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Chairman: FRANK HARTLEY

CHAIRMAN'S ADDRESS

STEROIDS IN PHARMACY AND MEDICINE

THE term steroid was originally applied to a group of naturally occurring secondary alcohols based upon perhydro*cyclo*pentenophenanthrene. It now has wider usage and embraces not only naturally occurring and synthetic derivatives of perhydro*cyclo*pentenophenanthrene but also related structures derived from the parent ring system by ring enlargement or contraction.

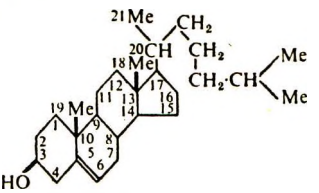
The group comprises not only the adrenocortical and sex hormones, and their synthetic analogues but also the bile acids, antirachitic vitamins, cardiac glycosides, certain alkaloids, saponins and toad poisons. Many steroids have already established their importance therapeutically. I believe that many more will do so with the increasing attention being devoted to chemical, biological and clinical studies of steroids and their derivatives and to their influence on the maintenance of health and even of life itself. And in the application of steroids to medicine there will continue to be need for the integrated efforts of organic and physical chemist, microbiologist, biochemist and pharmacologist as well as of the pharmacist and clinician. For many problems arise in the discovery and utilisation of steroids and many new techniques in consequence have been, and will continue to be, demanded.

Biosynthetic methods have been called forth to aid still further the skill of the organic chemist, as have new physico-chemical techniques. And while the usage and supervision of usage of steroids must be the responsibility of the clinician, the presentation of the materials for effective use rests with the chemist and pharmacist. The necessity for securing delayed or prolonged action in the therapeutic replacement of defective natural secretions, and the seeming versatility of action of many steroids have afforded the pharmacist many opportunities to develop new techniques and new forms of administration. Products suitable for implantation into tissues, microcrystalline suspensions, preparations effective topically without unwanted systemic effects, are some important examples of the contributions of the pharmacist to steroid development and usage. But pharmacy in its wider sense is concerned with all aspects of steroids and it seems appropriate at this Conference to consider some interrelationships in the steroid field, some recent developments and some future possibilities.

THE STEROLS, CHOLESTEROL

The sterols themselves are crystalline alcohols isolated from the unsaponifiable residues of lipoids derived from animals and plants. Most of them are compounds having 27 to 29 carbon atoms and one

secondary alcoholic group; some, like coprostanol, are completely saturated substances, others contain one, two or three double bonds as in cholesterol, stigmasterol and ergosterol respectively. Cholesterol (I) has long been known as the main constituent of human gall stones. It is widely distributed both in the free condition and as its fatty acid esters in the cells of the higher animals, particularly in brain and nerve tissue. Its structure was finally established by 1934 but its complete stereochemistry was not settled until 1947.



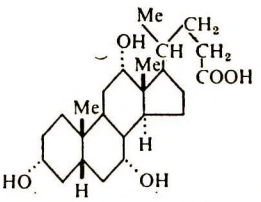
(I) Cholesterol

Cholesterol is of interest both in medicine and in pharmacy. It is elaborated in the liver from acetate. It was for many years regarded as the precursor of the bile acids and sex hormones. Indeed Bloch's isolation[1] of deuterated pregnane-3α:20-diol from the urine of a pregnant woman to whom he had fed deuterocholesterol was regarded as experimental proof that cholesterol was the biochemical precursor of the steroid hormones. It is now known, however, that while cholesterol can be converted into corticoids[2] and into androgens[3] it is not necessarily an obligatory precursor. This follows from experiments in which acetate containing ^{14}C-labelled carboxyl was incubated[4] with or perfused[5] through testis tissue and thereby converted to testosterone and cholesterol. The proportion of ^{14}C present in the testosterone was found to be higher than that present in the cholesterol, thus showing that the hormone did not originate solely from the cholesterol by oxidative degradation. Acetate is likewise a precursor of the oestrogens[6].

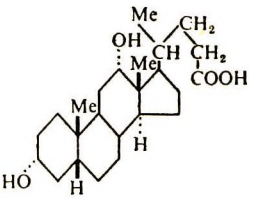
Cholesterol both free and esterified circulates in the blood stream, normal values in whole blood ranging from 20 to 100 mg. per 100 ml. for free cholesterol and from 60 to 200 mg. per 100 ml. for esterified cholesterol. Though the cholesterol level in blood varies within wide limits from one person to another it remains fairly constant in the individual. Its concentration is usually determined in serum or plasma and the values may increase above normal in nephritis, myxoedema, untreated diabetes, pregnancy and atherosclerosis[7]. Though much work has been done to link hypercholesterolaemia with atherosclerosis[8] recent studies reveal other complexities[9,10]. Whatever may be the precise significance of the high content of cholesterol in brain and spinal cord there can be little doubt that its metabolism will continue to engage the attention of biochemical workers for many years. Pharmaceutically cholesterol is used as such (U.S.P. XV) or in the crude form of wool alcohols (B.P. 1953) to increase the hydrophilic properties of soft paraffins, as in Hydrophilic Petrolatum, U.S.P. XV or Hydrous Ointment, B.P. Hadgraft[11] at the Conference in 1947 discussed the value of wool alcohols.

The Bile Acids

The liver transforms cholesterol into the bile acids which are then conjugated with glycine and taurine and the conjugates converted into their sodium salts. These sodium salts are known as the bile salts and are a major constituent of the solid matter of bile. They have the important function of promoting the emulsification and thus the absorption of fats in the intestinal tract. The chief bile acids are cholic acid (II) and deoxycholic acid (III). They occur in bile as the water soluble sodium salts of the peptide conjugates with glycine or taurine which are known as glycocholate and taurocholate, and glycodeoxycholate and taurodeoxycholate respectively.



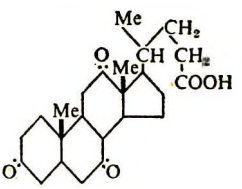
(II) Cholic acid



(III) De(s)oxycholic acid

Deoxycholic acid is of particular interest as it was from this bile acid that cortisone was first manufactured by a method based on the work of Sarett[12,13]. In fact, until microbiological methods for the introduction of hydroxyl at position 11 of progesterone were developed, deoxycholic acid remained the starting material of choice for the manufacture of cortisone. Its use in that way, involving as it did *inter alia*, the removal of a 12-hydroxy group and the introduction of hydroxyl groups at positions 11 and 17 as well as the transformations of the side chain at position 17 still represent an outstanding triumph of chemical skill.

The mixture of bile salts, chiefly sodium taurocholate and sodium glycocholate, are included in the B.P.C., under the title sodium tauroglycocholate, for use in cases of deficiency of biliary secretion to assist emulsification of fats and the absorption of certain water insoluble substances. The triketocholanic acid, dehydrocholic acid (IV), obtained by oxidation of cholic acid is much less toxic than cholic or deoxycholic acid and is preferred as a choleretic.



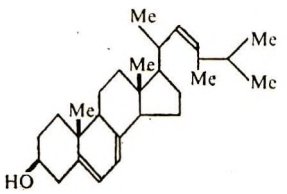
(IV) Dehydrocholic acid

The sodium salt, sodium dehydrocholate N.N.R., is used intravenously for increasing bile flow. It is also employed for the determination of arm-to-tongue circulation time, the time taken from injection into the arm to

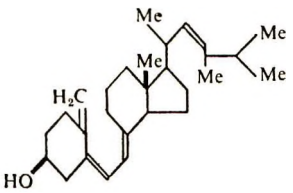
perception of a bitter taste aiding in distinguishing between right and left ventricular failure. Its use in this way, however, has been found to be more hazardous than the use of soluble saccharin.

Antirachitic Vitamins

Until thirty years ago cholesterol was regarded as the precursor of the antirachitic vitamin D. The fact that some, but not all, samples of purified cholesterol could be activated by exposure to sunlight or ultra-violet light, however, led to the discovery that a closely related sterol, ergosterol (V), gave on irradiation, a highly potent antirachitic substance named calciferol by its British discoverers[14] and vitamin D_2 almost simultaneously by its German discoverers[15] to distinguish it from an earlier preparation called vitamin D, and subsequently shown to be a molecular compound of vitamin D_2 and an isomer called lumisterol.



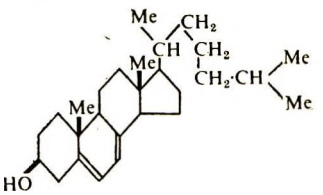
(V) Ergosterol



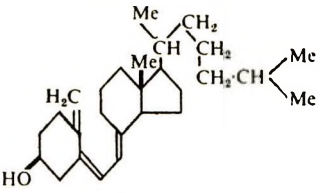
(VI) Calciferol (ergocalciferol)

Calciferol (VI), manufactured by ultra-violet irradiation of ergosterol obtained from yeast, is used in tablets or in oily solution to promote the absorption from foodstuffs of calcium and phosphorus, and in the prevention and cure of rickets in children. Calciferol is also used in the treatment of hypocalcaemia due to parathyroid deficiency. Prolonged high dosage of calciferol causes toxic effects[16] and may lead to hypercalcaemia causing abnormal calcium deposits in the arteries and kidneys, and even death. Calciferol as might be expected from the nature of the unsaturated linkages present, is unstable on exposure to air and light and in the solid form must be stored in sealed containers from which the air has been evacuated or replaced by an inert gas. Although physical constants are available for the standardisation of calciferol these are of little help in the standardisation of its preparations. Until recently only biological methods were recognised for the determination of calciferol and indeed are still the only ones applicable to natural and complex products containing vitamin D. Stross and Brealey at the 1955 Conference[17] however described the applicability of a chemical method for the determination of calciferol in the tablets and oily solution of the British Pharmacopoeia. The method cannot easily be used for the determination of the calciferol content of fish liver oils which still constitute an important source of vitamin D for prophylactic purposes. Calciferol is not the only steroid derivative to exhibit antirachitic activity[18]. Indeed it is only slightly active in the chick. This species, however, responds to a closely related substance called vitamin D_3 (VIII) probably the main form in which the vitamin is present in fish liver oils. Vitamin D_3 is

manufactured by the irradiation of 7-dehydrocholesterol (VII) and differs from cholesterol or vitamin D_2 in having the saturated side chain of cholesterol.



(VII) 7-Dehydrocholesterol



(VIII) Vitamin D_3 (cholecalciferol)

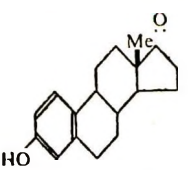
The Commission of Nomenclature of Biological Chemistry of the I.U.P.A.C. has adopted the names Ergocalciferol and Cholecalciferol for vitamin D_2 and vitamin D_3 respectively.

The Steroid Hormones

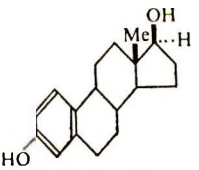
As mentioned already it was for long believed that cholesterol was the precursor of steroid hormones but it is now becoming clear that their biogenesis cannot be so simply explained. Together with cholesterol the steroid hormones form the most important group of phenanthrene derivatives, influencing demonstrably as some of them do the maintenance and propagation of life. They include among others the groups of substances referred to for convenience as androgens, oestrogens, progestational and adrenocortical hormones. Knowledge of their structure and methods for their synthesis followed directly from the final understanding of the structure of the sterols in 1932. Unlike the sterols which occur abundantly in plants and animals the steroid hormones occur in the organism in extremely small amounts and many triumphs of manipulative skill are represented by their isolation.

Oestrogens

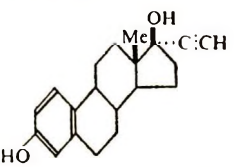
The first steroid hormone to be isolated in crystalline form was the oestrogenic hormone, oestrone (IX), so called[19] to indicate its ketonic nature and its characteristic physiological action exhibited by its ability to induce oestrus in animals. Originally regarded as the primary ovarian



(IX) Oestrone



(X) β-Oestradiol



(XI) Ethinyl oestradiol

hormone it was found that its more oestrogenically active dihydro-derivative is the primary oestrogenic hormone. Usually referred to as oestradiol (X), the more oestrogenically active epimer has been assigned the 17β-hydroxy-configuration[20]. The structure of oestrone was established in 1932[21,22] and the preparation of oestradiol from cholesterol was

accomplished in 1940[23]. Considerable improvements in the partial synthesis have since been effected[24] and total synthesis of natural oestrone was finally accomplished in 1948[25] a remarkable culmination to much brilliant work in this country, in the U.S.A. and in Europe, having regard to the fact that oestrone is one of sixteen possible stereoisomers.

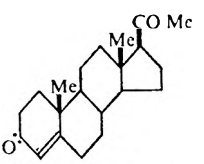
Essential for the proliferation of the uterine endometrium oestrone and oestradiol and their derivatives (e.g., ethinyl oestradiol, XI) have found considerable application in replacement therapy to correct the consequences of defects in the menstrual cycle, in the female climacteric and menopause, and for inhibition of lactation. But the importance of the oestrogens in the maintenance and restoration of health is not limited to their more immediately apparent physiological role. Despite the incidence of side effects, particularly mammary growth, oestrogens have been widely used in the control of prostatic carcinoma since Huggins' discovery of this application[26]. More recently their value in inhibiting the development of secondary metastases following mammary carcinomata, in post-menopausal women, has become established[27–30]. It seems possible that "oestrogens" may have an important role to play in the prevention and treatment of some cardiovascular irregularities[31]. Certain oestrogens also appear capable of influencing favourably the phagocytic activity of the reticulo-endothelial system and hence its role in combatting infection[32]. Though dietary as well as hormonal factors appear to be involved in atherogenesis[10], animal experiments have been sufficiently encouraging for long-term clinical studies to be initiated on the ability of oestrogens to prevent myocardial infarction and prolong life in males under 50 years of age who had recently experienced a proved myocardial infarction. Preliminary results have been considered by Stamler of the American Heart Association to "justify a guarded optimism"[31,33]. The possible value of oestrogens in the reversal of coronary lesions in the male naturally raises the question whether the several oestrogenic properties can be dissociated; that is to say, whether coronary lesions can be prevented without feminisation and alteration of plasma lipids. There are some grounds for believing that transformations of the steroid molecule may result in differentiating between those features of the molecular and stereochemical structures which influence physiology in the female and those features which appear to play an extragonadal role. Thus it now appears[34,35] that oestriol (oestra-1 : 3 : 5(10)-triene-3 : 16α : 17β-triol) and its epimer 16-*epi*oestriol (oestra-1 : 3 : 5(10)-triene-3 : 16α : 17α-triol) which were formerly considered to be merely excretory or breakdown products of oestradiol and oestrone exert a direct "protective" action against the more active oestrogens in the system. Other oestrane derivatives, particularly those oxygenated at positions 6 and 16, are also able to depress certain effects of oestrone. Such compounds have been called "impeded oestrogens"[35]. So that in addition to the naturally occurring oestrogens and the highly potent partial synthetic oestrogen, ethinyl oestradiol, the simpler synthetic analogues, stilboestrol, hexoestrol and dienoestrol, and related derivatives, we may also expect to see in the future "synthetic" oestrogens which have been "tailored" to accentuate specific biological

manifestations for therapeutic advantage. Dr. V. Petrow and his colleagues, in my own laboratories, in common with workers elsewhere, are actively engaged in such projects.

The value of oestrogen therapy in the treatment of premenopausal and menopausal disorders is well established and the therapy widely used. Its value in the elderly patient for the treatment of symptoms of both physical and psychogenic origin, particularly when used in conjunction with androgen is of more recent recognition[36–38]. Partly this is due to increased knowledge of the anabolic value of the oestrogens and androgens but perhaps more still to the knowledge that the fluid retention properties of the oestrogens can be avoided by the collateral use of androgens in suitable dosage.

Progestational Substances

An important physiological role of the oestrogens is the proliferation of the uterine mucosa; the further development of the uterus in preparation for reception of the fertilised ovum involves a secretory or progestational phase which was shown by Corner and Allen[39] to be stimulated by a hormone or hormones produced by the corpus luteum, a tissue so-called because of its yellow colour which is due to its abundant carotene content. The corpus luteum, developed in the ovary after the ripening and rupture of the follicle, performs the following functions after fertilisation of the ovum has occurred: it suppresses ovulation, it maintains the uterine mucosa in a secretory phase to nourish the developing embryo, it inhibits uterine motility and in conjunction with oestrogen it induces mammary gland development. The pure corpus luteum hormone, progesterone (XII), was isolated in 1934 in dimorphic interconvertible forms, first by Butenandt and almost simultaneously by three other teams. Its structure was established by its partial synthesis from the phytosterols, stigmasterol and sitosterol and it is now manufactured from steroid sapogenins not only for use as such therapeutically, but also as a material which on bio-oxygenation yields 11-hydroxyprogesterone, from which cortisone and its derivatives are manufactured.



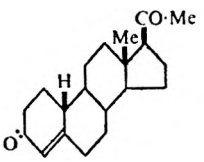
(XII) Progesterone

The characteristic biological properties of progesterone may be enhanced or modified by structural alterations. Thus both 9(11)-dehydroprogesterone and 17α-methylprogesterone are more potent progestational agents than progesterone itself. 17α-Hydroxyprogesterone seems devoid of progestational activity in the human female but is sixty times more active than progesterone in the Hooker-Forbes intra-uterine bioassay employing the mouse as test animal[40]. The hexanoate or caproate ester of 17α-hydroxyprogesterone, in contrast, has recently[41] been introduced

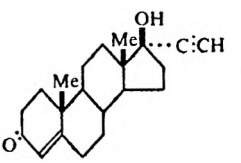
into medicine, as it exerts similar pharmacological responses to those of progesterone including its thermogenic effect, but its ability to induce secretory mucosa is more prolonged. It is therefore of particular value in continuing therapy as in the treatment of habitual and threatened abortion. As they are not active orally, progesterone and the ester of its 17-hydroxy derivative are usually administered by intramuscular injection in oily solutions. Progesterone has also been used by implantation but is liable to be extruded unless implanted deeply into the tissues and its use in this way is often not reliable. For oral use the 17β-hydroxyanhydro derivative, pregneninolone (17β-hydroxy-17α-pregn-4-en-20-yn-3-one or 17α-ethynyl-17β-hydroxytestosterone) or ethisterone (XIV), is widely used particularly in premenstrual tension, in the treatment of functional uterine haemorrhage and in threatened or more particularly habitual abortion.

Recent Development of Progestational Agents

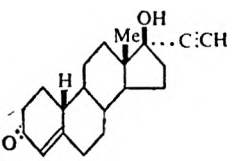
More recently it has been shown that the presence of the C(19) angular methyl group at position 10 is not essential for progestational activity. The 19-nor-steroids are, in fact, engaging attention not only for their progestational, but also for their androgenic and anabolic properties. 19-norprogesterone (XIII) has been shown to be more active than progesterone itself[42,43].



(XIII) 19-Norprogesterone

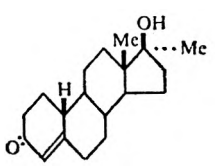


(XIV) Ethisterone

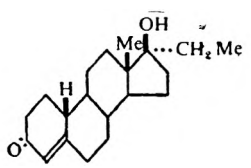


(XV) Norethindrone (19-Norethisterone)

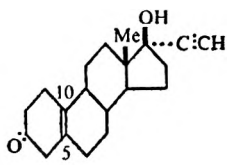
The corresponding 19-norethisterone (17α-ethynyl-19-nortestosterone) (XV) is orally active and of greater activity than ethisterone[44–46]. Other derivatives such as 17α-methyl-19-nortestosterone (XVI) and 17α-ethyl-19-nortestosterone (XVII) (referred to as norethandrolone) also appear to be more active than progesterone in certain respects[47–49] but as



(XVI) 17α-Methyl-19-nortestosterone



(XVII) 17α-Ethyl-19-nortestosterone



(XVIII) 17α-ethynyl-5(10)-oestrenolone

mentioned later they also have pronounced anabolic properties[50]. Another type of progestational agent is represented by 17α-ethynyl-5(10)-oestrenolone (XVIII), in which the unsaturated linkage is no longer $\alpha\beta$- to the 3-oxo group, but located at the juncture of Rings A and B. Surprisingly this material is more active orally than is ethisterone as a progestational agent[49].

From work in my own laboratories it also appears that the presence of alkyl groups at certain other positions can enhance the progesterone-like properties of ethisterone[51,52].

Though the therapeutic application of progesterone and its orally active analogue ethisterone has been mainly to produce a secretory phase in the endometrium and maintain a deciduum after pregnancy and more recently for the treatment of premenstrual tension[53], the ability of progesterone to inhibit ovulation in animals and in women has been recognised. It has now been demonstrated[54] that 17α-ethynyl-19-nortestosterone (norethisterone), 17α-ethynyl-5(10)-oestrenolone, and 17α-ethyl-19-nortestosterone (norethandrolone) on oral administration in dosages of 10 to 50 mg. daily are effective ovulation-inhibitors in women. The possible application of this property raises many interesting aspects and doubtless there will be much discussion of the ethical aspects of this use of these compounds. In the examples so far reported, fertility does not appear to have been impaired, for exposures after cessation of medication resulted in pregnancies in seven instances.

Adrenocortical Hormones

Progesterone is produced in the body not only by the corpus luteum but also by the adrenal cortex where it is almost certainly an intermediate in the biogenesis of the adrenocortical hormones. The importance of the adrenal or suprarenal gland and more particularly the physiological functioning of the adrenal cortex, to the maintenance of life has been known ever since Thomas Addison studied and described, in 1855, "the constitutional and local effects of diseases of the suprarenal capsules". Addison's name has long been associated with the "disease" resulting from adrenal cortical insufficiency. But it is only in the last 25 years that the variety and complexity of the steroids produced by the adrenal cortex, the so-called corticoids, have been recognised. More than twenty steroids have been isolated from extracts of the adrenal glands and though not all of them are biologically active and some may well be intermediate precursors of the biologically active steroids their chemical interrelationships are most interesting to note and are listed in Table I.

