthetised with urethane, the mean dose by intravenous infusion causing ventricular fibrillation was 477 μg./kg., compared with 469 μg. for digoxin and 245 μg. for ouabain. The doses to cause cardiac arrest were 600, 681 and 332 μg./kg. respectively. In the chick embryo heart, the potency compared with ouabain (= 1) was 2·09. In the guinea-pig afroside B was rapidly inactivated by passage through the liver *in situ* and the heart block produced by regular intra-atrial injections of adenosine were potentiated by non-toxic doses of afroside B. G. F. S.

Amiphenazole, Possible Mode of Action of. A. Shulman. (*Nature, Lond.*, 1956, **177**, 703.) Amiphenazole (2:4-diamino-5-phenylthiazole, Daptazole) has a mild respiratory stimulant action and has been used therapeutically to improve the respiration post-operatively and also in conditions such as pneumonia and asphyxia neonatorum. It has also been used in the treatment of barbiturate and morphine poisoning. When administered with morphine in cases of severe pain it minimises the degree of tolerance to the morphine and of addiction to the drug. It is effective orally and parentally, is free of side effects and it often improves the mental outlook of depressed patients. Since amiphenazole has such widespread effects in the body and since it has a very low toxicity the possibility that the drug might fit into some fundamental physiological process was investigated. As the structures of amiphenazole and the thiazole group of aneurine is similar and as aneurine is probably the only substance occurring naturally in the body which contains the thiazole nucleus, experiments were carried out in which rats were fed on an aneurine free diet and were given daily doses of amiphenazole and the pyrimidine part of aneurine in order to see if these two drugs would produce a substance capable of entering into aneurine metabolism. It was found that at suitable dose levels no signs of aneurine avitaminosis were apparent nine weeks after beginning the treatment, while all the control animals died by five weeks. Similarly aneurine deficiency could be reversed by the administration of amiphenazole and the pyrimidine part of the aneurine molecule. Such results suggest that amiphenazole may enter into the pathway of processes involving aneurine metabolism and that this may be at least a partial explanation of the mode of action of the drug. M. M.

Benactyzine Hydrochloride (Suavitil) in the Relief of Anxiety. E. B. Davies. (*Brit. med. J.*, 1956, **1**, 480.) Benactyzine hydrochloride is the hydrochloride of the diethylaminoethyl ester of benzilic acid, and has the formula:

$$HO{\cdot}C{\cdot}(C_6H_5){\cdot}_2COOCH_2{\cdot}CH_2{\cdot}N(C_2H_5)_2HCl.$$

It is claimed that the drug relieves anxiety, particularly in psychoneurotic patients and that no toxic effects are observed in therapeutic doses. (1–5 mg. up to 4 times a day.) It is rapidly eliminated, so that a dose of 4–5 mg. loses its effect after 3 to 5 hours. Moderate doses (1–4 mg.) produce a sense of divorce between outer reality and emotional reaction, which has been described as producing a barrier between the person and his problems. Larger doses (4–8 mg.) produce a more marked feeling of detachment, the muscles are relaxed, thoughts and feelings are retarded, and the power of concentration is felt to be diminished. There is a marked tendency to think of nothing and to react very slowly to stimulation from the outside world. With therapeutic doses the capacity to perform normal tasks may be little affected. It has little direct effect upon sleep, though it may have considerable indirect influence. This paper reports the use of the drug on 110 patients (56 men and 54 women); 25 were psychotics and 85 neurotics. It was used empirically as a means of relieving tension and anxiety, irrespective of the cause, in an attempt to assess its effect in all cases of tension. No controls were employed. Of the 110 patients, 67 were improved, 35 were unchanged, and 8 were worse, following a dosage of from 1–4 mg. three times a day. The drug is suitable for use with other sedatives, particularly phenobarbitone. A note of caution should be sounded concerning its use in patients with phobic obsessional disorders or feelings of depersonalisation or unreality. S. L. W.

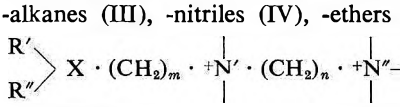
Benactyzine Hydrochloride as a Physical Relaxant. A. Coady and E. C. O. Jewesbury. (*Brit. med. J.*, 1956, **1**, 485.) The possible effect of benactyzine hydrochloride in relieving muscular rigidity, spasm or pain was investigated in a group of 80 neurological patients. No effect on muscle tone and no significant relief of symptoms were produced by a 2-weeks course of 2 mg. of benactyzine three times daily by mouth, and no temporary reduction of rigidity or of tendency to clonus was observed after individual doses. Although some patients reported diminished nocturnal spasms and cramps, greater mobility and confidence in walking, and increased general activity, these effects occurred almost equally both with benactyzine and inert control tablets. No systemic toxic effects were observed but transient side-effects occurred in 29 of 72 patients who received a dosage of 2 mg., and in all 8 patients who received 4 mg. The commonest side-effects were feelings of general apathy or detachment, and altered sensations in the limbs; other side-effects were dizziness, slight nausea, throbbing or tingling sensations, and blurred vision. The symptoms developed quickly and lasted from a few minutes to one and a half hours. With single oral dosage of more than 6 mg. normal control subjects showed increasing thought-blockage, impairment of concentration, and slowness and clumsiness in carrying out complicated procedures such as piano-playing. Because of these symptoms it would seem advisable that patients under treatment with the drug should not be allowed to drive. A striking suppression of the normal rhythm of the EEG was observed after subcutaneous injection of 5 and 7 mg. respectively in two normal subjects. S. L. W.

Cortisone Treatment of the Low-Salt Syndrome. I. G. Graber, P. Beaconsfield and O. Daniel. (*Brit. med. J.*, 1956, **1**, 778.) Post-operative patients

with a low plasma sodium and chloride usually respond well to the administration of salt. Occasionally abnormally low plasma sodium and chloride levels persist despite the establishment of positive salt-and-water balance. Details are given of three patients who developed a low-sodium syndrome following major surgical procedures and who failed to improve in spite of what was regarded as adequate replacement therapy. The condition was quickly remedied in all 3 patients by stopping administration of salt by mouth and administering cortisone acetate, 25 mg. every 6 hours. In 2 of the patients the plasma sodium and chloride returned to normal within 12 hours of commencing cortisone treatment. The rapid changes in plasma electrolytes following administration of cortisone in these cases is explained on the basis of cortisone altering cell permeability, allowing sodium and chloride to pass quickly from the cell into the plasma, thus restoring the normal plasma electrolyte picture and relieving intracellular œdema. This explanation is supported by the fact that cortisone administration was followed almost immediately by a greatly increased urinary output of sodium and a relatively diminished urinary output of potassium. S. L. W.

Ecolid, Action of, in Man. F. H. Smirk and M. Hamilton. (*Brit. med. J.*, 1956, **1**, 319.) The actions of Ecolid (4:5:6:7-tetrachloro-2(2-dimethylaminoethyl)*iso*indoline dimethochloride) a new hypotensive agent, have been studied and compared with pentolinium in 28 patients suffering from hypertension. The relative potency of the two drugs varied for each individual, but Ecolid appeared to be two to three times more potent than pentolinium. The effective oral dose was about fifteen times the parenteral dose. After a subcutaneous dose the blood pressure fall was maximal after one to two hours and was maintained for several hours. The effect on heart rate was slight and variable. The hypotensive action was greatest when standing and was increased by a meal. Patients showed a tolerance to Ecolid necessitating daily increases in dose in the early stages of treatment and there was a cross-toleration to pentolinium. Side effects observed were similar to those with other ganglionic blocking agents. The authors suggest that Ecolid merits further clinical trial. G. F. S.