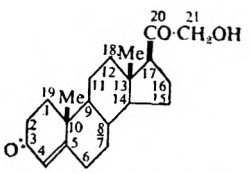
The series of pregnane derivatives, some active, some inactive biologically, which have been isolated indicate the nature of the biosynthetic reactions occurring in the adrenal gland, many of which have been demonstrated by perfusion studies with isolated glands or homogenates using selected steroid substrates[55].

Biogenesis of pregnane derivatives in the adrenals seems to proceed via progesterone which is then hydroxylated at positions 17 or 21. The 17α-hydroxyprogesterone, which has been isolated as such from adrenocortical extracts[56], is then oxidised to hydrocortisone, whilst the 21-hydroxy derivative is converted into corticosterone and presumably into aldosterone. Many of the possible hydroxylations of the pregnane nucleus have been effected by microbiological means. Thus with the aid of appropriate microorganisms, bacteria, yeasts and other fungi, hydroxylations have been effected at positions 6, 7, 8, 11, 14, 16 and 17 in the

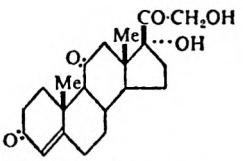
TABLE I

ADRENOCORTICAL STEROIDS

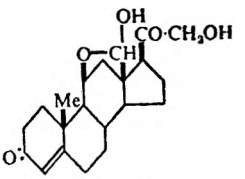
Biologically active steroids:	
Corticosterone	11β:21-Dihydroxypregn-4-ene-3:20-dione (Kendall's compound B; Reichstein substance H)
Cortisone (XX)	17α-Hydroxy-11-dehydrocorticosterone or 17α:21-dihydroxypregn-4-ene-3:11:20-trione (Kendall's compound E)
Deoxycortone (XIX) (deoxycorticosterone)	21-Hydroxypregn-4-ene-3:20-dione
11-Dehydrocorticosterone	21-Hydroxypregn-4-ene-3:11:20-trione (Kendall's compound A)
Deoxycortisol	17α-Hydroxydeoxycorticosterone
	17α:21-Dihydroxypregn-4-ene-3:20-dione
Hydrocortisone (cortisol)	17α-Hydroxycorticosterone
	11β:17α:21-Trihydroxypregn-4-ene-3:20-dione
Aldosterone (XXI)	11:18-Hemiacetal of 11β-21-Dihydroxy-18-oxa-pregn-4-ene-3:20-dione



(XIX) Deoxycortone



(XX) Cortisone

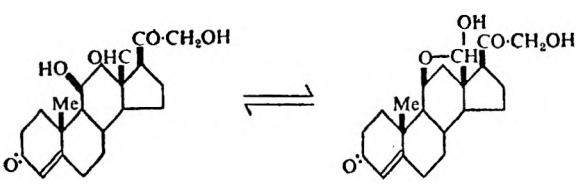


(XXI) Aldosterone

Biologically inactive steroids:	
Reichstein Substance A:	3β:11β:17α:20:21-Pentahydroxyallopregnane
,, ,, V:	3β:11β:17α:21-Tetrahydroxyallopregnan-20-one
,, ,, C:	3α:11β:17α:21-Tetrahydroxyallopregn-20-one
,, ,, D:	3β:17α:21-Trihydroxyallopregnan-11:20-dione
,, ,, E:	11β:17α:20:21-Tetrahydroxypregn-4-en-3-one
,, ,, U:	17α:20:21-Trihydroxypregn-4-en-3:11-dione
,, ,, K:	3β:17α:20:21-Tetrahydroxyallopregnane
,, ,, P:	3β:17α:21-Trihydroxyallopregnan-20-one
,, ,, J:	3β:17α:20-Trihydroxyallopregnane
,, ,, R:	3β:11β:21-Trihydroxyallopregnan-20-one
,, ,, N:	3β:21-Dihydroxyallopregnane-11:20-dione
,, ,, T:	20:21-Dihydroxypregn-4-en-3:11-dione
,, ,, O:	3β:17α:20-Trihydroxypregnane
,, ,, L:	3β:17α-Dihydroxyallopregnan-20-one

pregnane nucleus[57]. And by the use of appropriate homogenates hydroxylation has been accomplished in the side chain at position 21 and in the angular methyl groups, that is on carbon atoms C(18) and C(19)[58,59]. These many possibilities of hydroxylation foreshadow the natural existence of a whole galaxy of minor steroids, all pregnane derivatives. Many of them are however as yet of unestablished structure and of unknown function. Hydroxylation at position 6 is perhaps of some interest as it occurs mainly in the liver and may in part explain the relatively low activity of progesterone when administered orally.

The accomplishment of hydroxylation biologically at C(18) is of particular interest in relation to the mode of biogenesis of aldosterone, which is characterised by a unique aldehyde group in place of C(18) methyl group.



(XXI) Aldosterone

The existence of 19-hydroxylating systems[59] portends the possible natural occurrence of the 19-nor-steroids as well as indicating a likely route whereby androgens may be converted into oestrogens.

In addition to the so-called corticoids, other steroids have been isolated from adrenal cortical extracts namely oestrone[60], progesterone[61] and three androgens, derivatives of androsterone: adrenosterone or androst-4-en-3:11:17-trione[62], androstenedione or androst-4-en-3:17-dione, and 3β:11β-dihydroxyandrostan-17-one[63]. While, as stated, progesterone and 17-hydroxyprogesterone seem likely to be biological precursors of the "corticoid" hormones, the androsterone derivatives and oestrone seem likely to arise by subsequent biological oxidations and dehydrogenation of the corticoids.

The physiological functions of the secretions of the adrenal cortex, which are stimulated by the peptide called corticotrophin or adrenocorticotrophic hormone (ACTH) secreted by the anterior lobe of the pituitary gland, are necessary for the preservation of life and the maintenance of health. They are concerned with nitrogen metabolism, carbohydrate metabolism, and the control of electrolyte balance of the blood. But such a simplification scarcely does justice to the remarkable properties and range of therapeutic applications that have been discovered or advocated since manufacture became possible of many of the cortical hormones and their subsequent modifications.

Extract of suprarenal cortex for injection is still included in the B.P.C. and is still used in some cases for the treatment of Addison's disease and other types of adrenal insufficiency. Its continued use reflects the complexity of adrenocortical function. Deoxycortone which, as its acetate, was the first of the corticoids to be manufactured in quantity has not proved adequate for all cases of Addison's disease. It influences electrolyte balance, and is therefore a so-called mineralocorticoid, by controlling loss of sodium, and retention of potassium, with consequent modification of the sodium to potassium ratio, but it has only limited effect on carbohydrate metabolism, the so-called glucocorticoid activity. The expectation at the outbreak of the second world war that the sodium-retaining properties of deoxycortone might be applied to the prevention of fluid loss and the avoidance of secondary shock was not fulfilled. Whether corticosterone itself would have proved much more valuable had it been available in quantity now seems less likely. Although corticosterone, first obtained by partial synthesis by Reichstein in 1941, has a more profound influence on sodium retention than deoxycortone, it too has little effect upon carbohydrate or nitrogen metabolism. Interest in the therapeutic possibilities of corticosterone has waned with the development of cortisone and its derivatives, and with the isolation and investigation of aldosterone.

Aldosterone

Aldosterone (XXI) is the most active electrolyte-regulating hormone secreted by the adrenal cortex having a potency about one hundred times that of deoxycortone when measured by the sodium:potassium urinary

ratio in adrenalectomised rats. Unlike deoxycortone it has an appreciable effect upon organic metabolism having a potency of about one third of that of cortisone in the liver glycogen deposition assay. The isolation of aldosterone, then called electrocortin, by Simpson and Tait in 1953, followed by elucidation of its chemical structure[64] aroused considerable interest matched only by its synthesis in a remarkably short time[65]. Aldosterone was unusually interesting among adrenal cortical hormones not merely because of its uniqueness in possessing an aldehyde group in place of the 18-methyl group which suggested hitherto unsuspected biosynthetic possibilities in the adrenal, but because the daily output (micrograms per day) in normal man was so very much less than that of the corticoids (milligrams per day). Adrenal tumours however cause a considerable rise in the production and excretion of aldosterone and its determination has provided a new diagnostic test for the chemical pathologist. Aldosterone, however, does not merely influence sodium retention. It also accentuates potassium loss. In addition, some kinds of hypertension appear to derive from chronic hypersecretion of aldosterone[66].

Although the therapeutic application of aldosterone now seems likely to be strictly limited, much work continues to be carried out both biologically and clinically to assess the significance of aldosterone output and secretion. A clearer understanding of the part played by the adrenal gland in health and disease seems likely to result. There can be little doubt about the importance of mineralocorticoid activity to health but its control must await further elucidation. In this connection mineralocorticoid antagonists[67] may have their part to play.

The Glucocorticoids

Although the study of steroid hormones has yielded many fascinating and even dramatic results, none of these have so captured the imagination of the scientific world as Hench's revelation in April 1949 of the influence of cortisone on the suppression of rheumatoid arthritis. Following publication of the results of the work at the Mayo Clinic[68] widespread interest in Kendall's compound E subsequently named cortisone was aroused. Its anti-inflammatory, anti-allergic and anti-fibroblastic properties were gradually revealed as also were those other properties which led to undesirable effects during continuing therapeutic use. First obtained by isolation from adrenal extracts, cortisone was subsequently manufactured chemically from deoxycholic acid and later from such steroid sapogenins as diosgenin and hecogenin. The total synthesis of cortisone could formally be said to be based on the Robinson and Woodward synthesis of non-aromatic steroids accomplished in 1951, but the first *de facto* total synthesis of cortisone was accomplished in 1952[69].

Cortisone was found to suppress rheumatoid arthritis but not to reverse or even check the underlying disease. During treatment, however, it afforded much relief to the patient by its anti-inflammatory properties, pain and stiffness being diminished and the patient acquiring a greater range of movement. The use of cortisone for the treatment of rheumatoid

arthritis, however, was found to be not without risk of serious side effects. Electrolyte balance was affected leading even to signs of congestive heart failure in some cases and, in consequence, potassium chloride needs to be given daily during treatment to prevent hypokalaemia. Carbohydrate metabolism was affected leading to glycosuria and increased insulin requirements in diabetes mellitus. Protein metabolism was affected leading with continuing large doses to a negative nitrogen balance. Resistance to bacterial infection was diminished and wound healing delayed. There was found to be a risk of peptic ulceration and of perforation in patients with symptoms of past peptic ulceration. Replacing as it did an important natural secretion of the adrenal, its withdrawal led to the symptoms of adrenal exhaustion which in many cases resulted in worsening of the disease and in some instances proved fatal. Large scale trials organised by the Medical Research Council led to the conclusion that for the treatment of rheumatoid arthritis, cortisone was not significantly more beneficial in longstanding cases than aspirin, and the incidence of side effects circumscribed continuing therapy. Though it might be fair to say that cortisone has not fulfilled the early expectations that its introduction to therapy aroused, it is still a valuable drug and its use has hastened the search for improvements and consideration of the anticortisone properties of steroids[70]. Cortisone is of value and is used as an anti-inflammatory agent in self-limiting conditions, in diseases of the eye, in acute disseminated lupus erythematosus and acquired haemolytic anaemia. It is also valuable in maintenance in Addison's disease and Simmonds' disease with secondary hypoadrenalism and in Addisonian crisis. Its value in acute rheumatic fever is less clear[71].

The corresponding secondary alcohol, hydrocortisone, in which the 11-keto group of cortisone has been reduced, represents the major glucocorticoid secreted by the adrenals. It is now manufactured chemically from bile acids but more commonly from the steroid sapogenins. The application of biooxygenation to convert progesterone into 11α-hydroxyprogesterone has considerably facilitated the accessibility of hydrocortisone. Both this substance and its 21-acetoxy derivative, hydrocortisone acetate, are of considerable value dermatologically and opthalmologically. For topical use there is as yet no general agreement about which form is preferable or what is the most effective ointment base. In addition both forms are of value parenterally. Hydrocortisone acetate in contrast to cortisone acetate is effective when injected intra-articularly. Hydrocortisone is more effective orally than intramuscularly and its use is frequently attended by less risk of the side effects that are encountered with cortisone.

For oral use in rheumatic diseases a considerable advance on cortisone and hydrocortisone appears likely to follow the introduction and usage of the corresponding compounds having an additional unsaturated linkage between carbon atoms 1 and 2 namely the Δ^1 compounds: 17α:21-dihydroxypregna-1:4-diene-3:11:20-trione (Prednisone) and 11β:17α:21-trihydroxypregna-1:4-diene-3:20-dione (Prednisolone)[72–74]. Prednisone and prednisolone appear to be some three to five times more potent

as glucocorticoids than cortisone and hydrocortisone, and seem likely to become, at least for a time, the "glucocoids" of choice for oral use. The availability of the corresponding acetates as well as the free alcohols reflects the use of different routes of manufacture, the free alcohol being directly obtained by one process and the acetate by the other. Conversion of acetate into the free alcohol results in some loss of yield. There appear to be no differences in therapeutic value observable when equivalent molecular quantities of the acetates and free alcohols are used orally.

Recent Developments

Still further advances are likely to result from studies of further modifications of the steroid molecule in the glucocorticoids. For example, the introduction of an unsaturated linkage between C(6) and C(7) into prednisone and prednisolone yields 17α:21-dihydroxypregna-1:4:6-triene-3:11:20-trione and 11β:17α:21-trihydroxypregna-1:4:6-triene-3:20-trione which are stated[75] to be considerably more potent than the parent steroids. Further, halogenation at position 9 by fluorine appears to provide advantages. 9α-Fluorohydrocortisone has received some attention for its value as a glucocorticoid particularly in maintenance therapy in hypoadrenalism. Its mineralocorticoid action, however, leading to fluid retention and to hypertension has limited its value in the treatment of collagen diseases[76,77]. 9α-Fluoro-17α:21-dihydroxypregna-1:4-diene-3:11:20-trione or 9-fluoroprednisone, as it might be termed[78] seems more likely to be valuable and it will be of interest to see whether the corresponding Δ^6 compound proves even more potent. Then again the value of alkylation has been studied and though cortisone derivatives having a methyl group at position 1, 2 or 4 are less active as glucocorticoids than cortisone, enhancement of activity is stated to occur when the methyl group is present at position 6 namely in 6-methyl-11β:17α:21-trihydroxypregna-1:4-diene-3:20-dione or 6-methylprednisolone[79]. But the greatest enhancement of glucocorticoid activity so far reported occurs in the 9α-fluoro derivative of that compound. 9α-Fluoro-6α-methyl-11β:17α:21-dihydroxypregna-1:4-diene-3:20-dione or 9α-fluoro-6α-methylprednisolone is stated to have a glucocorticoid activity some fifty times that of prednisolone and nearly two hundred times that of hydrocortisone[80,81]. Potential glucocorticoids carrying additional hydroxyl groups at position 2[82], position 5[83], position 12[84], position 14[85], position 16[86,87] have been reported. The 19-nor analogues of hydrocortisone and cortisone have also been prepared[88] but surprisingly, loss of the C(19)-angular methyl group is accompanied by marked loss of glucocorticoid activity.

The adrenocortical hormones and their chemical modifications have perhaps suffered to some extent in their therapeutic status by over-enthusiastic use and misuse. Now that the limitations, side effects and definite contraindications for use are becoming accepted, the fields of therapeutic usefulness are becoming more clearly defined. Systemically, the "corticoids" can no longer be regarded as first choice in the treatment

of rheumatoid arthritis or chronic bronchial asthma. They may help prolong life in progressive potentially fatal diseases such as the leukaemias and they are, of course, capable of providing the replacement therapy necessary in Addison's disease and after adrenalectomy. Because of their influence on sodium retention with concomitant potassium loss, their use needs to be linked with restricted sodium chloride intake and with supplementary potassium chloride. For the treatment of skin lesions and of inflammatory conditions of the eye hydrocortisone ointments and drops provide dramatic evidence of the antiphlogistic, anti-allergic, and anti-pruritic properties of hydrocortisone though when infection is present, specific collateral treatment to eliminate the infection is also required. Whether prednisolone will prove more useful than hydrocortisone dermatologically is at present uncertain, conflicting results having so far been obtained[89,90].

Steroids as Anaesthetics

Another interesting aspect of the properties of cortical hormones and their derivatives arises in connection with their potential anaesthetic properties. It has been known for some time that certain steroids and related compounds were capable of exerting general anaesthetic effect on intraperitoneal injection into animals. In 1942 Selye of McGill University published a paper which attracted much interest, giving the results of his examination of some seventy-five steroid compounds and showing the anaesthetic properties possessed by many of them, and somewhat surprisingly, by the non-steroid, stilboestrol. In his "Correlations between the chemical structure and the pharmacological actions of steroids"[91], Selye drew attention to the molecular features which appeared to lead to increased anaesthetic properties, but at that time the practical application of the results did not seem feasible owing to the corticoid, folliculoid, luteoid or androgenic properties which to greater or less extent were always also exhibited by the active compounds in the dosages required. Recently, however, Selye's pioneer studies have borne fruit and have led to the clinical evaluation of sodium 21-succinoyl-pregnane-3:20-dione as an anaesthetic[92–94]. As a basal anaesthetic, this compound proved acceptable and is now generally available. Its behaviour as an anaesthetic resembles that of thiopentone, though its influence on pulse rate and blood pressure have been commented upon adversely[95]. Whether this reflects mineralocorticoid activity in the compound is not clear.

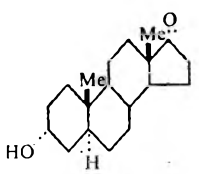
The influence of adrenocortical steroids on brain function and metabolism is attracting increasing attention and it seems reasonable to expect that clarification of neuroendocrinological actions and possibilities will be forthcoming in the near future[96]. The 3:5-*cyclo*steroids are also likely to be of interest in this connection[97].

Androgens

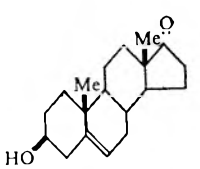
If biogenesis of hydrocortisone in the adrenals proceeds via 17α-hydroxyprogesterone, then it might well be expected that degradation of the latter intermediate to androstane derivatives should take place. This

is indeed found to be the case. It is established that androgens occur not only in the testis but also in the adrenal cortex and ovary. And it has already been mentioned that three androgens androst-4-ene-3:17-dione, adrenosterone and androstane-3β:11β:-diol-17-one have been isolated in crystalline form from adrenal cortical extracts. Furthermore, urinary androgen concentration is considerably increased in certain patients with adrenocortical hyperplasia or tumour, and is diminished in conditions of adrenal hypofunction. The elaboration of androgens by the adrenal gland, irrespective of sex, makes it seem likely that adrenocortical androgens are the biological precursors of oestrogens in the ovary. Though no pure androgen has yet been isolated from ovarian tissue, androgenic extracts have been obtained. There is a great tendency to think of androgens as primarily of importance in a gametogenic function but in fact the biological effects of the androgens include not only effects on sex specific tissues but also on general body mass, kidney, muscle and hair growth, on nitrogen metabolites, on water and electrolyte metabolism and on certain enzyme systems. Not only have androgens an influence on behaviour and characteristics but they play an important part in development, metabolism and ageing. Clinically the androgens have important applications both for male and female use, in correcting deficiencies due to hypogonadism, as anabolic agents both during the menopause and during ageing, and in inhibiting neoplastic developments; proving especially valuable in advanced mammary cancers[98].

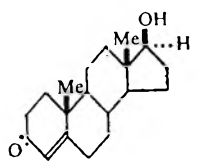
Androgens were first isolated from urine. Androsterone, the first androgenic hormone to be obtained in crystalline form, was isolated in 1931 by Butenandt who used increase of capon comb as a means of measurement of activity to guide the work. The structure of androsterone was deduced by Butenandt in 1932, when only 25 mg. of substance had been available for study and was confirmed by its preparation from cholesterol by Ruzicka, by Butenandt, and by Callow and Deanesly[99]. In currently agreed steroid nomenclature[100] its structure is 3α-hydroxy-androstan-17-one.



(XXII) Androsterone



(XXIII) Dehydroepiandrosterone



(XXIV) Testosterone

A second weakly active androgen, dehydroepiandrosterone, was subsequently isolated from urine in 1934 by Butenandt although this substance which has also been called dehydroisoandrosterone or simply dehydroandrosterone is not itself of importance as an androgen. It is particularly important, however, as it forms an intermediate which is readily obtained by oxidative degradation of cholesterol and of certain sapogenins, and which is widely used for the manufacture of therapeutically important steroids. Molecularly isomeric with dehydroepiandrosterone is the much

more potent androgen, testosterone, which was first isolated from testis tissue in 1935 by Laqueur[101]. The discovery of testosterone stemmed from inexplicable discrepancies in the biologically assayed potencies of androgenic extracts prepared respectively from urines and from testis tissue and provides another reminder of the importance of quantitative biological work in the discovery and development of steroids. Though the original preparative and manufacturing methods for the conversion of 3β-hydroxyandrost-5-en-17-one (dehydroepiandrosterone) into 17β-hydroxyandrost-4-en-3-one (testosterone) involved no fewer than five chemical stages with consequential losses in overall yield, the development of methods of steroid oxidation with the aid of suitable microorganisms has now made it possible to effect the conversion in two stages with but little loss of yield.

Within the last five years the total synthesis of dehydroepiandrosterone has been accomplished independently and by quite different routes by both British and American teams. The structure and stereochemistry of the steroid hormones may consequently be regarded as finally established[69,102–104].