Ganglion-blocking Agents, New Series of. D. W. Adamson, J. W. Billinghurst and A. F. Green. (*Nature Lond.*, 1956, **177**, 523.) Marked ganglion-blocking action was found in series of diquaternary-amino-carbinols (I), -alkenes (II), -alkanes (III), -nitriles (IV), -ethers (V), and esters (VI).



I; X = $C(OH)CH_2$ IV; X = $C(CN)CH_2$

II; X = C:CH V; X = CH:O

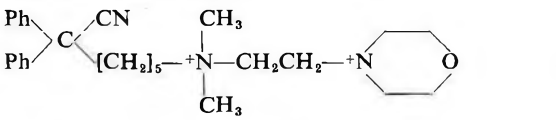
III; X = $CH{\cdot}CH_2$ VI; X = $CH{\cdot}CO{\cdot}O$

R′ and R″ = phenyl or *cyclo*hexyl groups.

N′ and N″ are fully substituted quaternary nitrogens.

Compounds I to III are related to series of spasmolytics, antihistamines and analgesics previously studied, e.g., tricyclamol, procyclidine, triprolidine and thiambutene; series IV is related to methadone, V to diphenhydramine and VI to the spasmolytic Trasentin. By introducing into the molecules a group consisting of two quaternary nitrogens linked by a short polymethylene chain, in place of the tertiary or quaternary nitrogens in the parent compounds,

ganglion-blocking activity was obtained in all the series, while the original types of activity were reduced or disappeared. Ganglion-blocking activity was tested for by antagonism of the pressor action of *N'N''*-dimethyl-*N''*-phenylpiperazinium, fall in blood pressure, blockade of vagal bradycardia, and augmentation of the pressor action of adrenaline and noradrenaline in cats and dogs. These effects were obtained with doses smaller than those necessary for blockade of transmission through the superior cervical ganglion of the cat. Some of the compounds were powerful inhibitors of gastric secretion in pyloric-ligated rats, in doses producing little mydriasis. The peristaltic reflex of isolated guinea-pig ileum was inhibited readily by the compounds. The main toxic action was associated with neuromuscular block, in doses up to 100 times ganglion-blocking doses. Some of the compounds were much more active ganglion-blocking agents than hexamethonium or pentolinium and action lasted longer. Of the compounds used successfully clinically in hypertension 356C54 (below) was one of the most promising.



Subcutaneous injection of an effective dose lowered blood pressure for 7 to 12 hours. Side effects were minimal, paralytic ileus or severe constipation being absent; gastric secretion and motility were inhibited suggesting a use in peptic ulcer therapy. G. P.

Mestinon Bromide in the Treatment of Myasthenia Gravis. J. E. Tether. (*J. Amer. med. Ass.*, 1956, **160**, 156.) Mestinon bromide (a dimethylcarbamate of 3-hydroxy-1-methyl pyridinium bromide) was given to 165 patients with myasthenia gravis for periods of from 3 to 17 months. The dosage requirements varied widely. Thirty-three per cent. of the patients took less than 200 mg. daily (in tablets containing 60 mg.), 30 per cent. took 200 to 399 mg., 29 per cent. took 400 to 1399 mg., and 8 per cent. took 1400 mg. or more. One patient took 6000 mg. of the drug as well as 1500 mg. of neostigmine, without apparent beneficial effect and without side-effects. Most of the milder cases required under 200 mg. daily, and obtained almost complete relief of symptoms. Patients requiring more than 1200 mg. daily did not obtain as good control of symptoms as those in the lower dosage groups. No organic toxic effects were noted regardless of the dosage, but when given in excess it produces, in addition to sweating, salivation and mild abdominal symptoms, "nicotinic" side effects, such as muscle fasciculations, blurring of vision with mild vertigo, and occasionally a "thick tongue" sensation, with dysarthria and dysphagia. These symptoms are usually accompanied by increased weakness. If they are ignored, and medication is continued, this weakness may include the muscles of respiration resulting in a cholinergic crisis. Occasionally, cholinergic weakness may precede all other signals of overdosage. In the majority of cases symptoms of overdosage are clear enough to serve as warning signals. The author considers Mestinon to be an advance in the treatment of myasthenia and to be preferred to neostigmine bromide because of its ability to provide effective smooth control of myasthenic symptoms without distressing side-effects or wide fluctuations in the intensity of the drug's action. S. L. W.

Primidone; Megaloblastic Anæmia during Therapy. R. H. Girdwood and J. A. R. Lenman. (*Brit. med. J.*, 1956, **1**, 146.) An account is given of a

patient who developed megaloblastic anæmia while receiving primidone and phenobarbitone for epilepsy. The patient had been receiving phenobarbitone ½ gr. (32 mg.) three times daily, and primidone 250 mg. twice daily for a period of seven months before admission to hospital. There was no response to injections of cyanocobalamin but a good response occurred to folic acid therapy. Tests for folic acid excretion gave no evidence of malabsorption of synthetic folic acid. Epilepsy is a common disorder and anticonvulsant drugs are used in large quantities; megaloblastic anæmia from the use of phenobarbitone alone has not been recorded and its occurrence after phenytoin or primidone therapy is very rare. It is possible, however, in view of the similarities in chemical structure that these two substances act as competitive inhibitors of some enzyme system normally involving folic acid as a co-factor. S. L. W.

Progesterone and 17α-Hydroxyprogesterone, Comparative Anti-œstrogenic Potencies of. E. Mardones, D. Jadrijevic and A. Lipschutz. (*Nature, Lond.*, 1956, **177**, 478.) Progesterone is known to prevent the induction by œstrogens of abdominal fibroid formation, myometrial growth and excessive luteinization. This antagonism of œstrogens is decreased by oxidation of the progesterone at the C(21), C(11) and C(17) positions, but evidence for loss of anti-œstrogenic potency in the C(17) compound has previously been indirect. Pellets of 17α-hydroxyprogesterone and œstradiol were implanted in castrated female guinea-pigs. Neither œstrogen-induced abdominal fibroids nor uterine growth was prevented by as much as 136 μg. of 17α-hydroxyprogesterone per day; 15 μg. of progesterone per day, in similar circumstances, had pronounced anti-œstrogenic activity. 11β-Hydroxyprogesterone, 11-ketoprogesterone and deoxycorticosterone also were more active than 17α-hydroxyprogesterone. G. P.

Tricyclamol, Studies on. W. H. Bachrach and H. Schapiro. (*Amer. J. med. Sci.*, 1956, **231**, 192.) The physiological and clinical effects of trycyclamol sulphate (1-*cyclo*hexyl-1-phenyl-3-pyrrolidino-1-propanol methyl sulphate Elorine sulphate), an anticholinergic drug, have been studied in 150 individuals. Doses of 10 to 20 mg. parenterally and 150 to 200 mg. orally, inhibited gastrointestinal motility and both normal and histamine induced secretion of gastric juice. Oral doses up to 250 mg. did not prevent the gastric secretory response to a standard meal or to injected insulin. Clinically, tricyclamol sulphate did not relieve functional dyspepsia or pancreatitis. In peptic ulcer therapeutic results were good in 60 per cent. of patients receiving in addition adequate doses of antacids. Side effects, such as dryness of the mouth, were present, but were rarely troublesome at therapeutic doses. About 10 per cent. of patients showed an idiosyncrasy to the drug. Its actions are comparable to the natural and synthetic anticholinergic drugs. G. F. S.