During early studies of the biological properties and clinical applications of testosterone it was found to be relatively inactive on oral administration. That it was absorbed orally and metabolised was shown by recovery of its metabolic products in appropriate yield from the urine[105]. Its relative oral inactivity, as low as one-tenth of that by injection[106] appears to be due to oxidation and esterification on passage through the liver[107] the less active androsterone, among other products, together with the highly stable water soluble sulphates and glycuronates, being formed. Though active on injection in oily solution, testosterone was found to have a less prolonged action than that of its esters[108], and much attention was devoted to the correlation of the nature of the group used to esterify the secondary alcoholic group at position 17 and the resultant potency and duration of action. Among ester groups studied were formate, acetate, propionate, butyrate, *iso*butyrate, valerate, *iso*valerate, palmitate, stearate and benzoate, the most effective clinically being found to be the propionate. In recent years interest has been revived in the modification of the esterifying group and among alternatives to testosterone propionate the esters of the following acids have been advocated as having more prolonged action on intramuscular injection in oily solution, heptanoic or oenanthic ($C_7H_{15}COOH$); decanoic ($C_9H_{19}COOH$); *cyclo*pentylpropionic ($CH_2CH_2CH_2CH_2CH{\cdot}CH_2CH_2COOH$); *cyclo*hexylpropionic ($CH_2CH_2CH_2CH_2CH_2CH{\cdot}CH_2CH_2COOH$); phenylpropionic ($C_6H_5{\cdot}CH_2CH_2COOH$). For water soluble use, the hydrochloride of the testosterone ester of β-diethylaminoethyl carbonic acid ($(C_2H_5)_2N{\cdot}CH_2CH_2{\cdot}O{\cdot}CO{\cdot}OH$) has been proposed. Hydroxylated testosterones have also been prepared[109].

Testosterone esters have been widely used clinically by intramuscular injection in oily solutions, the choice of oil influencing to some extent the

rate of onset of effect and the duration of action, though greater variations in the duration of action result from varying the ester group. Prolongation of action has also been achieved by the use intramuscularly of suspensions of free testosterone in microcrystalline form in aqueous vehicles. Solutions of testosterone in propylene glycol have been used intravenously in high dosage without ill effects[110] but the absence of clinical advantage has apparently discouraged the routine use of such solutions. Where however considerably prolonged action is required, as for example, in the treatment of advanced mammary carcinoma, free testosterone is implanted, usually in the thigh or abdomen, in up to 300 mg. units in the form of sterile pellets. These are prepared by fusion and extrusion from suitable moulds, or by appropriate compression methods, under aseptic conditions. Implantation therapy, which has been studied with oestrogens, progestational substances, corticoids, as well as with androgens is of course more appropriate when continued steady administration is required. With oestrogens it is not a particularly desirable method of administration because of the risks of excessive absorption and the greater value of interrupted therapy. With androgens, continuous absorption is sometimes necessary. Free testosterone is absorbed from implants more rapidly than is testosterone propionate and is the form commonly preferred, some 3 to 5 mg. being absorbed daily and proving equivalent to 25 mg. of testosterone propionate given by injection three times weekly.

TABLE II

RATE OF ABSORPTION AND DURATION OF ACTION OF IMPLANTED PELLETS OF ANDROGENS

Hormone	µg./ sq. mm. absorbed per day	Pellet weight (mg.)	Mg. absorbed per day per pellet	Proportion absorbed per month (per cent of pellets)	Effective life of pellets (months)	Number of pellets for replacement therapy in man
Testosterone	6–9	<5	<0·1	>90	<1	>50
		30–50	0·5	40	2	8–15
		75–225	1·5	30	3	3–6
Testosterone propionate	3–4	<10	<0·2	60	<2	>25
		20–50	0·3	24	4	15–30
		100–300	1·0	16	5	4–10
Methyltestosterone ..	4–5	<5	<0·1	>90	<1	>50
		5–10	0·2	70	1	25–50
		40–100	0·7	30	3	5–12

The rate of absorption of steroid from an implanted pellet is, of course, dependent not only on the weight of the pellet but on its surface area and Table II, due to Dorfmann[111], has been compiled from studies by various workers. Although early experiments showed that androgens were absorbed through the intact skin and free testosterone was more effective percutaneously than its equivalent concentration of testosterone propionate[112], the use of testosterone ointments has not found any considerable favour in clinical practice; implantation, injection, or oral therapy being preferred.

As already mentioned, testosterone is relatively inactive by the oral route, the 17α-methyl derivative, methyltestosterone, being preferred. Miescher and Tschoff in 1938[113] first discovered the oral activity of methyltestosterone and its clinical applicability was established by Foss in 1939[114].

As an androgen, methyltestosterone by mouth has an activity of about one-third of that of the same dose of testosterone propionate administered by injection. It appeared to be relatively more potent when administered to animals sublingually[115], a study made following the observation that the activity of deoxycorticosterone could be demonstrated after sublingual administration[116]. The observed enhancement of androgenic potency on administering methyltestosterone sublingually in animals has led to clinical usage by this route. Various formulae have been proposed for tablets to enable them conveniently to be used by placing in the groove between the lower cheek and gum (the so-called buccal position). Unfortunately, the arbitrary disintegration time of not more than fifteen minutes at present required by the British Pharmacopoeia was fixed for tablets intended to be swallowed and is not adequate for satisfactory sublingual use. It would appear desirable for a longer time to be allowed to accommodate a choice of route. Free testosterone and its esters have been claimed also to be effective when administered sublingually[117] but to be less potent than methyltestosterone[118].

Anabolic Agents

As already mentioned, androgens play an important part in metabolic processes especially in relation to the ageing process. Their influence on nitrogen metabolism and in consequence on calcium metabolism is being increasingly applied therapeutically and it is possible that their protein-building or anabolic influence may prove to be their most important property. It seems clear that anabolic applications are of value in underdeveloped children, in convalescence and in geriatrics. An important consequence of the anabolic properties of the androgens appears to be their assistance in preventing, and in promoting the healing of, bone fractures in the elderly. The value of androgens in osteoporosis has been considered recently by Wheddon[119]. Understandably the masculinising effects of androgens have somewhat limited hitherto the application of their anabolic properties, a limitation that has to some extent been overcome by the simultaneous use of oestrogen and androgen. The combined use of oestrogen and androgen which is widely preferred for menopausal treatment has, for example, been found effective in the treatment of acute osteoporosis with hypercalcaemia[120]. There seems little doubt that the anabolic properties of the androgens can overcome the demineralisation of bone that accompanies defective protein synthesis. The ability of simple amino acids such as alanine to dissolve calcium phosphate can, of course, readily be demonstrated in the test tube so that anabolic measures to overcome the catabolic processes especially in ageing would be expected to limit the continuing demineralisation of bone.

It is the recognition of the anabolic applications of androgens that has stimulated the search for modified steroids in which the ratio of anabolic to androgenic potency is increased. Androgenic properties which were formerly assessed by comb growth tests in capons are now generally evaluated by their effect on the seminal vesicle and prostate weight in rats or guinea pigs. The anabolic properties manifest themselves by

nitrogen retention and can be evaluated by determining intake and output of nitrogenous materials, or by direct determination of nitrogen content of killed animals compared with controls. But nitrogen retention by enhanced protein synthesis also manifests itself by increased muscle growth and hence the gain in weight of suitably dissectable muscles of test animals compared with those of untreated controls provides a convenient method. The change in weight of the levator ani muscle of the castrated rat has been suggested by Eisenberg and Gordan as a suitable indicator of steroid influence on protein anabolism[121], and has since been widely used in assessing the anabolic properties of recently discovered variants of the testosterone molecule. The levator ani which atrophies in the castrated animal shows marked weight increase in animals receiving an anabolic agent.

17-Methylandrost-5-ene-3β:17β-diol (methylandrostenediol) the penultimate product obtained in the manufacture of methyltestosterone, was among the first alternatives to methyltestosterone to attract some attention as a potentially clinically valuable non-virilising anabolic agent. As an androgen its activity is about one-twentieth only of that of methyltestosterone[122]. Although in animals it appeared to have a somewhat greater anabolic activity than methyltestosterone, and hence a considerably greater anabolic to androgenic ratio than the latter[123], its clinical use as an anabolic agent has generally been disappointing[124]. Greater interest has been aroused in the therapeutic possibilities of derivatives of testosterone lacking the C(19) angular methyl group, namely 19-nortestosterone. 19-Nortestosterone itself, first synthesised by Birch in 1950[125], was stated to have about one-fifth of the androgenic activity of testosterone[126] and was subsequently shown by Hershberger[123] to have about the same anabolic potency as testosterone. It has not yet been made available for therapeutic use though some of its derivatives having the same order of anabolic to androgenic potency ratio, and patented as new products, have been made available. For oral use the 17α-methyl-19-nortestosterone might have been expected to be more active than 19-nortestosterone and this indeed appears to be the case. Other homologues, however, showed a similar activity and ratio[127]. 17α-Ethyl-19-nortestosterone has been judged to be more active orally than the 17α-methyl compound which appeared to inhibit the appetite of the animals used. The 17α-ethyl compound has recently been made available and described as norethandrolone[128]. Although the 17α-methyl compound, first prepared by Djerassi in 1954[129] had first been considered primarily for its potential interest as an oral anabolic agent, clinical studies have revealed its progestational activity[47,48]. As an oral progestational agent, 17α-methyl 19-nortestosterone (also described as methandrone and as methyl-(o)estrenolone) appears to merit closer study. The homologous compound, norethandrolone likewise appears to possess progestational properties[49] and its ability to inhibit ovulation, to cause endometrial proliferation, and the retention of salt and fluid will demand caution in its use as an anabolic agent. In fact, it now seems likely that the 19-nortestosterone derivatives may find their most important clinical application

as oral progestational agents and not as oral anabolic agents. Indeed 17α-methyl- and 17α-ethynyl-19-nortestosterone (19-norethisterone or norethindrone) have already been made available for use as oral progestational agents.

The anabolic action of 19-nortestosterone can be prolonged by esterification, for example by benzoic, *cyclo*pentylpropionic[130] or phenylpropionic acids[131], but the esters so obtained must be administered intramuscularly in oily solution.

In addition, research groups in Europe and the U.S.A. are actively engaged in the preparation of testosterone derivatives containing nuclear substituents such as alkyl, hydroxyl or halogeno[132–134]. Their biological study seems likely to reveal many more compounds of potential therapeutic interest. Thus for example, we already know that 4-chlorination or 6-methylation can increase the anabolic/androgenic index of testosterone derivatives[135]. Furthermore, it is interesting to note that while introduction of a 9α-fluoro substituent into hydrocortisone increases mineralocorticoid and, to a lesser extent, glucocorticoid activity, the introduction of such a substituent into the 11β-hydroxy derivative of methyltestosterone considerably enhances anabolic and androgenic potency. The androgenic activity of 11β:17β-dihydroxy-9α-fluoro-17α-methylandrost-4-en-3-one (fluoxymestrone) is stated to be some ten times greater than that of methyltestosterone[136] and the presence of the 11β-hydroxy group apparently does not give rise to oedema or to hypertension[137].

If it appears that the so-called androgens and their derivatives are at present attracting a disproportionate amount of attention, it must be borne in mind that part of the explanation rests in the fact that the investigation of human steroid metabolism still depends to a considerable extent on knowledge, understanding and interpretation of the 17-ketosteroid output revealed by determination in blood, plasma and urine. Although 17-ketosteroids arise by oxidative degradation of naturally occurring steroids of widely varying physiological significance, nevertheless they are most readily produced from the androgens[138]. Though it is well established that changes in steroid metabolism occur with age[139–141] much remains to be clarified before the full potentialities of the use of steroids in the maintenance of health can be realised. With developments in our knowledge of gerontology[142] and in neuroendocrinology[143] it seems certain that the therapeutic importance of steroids and their synthetic modifications must become increasingly recognised. And with a greater recognition of the importance of steroids there must also be increasing attention devoted to increasing our knowledge of their precise structure, stereochemistry and conformation[144]. The newer applications of spectrology[145,146] and of spectropolarimetry over the wavelength range 230 to 700 mμ[147] are proving extremely valuable in this connection.

Our understanding of the relationship between chemical structure and biological properties seems likely to be increased by the development of newer physical techniques and their physico-chemical application and eventually to replace empirical approaches, however enlightened, by a greater certainty.

Conclusions

An attempt has been made to present some interrelationships in the steroid field, some recent developments and some future possibilities. Steroid chemistry virtually began with the isolation and characterisation of the natural hormones and the preparation of simpler derivatives therefrom. It moved on with the study of their detailed biological properties and the recognition of the multiplicity of their biological properties. Every natural steroid hormone possesses not one but a whole range of biological activities and not all of them are necessary or even desirable in their application therapeutically.

Some of the properties, in fact, seem to be undesirable. The need has thus arisen for steroids "tailored" to a particular therapeutic requirement and this need is now slowly and systematically being met by the various alkyl-, halogeno-, nor- and dehydro-steroids that are being evolved in laboratories throughout the world. Such artificially-created steroids, moreover, are often many times as active, weight for weight, as the natural products they seek to replace. What of the future? The most important future applications of steroids would seem likely to be in the fields of gerontology, neuroendocrinology, and population control. The revolutionary technical advances of this century and even of the past two decades have changed the balance between health and disease and between life and death. The steroid chemist, in collaboration with biologists, pharmacists and clinicians, seems likely to assist in still further alteration of that balance.

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RESEARCH PAPERS

PHARMACOLOGY OF (±)-, (+)- AND (−)-2:2-DIPHENYL-3-METHYL-4-MORPHOLINO-BUTYRYLPYRROLIDINE

BY D. K. DE JONGH AND E. G. VAN PROOSDIJ-HARTZEMA

From the Nedchem Institute for Pharmaco-Therapeutic Research, Amsterdam

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(±)-2:2-Diphenyl-3-methyl-4-morpholino-butyrylpyrrolidine (R610) is a more active analgesic agent in mice, rats and guinea pigs than morphine. The analgesic activity is due to the (+)-isomer (R875). The (−)-isomer (R898) is nearly inactive. R610 lowers the body temperature to below control levels in immobilised rats, inhibits the respiration in unnarcotised rats, lowers the blood pressure in unnarcotised rats with experimental renal hypertension, and causes mydriasis in mice. It also augments the action of barbiturates in mice. In all these respects the (+)-isomer is the active compound of the racemic mixture. The (−)-isomer is almost as toxic as the (+)-isomer, which has a broader therapeutic margin than the (±)-form. The dosage-toxicity relation of highly active analgesic agents in mice and rats is irregular especially in the lower dosage range.

IN previous papers[1,2] the analgesic activities of a series of butyramide derivatives were described. In this paper the pharmacology of the most active of these compounds (±)-2:2-diphenyl-3-methyl-4-morpholino-butyrylpyrrolidine (R610) and of its (+) and (−) forms (R875 and R898) is dealt with in more detail.

EXPERIMENTAL METHODS

Toxicity in Mice and Rats

Acute Toxicity

Intravenous toxicity was estimated in mice by injection of the drugs in saline 0·1 ml./10 g. given over 20 ±2 seconds into the tail veins, the animals being previously subjected to a temperature of 36 ±1° for 10 minutes to dilate the tail veins. Subcutaneous toxicity in mice was determined by injecting the drugs in saline 0·1 ml./10 g. into the neck. For oral toxicity estimations, the drugs were given in a mucilage in 0·1 ml./10 g. weight by stomach tube to mice, the animals being fasted for 14 hours previously. After administration of the drugs the animals were kept at a temperature of 24 ±2° in groups of 10, each group being in a container of 3·5 litre capacity. The observation period was 3 days.

Acute intraperitoneal and subcutaneous toxicities were estimated by injection of the drugs in saline 0·1 ml./100 g. weight into white rats from an inbred strain, weighing from 120–180 g. using 40 to 120 rats for each compound. The animals were kept in groups of 5 at a temperature of 24 ±2° and observed over 3 days. For intravenous toxicity tests the injections were given in saline 0·1 ml./100 g. into the tail vein after the tail had been kept for 30 seconds in water at a temperature to 42–44° to dilate the veins.

Chronic Toxicity

20 female rats weighing 70 ±15 g. received a daily intraperitoneal injection of 1·25 mg. of R875 per kg. weight during a period of five months. Two other groups of 20 were given saline or 10 mg. of morphine per kg. body weight respectively. Growth curves were drawn from weekly weights. At the end of the experiment the rats were killed and the organs examined histopathologically.

Analgesic Activity

Observations on rats

We used a modification of the method of d'Amour and Smith[3] described in a previous paper from our laboratory[4]. A criterion of analgesic activity was derived as follows. In 234 rats the reaction time to a radiant heat stimulus was estimated immediately before and one hour after the intraperitoneal injection of saline 0·1 ml./100 g. body weight. The frequency distribution of the difference between both values was calculated. The mean value amounted to 0·25 seconds and a value of 3 or more was observed only five times in this series. According to the Camp-Meidell inequality[5] an augmentation of the resting threshold value after the injection of the drug of at least three seconds, could be considered as an analgesic effect at the 95 per cent level of significance. The drugs were injected in saline, 0·1 ml./100 g. weight. The frequency of this all-or-none response in series of 10 animals given geometrically progressing doses of the compounds was the basis for the estimation of ED50 values previously described.

Observations on guinea pigs

We used the thermal radiation method of Winder[6] modified by us[7]. The reaction time to a radiant heat stimulus was estimated immediately before and one hour after the intraperitoneal injection of the compounds in saline 0·1 ml./100 g. body weight. In 246 observations in which only saline was injected, the difference surpassed the value of one watt only in 4 instances. According to the Camp-Meidell inequality[5] a difference of at least one watt could be considered as an analgesic effect, at the 95 per cent level of significance.

Tests on mice

These were made with the methods of Haffner[8] and Eddy[9].

Haffner's method

The modification described by Bianchi and Franceschini[10] was used, replacing the artery clip by small clamps adapted from alligator clips.* These have the teeth of the clip replaced by a flat copper surface. Clips were applied to the root of the tail for thirty seconds or until a reaction occurred within this period. Only actual biting the clip was considered as a reaction. Before the experiments the non-reactors were eliminated. In our experience mice which once reacted in the described manner to the

* Mueller Electric Co., Cleveland, 60 series.

application of the clip usually did so again. Only 5 out of 200 mice failed to do so. Disappearance of the biting reaction after the administration of the drug was used as an all-or-none analgesic effect. The frequency of this response in series of ten aminals given geometrically progressing doses of the compounds was again used for the estimation of ED50 values. The drugs were given intravenously, subcutaneously and orally; the interval between the administration of the drug and the application of the clamp was 30 minutes in the first two, and one hour after oral doses.

Eddy's hot plate method[9]

This was slightly modified. The height of the glass cylinder was reduced to 4·5 cm. and covered with a plastic plate, which could be switched on and off by a simple movement. This prevented the mice from walking on their hind legs or from jumping out of the container. The reaction time was the time elapsing between contact with the plate and the licking response. It was determined twice before, with an interval of five minutes, and 10, 20, 30, 40, 50, and 60 minutes after drug administration. The frequency distribution of the sum of the differences between the reaction times after the drug and the average control value was investigated in 444 mice. As the distribution of this value was not normal[11], a criterion for analgesia was derived by using the Tchebycheff inequality[5] (mean value = 0·27 seconds; S.D. = 15·5; a variation of at least 50 seconds being significant at the 19/20 significance level). With this criterion defining a quantal response we used the graphical method of Litchfield and Wilcoxon[12] for the estimation of ED50 values.

Influence on body temperature of immobilised rats

White rats weighing 150 to 200 g. were placed in a container made from small-mesh brass netting divided into ten longitudinal compartments for individual rats. The animals were provided with rectal thermocouples, and temperatures recorded once before and 10 times after the subcutaneous injection of the drugs (in saline 0·1 ml./100 g. body weight) with intervals of 15 minutes. The frequency distribution of the average temperature variation was studied in a series of 221 observations. This average was negative (0·38°), because of the fall of body temperature under the circumstances. The average fall in the ten measurements exceeded a value of 1·5° only 8 times. If this value was exceeded a hypothermic effect was considered to have occurred. The frequency of such an effect in series of ten observations was used for the estimation of ED50 values according to Litchfield and Wilcoxon[12].

Respiration in unnarcotised rats

The rat was placed in a cage of mesh wire with a springsteel tape encircling the thorax. One end of the tape was attached to the top of the cage and the other to the voice coil of a modified loudspeaker system,* functioning as a transducer. The cone was removed from

* Philips AD3500M.

the voice coil to prevent it from picking up noise. By this apparatus the thorax movements were converted into electrical potentials which were led to a penwriter, via a symmetrical R.C. filter circuit with a time constant of 0·1 second.

Four rats weighing from 250–300 g. were put in the apparatus and alternately connected with the recording unit in a fixed sequence. The respiratory movements were recorded for 30 seconds in each animal. If struggling occurred the recording was continued until an uninterrupted respiration curve of 30 seconds duration was obtained. Then the transducer of the next animal was connected with the recording unit. Each five minutes the complete cycle of 4 animals was repeated, until a total of 7 cycles was obtained. Then the intraperitoneal injections were given in saline per 0·1 ml./100 g. body weight. Immediately afterwards the recording was restored until a run of 20 cycles after the injection was completed. In 42 cases in which only the saline was given, the frequency distribution of the difference between the average respiratory frequencies in both periods, expressed as percentage of the average pre-injection frequency, was studied ($\bar{x} = -1{\cdot}2$, S.D. $= 3{\cdot}9$). Applying the Camp-Meidell Inequality[5] to these data, an all-or-none criterion for respiratory depression could be derived. We found that a diminution of the average frequency to below 85 per cent of the pre-injection value was statistically significant at the 95 per cent level. The frequency of this effect in series of ten observations under different dosages was used for the estimation of ED50 values as described above.

Influence on the blood pressure in unnarcotised rats with experimental renal hypertension.

The blood pressure was taken with a modification of the tail plethysmograph described elsewhere[13]. Hypertension was induced by constriction of one renal artery and ablation of the other kidney[14]. Influence with drugs were investigated as previously described[15].

Antidiuretic activity in rats

Female rats weighing about 150–250 g. were kept in metabolic cages constructed to collect urine without faeces. Urine not voided spontaneously was obtained by manual suprapubic pressure. This method is practically quantitative, as was demonstrated previously[17]. The rats were fasted for 24 hours but allowed water *ad libitum*. Two hours before the injection of the drug (t_1) the bladder was emptied as described and water 2·5 ml./100 g. body weight was given by stomach tube. Two hours later (t_2) the bladder was again emptied. The total of the volume of urine thus obtained and voided spontaneously during the two hours period was noted. A quantity of 5 ml./100 g. body weight of tepid water was then given by stomach tube and the drug was injected subcutaneously in saline 0·2 ml./100 g. body weight. Urine was collected during one hour and expressed at the end of the experiment (t_3).

The total excretion ($t_1 + t_2 + t_3$) was expressed as the percentage of the water load. This amounted to 7·7 per cent of the body weight minus the quantity of the urine produced between t_1 and t_2.

We studied the frequency distribution of this statistic in 78 observations with saline, 18 with morphine and 15 with R875. A diuresis of less than 10 per cent of the load never occurred in the controls. It was easily obtained with suitable dosages of morphine and R875. We considered urine production of less than ten per cent of the water load to be an antidiuretic effect. ED50 values could thus be obtained as described above.

Mydriatic response in mice

We used the method described by Pulewka[16]. The pupil diameter in white mice of an inbred strain, weighing 16–19 g., was measured by means of a microscope with an eye-piece micrometer. Diameters were measured immediately before and 10 minutes after the intravenous injection of the compounds, in saline 0·1 ml./10 g. body weight. The frequency distribution of the difference between both measurements in 100 mice injected with the saline only, was studied. Application of the Camp-Meidell Inequality[5] enabled us to define an all-or-none mydriatic response, significant at the 95 per cent probability level. We estimated ED50 values in the usual manner.

Augmentation of the effect of barbiturates in mice

White mice weighing 18–23 g. were injected subcutaneously with the analgesic. Fifteen minutes later 10 mg. pentobarbitone sodium per kg. body weight was given intravenously in 0·1 ml. saline per 10 g. body weight. The injection time was standardised at 20 $\pm$1 seconds. The animals were then put on their back on a warmed surface (30 $\pm$1°). Mice turning on their feet within 30 seconds were again put on their back. This was eventually repeated twice. If a loss of righting reflexes lasting at least 30 seconds occurred, this was considered as an augmentation of the barbiturate effect. In a series of 80 control observations with saline this all-or-none response never occurred. We computed ED50 values as described above.

TABLE I

ACUTE INTRAVENOUS TOXICITY IN MICE AND RATS
LD50 VALUES IN MG./KG.

Compound	Mice	Rats
R610	25·0 $\pm$4·3 (80)*	12·2 $\pm$2·0 (55)
R875	21·0 $\pm$2·1 (85)	13·0 $\pm$4·1 (80)
R898	30·5 $\pm$5·5 (60)	37·0 $\pm$4·8 (55)
Morphine	151·0 $\pm$20·0 (130)	120 (45)

* Number of animals.

RESULTS

Acute Toxicity

The results of acute toxicity experiments are reported in Table I and Figure 1. The phenomena observed during the work are qualitatively alike for all 4 compounds investigated. Alertness, excitation alternating

with depression, the Straub phenomenon, salivation, convulsions, diarrhoea, haematuria and cyanosis were regularly observed. Death seemed to be due to asphyxia. A rather striking difference between morphine and the synthetics was the degree of katatonia caused in rats. In the case of morphine some rigidity was regularly seen, but high doses of

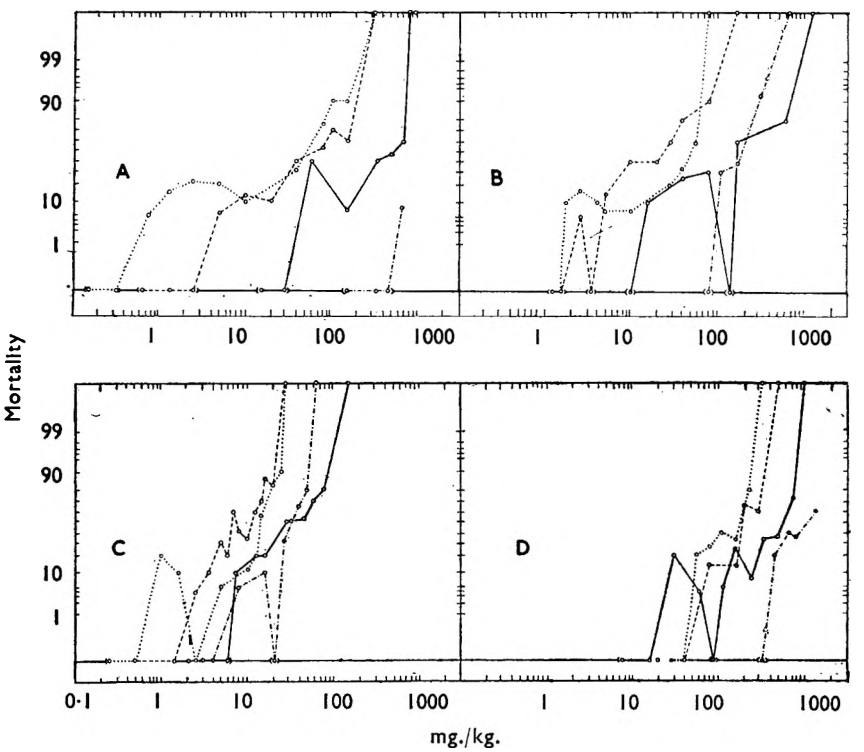

FIG. 1. The acute (A) subcutaneous (B) intraperitoneal toxicity in rats and (C) subcutaneous and (D) oral toxicity in mice. LD50 values. Number of animals in parentheses.

	A	B	C	D
— Morphine	600 (123)	100 (125)	318 ± 130 (140)	450 ± 150 (344)
– – – R610	75 ± 52 (120)	21·3 ± 112 (120)	125 ± 50 (255)	220 ± 75 (135)
. . . R875	50 ± 30 (178)	(179)	140 ± 60 (160)	168 ± 36 (165)
-.- R898	> 640 (55)	162 ± 13 (97)	330 ± 100 (115)	1000 (90)

R610 and its optical isomers made the animals look as if they were carved from wood.

We also injected a number of unnarcotised dogs and cats with larger than analgesic dosages. Dogs were very much depressed, they showed salivation and bradypnoea, while clonic rapidly reversible convulsions also occurred. Cats became restless and excited, with extreme mydriasis and severe convulsions.

Chronic toxicity

The average growth of the rats is shown in Figure 2. In the control group one rat died, and three in each of the experimental groups. In both experimental groups the animals became progressively aggressive towards the end of the experiments. The growth was slightly inhibited by 10 mg. morphine per kg. body weight and by 1·25 mg. R875 per kg. body weight. No gross or miscroscopic alterations were found in a pathological investigation of liver, kidneys, brain, bone-marrow, spleen and heart muscle.

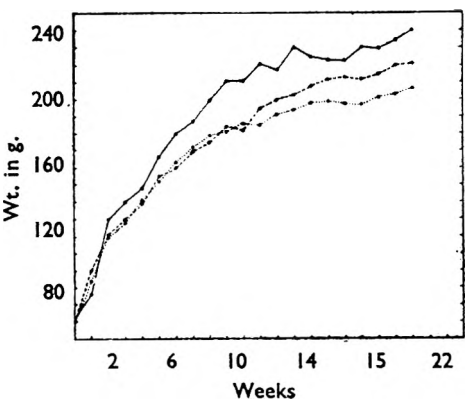

FIG. 2. Growth curves of rats in a subacute toxicity experiment. — saline; - - - R875, 1·25 mg./kg. i.p.; . . . morphine 10 mg./kg. i.p.

Analgesic activity

The results obtained are given in Table II. According to each of the four criteria used R610 is much more active than morphine. The activity of the racemic mixture is to be ascribed to the (+)-isomer, its optical isomer being practically inactive.

TABLE II

ANALGESIC ACTIVITY IN MICE, RATS AND GUINEA PIGS
ED50 VALUES IN MG./KG.

Compound	Hot plate Eddy mice s.c.	Haffner method mice i.v.	d'Amour and Smith rats i.p.	Winder guinea pigs i.p.
R610	1·7 ±0·5 (120)*	0·62 ±0·3 (60)	2·25 ±0·8 (90)	1·4 ±0·5 (80)
R875	0·87 ±0·3 (140)	0·35 ±0·3 (40)	1·3 ±0·4 (120)	0·79 ±0·3 (85)
R898	85 ±19·6 (80)	18 ±5 (40)	116 ±48·7 (54)	81 ±35·6 (35)
Morphine	10·2 ±1·2 (389)	3·79 ±0·7 (200)	7·58 ±0·9 (340)	9·85 ±3·0 (138)

* Number of animals.

Influence on the body temperature, diuresis and respiration in rats, on the pupil diameter in mice and on the pentobarbital effect in mice

The results are given in Table III. The potency ratios are the same as in the analgesia experiments. The pharmacological activity of R610 is to be ascribed to its (+)-isomer.

The activity of the (−)-isomer was greater in the very sensitive mydriatic test than by the other criteria. A dose of 0·11 mg. per kg. body weight R875 (ED50) was given to 60 mice, 8·0 mg. R898 per kg. body weight (ED50) to a second group, and 0·055 mg. R875 per kg. body weight (½ ED50) together with 4·0 mg. R898 per kg. body weight (½ ED50) to a third group also of 60 mice. The numbers of mydriatic responses in the three groups were 26, 31 and 39 respectively. According to the

TABLE III

ED50 VALUES (IN MG./KG. OF THE BASE) OF MORPHINE AND R875 AND R898

Compounds	Hypothermic activity in rats	Depression of respiration in rats	Antidiuretic activity in rats	Augmentation of pentobarbitone effect in mice	Mydriatic activity in mice
610	1·28 ±0·4 (48)*	1·8 ±0·8 (58)	—	1·25 ±0·4 (76)	0·24 ±0·1 (59)
875	0·87 ±0·2 (62)	1·3 ±0·4 (77)	0·37 ±0·06 (59)	1·1 ±0·2 (182)	0·11 ±0·1 (120)
898	inactive up to 40 mg./kg. (30)	inactive up to 40 mg./kg. (20)	—	inactive up to 16 mg./kg. (40)	8·0 ±1·5 (69)
orphine	13·6 ±3·7 (50)	7·2 ±2·9 (50)	2·7 ±0·4 (88)	21·2 ±5·3 (79)	0·56 ±0·15 (130)

* Number of animals.

χ square test this result does not contradict the supposition of simple addition ($P \approx 0{\cdot}15$).

Effect on the blood pressure of unnarcotised rats with experimental renal hypertension

The results are given in Figure 3. R898 was again nearly inactive. All compounds lowered the blood pressure.

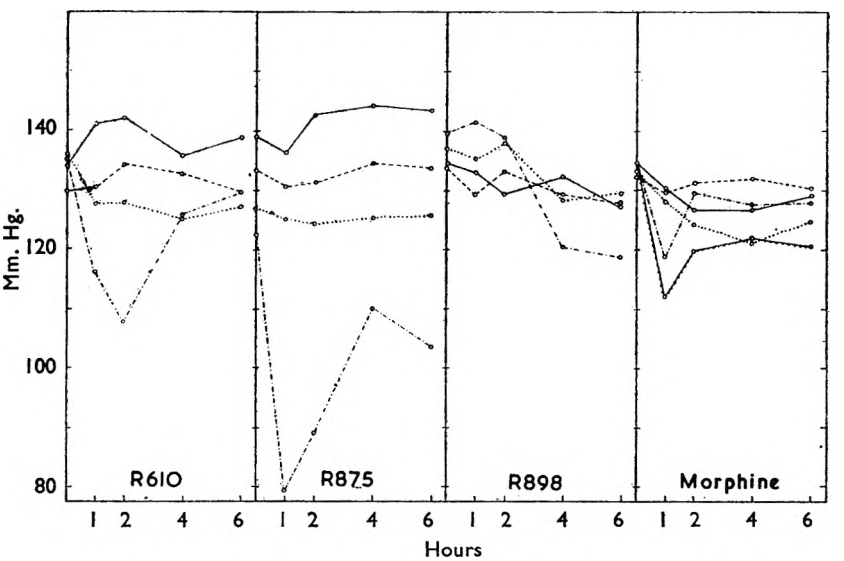

FIG. 3. Influence on blood pressure of rats with experimental renal hypertension. Number of animals in parentheses.

R610, - - - saline (16); — 0·625 mg./kg. (7); . . . 1·25 mg./kg. (8); - · - 2·5 mg./kg. (8)
R875, „ „ (31); „ „ „ (7); „ „ „ (15); „ „ „ (16)
R898 „ „ (8); „ 10 mg./kg. (8); „ 20 mg./kg. (8); „ 40 mg./kg. (8)
Morphine „ (39); „ 5 „ „ (8); „ 10 „ (20); „ 20 „ (20); 40 mg./kg. (13).

Effect on the blood pressure and respiration in allobarbitone narcotised cats and urethane narcotised rats; Nalorphine antagonism

Morphine and R875 both depressed the respiration and caused a fall of the blood pressure. Nalorphine in suitable dosages antagonised these effects.

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THE ASSAY AND IDENTIFICATION OF PYRIMETHAMINE AND ITS PREPARATIONS

By R. E. A. Drey

From the Wellcome Chemical Works, Dartford, Kent

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A gravimetric method of assay for pyrimethamine, using phosphotungstic acid as precipitant, is described. Assay procedures are given for three preparations of the drug, viz. Tablets of pyrimethamine, Tablets of pyrimethamine and quinine, and Powder of pyrimethamine and sulphaguanidine. Tests of identity for pyrimethamine, additional to those described in the British Pharmaceutical Codex, are also given.

Pyrimethamine (2:4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine) is one of a series of substituted 2:4-diaminopyrimidines synthesised by Hitchings and his co-workers in the course of a study of folic acid antagonists[1–4].

The compound is active against a number of micro-organisms; in particular it is a powerful inhibitor of *Plasmodium falciparum* and of *Eimeria tenella*, and is thus both an antimalarial and a coccidiostatic agent.

Pyrimethamine is the subject of a monograph in the British Pharmaceutical Codex 1954. Goodwin[5] and Schmidt, Hughes and Schmidt[6] estimated trace amounts of the drug in biological materials by a colorimetric method depending on complex formation with a suitable dye. A search of the literature failed to elicit any other description of the analytical chemistry of pyrimethamine.

The present investigation arose from a need for a method of assay for certain preparations of the drug; at the same time the opportunity was taken of developing a gravimetric method of assay and of supplementing the tests of identity given in the British Pharmaceutical Codex.

Purity of Reagents

All reagents were of "Analar" or B.P. grade, or other suitable standard of purity. Two samples of pyrimethamine B.P.C. were used for the determination of the gravimetric factor and the specific extinction coefficients (1, loss on drying to constant weight at 105° nil; 2, recrystallised from ethanol and dried to constant weight at 60°). Both gave identical results.

Gravimetric Assay of Pyrimethamine

Preliminary Experiments. As in earlier work with polymyxin[7], preliminary experiments were made to find the most suitable reagent for the gravimetric assay. Of the precipitants examined, phosphotungstic acid gave the best results. In particular it was found that (i) the complex formed is virtually insoluble in dilute acid medium, (ii) the complex is readily obtained in a form suitable for filtering and washing free from excess precipitant; and (iii) the results are highly reproducible and are independent of the batch of precipitant used.

To establish optimum conditions of precipitation, washing and drying of the phosphotungstate complex, the effect of systematic changes in

the following variables was studied: (i) acidity of precipitation medium; (ii) optimum quantity of precipitant; (iii) volume of liquid to be filtered; (iv) conditions of heating before addition of precipitant; (v) acidity of wash-liquid; (vi) volume of wash-liquid required; (vii) optimum drying conditions for the phosphotungstate complex.

The solubility of pyrimethamine phosphotungstate in water at 20° is 0·0005 per cent, whilst in acid medium it is virtually nil; the complex is sparingly soluble in cold ethanol and readily soluble in methanol, acetone and hot ethanol. The complex is readily obtained in the anhydrous state, being unaffected by heating for prolonged periods at 100° or 110°.

As a result of these experiments, the following procedure was adopted.

Assay Process. Weigh out accurately about 50 mg. of pyrimethamine and dissolve in 80 ml. of 5 per cent w/v sulphuric acid. Add 40 ml. of water, heat to boiling and add slowly, with constant stirring, 8 ml. of a 5 per cent w/v aqueous solution of Analar phosphotungstic acid, previously filtered through a Whatman No. 5 paper. Heat gently for a further two minutes, stirring constantly, then allow to stand for one hour and cool to room temperature. Filter through a tared No. 4 sintered glass crucible and transfer the precipitate completely on to the filter with three 20 ml. portions of 2 per cent w/v sulphuric acid. Finally wash the residue on the filter with three 20 ml. portions of water. Dry at 50° for two hours, or over phosphorus pentoxide *in vacuo* for not less than four hours, then heat at atmospheric pressure at 110° for 1½ hours, cool and weigh.

Each g. of residue is equivalent to 0·2040 g. of $C_{12}H_{13}N_4Cl$.

In 22 determinations using three samples of Analar phosphotungstic acid (loss on drying to constant weight at 110° = 6·00, 7·79 and 10·78 per cent, respectively), the standard error of the mean ($100s/\bar{x}\sqrt{n}$) was found to be 0·094 per cent. Results of comparative assays by the B.P.C. method and the gravimetric method are given in Table I.

TABLE I

ASSAY OF PYRIMETHAMINE BY GRAVIMETRIC AND B.P.C. METHODS

	Per cent $C_{12}H_{13}N_4Cl$ recovered	
	Gravimetric method	B.P.C. method
Sample A, determination 1 ..	100·5	99·3
" " " 2 ..	99·97	99·2
" " " 3 ..	99·77	98·7
" " " 4 ..	100·2	99·0
Sample B, determination 1 ..	99·86	98·6
" " " 2 ..	100·0	99·2
" " " 3 ..	100·2	98·9
" " " 4 ..	100·2	98·4

ASSAY OF PREPARATIONS OF PYRIMETHAMINE

Tablets of Pyrimethamine

Assay Process. Weigh and powder 20 tablets. Weigh out accurately a quantity of powder, equivalent to about 50 mg. of pyrimethamine and add 40 ml. of 5 per cent w/v sulphuric acid. Heat to approximately

50° and maintain at this temperature for 30 minutes, stirring intermittently. Cool, filter through a Whatman No. 2 paper and wash the filter with two 20 ml. portions of 5 per cent w/v sulphuric acid. Complete the determination as described under the assay for pyrimethamine, commencing with the words ". . . add 40 ml. of water, heat to boiling and . . ."

Results of assays of two production batches of tablets of pyrimethamine, each tablet containing 25 mg. of pyrimethamine and 100 mg. of a mixture of inert (i.e. non-nitrogeneous) tablet bases, are given in Table II.

TABLE II

ASSAY OF TABLETS OF PYRIMETHAMINE (25 MG.)

Tablet sample No.	Sample of phosphotungstic acid used	Weight of pyrimethamine found per average tablet (mg.)	Per cent of stated strength
Batch I, container 1 ..	A	25·08	100·3
" " " " ..	"	25·28	101·1
" " " 2 ..	"	24·92	99·7
" " " " ..	B	25·13	100·5
Batch II, " 1 ..	"	25·22	100·9
" " " " ..	"	25·27	101·1
" " " 2 ..	"	25·34	101·4
" " " " ..	A	25·41	101·6

Tablets of Pyrimethamine and Quinine

The tablets for which a method of assay was required contained: pyrimethamine 5 mg.; quinine dihydrochloride B.P. 300 mg.; tablet bases 8 mg. The tablets were available both in a sugar-coated and an uncoated form.

Preliminary Experiments. The proposed method of assay is based on the observation that in 0·1 N hydrochloric acid solution the wavelength of maximum absorption for pyrimethamine coincides closely with the wavelength of minimum absorption for quinine dihydrochloride, whilst the latter compound has a characteristic absorption band at 347 mμ, at which wavelength pyrimethamine is virtually transparent. The absorption curves are shown in Figure 1.

The extinction coefficients with reference to the anhydrous compounds are as follows:—

	270·5 mμ	347 mμ
Pyrimethamine B.P.C.	317	0·06
Quinine Dihydrochloride B.P.	17·1	134·6
equivalent to Quinine	20·95	164·8

The values obtained for the specific extinction coefficient of quinine alkaloid at 270·5 mμ and 347 mμ (minimum and maximum, respectively), may be compared with the corresponding values given in the literature[8]: Quinine 270 mμ, 21·12; 347 mμ, 164·1.

The extraction of quinine dihydrochloride from the tablets with 0·1N hydrochloric acid presents no difficulty. On the other hand the extraction of pyrimethamine with this solvent is a slow process in the cold, since the solubility at 20° is only 0·10 per cent. By heating to about 50°, solution is greatly facilitated and it was found that so heating the tablets afforded a

convenient means of extraction. Prolonged heating at 55° had a negligible effect on the ultra-violet absorption characteristics of standard solutions of both drugs in 0·1N hydrochloric acid.

Solutions of pyrimethamine in 0·1N hydrochloric acid obey Beer's law over the range of concentration investigated (0 to 1·5 mg./100 ml. for pyrimethamine) so do solutions of quinine in 0·1N hydrochloric acid[8–10]. To test the validity of the assumption that in a solution of pyrimethamine and of quinine dihydrochloride in 0·1N hydrochloric acid the contributions to the total absorption made by the respective components are additive, a number of solutions containing known amounts of pyrimethamine and of quinine dihydrochloride in the ratio of about 1:60 were prepared. These solutions were suitably diluted and their optical densities at 270·5 mμ and 347 mμ determined. Results of two determinations are given in Table III.

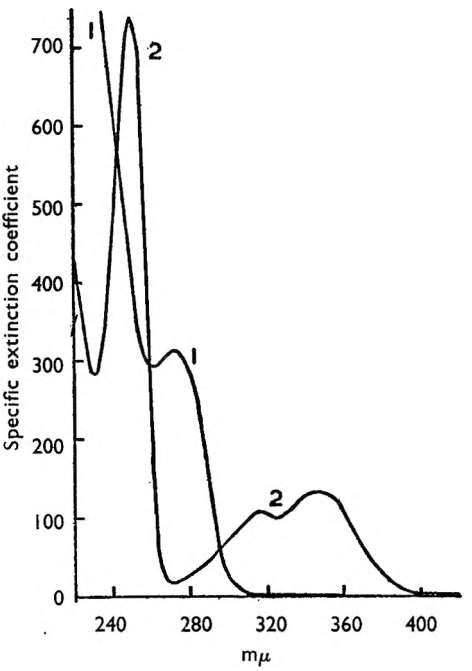

FIG. 1. Ultra-violet absorption spectra of pyrimethamine and of quinine dihydrochloride in 0·1N hydrochloric acid.