Triiodothyronine; Metabolic and Therapeutic Effects. T. F. Frawley, J. C. McClintock, R. T. Beebe and G. L. Marthy. (*J. Amer. med. Ass.*, 1956, **160**, 646.) Fourteen myxœdematous patients were observed over a two-year period during which four types of medication were compared as follows: (1) 3:5:3′-DL-triiodothyronine, in a total daily dose of from 25 to 400 μg.; (2) L-triiodothyronine, in a daily dose of from 100 to 200 μg.; (3) L-thyroxine sodium, in a daily dose of 100 to 500 μg.; (4) desiccated thyroid, in an average daily dose of 86 mg. The four substances were all effective in raising the body temperature, accelerating the pulse, increasing the rate of oxygen consumption, decreasing the body weight, reducing the serum cholesterol and carotenoid levels, and improving the mental state of the patient. With DL-triiodothyronine the average daily dose to maintain euthyroidism is about 200–250 μg.; with L-tri-

iodothyronine a dose of 75–100 μg. is sufficient. This suggests that the effectiveness of the DL-form is due to its L-triiodothyronine content. The equivalent dosage of L-thyroxine sodium was between 200–300 μg. daily, and of thyroid extract was between 96–192 mg. Side-effects attributed to triiodothyronine included palpitation, angina, dyspnœa and headache. An unexpected syndrome was the sudden and marked relapse following the withdrawal of triiodothyronine. Within 24 hours patients complained of being weak and tired and of a return of their hypothyroid symptoms. The cause of this rapid loss of effectiveness is related to the speed with which the compound disappears from the tissues. The duration of activity after cessation of treatment or reduction in dosage is judged to be less than 3 days and in order to avoid a relapse it is necessary to start therapy with thyroid extract or thyroxine several days prior to the withdrawal of the triiodothyronine. On the other hand, triiodothyronine has the advantages that it is a chemically pure synthetic compound not requiring standardisation, that it acts rapidly in very small doses, and that it produces a calorigenic response when the patient's condition is refractory to thyroid extract or thyroxine. S. L. W.

Zoxazolamine for Cerebral Palsy in Children. E. H. Abrahamsen and H. W. Baird, III. (*J. Amer. med. Ass.*, 1956, **160**, 749.) After a preliminary trial in 10 children who had a marked increase in muscle tone of the extremities and were severely mentally retarded, zoxazolamine (Flexin) was given to 28 children who had in addition other abnormalities including orthopædic deformations, behaviour disorders, convulsive disorders, or growth failure. Initial dosage was 50 mg./kg. daily in 3 or 4 divided doses by mouth, the amount being increased by 25 to 50 per cent. at intervals of 1–2 weeks until side effects were observed or muscular relaxation was demonstrable. The dose of the drug necessary to produce a definite decrease in muscle tone was from 30 to 140 mg./kg. daily. The drug was given for from 10 to 210 days and a decrease in muscle tone was demonstrable in every patient, with definite clinical improvement in 15. An effective dose produced relaxation within an hour, the effect reaching a peak within 2 hours and waning within 4 hours. Occasionally some relaxation was still present after 24 hours. Side effects occurred in 15 patients; they included a burning taste, anorexia, vomiting, hypotonia and bleeding from a duodenal ulcer. Children who chewed the tablets complained of the taste and in these cases the crushed tablet was given in honey or treacle. H. T. B.

Zoxazolamine in Rheumatic Diseases. R. T. Smith, K. M. Kron, W. P. Peak and I. F. Hermann. (*J. Amer. med. Ass.*, 1956, **160**, 745.) Zoxazolamine (Flexin) was tried in 100 patients with rheumatic disease in whom acute or chronic fibrositic symptoms predominated, including rheumatoid spondylitis, fibrositis, peripheral rheumatoid arthritis, acute torticollis and post-traumatic muscle spasm of the low back. The usual dose was 500 mg. 3 or 4 times a day in the form of tablets. In 41 patients the response was excellent, relief of the stiffness occurring within 20–30 minutes and continuing throughout the day. In 44 the response was good, only mild stiffness persisting 30–60 minutes after the dose. 7 patients claimed slight benefit. The best over-all relief of muscle spasm occurred in patients with rheumatoid spondylitis. Toxic effects occurred in 43 patients, the most common being gastrointestinal irritation and disturbance of equilibrium. Fever, burning of the eyes, and skin rash also occurred. In 9 the symptoms gradually disappeared without altering the dose and 21 tolerated half the previous dose, but in the remainder treatment had to be discontinued. H. T. B.