1. Pyrimethamine B.P.C.
2. Quinine dihydrochloride B.P.

Tablet bases and the coating material (sugar) had no effect on the spectrophotometric determinations; thus the specific extinction coefficient of sucrose B.P. in 0·1 N hydrochloric acid at the respective wavelengths was found to be:

Wavelength	*E*(1 per cent, 1 cm.)
270·5 mμ	0·0028
347 mμ	0·0003

Thus, the spectrophotometric assay should be applicable to both coated and uncoated tablets, and this was proved in practice.

Assay Process. To 20 tablets add approximately 300 ml. of 0·1N hydrochloric acid. Heat to about 50°, and maintain at this temperature until the tablets have disintegrated, then continue heating still at 50° for a further 1½ hours. Cool and filter through a sintered glass filter of No. 3 porosity. Transfer all the residual solid matter from the flask on to the filter with small portions of 0·1N hydrochloric acid. Wash the filter with the same acid and dilute to 500 ml.

TABLE III

ASSAY OF STANDARD SOLUTIONS OF PYRIMETHAMINE AND QUININE DIHYDROCHLORIDE

	Solute	Taken (mg./100 ml.)	Found (mg./100 ml.)	Per cent Recovered
Determination 1.	Pyrimethamine	0·3856	0·3864	100·2
	Quinine dihydrochloride	23·63	23·69	100·2
Determination 2.	Pyrimethamine	0·39105	0·3865	98·86
	Quinine dihydrochloride	23·58	23·56	99·98

Dilute 10 ml. of filtrate to 500 ml. with the same acid and determine the optical density of this solution at 270·5 mμ in 1 cm. quartz cells, using 0·1N hydrochloric acid as reference liquid.

Let the optical density at this wavelength be d_1.

Transfer 25 ml. of the diluted solution to a graduated 200 ml. flask and make up to volume with 0·1N hydrochloric acid. Determine the optical density at 347 mμ in 1 cm. quartz cells.

Let the optical density at this wavelength be d_2.

Since the contribution made by the pyrimethamine to the total absorption at 347 mμ is negligible, the weights per average tablet of quinine dihydrochloride and of pyrimethamine are given by the following expressions:—

Weight of quinine dihydrochloride (anhydrous), in mg. per average tablet $= 742{\cdot}9\ d_2$.

Weight of pyrimethamine (anhydrous), in mg. per average tablet $= 39{\cdot}432\ d_1 - 40{\cdot}076\ d_2$.

Assay results for three samples of Tablets of Pyrimethamine and Quinine are given in Table IV.

TABLE IV

ASSAY OF TABLETS OF PYRIMETHAMINE AND QUININE

	Constituent determined	Weight of constituent found per average tablet (mg.)	Per cent of stated strength
Sample 1 (uncoated)	Pyrimethamine	5·02	100·4
	Quinine dihydrochloride	296·7	98·9
Sample 2 (sugar-coated)	Pyrimethamine	5·08	101·6
	Quinine dihydrochloride	300·9	100·3
Sample 3 (sugar-coated)	Pyrimethamine	5·07	101·4
Determination 1 ..	Quinine dihydrochloride	293·5	97·8
Sample 3 (sugar-coated)	Pyrimethamine	5·08	101·6
Determination 2 ..	Quinine dihydrochloride	295·6	98·5

If a smaller number of tablets is to be assayed, or if the tablets are of a different strength, or have a different pyrimethamine: quinine dihydrochloride ratio, it will be necessary to modify the dilutions. The appropriate expressions for relating the assay results to the optical densities can be readily computed from the extinction coefficients at 270·5 mμ and 347 mμ.

Powder of Pyrimethamine and Sulphaguanidine

This preparation is used in veterinary practice as a coccidiostatic agent in admixture with a suitable diluent. The powder is made up of

pyrimethamine 6 parts (1·186 per cent w/w), sulphaguanidine B.P. 500 parts (98·814 per cent w/w).

Preliminary Experiments. The spectral characteristics of the constituents of the mixture are not sufficiently distinct to permit their estimation by measurement of total ultra-violet absorption at two wavelengths. Pyrimethamine could not be assayed without at least a partial preliminary separation of the constituents of the mixture. A quantitative separation can be made by direct extraction of the pyrimethamine from the powder with chloroform, in which approximate solubilities at room temperature are: pyrimethamine, 0·75 per cent; sulphaguanidine B.P. 0·0010 per cent.

TABLE V

ASSAY OF PYRIMETHAMINE IN POWDER OF PYRIMETHAMINE AND SULPHAGUANIDINE

	Pyrimethamine found (per cent)	Per cent of stated strength
Sample A, determination 1 ..	1·194	100·7
" " " 2 ..	1·200	101·2
Sample B, determination 1 ..	1·150	97·0
" " " 2 ..	1·144	96·5

TABLE VI

ASSAY OF SULPHAGUANIDINE IN POWDER OF PYRIMETHAMINE AND SULPHAGUANIDINE

	Titration with 0·1M sodium nitrite		Micro-determination of S		
	Sulphaguanidine B.P. found (per cent)	Per cent of stated strength	S found (per cent)	Equivalent to sulphaguanidine B.P. (per cent)	Per cent of stated strength
Sample A, determination 1	97·68	98·9	13·75	99·59	100·8
" 2	97·92	99·1	13·58	98·38	99·6
Sample B, determination 1	99·11	100·3	13·54	98·08	99·3
" 2	98·17	99·4	13·70	99·24	100·4

Assay for pyrimethamine. Weigh out accurately approximately 0·6 g. of powder into a glass-stoppered flask and shake mechanically for 30 minutes with 40 ml. of previously dried and redistilled chloroform. Filter through a dry Whatman No. 1 paper and wash the flask and filter with one 20 ml. and one 10 ml. portion of redistilled chloroform. Combine the filtrates and evaporate to dryness. Take up the residue in a mixture of 100 ml. of 0·1N hydrochloric acid and 10 ml. acetic acid B.P., and dilute to 500 ml. with 0·1N hydrochloric acid. Determine the optical density of the resulting solution at 272·5 mμ in 1 cm. quartz cells, using 0·1N hydrochloric acid as reference liquid. The specific extinction coefficient of pure pyrimethamine in 0·1N hydrochloric acid is E(1 per cent, 1 cm.) (272·5 mμ) = 320.

Assay for sulphaguanidine. Determine the sulphaguanidine content of the powder either by the B.P. method for sulphaguanidine (each ml. of 0·1M sodium nitrite is equivalent to 0·02323 g. of $C_7H_{10}O_2N_4S,H_2O$), or from the sulphur content of the powder (each g. of S is equivalent to

7·243 g. of $C_7H_{10}O_2N_4S,H_2O$). Pyrimethamine does not interfere in either determination.

Results of assays for two samples of Powder of pyrimethamine and sulphaguanidine are given in Tables V and VI.

IDENTIFICATION

The following tests of identity, in addition to those in the British Pharmaceutical Codex, are applicable to the characterisation of pyrimethamine.

Action of Alkaloidal Precipitants

A 0·2 per cent w/v solution of pyrimethamine in 5 per cent w/v sulphuric acid gives a positive reaction to the usual alkaloidal precipitants

Ammonium reineckate is particularly useful for characterising the drug, since it affords a crystalline derivative from dilute acid solution. The test is conveniently carried out as follows:—

Dissolve 10 mg. of pyrimethamine in 30 ml. of 0·5N sulphuric acid and add 10 ml. of previously-filtered 1 per cent w/v aqueous solution of ammonium reineckate. The complex crystallises out slowly in the form of fine pale red needles. Allow to stand for one hour, filter at the pump and wash the crystals with two 20 ml. portions of water. The complex decomposes at about 138°.

The phosphotungstate and picrate complexes of pyrimethamine may be useful in the identification of pyrimethamine in biological and toxicological specimens. These complexes are appreciably less soluble in water and in dilute mineral acids than the reineckate and are thus suited for the identification of small amounts of the drug; the approximate decomposition temperatures are: phosphotungstate 211° (no charring); picrate 239°.

2:4-*Diacetyl Derivative*

Heat under reflux for 30 minutes 1 g. of pyrimethamine with 2 ml. of a 1:1 glacial acetic acid-acetic anhydride mixture. Cool, add 50 ml. of water, shake, allow to stand for five minutes and decant the supernatant liquid. To the residual solid in the flask add 40 ml. of a 2 per cent w/v aqueous solution of sodium bicarbonate and heat gently for a few minutes. Cool thoroughly and allow to stand for not less than thirty minutes. Filter at the pump, wash well with water and recrystallise from 50 per cent ethanol.

Melting point of the crystals after drying at 100°: 172°. Found: C, 57·75; H, 5·17; N, 16·88; Cl, 10·66; $C_{16}H_{17}O_2N_4Cl$ requires C,57·74; H, 5·15; N, 16·84; Cl, 10·66 per cent.

Identification of Pyrimethamine in Preparations

Tablets of pyrimethamine. Powder one or more tablets and weigh out a quantity of material equivalent to 10 mg. of pyrimethamine. Add 30 ml. of 0·5 N sulphuric acid and heat at 50° for 30 minutes. Cool, filter and complete the test as described for pyrimethamine, commencing

with the words ". . . add 10 ml. of a previously-filtered 1 per cent w/v aqueous solution of ammonium reineckate. . . ."

Powder of pyrimethamine and sulphaguanidine. Extract 0·85 g. of powder (equivalent to 10 mg. of pyrimethamine) as described in the assay for pyrimethamine. Treat the residue obtained on evaporation with 1 per cent w/v ammonium reineckate solution and isolate the complex as described for pyrimethamine.

No method has so far been developed for the identification of the drug in tablets of pyrimethamine and quinine beyond computation of the ratio of the optical densities at 270·5 mμ and 347 mμ in the assay process, viz.

$$\frac{d_1}{d_2} = 1{\cdot}33$$

Chromatography

Pyrimethamine may be identified by its R_F value on a paper chromatogram. The system *n*-butanol (50 ml.)—water (50 ml.)—citric acid (1 g.), used with Whatman No. 1 papers impregnated with a 5 per cent w/v aqueous sodium dihydrogen citrate solution, proposed by Curry and Powell[11] for the toxicological examination of alkaloidal extracts, is suitable for this purpose and may be used to separate pyrimethamine from quinine. The spots are revealed by dipping the papers in a tartaric acid solution of potassium iodobismuthate[12].

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GANGLIONIC BLOCKING ACTION OF DIMETHYLPHENYL-PIPERAZINIUM (DMPP)

By G. D. H. Leach

From the Department of Pharmacology, University of London, King's College

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Dimethylphenylpiperazinium iodide (DMPP) blocks transmission of preganglionic nerve impulses at the superior cervical ganglion of the cat. This effect is augmented by subsequent addition of acetylcholine. It also depresses the emptying reaction, but has no effect on the longitudinal muscle contraction, caused by transmural stimulation of the guinea pig ileum. DMPP appears to block transmission in the same way as does tetramethylammonium, and large doses of acetylcholine.

Experimental

Nictitating Membrane. Cats were anaesthetised with ether and chloralose, 65 mg./kg. Injection to the superior cervical ganglion was retrograde from the right external carotid artery. The post-ganglionic nerves of the ganglion were exposed for stimulation by cutting away the

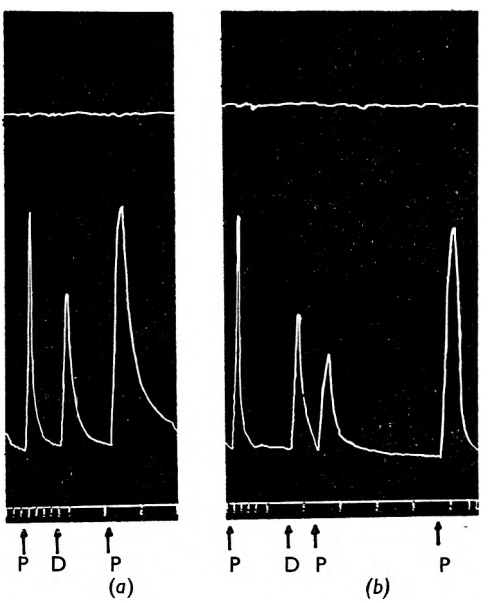


Fig. 1. Cat 3 kg. Responses of right nictitating membrane (lower record) to preganglionic stimulation (P) for five seconds at 3/sec. every two minutes. (*a*) Close arterial injection of 10 μg. D.M.P.P. (D). (*b*) Close arterial injection of 20 μg. D.M.P.P. Control responses of opposite membrane upper record. Time = 30 sec.

tympanic bulla, and the pre-ganglionic nerves by exposing the peripheral end of the cut sympathetic nerve. Movements of both nictitating membranes were isotonically recorded.

Stimulation was by square wave stimuli of 0·03 msec. duration, at 4 to 10 volts, at a frequency shown in the figures.

Guinea pig ileum. The ileum was prepared by Paton's method[2]. The longitudinal movements were measured by an isometric mirror-lever; movements were amplified by photocell d.c. amplifier and recorded on smoked paper by a direct-writing galvanometer.

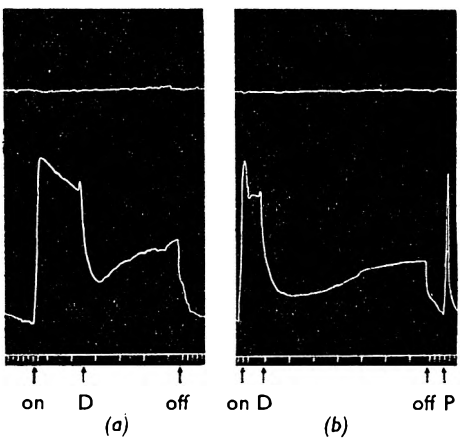


FIG. 2. Cat 3 kg. Effect of D.M.P.P. (D) on the continuously stimulated preganglionic nerve to right nictitating membrane at 3/sec. (*a*) Close arterial injection of 10 μg. D.M.P.P. (*b*) Close arterial injection of 20 μg. D.M.P.P. End of stimulation. Control membrane, upper record. Time = 30 sec.

Changes in volume were recorded hydraulically with a sensitive float recorder; in this way simultaneous records of longitudinal and volume changes were made.

Emptying reaction. An emptying reaction was obtained by distending the ileum with a pressure of about 1.5-2 cm. of water, and stimulating transmurally with square waves of 0·03 msec. duration, at 15 volts, and 1 sec. frequency, for a period of five seconds.

Peristaltic reflex. By raising the hydrostatic pressure for periods of 30 seconds to about 6 to 8 cm. of water from a constant head device, a peristaltic reflex was developed.

Blood pressure. The carotid blood pressure was recorded by mercury manometer and the splanchic nerves to the suprarenal medulla stimulated by an electrode inserted through an opening in the abdominal wall.

RESULTS

Nictitating membrane. 3 kg. cat. The retrograde intra-arterial injection of 5 to 10μg. of DMPP iodide stimulates the ganglion causing retraction of the nictitating membrane (Fig. 1*a*). Doses of 20 μg. stimulate, and produce an immediate blockade of preganglionic nerve stimulation (Fig. 1*b*).

A dose of 10 μg. of DMPP lyses the contracture of the nictitating membrane stimulated preganglionically (Fig. 2*a*); with 20 μg. the blockade is more evident (Fig. 2*b*).

The lysis of contracture of the nictitating membrane stimulated through its preganglionic nerve by 5 μg. DMPP is added to by a dose of 10 μg. of acetylcholine (Fig. 3*a*); under the same conditions 10 μg. of acetylcholine opposes the block caused by 2·5 μg. of hexamethonium.

Guinea pig Ileum. A dose of 1·0 to 1·5 μg./ml. increased the tone and rhythmic movements of the gut subjected to an internal hydrostatic pressure of 1 to 1·5 cm. of water, larger doses depressed (Fig. 4).

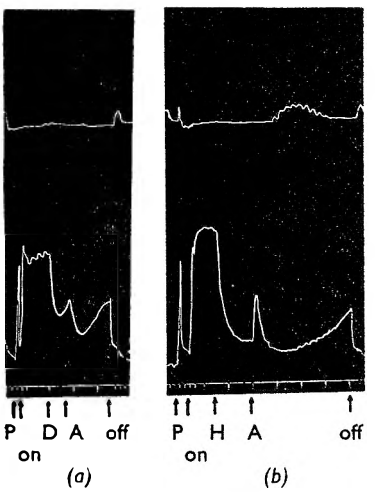


FIG. 3. Cat 3 kg. Effect of subsequent close arterial injections of acetylcholine (A) after ganglionic blockade to the preganglionically stimulated right nictitating membrane (lower record) at 0·5/sec. (*a*) Close arterial injection of 5 μg. D.M.P.P. (D) and response of ganglion to 10 μg. acetylcholine. (*b*) Close arterial injection of 2·5 μg. hexamethonium (H) and response of ganglion to 10 μg. acetylcholine. Animal previously treated with 500 μg. tetra-ethyl pyrophosphate. Time = 30 sec.

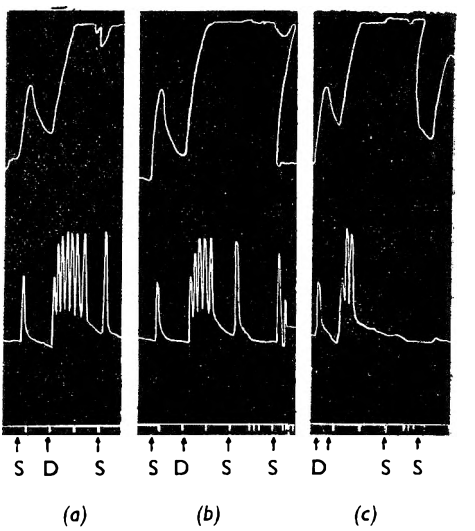


FIG. 4. Effect of D.M.P.P. (D) on the emptying reaction of guinea pig ileum suspended in Krebs's at 32°. Hydrostatic pressure inside lumen 2 cm. of water, transmural stimulation (S) at 1/sec. for five seconds. Upper record longitudinal muscle movements, lower one changes in intestinal volume. (*a*) D.M.P.P. 1·5 μg./ml. (*b*) D.M.P.P. 2·0 μg./ml. (*c*) D.M.P.P. 3·0 μg./ml. Preparation washed between each addition of drug. Time = 30 sec.

Emptying Reaction. Guinea pig ileum. The emptying reaction of the transmurally stimulated ileum is first stimulated by 1·5 to 2·0 μg./ml. and then blocked by 2·5 to 3·0 μg./ml. of DMPP (Fig. 4).

Peristaltic Reflex. Guinea pig ileum. The peristaltic reflex is blocked by 3·0 to 5·0 μg./ml. (Fig. 5).

Blood Pressure responses. Chloralose 65 mg./kg. cat. Atropine 1·5 mg./kg.

With doses of 5 to 10 μg./kg. there is seen a biphasic response; first depressor then pressor (Fig. 6*a*); larger doses of up to 2 mg./kg. still show evidence of the depressor response but with high secondary pressor

components. In the range 0·5 to 2 mg./kg. there is seen some evidence for the onset of a nicotine-like block of the pressor response.

Stimulation of the greater splanchic branch to the left adrenal medulla showed no diminution in pressor effect with increasing doses of DMPP until the range of 1 to 2 mg./kg. was reached (Fig. 6*b*).

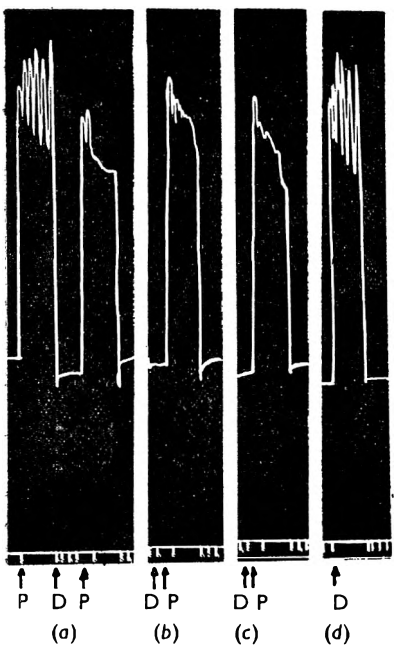


FIG. 5. Effect of D.M.P.P. (D) on the peristaltic reflex (P). Hydrostatic pressure increased to 9 cm. of water. (*a*) Control response and effect of 3·0 μg./ml. D.M.P.P. (*b*) D.M.P.P. 4·5 μg./ml. (*c*) D.M.P.P. 6·0 μg./ml. (*d*) Control response after washing. Time = 30 sec.

DISCUSSION

Small doses of DMPP injected intra-arterially into the superior cervical ganglion stimulates the ganglion. Larger doses, themselves stimulatory, block the effects of stimulating the preganglionic nerves; that the effect is located at the synapse is shown by the integrity of the post-ganglionic stimulation.

The ganglionic block by DMPP differs from that by hexamethonium in being added to by the acetylcholine. These two pieces of evidence, taken together, suggest that DMPP stimulates and depresses ganglionic transmission at the superior cervical synapse in the same way as does acetylcholine and tetramethylammonium[3].

DMPP stimulated both the longitudinal and circular muscle of the guinea pig ileum; the effect on the circular muscle was abolished by hexamethonium, and also by increasing the dose of DMPP. Both observations are consistent with ganglion stimulating and blocking actions.

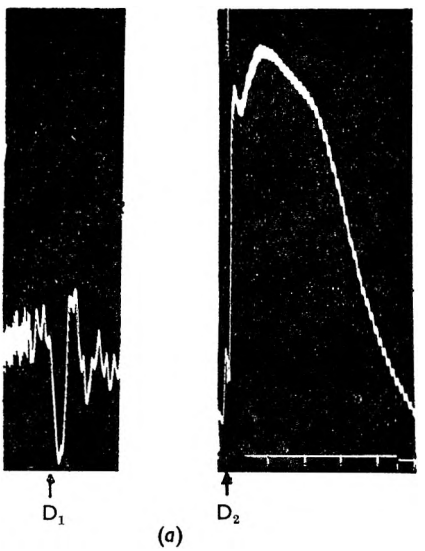
The electrically stimulated emptying reaction of the guinea pig ileum is readily blocked by DMPP, whilst the response of longitudinal muscle is unaltered. To depress the longitudinal contraction, drugs affecting responses at post-ganglionic cholinergic synapses have to be used[2]. Larger doses of DMPP also depress the peristaltic reflex, an effect shared by other drugs which depress ganglionic transmission.

The effects of DMPP on the blood pressure are overwhelmingly stimulatory; very large doses are needed before diminution in response to repeated injections is seen. The effect is reminiscent of nicotine but the stimulatory component is more pronounced.

The adrenal medulla seems to be more easily affected in the cat than the other sympathetic abdominal ganglia; the rise of blood pressure seen after

stimulating the left sympathetic nerve to the adrenal gland is reduced after large intravenous doses of DMPP.

In the atropinised cat (1·5 mg./kg.) small and large doses showed a marked vasodilator component which preceded the rise of blood pressure.



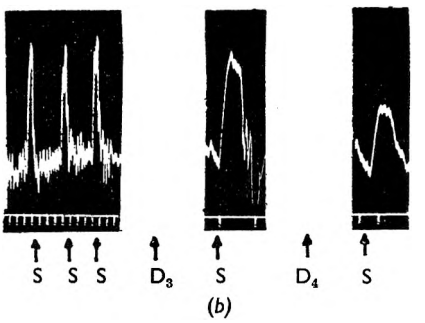


FIG. 6. Cat 3 kg. Blood pressure responses after atropine 1·5 mg./kg. (*a*) 10 μg./kg. and mg./kg. D.M.P.P. (D_1 and D_2), injected intravenously. (*b*) Stimulation of left splanchnic nerve at 6/sec. for thirty seconds (S) before and after 250 and 500 μg./kg., D.M.P.P. (D_3 and D_4) injected intravenously. Time = 30 sec.

It is concluded that DMPP stimulates and depresses ganglia. The drug must be injected directly into the ganglion or used in large doses to demonstrate its blocking action. Its mode of action seems to be similar to that of other drugs which stimulate before depressing the activity of ganglion cells.

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MICROCHEMICAL IDENTIFICATION OF SOME ANTIHISTAMINE DRUGS

By E. G. C. Clarke

From the Department of Physiology, Royal Veterinary College, London, N.W.1

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Crystal and colour tests are described for 50 antihistamine drugs.

Since the discovery[1] some 20 years ago that certain substituted alkylene diamines and alkamine ethers were able to antagonise the effects of histamine *in vivo*, many hundreds of substances have been tested for antihistamine activity. Of these about 50 have come into clinical use.

Chemically these antihistamine drugs are all basic nitrogenous substances. In general the free bases are soluble in chloroform, and the salts soluble in water or alcohol. They can thus be extracted from viscera by the Stas-Otto process, and since they give crystal and colour tests similar to those given by natural alkaloids, their absolute identification is a matter of some importance.

Tests for a number of these substances have been described by Auterhoff[2–4], Haley and Keenan[5,6], Osol and Sideri[7], and Eijkel, Hosfstra and Nauta[8]. It is the purpose of this paper to describe crystal and colour tests for μg. quantities of 50 of these compounds. Because of their similarity in chemical structure, certain substances such as chlorpromazine and ethopropazine, which are not normally used as histamine antagonists, have been included.

Experimental Procedure

Microcrystalline Tests

The hanging microdrop technique described by Clarke and Williams[9] was used. Increased experience with this method has shown that, unless speed is a primary consideration, the pedestal slide originally described is not necessary. The cover slip may be mounted directly on an ordinary cavity slide; evaporation takes place slowly by diffusion through the gum, crystals forming usually within 24 hours. When non-aqueous solvents are used, it is advisable to add the microdrop of the test material to the microdrop of the reagent in order to avoid spreading.

Solutions were made in 1 per cent HCl with the following exceptions: Histaphene and Alfadryl were dissolved in water; phenindamine, Pacatal and Hystryl in 1 per cent acetic acid; Kolton, diethazine and pyrrobutamine in ethanol; and meclozine, buclizine and prochlorperazine in glacial acetic acid. Incidal proved too insoluble in the ordinary reagents to give satisfactory tests. It was therefore treated as follows: 50 mg. of the substance was triturated with 2 ml. of 2N sodium hydroxide solution for several minutes. Anhydrous sodium sulphate 5 g. was then added, and the whole extracted with 25 ml. of chloroform. After filtration the chloroform was evaporated, and the residue dissolved in 2N acetic acid.

TABLE I

Substance	Reagent†	Crystals	Sensitivity μg.
Alfadryl (α-methylbenzhydryl β-dimethylaminoethyl ether HCl)	Lead iodide	Branching rods	0·1
	Potassium tri-iodide (3)	Rods and irregular plates	0·1
Allercur (1-*p*-chlorobenzyl-2-pyrrolidinomethyl-benziminazole HCl)	Zinc chloride	Rosettes or fans of needles	0·1
	Potassium cyanide	Plates	0·5
Ambodryl (Bromazine, 2-dimethylaminoethyl *p*-bromo benzhydryl ether HCl)	Gold chloride	Plates and rods	0·1
	Lead iodide	Plates, sometimes in small rosettes	0·1
Antadril (2-imidazolinylmethyl benzhydryl ether HCl)	Lead iodide	Branching rods	0·1
	Picric acid	Curved or serrated needles	0·1
Antazoline (Antistin, 2-(*N*-benzyl-*N*-phenylaminomethyl) imidazoline HCl)	Sodium carbonate	Needles	1·0
	Potassium cyanide	Needles and rods	0·5
Antergan (*N'*-phenyl-*N'*benzyl *NN*-dimethyl ethylenediamine HCl)	Gold bromide/HCl	Dumb-bells	0·1
	Picric acid	Feathery rosettes	0·1
Anthallan (3-dibutylaminoethyl-4 : 5 : 6-trihydroxybenzo (1 : 2-c) furan-1-one)	Potassium tri-iodide (1)	Rods	0·5
	Picric acid	Bunches of plates	0·5
Buclizine (Longifene, 1-*p*-chlorobenzhydryl-4-*p*-*tert*-butylbenzyl-piperazine HCl)	Gold bromide	Smudge rosettes	0·5
	Mercurous nitrate/HNO_3	Small splinters	0·5
Carbinoxamine (Clistin, *p*-chlorophenyl-2-pyridylmethyl 2-dimethylaminoethyl ether maleate)	Gold chloride	Bunches of rods	0·25
	Lead iodide	Dense rosettes	1·0
Chlorcyclizine (Histantin, Diparalene, 1-(*p*-chlorobenzhydryl)-4-methylpiperazine HCl)	Platinum chloride	Small oily needles	0·1
	Potassium bismuth iodide	Small needles	0·1
•Chlorpromazine (Largactil, 3-chloro-10-(3'-dimethylamino-*n*-propyl) phenothiazine HCl)	Gold bromide/HCl	Bundles of curved needles	0·25
	Gold chloride	Bunches of plates	0·1
Chlorprophenpyridamine (Chlortrimeton, Piriton, 1-(*p*-chlorophenyl)-1-(2-pyridyl)-3-dimethylaminopropane maleate)	Gold chloride	Irregular rods	0·1
	Lead iodide	Dense rosettes	0·1
Chlorthenylpyramine (Tagathen, Chlorothen, *N'*-2-pyridyl-*N'*-5-chloro-2-thenyl-*NN*-dimethylethylene diamine citrate)	Gold bromide	Oily plates or rods	0·1
	Gold chloride	Snowflake rosettes	0·1
Chlortripellenamine (Synopen, *N'*-*p*-chlorobenzyl-*N'*-2-pyridyl-*NN*-dimethylethylene diamine HCl)	Gold bromide/HCl	Irregular rosettes or rods	0·1
	Lead iodide	Rosettes of needles	0·1
•Cyclizine (Marzine, 1-benzhydryl-4-methyl-piperazine HCl)	Potassium bismuth iodide	Bunches of small irregular needles	0·25
	Sodium carbonate	Long plates	0·5
Diethazine (Diparcol, Casantin, 10-(2'-diethylaminoethyl)phenothiazine HCl)	Platinum chloride	Small serrated needles	0·1
	Platinum bromide	Small wedges	0·25
Diphenhydramine (Benadryl, 2-dimethylaminoethyl benzhydryl ether HCl)	Gold bromide/HCl	Needles, some curved	0·1
	Potassium tri-iodide (1)	Plates	0·1
Diphenylpyrilene (Hystryl, 1-methyl-4-piperidyl benzhydryl ether HCl)	Platinum chloride	Rosettes of oily blades	0·5
	Styphnic acid	Feathery rosettes	0·25
•Disipal (2-dimethylaminoethyl-2-methyl benzhydryl ether HCl)	Gold bromide/HCl	Small blades or plates	0·1
	Lead iodide	Sheaves of oily needles	0·1
Doxylamine (Decapryn, 2-dimethylamino-1-2'-pyridyl-1-phenyl ethyl ether succinate)	Lead iodide	Irregular dense rosettes	0·1
	Potassium cadmium iodide	Serrated needles	0·1
•Ethopropazine (Lysivane, 10-(2'-diethylamino-*n*-propyl)phenothiazine HCl)	Mercuric chloride	Curved needles	0·1
	Ammonium thiocyanate	Bunches of plates	0·1
Hibernon (*N'*-*p*-bromobenzyl-*N'*-2-pyridyl-*NN*-dimethylethylene diamine HCl)	Gold bromide/HCl	Rosettes of blades	0·1
	Potassium chromate	Long plates	1·0
Histaphene (2-dimethylaminoethyl-*p*-methoxybenzhydryl ether HCl)	Gold bromide	Needles and plates	0·1
	Gold chloride	Rods and plates	0·25
Incidal (3-*N*-methyl-9-benzyl-tetrahydro-γ-carboline naphthalene-1 : 5-disulphonate)	Mercuric chloride	Rosettes of needles sometimes dense	0·25
	Ammonium thiocyanate	Rosettes	0·5
Linadryl (2-morpholinoethyl-benzhydryl ether HCl)	Picrolonic acid	Rosettes of rods	0·1
	Gold cyanide	Segmented blades	0·1
Luvistin (1-(2-*N*-benzyl-*N*-phenylaminoethyl)pyrrolidine HCl)	Platinum iodide	Needles and plates	0·5
	Gold bromide/HCl	Needles	0·25
Meclozine (Meclisine, Postafene, 1-*p*-chlorobenzhydryl-4-methyl benzylpiperazine HCl)	Gold bromide	Smudge rosettes and oily blades	1·0
	Picrolonic acid	Rosettes of needles	1·0
Mepyramine (Pyrilamine, Anthisan, *N'*-*p*-methoxybenzyl-*N'*-2-pyridyl-*NN*-dimethylethylene diamine maleate	Gold bromide/HCl	Small serrated needles or rods	0·1
	Platinum chloride	Rosettes or fans of needles	0·25

TABLE I—(*continued*)

Substance	Reagent	Crystals	Sensitivity µg.
Methaphenilene (Diatrin, *N'*-phenyl-*N'*-2-thenyl-*NN*-dimethylethylenediamine HCl)	Platinum chloride Picrolonic acid	Serrated plates Rosettes of needles	0·1 0·1
Methapyrilene (Thenylene, Thenylpyramine, *N'*-2-pyridyl-*N'*-2-thenyl-*NN*-dimethylethylenediamine HCl)	Platinum chloride Styphnic acid	Branching needles Stout blades	0·1 0·1
Neo-benoidine (2-dimethylaminoethyl-*p*-methylbenzhydryl ether HCl)	Gold chloride Potassium tri-iodide (3)	Curved needles or rods Segmented rods	0·1 0·1
*Notensil (3-acetyl-10(3'-dimethylamino propyl phenothiazine maleate)	Gold chloride Potassium tri-iodide (1)	Masses of very small rods Small plates	0·1 0·1
*Pacatal (Paxital, 10(*N*-methyl-3-piperidyl-methyl-phenothiazine HCl)	Potassium iodide Ammonium thiocyanate	Small irregular crystals Small blades in bundles	0·1 0·1
Phenindamine (Thephorin, 2-methyl-9-phenyl-1 : 2 : 3 : 4-tetrahydro-2-pyridindene hydrogen tartrate)	Mercuric chloride Potassium tri-iodide (1)	Rosettes, often dense Branching needles	1·0 0·1
Phenyltoloxamine (Bristamine, 2-dimethylaminoethyl *o*-benzylphenyl ether citrate)	Potassium tri-iodide (1) Styphnic acid	Plates and needles Rods, often curved	0·25 0·1
*Prochlorperazine (Stematil, 3-chloro-10[3-(4-methyl-piperazine-1-yl)propyl] phenothiazine maleate)	Gold bromide/HCl Picric acid	Needles Smudge rosettes	0·1 0·1
*Promazine (Sparine, 10(3'-dimethylamino-*n*-propyl)phenothiazine HCl)	Gold chloride Potassium permanganate	Rods Branching needles	0·25 0·25
Promethazine (Phenergan, 10-(2'-dimethylamino-*n*-propyl-phenothiazine HCl)	Platinum bromide Gold cyanide	Very small dense rosettes Plates and rods	0·1 0·1
Prophenpyridamine (Trimeton, 1-phenyl-1-(2-pyridyl)-3-dimethylaminopropane maleate)	Gold bromide/HCl Gold chloride	Small stout needles Serrated blades	0·1 0·1
Pyrathiazine (Pyrrolazote, 10-(2'-pyrrolidinoethyl) phenothiazine HCl)	Platinum chloride Platinum bromide	Small irregular needles Very small irregular needles	0·1 0·1
Pyrrobutamine (Pyronil, 1-(*p*-chlorophenyl)-2-phenyl-4-pyrrolidino butene diphosphate)	Potassium iodide Potassium tri-iodide (2)	Transparent blades Branching needles	0·1 0·1
Soventol (4-(*N*-benzyl-*N*-phenylamino)-1-methylpiperidine HCl)	Potassium chromate Sodium carbonate	Long plates Segmented rods	0·1 0·1
Thenalidine (Sandosten, 1-methyl-4-*N*-(2-thenyl)anilinopiperidine tartrate)	Picric acid Styphnic acid	Branching needles Small rosettes	0·1 0·1 0·1
Thenyldiamine (Thenfadil, *N'*-2-pyridyl *N'*-3-thenyl-*NN*-dimethylethylenediamine HCl)	Gold bromide Platinum chloride	Plates and needles Feathery rosettes	0·1
Thonzylamine (Neohetramine, 2-[(2-dimethylaminoethyl) (*p*-methoxybenzyl)amino] pyrimidine HCl)	Lead iodide Gold chloride	Feathery rosettes Serrated needles	0·25 0·1
Tripelennamine (Pyribenzamine, *N'*-benzyl-*N'*-2-pyridyl-*NN*-dimethylethylene diamine HCl)	Lead iodide Platinum chloride	Snowflake rosettes Rosettes of plates	0·1 0·1
Triprolidine (Actadil, *trans*-1(4-methylphenyl)-1-(2-pyridyl)-3-pyrrolidino prop-1-ene HCl)	Picric acid Platinum bromide	Rosettes Bunches of blades	0·1 0·1

* These substances are not normally employed as histamine antagonists.
† See refs. 9–11 for reagents.

The results are recorded in Table I. The reagents used are those described previously[9–11]. The lead iodide reagent is made by saturating potassium acetate solution (*not* lead acetate solution) with lead iodide. Two further reagents[9–11], namely saturated solutions of mercurous nitrate in water and in N nitric acid, have been introduced. Most of these substances give crystalline precipitates with gold and platinum halides, and with lead iodide, but with few other reagents. As many of the bromaurates and chloraurates are very similar in appearance, it is essential to compare the test crystals with those obtained from an

TABLE II

Substance	Colour	Sensitivity µg.
Formaldehyde-sulphuric acid test 'Marquis'		
Alfadryl	Yellow—green	0·25
Ambodryl	Yellow	0·1
Antadril	Yellow	0·1
Avomine	Purple	0·1
Chlorpromazine	Purple	0·1
Chlorthenylpyramine	Purple	0·1
Diethazine	Purple	1·0
Dimenhydrinate	Yellow	0·1
Diphenhydramine	Yellow	0·1
Diphenylpyrilene	Yellow	0·1
Disipal	Yellow-orange	0·25
Doxylamine	Purple	0·25
Ethopropazine	Purple	0·25
Histaphene	Yellow	0·1
Incidal	Grey-blue	1·0
Kolton	Yellow	0·1
Linadryl	Yellow	0·1
Mepyramine	Mauve	1·0
Methaphenilene	Dull-purple	0·25
Methapyrilene	Black-violet	0·1
Neo-benoidine	Yellow	0·1
Notensil	Yellow—purple	0·1
Pacatal	Purple	0·1
Phenindamine	Grey-green	0·1
Phenyltoloxamine	Purple	0·1
Prochlorperazine	Purple	0·1
Promazine	Purple	0·5
Promethazine	Purple	0·1
Pyrathiazine	Purple	0·25
Pyrrobutamine	Grey-purple	0·5
Thenalidine	Grey-purple	0·5
Thenylidiamine	Black-violet	0·1
Tripelennamine	Red-brown	0·1
Ammonium vanadate test		
Alfadryl	Yellow—green	0·25
Ambodryl	Yellow	0·1
Antadryl	Yellow	0·1
Antazoline	Reddish purple	0·1
Antergan	Reddish purple	0·1
Anthallan	Grey	0·5
Avomine	Green—purple	0·1
Carbinoxamine	Orange	1·0
Chlorpromazine	Green—purple	0·25
Chlorthenylpyramine	Purple—violet—orange	0·1
Chlortripellenamine	Orange	0·5
Cyclizine	Orange	1·0
Diethazine	Green—purple	0·1
Dimenhydrinate	Yellow	0·1
Diphenhydramine	Yellow	0·1
Diphenylpyramine	Yellow	0·1
Disipal	Yellow-orange	0·25
Ethopropazine	Green—purple	0·1
Hibernon	Red-orange	0·25
Histaphene	Yellow	0·1
Incidal	Bright blue	0·5
Kolton	Yellow	0·1
Linadryl	Yellow	0·1
Luvistin	Red-purple	0·1
Mepyramine	Purple	0·25
Methaphenilene	Orange—grey-purple	0·1
Methapyrilene	Black-purple	0·1
Neo-benoidine	Yellow	0·1
Notensil	Brown—purple	0·1
Pacatal	Green—purple	0·1
Phenindamine	Green	0·1
Phenyltoloxamine	Green	1·0
Prochlorperazine	Brown—purple	0·1
Promazine	Green—purple	0·1
Promethazine	Green—purple	0·25
Pyrathiazine	Green—purple	0·1
Pyrrobutamine	Purple	0·5
Soventol	Purple rim	0·5
Thenalidine	Orange—brown	0·5
Thenyldiamine	Olive	0·1
Thonzylamine	Red-violet	0·5
Tripelennamine	Yellow-brown	0·5

TABLE II—(*continued*)

Substance	Colour	Sensitivity µg.
Ammonium molybdate test		
Alfadryl	Yellow—green—blue	0·25
Ambodryl	Yellow	0·1
Antadril	Yellow	0·1
Anthallan	Blue	0·5
Avomine	Black-violet—purple	0·1
Chlorpromazine	Blue-violet—purple	0·25
Chlorthenylpyramine	Purple	0·1
Diethazine	Blue-violet—purple	0·1
Dimenhydrinate	Yellow	0·1
Diphenhydramine	Yellow	0·1
Diphenylpyraline	Yellow	0·1
Disipal	Yellow-orange	0·25
Ethopropazine	Blue-violet—purple	0·1
Histaphene	Yellow	0·1
Incidal	Bright blue	0·5
Kolton	Yellow	0·1
Linadryl	Yellow	0·1
Mepyramine	Violet—brown	0·1
Methaphenilene	Faint brown	1·0
Methapyrilene	Black—green—purple	0·1
Neo-benoidine	Yellow	0·1
Notensil	Brown—purple	0·1
Pacatal	*Purple	0·1
Phenindamine	Green	0·1
Phenyltoloxamine	Green—blue—yellow	0·5
Prochlorperazine	Blue-violet—purple	0·1
Promazine	Dull green—purple	0·1
Promethazine	Black-violet—purple	0·1
Pyrathiazine	*Violet—purple	0·1
Pyrrobutamine	Blue—yellow	0·1
Thenalidine	Grey	1·0
Thenyldiamine	Olive	0·25
Thonzylamine	Red-violet	0·25
Tripelennamine	Yellow-brown	0·1
Selenium dioxide test		
Alfadryl	Yellow—green—grey	0·25
Antadril	Yellow	0·1
Antazoline	Purple	0·1
Anthallan	Grey	0·5
Avomine	*Purple	0·1
Chlorcyclizine	Yellow	1·0
Chlorpromazine	Purple	0·1
Chlorthenylpyramine	Purple	0·25
Diethazine	Purple	0·1
Dimenhydrinate	Yellow	0·1
Diphenhydramine	Yellow	0·1
Diphenylpyrilene	Yellow	0·1
Disipal	Orange—yellow	0·25
Ethopropazine	Purple	0·1
Histaphene	Yellow	0·1
Incidal	Blue-black	0·5
Kolton	Yellow	0·1
Linadryl	Yellow	0·1
Mepyramine	Purple	0·1
Methaphenilene	Purple	0·1
Methapyrilene	Purple	0·1
Neo-benoidine	Yellow	0·1
Notensil	Brown—purple	0·1
Pacatal	*Purple	0·1
Phenindiamine	Brown-orange	0·5
Phenyltoloxamine	Orange	1·0
Prochlorperazine	Purple	0·1
Promazine	*Purple	0·1
Promethazine	*Purple	0·1
Pyrathiazine	*Purple	0·1
Pyrrobutamine	Brown—grey	0·25
Thenalidine	Purple	0·5
Thenyldiamine	Olive	0·5
Thonzylamine	Red-purple	1·0
Tripelennamine	Yellow-brown	0·1
Vitali's test		
Allercur	—/yellow rim/brown rim	1·0
Antazoline	Red/yellow-brown/orange-brown	0·25
Antergan	Orange/yellow/brown	0·5
Anthallan	Purple/brown/brown	0·5

TABLE II—(*continued*)

Substance	Colour	Sensitivity μg.
Avomine	Purple—yellow/yellow/yellow	0·5
Chlorpromazine	Purple—yellow/yellow/yellow	0·25
Diethazine	Purple—yellow/yellow/yellow	1·0
Ethopropazine	Purple—yellow/yellow/yellow	1·0
Incidal	Green—yellow/yellow/yellow	1·0
Luvistin	—/yellow/yellow	0·25
Methaphenilene	Brown/brown/brown	0·5
Methapyrilene	Purple/purple/purple	0·1
Notensil	Purple—yellow/yellow/yellow	0·1
Pacatal	Purple—yellow/yellow/yellow	0·1
Prochlorperazine	Purple—yellow/yellow/yellow	0·1
Promazine	Purple—yellow/yellow/yellow	0·25
Promethazine	Purple—yellow/yellow/yellow	1·0
Pyrathiazine	Purple—yellow/yellow/yellow	0·5
Soventol	—/yellow/yellow	0·5

* A transient green colour may sometimes be noticed.

authentic sample of the drug, and to confirm any tentative diagnosis by means of the colour tests given below.