Zoxazolamine in the Treatment of Spasticity. M. Rodriguez-Gomez, A. Valdes-Rodriguez and A. L. Drew. (*J. Amer. med. Ass.*, 1956, **160**, 752.) Zoxazolamine (Flexin) is a muscle relaxant resembling mephenesin in acting centrally; it appears to exert a somewhat selective depressing effect on subcortical and spinal polysynaptic pathways. The compound was given to 70 patients with increased muscle tone, of whom 35 were followed sufficiently closely and for a long enough time to permit evaluation. The patients belonged to two groups; in one the spasticity resulted from interruption of the cortico-spinal pathways at spinal cord levels while in the other the spasticity was secondary to disease above the spinal cord. 18 patients were in the first group, 10 suffering from multiple sclerosis, 6 from transverse myelitis and 2 from unclassified spinal cord disease. Of these, 14 showed undoubted reduction in spasticity. Patients with multiple sclerosis were given 500 mg. 3 to 5 times a day and improvement occurred in all except two within a period varying from a few days to 4 weeks. 5/6 of the transverse myelitis cases also showed marked improvement. The second group of patients responded less satisfactorily. In one case of paralysis agitans treatment with zoxazolamine noticeably improved rigidity and voluntary movement but no change in tremor was observed; in two other cases there was no improvement. Drowsiness and mild gastric irritation were the only side effects observed. H. T. B.

BACTERIOLOGY

Nicotin Hydrazide, Effect of, on the Oxidative Metabolism of *Mycobacterium tuberculosis* var. *bovis* BCG. P. Meadow and R. Knox. (*J. gen. Microbiol.*, 1956, **14**, 414.) This paper reports studies on the effects of isoniazid on the oxidation of acetate in the Warburg apparatus by mycobacteria and by other organisms. Only oxidation by the mycobacteria was found to be inhibited by isoniazid. *Myco. tuberculosis* var. *bovis* BCG and *Myco. tuberculosis* var. *hominis* were inhibited irrespective of the inclusion of glucose or glycerol in the medium, whereas *Myco. smegmatis* was inhibited only when grown in the absence of both glycerol and glucose. *Staph. aureus*, *B. cereus* and *E. coli* were not inhibited under any growth condition used. The acetate inhibition system was studied in further detail with BCG, when it was found that inhibition of acetate oxidation was not altered by varying the medium on which the organisms were grown. The concentration of isoniazid required to give 50 per cent. inhibition was found to depend on the cell concentration of BCG, the quantities of isoniazid required being related to sensitivity as determined by the test tube method. Reputed antagonists of the action of isoniazid: pyridoxine, manganous chloride, biotin, oleic acid derivatives, hæmin and catalase, were tested on the inhibition of acetate oxidation by BCG None was effective. Several disinfectants were found to cause complete inhibition of acetate oxidation by BCG, e.g., phenol (0·01 per cent.). Lastly, mixtures of antituberculous drugs as inhibitors of the acetate system were studied. Mixtures of subinhibitory concentrations of isoniazid with streptomycin, *p*-aminosalicylic acid or terramycin or of streptomycin with *p*-aminosalicylic acid all gave 50 per cent. inhibition of acetate oxidation by BCG. Mixtures of isoniazid with aureomycin, tetracycline, thiosemicarbazone and cyanacetic acid hydrazide were not effective. The authors suggest that their method might be applied usefully to studies of the actions of other drugs or mixtures of drugs, particularly since the selection of resistant strains is largely eliminated by the short duration of the experiments. B. A. W.

KENNETH BULLOCK

Chairman, 1956