A number of these crystals will not form if the solution is too concentrated; this is particularly true of the picrates.

Colour Tests

These are made with microdrops on opal glass as previously described[9]. The results obtained are shown in Table II. Most of these substances give colours with all the reagents used, but many of the tests serve only to allocate a compound to a certain class. For example the derivatives of benzhydral ether give bright yellows with the sulphuric acid reagents, while phenothiazine compounds give pinks or purples. Slight differences of shade which may be of value when identifying milligram quantities are of little use when fractions of a microgram only are available.

Discussion

Special mention must be made of diphenhydramine (Benadryl) and dimenhydrate (Dramamine). The former is the hydrochloride and the latter the 8-chlorotheophyllinate of the same base, 2-dimethylaminoethyl benzhydryl ether. They give the same colour reactions and similar microcrystals with the usual reagents. They differ in solubility, the hydrochloride being readily soluble in water, the 8-chlorotheophyllinate insoluble. They may also be distinguished by the following test. A microdrop of a saturated aqueous solution of mercurous nitrate is added to a microdrop of an ethanolic solution of the drug. Dimenhydrate gives bundles of rods, while diphenhydramine gives an amorphous precipitate. The same test may be used to distinguish between the analogous pairs of substances Phenergan (promethazine HCl) and Avomine (promethazine 8-chlorotheophyllinate), and Hystryl (diphenylpyrilene HCl) and Kolton (diphenylpyrilene 8-chlorotheophyllinate).

The identification of meclozine and buclizine also presents certain problems. They give no characteristic colour reactions, and amorphous precipitates only are formed from solutions in water, dilute acids, methanol

or ethanol. The crystals described in Table I were obtained with glacial acetic acid as the solvent, alkaloidal precipitates in general being more soluble in this substance than in water, thus tending to crystallize more readily (Fulton[12]). Another way in which these two substances may be distinguished is by a paper chromatographic separation. If run on a citrate buffered paper with a butanol-water-citric acid solvent[13] they travel much faster than any other drugs included in this study, meclozine having an R_F value of 0·92 and buclizine of 0·94.

The results recorded above were obtained with pure material. All tests were made on the microscale. No quantity greater than 1 μg. was used for any test, nor was any solution employed more concentrated than 1 per cent.

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THE STABILITY OF VITAMIN B_{12}

Protection by Iron Salts Against Destruction by Aneurine and Nicotinamide

By S. L. Mukherjee and S. P. Sen

From the Microbiological Section, Albert David (Laboratories) Ltd., Calcutta

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Vitamin B_{12} has been found to deteriorate progressively in association with aneurine and nicotinamide in solution over a pH range of 4 to 4·5. The deterioration can be satisfactorily prevented by the use of iron salts, in ferrous, ferric and complex form. Salts of cobalt, manganese, copper and lead do not protect the vitamin from the combined destructive influence of aneurine and nicotinamide in solution.

Whereas solutions of pure crystalline vitamin B_{12} have been found to be stable over a period of eighteen months at an optimum pH range between 4 to 4·5 and under normal storage conditions, we have observed, with others, that vitamin B_{12} progressively deteriorates when mixed with other vitamins. In some instances the loss is complete in three months in sterile vitamin B-Complex and liver extract solutions prepared according to prescribed specifications[1] and stored under similar conditions. Light, oxygen or air in the containers, temperature[2], reducing agents[3], and ascorbic acid[4,5] have been shown to be possible factors responsible for the deterioration of vitamin B_{12} potency in liver extract and other pharmaceutical preparations. Blitz, Eigen and Gunsberg[6] in an elaborate study of various commercial B-Complex preparations containing vitamin B_{12} have found it unstable, steadily losing its potency at pH 4·25 in B-Complex solution containing aneurine and nicotinamide, and that perhaps oxidation is not a possible factor for such loss. They have also found that loss of Vitamin B_{12} is a function of the concentration of both aneurine and nicotinamide present. Dony and Conter[7] and Feller and Macek[8] also confirmed the observation of Blitz and his colleagues[6], and have shown that stability of vitamin B_{12} in solution with aneurine and nicotinamide is affected, particularly at elevated temperature when the destruction of B_{12} is at a maximum. Feller and Macek[8] have presented evidence that this destruction of vitamin B_{12} may be due to aneurine decomposition products or to the thiazole moiety.

The role of iron as a stabiliser of vitamin B_{12}, particularly in liver extract solutions, has been elaborated by Shenoy and Ramsarma[9], who have stabilised B_{12}-activity in fractionated liver extract solutions, containing insufficient naturally present iron salts, by the addition of ferric chloride over wide ranges of pH. Smith[10] has also suggested that the presence of iron salts in liver extract exerts a protective action on vitamin B_{12}.

The authors of the present paper, while confirming the work of Blitz and others[6] on the destructive influence of aneurine and nicotinamide on vitamin B_{12}, have also observed that iron salts in general exert a satisfactory protective action in stabilising vitamin B_{12} in B-Complex solution,

containing aneurine and nicotinamide[12]. The concentration of iron salts required is so low that their adoption to stabilise vitamin B_{12} in pharmaceutical preparations like vitamin B-Complex or liver extract solution seems practical.

The procedures, methods, technique and experimental findings are described below.

Experimental

The simple elevated temperature test proposed by Gakenheimer[11] was used to assess the compatibility of the various substances with vitamin B_{12}. This consisted of heating the samples in a suitable buffer of pH 4–4·5 for 4 hours at 100°. Blank compatibility experiments were made with the vials, caps and the individual components before use. Three to 4 ml. of a 2 per cent sodium acetate (analar) solution adjusted to pH 4–4·5 with glacial acetic acid was used as buffer in 10 ml., and care was taken that the pH remained within this range.

TABLE I

Stability of vitamin B_{12} in vitamin B-complex injectable solutions* stored in room temperature, and also in the refrigerator

Time stored at room temperature (27°—33°) months	Vitamin B_{12} μg./ml.	Time stored in the refrigerator (0°—4°) months	Vitamin B_{12} μg./ml.
1	5	1	5
2	2·6	2	5
3	0·5	3	5
4	none	4	5

* 10 ml. in rubber-capped vials: aneurine, 15; riboflavine, 1·5; pyridoxine, 5; nicotinamide, 100; panthenol, 5; choline HCl, 10 mg./ml., vitamin B_{12}, 5 μg./ml., benzyl alcohol, 1·5 per cent.

Room temperature (27–33°) and refrigerator (0–4°) storage stability experiments for a period of 4 months were made. Storage at room temperature was found to affect the stability of vitamin B_{12} similarly to heating at 100° for 4 hours.

The microbiological potency of vitamin B_{12} was determined by the "Cup Plate Assay Method" using *E. coli* Mutant M200 as test organism, developed by Bessel and others[12] and Cuthbertson and others[13]. The accuracy of this microbiological method is ± 10 per cent. Aneurine was assayed fluorimetrically by the thiochrome method of the U.S.P. XV.

Results

From Table I it can be seen that potency of vitamin B_{12} in B-Complex solution on storage at room temperature progressively deteriorates and is destroyed completely within 3 to 4 months, but its potency is stable when kept at 0–4° for 4 months. After heating a similar preparation at 100° for 4 hours the initial B_{12}-activity of 5 μg./ml. was reduced to no activity, and the initial aneurine content from 15 mg./ml. to 11 mg./ml.

In an attempt to ascertain the effect of individual constituents of a vitamin B-Complex solution, 5 μg. of vitamin B_{12} was heated at 100° for 4 hours with, respectively, aneurine, 15 mg./ml., riboflavine 1·5

mg./ml., pyridoxine, 5 mg./ml., nicotinamide, 100 mg./ml., panthenol, 5 mg./ml., choline HCl, 5 mg./ml., and benzyl alcohol, 15 mg./ml. The B_{12}-activity after this treatment was found to be approximately unchanged. Some aneurine loss occurred, the original 15 mg. assaying at 10·6 mg.

TABLE II

EFFECT OF DIFFERENT CONCENTRATIONS OF ANEURINE AND NICOTINAMIDE ON THE STABILITY OF VITAMIN B_{12} IN AN ELEVATED TEMPERATURE TEST

Aneurine mg./ml.	Nicotinamide mg./ml.	Vitamin B_{12} before test μg./ml.	Vitamin B_{12} after test μg./ml.	Loss of Vitamin B_{12} activity per cent	Aneurine after test mg./ml
15	5	5	4	20	11·0
15	10	5	3·75	25	10·8
15	20	5	0·25	95	10·7
15	100	5	none	100	10·8
7·5	100	5	none	100	5·6
2	100	5	0·2	96	1·44
1	100	5	2·0	60	0·8

The results in Table II indicate the effect on vitamin B_{12} potency of varying the concentration of aneurine and nicotinamide in the original solution. It appears that over 95 per cent loss of B_{12} occurs if the aneurine content is between 2–15 mg./ml. and the nicotinamide content 20–100 mg./ml.

To investigate the protective action of iron salts in the elevated temperature test, eight iron salts were heated separately with 15 mg./ml. of aneurine, 100 mg./ml. of nicotinamide and 5 μg./ml. of vitamin B_{12}. The salts used were (0·5 mg./ml.) iron and ammonium citrate, ferrous gluconate ferric alum, ferrous alum, ferric chloride, potassium ferro- and ferricyanides, and ferrous sulphate. There was no loss in B_{12} activity after the test. The aneurine content, however, fell to between 10·6 and 11 mg./ml.

TABLE III

EFFECT OF FERRIC CHLORIDE SOLUTION ON THE STABILITY OF VITAMIN B_{12} IN VITAMIN B-COMPLEX INJECTABLE SOLUTION* AT THE ELEVATED TEMPERATURE TEST AND WHEN STORED AT ROOM TEMPERATURE

Ferric chloride mg./ml.	Initial vitamin B_{12} activity μg./ml.	Vitamin B_{12} activity (μg./ml.) after storage at room temperature for				Vitamin B_{12} activity after test μg./ml.
		1 month	2 months	3 months	4 months	
0·5	5	5	5	5	5	5
0·25	5	5	5	5	5	5
0·1	5	5	5	4	3·5	4
0·05	5					3·5
0·02	5					0·5

* Composition same as in Table I.

Table III shows that the ferric chloride solution of about 0·25 mg./ml. gave protection to vitamin B_{12} over a period of 4 months or under elevated temperature conditions.

The protective effect of 0·5 mg./ml. of the sulphates of cobalt, manganese and copper and also lead acetate was investigated, using the same quantities and conditions as those for iron salts. The B_{12}-activity was completely destroyed.

Discussion

According to Feller and Macek[8] decomposition products of aneurine are likely to cause deterioration of vitamin B_{12}. Our observations indicate that the total loss of aneurine is more or less the same when it is subjected to elevated temperature test conditions in association with vitamin B_{12} alone or in combination with nicotinamide with or without the presence of iron salts, whereas B_{12} potency deteriorates only in presence of aneurine and nicotinamide (Table II). Hence it is felt that the decomposition products of aneurine and nicotinamide are different from those of aneurine alone. Iron salts in association with aneurine and nicotinamide without preventing the decomposition of aneurine protect vitamin B_{12} in a specific way.

Further studies on the mechanism of action of iron salts in preventing or arresting decomposition of vitamin B_{12} in combination with aneurine and nicotinamide are in progress.

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THE PHARMACOGNOSY OF THE ASPIDOSPERMA BARKS OF BRITISH GUIANA*

PART V. THE MICROSCOPY OF THE BARKS OF *Aspidosperma megalocarpon* MUELL. ARG. AND *Aspidosperma quebracho-blanco* SCHLECHT.

BY J. D. KULKARNI†, J. M. ROWSON AND G. E. TREASE

From the Department of Pharmacy, University of Nottingham, and the Museum of the Pharmaceutical Society of Great Britain

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Three samples of barks of each of the two species *Aspidosperma megalocarpon* Muell. Arg. and *Aspidosperma quebracho-blanco* Schlecht. have been examined histologically. The arrangement of tissues, cell structure and cell contents in each bark are accurately described and illustrated. The diagnostic characters by which the two barks may be identified and distinguished from each other are tabulated. Those characters by which the bark of *A. megalocarpon* differs from those of *A. album*, *A. excelsum* and *A. ulei* are also discussed.

THE macroscopical characters of the bark of *Aspidosperma megalocarpon* Muell. Arg. have already been described, illustrated and compared with those of the bark of *Aspidosperma quebracho-blanco* Schlecht[1]. The structure of the latter bark has been incompletely described by several authors[2–8], it was official in the B.P.C. 1934[9] and the Dispensatory of the U.S.A. 1943[10]. The detailed histology of the bark of *A. megalocarpon* is described in this paper, together with a more complete series of drawings than has been presented by earlier workers, to illustrate the structural characters of the bark of *A. quebracho-blanco*.

MATERIALS

Our samples of the bark of *A. megalocarpon* were those previously designated 3A, 3B and 3C collected in British Guiana in 1949, 1950 and 1954 respectively[1]. Our authenticated samples of the bark of *A. quebracho-blanco* were: 6A, collected by Dr. Martin Cardenas in 1953 at Cochabamba in Bolivia; 6B, obtained from the Museum of the Pharmaceutical Society (used by G. R. A. Short for his investigations in 1926[11]); and 6C, collected by the Conservator of Forests, British Guiana in British Guiana in 1954.

EXPERIMENTAL

Line drawings to illustrate the diagnostic characters of outer and inner surfaces and of tissue distribution as seen in smoothed transverse sections of the barks of these two species are given in Figures 1 and 5, A, B and C.

Histology of the Bark of A. megalocarpon (Figs. 1, 2, 3 and 4)

The histological characters of this bark were in general agreement with those of the three other *Aspidosperma* species previously described[1,12,13]

* The subject matter of this communication forms part of a thesis by one of us (J.D.K.) accepted by the University of Nottingham for the degree of Doctor of Philosophy in Pharmacy.

† Nottingham University Research Scholar.

but specific differences were found. The thick cork comprised some fifteen to fifty layers of tangentially-elongated cells, with inner and outer tangential walls thickened and lignified (Fig. 1, D, *ck* and Fig. 3, A, *ck*). A few groups of sclereids, similar to those present in the phelloderm (Fig. 1, C, *g.sc*), were also enclosed in the cork. One to three layers of thin-walled and tangentially-elongated cells formed the phellogen. A well-marked phelloderm contained many sclerenchymatous cells arranged in small groups (Fig. 1, D, *phe* and Fig. 3, A, *phe*), together with thin-walled slightly tangentially-elongated parenchymatous cells. Individual sclereids were isodiametric to tangentially-elongated with thick, lignified walls, traversed by simple or branched pits and with small to somewhat large lumen. Cortex of bands of parenchyma alternating with four to six more or less continuous bands of sclereids (Fig. 1, C and E, *b.sc* and Fig. 3, B, *b.sc*); parenchymatous cells with large intercellular spaces; sclereids similar to those of the phelloderm, a few of which contain a granular material which stained yellow with iodine solution and pinkish-red with Millon's reagent. Towards the inner region of the cortex were discontinuous groups of sclereids and at times a few isolated thick-walled fibres; no defined endodermis or pericycle were found.

Up to about 50 per cent of the bark is of phloem consisting of sieve tissue, parenchyma, fibres, medullary rays and sclereids. In the outer half of the phloem are groups of sclereids, and the cells of the medullary rays are sclerotic when traversing these groups; the inner half of the phloem contains no sclereids. The sieve tubes have oblique, compound sieve plates on the end walls (Fig. 2, D, E and F, *s.p*, Fig. 3, E, *s.p* and Fig. 4, A, *s.p*) and are less readily distinguished in the outer part of the phloem. Phloem parenchyma consists of thin-walled cells, with a few intercellular spaces, some vertical walls reticulately thickened and with compound pits. Fibres are scattered throughout the phloem and a few are embedded in the sclereid groups of the pericyclic region. The greater number of fibres with narrow lumen are mostly isolated (Fig. 2, C, D, E and F, *p.f* and Fig. 3, C, D and E, *p.f*) or very rarely in groups of two to three fibres (Fig. 2, B, *g.p.f*); a smaller number with large lumen are always isolated (Fig. 2, E, *p.f.l* and Fig. 4, A, *p.f.l*). Both types of fibres, which measure R and T = 28 to **80** to 134 μ and H = 600 to **1140** to 1650 μ are spindle shaped, with bluntly pointed ends and thick, lignified, stratified walls traversed by a few simple pits along which splitting may have occurred. A crystal sheath surrounds the isolated phloem fibres and groups of fibres, except when these are embedded in groups of sclereids. The medullary rays are very wavy, are one to two cells in width but becoming up to three cells wide (Fig. 2, A, *e.m.r*) towards the periphery of the phloem; they are 25 to 45 cells in height; individual cells are straight to somewhat wavy in outline and contain starch granules. When these medullary rays pass through groups of sclereids, the cells become sclerotic (Fig. 2, A and B, *sc.m.r* and Fig. 3, C, *sc.m.r*) with thick pitted walls and narrow lumen.

In the cortical and phloem parenchyma are abundant simple or 2- to 4-compound starch granules; individual granules with eccentric hilum

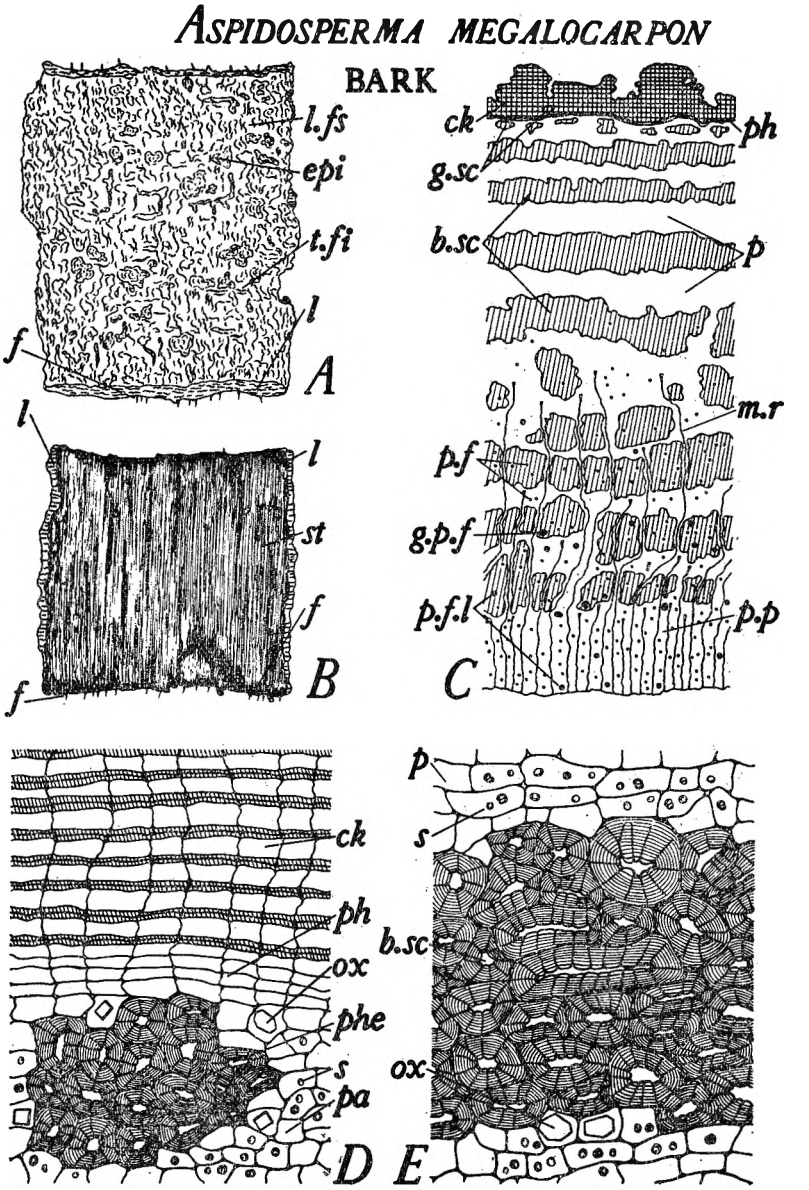

FIG. 1. *Aspidosperma megalocarpon* bark, macroscopical characters and T.S.:— A, outer surface × ¼; B, inner surface × ¼; C, smoothed T.S. × 12; D, cork, phellogen and phelloderm; E, cortex; D and E, × 200; *b.sc*, band of sclereids; *ck*, cork; *epi*, epiphyte; *f*, fibre; *g.p.f*, group of phloem fibres with narrow lumen; *g.sc*, group of sclereids; *l*, laminations; *l.fs*, longitudinal furrow; *m.r*, medullary ray; *ox*, crystal of calcium oxalate; *p*, cortical parenchyma; *pa*, cortical parenchyma found associated with phellodermic sclereids; *ph*, phellogen; *phe*, phellodermic sclereids; *p.f*, isolated phloem fibre with narrow lumen; *p.f.l*, isolated phloem fibre with large lumen; *p.p*, phloem parenchyma; *s*, starch; *st*, longitudinal striation; *t.fi*, transverse fissure.

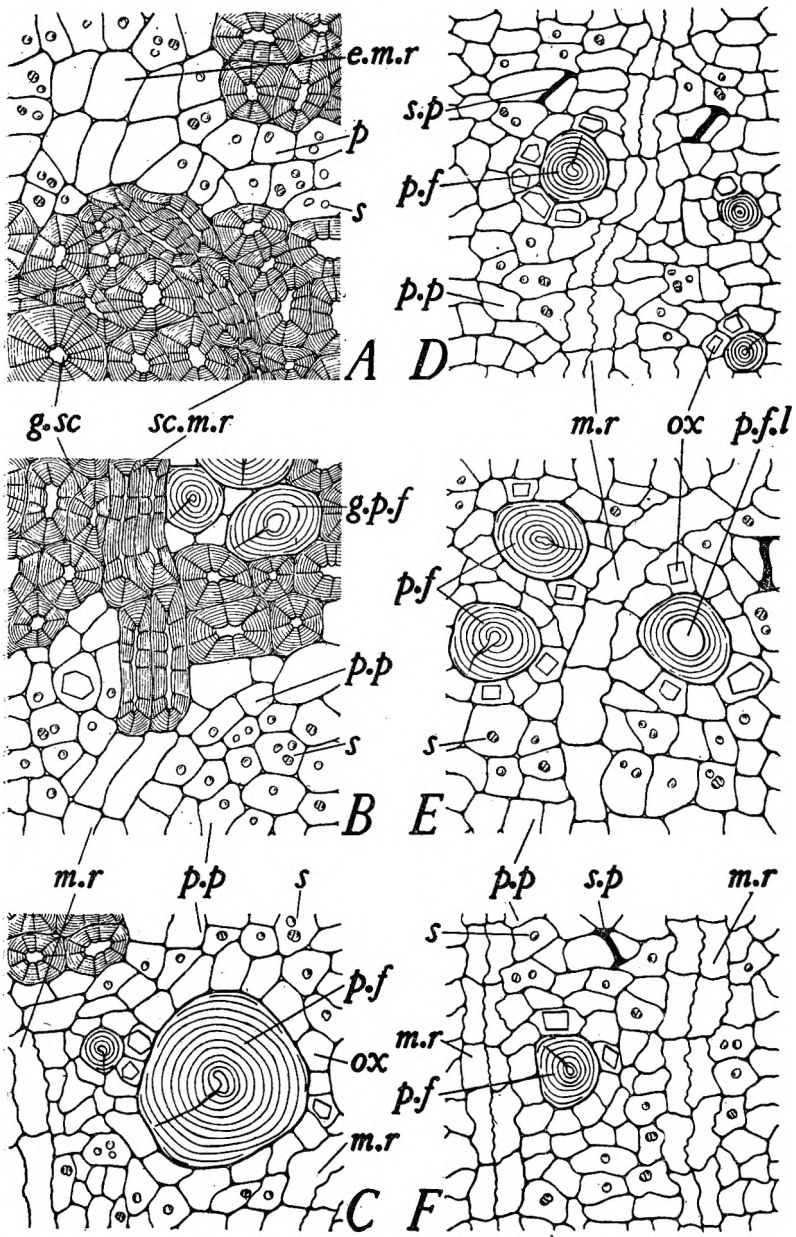

FIG. 2. *Aspidosperma megalocarpon* bark in T.S.:—A, innermost cortex and outermost phloem; B, outer phloem; C, D and E, phloem; F, innermost phloem; all × 200; *e.m.r*, end of medullary ray; *g.p.f*, group of phloem fibres with narrow lumen; *g.sc*, group of sclereids; *m.r*, medullary ray; *ox*, crystal of calcium oxalate; *p*, cortical parenchyma; *p.f*, isolated phloem fibre with narrow lumen; *p.f.l*, isolated phloem fibre with large lumen; *p.p*, phloem parenchyma; *s*, starch; *sc.m.r*, sclerotic medullary ray; *s.p*, sieve plate.

are spherical, ovoid or plano-convex and up to 32 μ in diameter (Figs. 1, 2, 3 and 4, *s*). Calcium oxalate, in square, rectangular or obliquely rectangular prisms or small cubes up to 55 μ, occurs as a crystal sheath around the fibres or groups of fibres (Figs. 1, 2, 3 and 4, *ox*) and, at times, in association with the sclereid groups.

Powder. The powder is very light brown in colour and exhibits characters typical of those structures described above. These include the cork cells which are polygonal in surface view and slightly reddish brown in colour, with thick, lignified walls. The sclereids occur in masses the individual cells being 25 to **75** to 115 μ in length and 15 to **45** to 60 μ in width; they are thick walled and lignified, the lumen is narrow but is somewhat larger in those sclerotic cells containing granular contents (Fig. 4, B, *sc.g*) which stain with iodine solution and with Millon's reagent. The phloem fibres are usually broken during the powdering of the bark; fragments with very narrow lumen, either isolated or in groups of two to three, preponderate; fewer fragments of isolated fibres with large lumen are present, which may be associated with sclereids. The calcium oxalate crystal sheath surrounding these fibre fragments or groups of fragments is readily detected (Fig. 4, B, *f.l.s*, *g.p.f* and *p.f*). Calcium oxalate crystals (Fig. 4, B, *ox*) are also present in association with a few of the groups of sclereids. Phloem parenchyma (Fig. 4, B, *p.p*) associated with cells of the medullary rays (Fig. 4, B, *m.r*) and cortical parenchyma (Fig. 4, B, *p*) of tangentially-elongated cells all contain starch granules.

Detailed Histology of the Bark of A. quebracho-blanco (Figs. 5, 6 and 7)

The general histological characters of this bark were in agreement with those exhibited by the three barks of the genus, described previously and already compared with the bark of *A. quebracho-blanco*[1,12,13]. The distribution of the tissues in *A. quebracho-blanco* differs from those described in this present paper for *A. megalocarpon* in a number of important respects. Thus, the cork is composed of about twenty to seventy-five layers of cells which are pronouncedly elongated (Fig. 5, C, *ck*, Fig. 6, A, *ck* and Fig. 7, A, *ck*). The phelloderm is well marked as a somewhat discontinuous band of sclereids about eight to twelve cells in radial breadth, together with a small amount of parenchyma. In the cortex the abundant sclereids are arranged in a number of masses (Fib. 5, C, *g.sc*, Fig. 6, B, *g.sc* and Fig. 7, B, *g.sc*). The phloem, comprising up to about 70 per cent of the thickness of the bark, may be divided into three very unequal zones. The greater part contains abundant sclereid groups, also fibres with narrow lumen which are mostly isolated or very rarely in groups of two fibres and at times embedded in the sclereid groups; medullary rays become sclerotic when passing through these sclereid groups. Towards the inner region of phloem, isolated fibres with narrow lumen are present but sclereid groups are absent. The innermost region contains isolated fibres with narrow lumen and a few isolated fibres with large lumen, sclereid groups are absent. The medullary rays (Fig. 6 and 7, *m.r*) are very wavy, due to displacement by groups of sclereids; they are one to three cells in width but becoming up to five cells wide (Fig. 6,

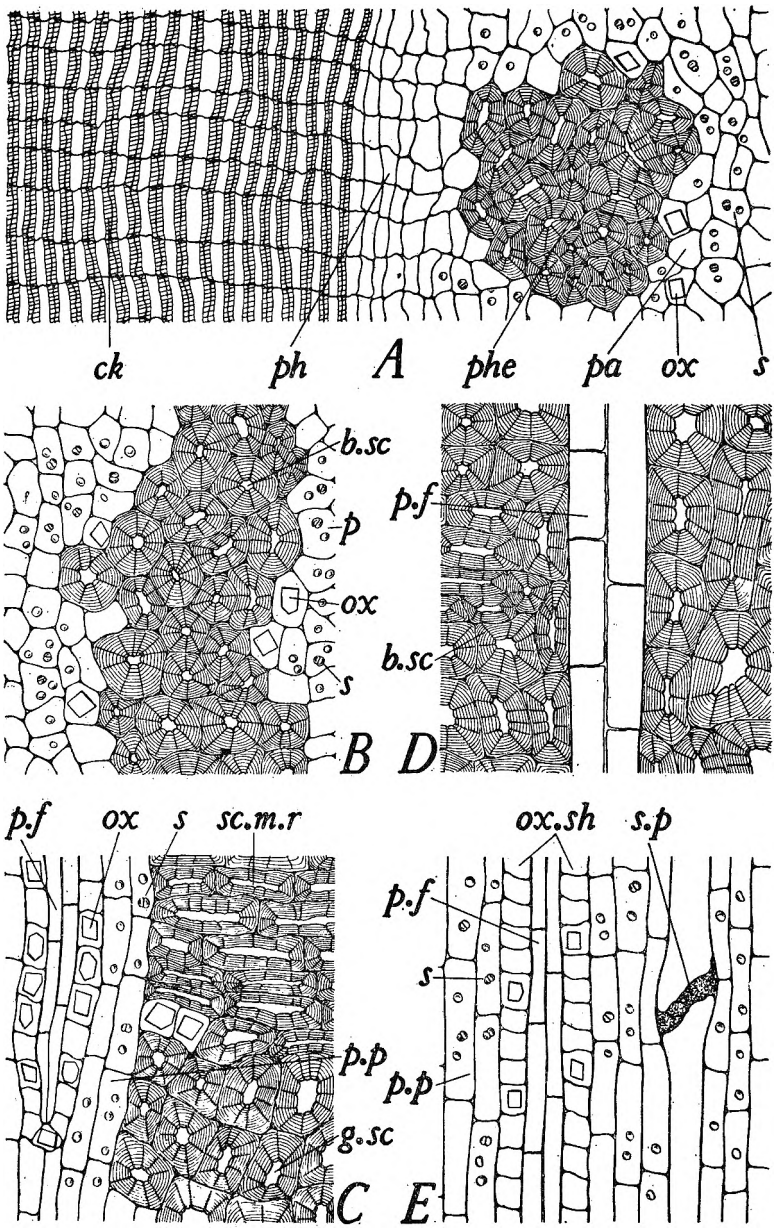

FIG. 3. *Aspidosperma megalocarpon* bark in L.S.:—A, cork phellogen and phellodermi; B, cortex, C, outer phloem; D and E, phloem; all × 200; *b.sc*, band of sclereids; *ck*, cork; *g.sc*, group of sclereids; *ox*, crystal of calcium oxalate; *ox.sh*, calcium oxalate crystal sheath; *p*, cortical parenchyma; *pa*, cortical parenchyma found associated with phellodermic sclereids; *ph*, phellogen; *phe*, phellodermic sclereids; *p.f*, isolated phloem fibre with narrow lumen; *p.p*, phloem parenchyma; *s*, starch; *sc.m.r*, sclerotic medullary ray; *s.p*, sieve plate.

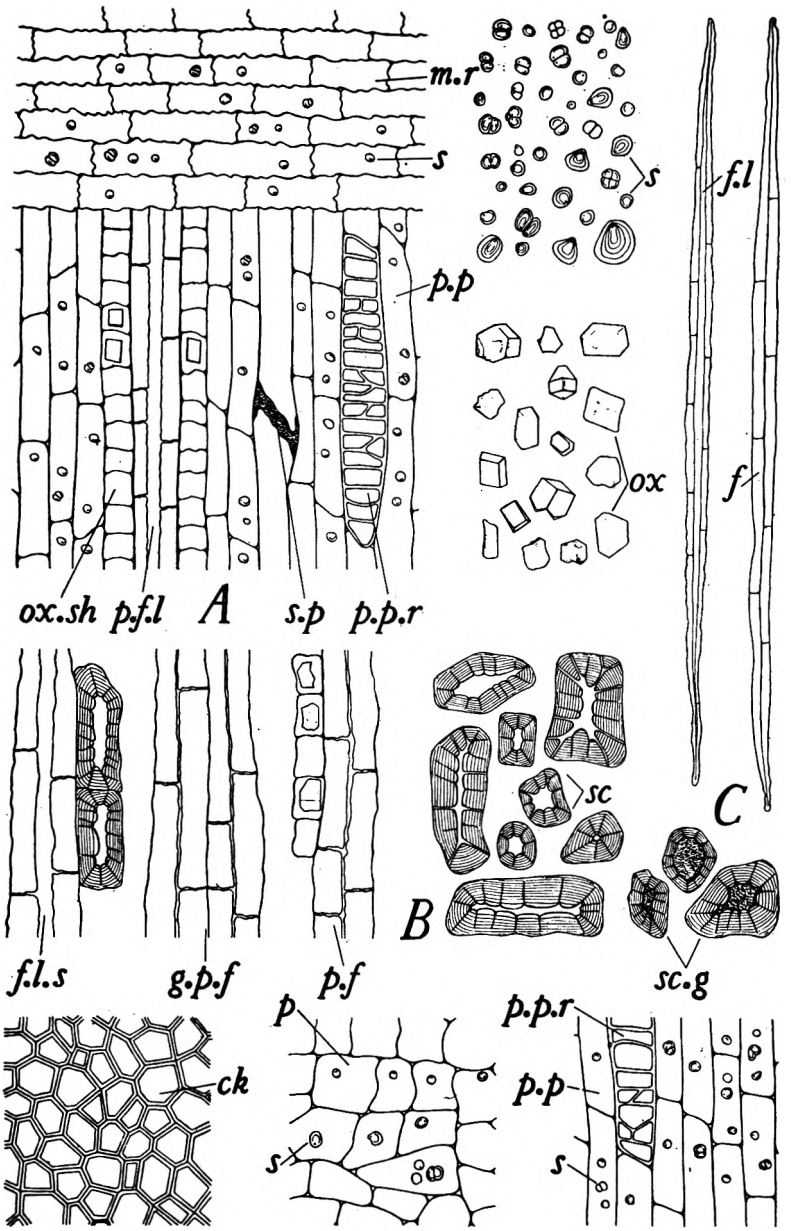

FIG. 4. *Aspidosperma megalocarpon* bark in L.S., powder and macerate:—A, innermost phloem × 200; B, various components as seen in powder × 200; C, fibres, isolated by maceration × 50; *ck*, cork; *f*, phloem fibre with narrow lumen; *f.l*, phloem fibre with large lumen; *f.l.s*, sclereids attached to large lumened fibre; *g.p.f*, group of phloem fibres with narrow lumen; *m.r*, medullary ray; *ox*, crystal of calcium oxalate; *ox.sh*, calcium oxalate crystal sheath; *p*, cortical parenchyma; *p.f*, isolated phloem fibre with narrow lumen; *p.f.l*, isolated phloem fibre with large lumen; *p.p*, phloem parenchyma; *p.p.r*, phloem parenchyma with vertical walls reticulately-thickened; *s*, starch; *sc*, sclereids; *sc.g*, sclereids with granular material; *s.p*, sieve plate.

C, *e.m.r*) towards the periphery of the phloem and are 16 to 25 cells in height.

The cells of the cork of *A. quebracho-blanco* differ from those of *A. megalocarpon* in being more pronouncedly tangentially-elongated and have walls which are thin and are either unlignified or only slightly lignified. The other cell forms are similar in the two barks: these include the phellogen; the sclereids of phelloderm, cortex and phloem together with those sclereids containing granular contents which stain with iodine and Millon's reagent; the two types of phloem fibres with narrow or wide

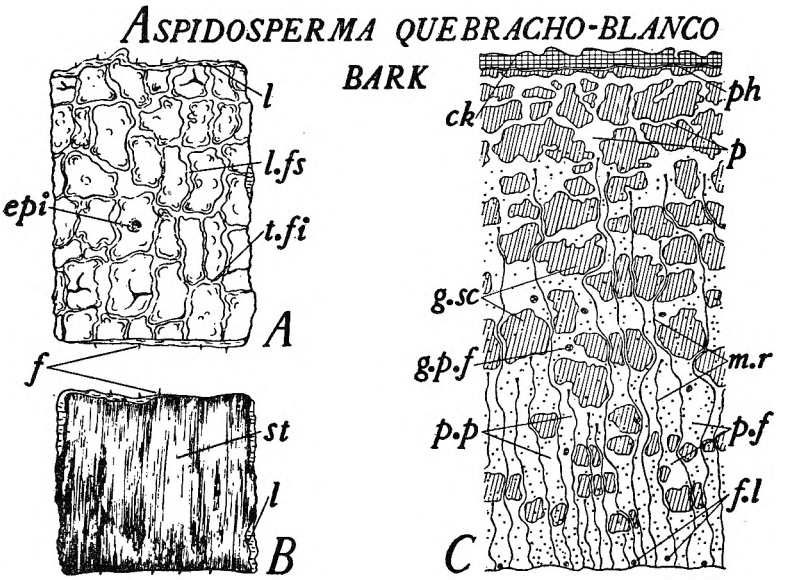

FIG. 5. *Aspidosperma quebracho-blanco* bark, macroscopical characters and T.S.:— A, outer surface × ¼; B, inner surface × ¼; C, smoothed T.S. × 8; *ck*, cork; *epi*, epiphyte; *f*, fibre; *f.l*, isolated phloem fibre with large lumen; *g.p.f*, group of phloem fibres with narrow lumen; *g.sc*, group of sclereids; *l*, laminations; *l.fs*, longitudinal furrow; *m.r*, medullary ray; *p*, cortical parenchyma; *ph*, phellogen; *p.f*, isolated phloem fibre with narrow lumen; *p.p*, phloem parenchyma; *st*, longitudinal striation; *t.fi.*, transverse fissure.

lumen; the sieve tubes; the parenchyma of phelloderm, cortex and phloem, also of the medullary rays which become sclerotic when associated with the sclereid groups. The calcium oxalate crystals and starch grains present agree in their structure and distribution in the two species.

Powder. The powder is light pink to light brown in colour and exhibits the characters described above for the entire bark. The powder resembles that of powdered *A. megalocarpon* bark in the presence of the following: characteristic sclereids in masses, some of which have granular contents that stain yellow with iodine or pinkish-red with Millon's reagent; fragments of two types of phloem fibres with either narrow or wide lumen; sieve tissue; parenchyma containing either calcium oxalate or starch. Calcium oxalate crystals are up to 32 μ in maximum length; starch

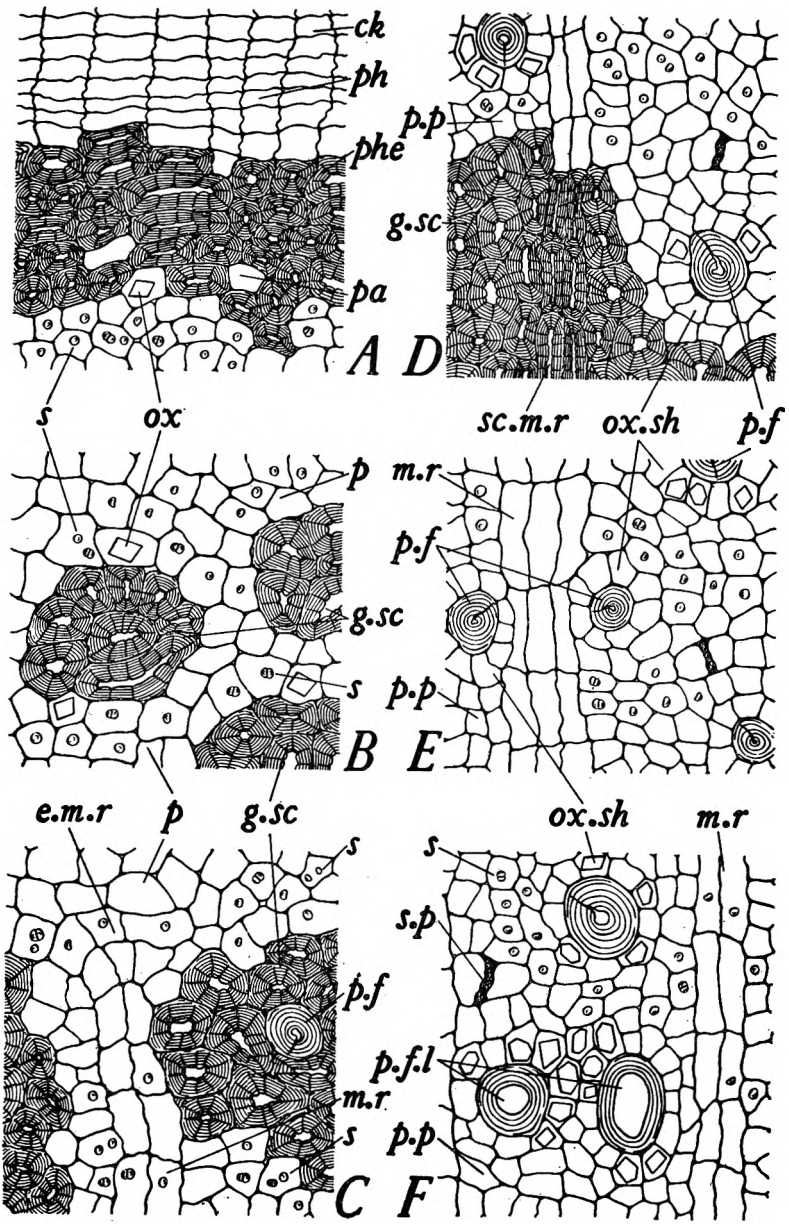

FIG. 6. *Aspidosperma quebracho-blanco* bark in T.S.:—A, cork, phellogen and phelloderm; B, cortex; C, innermost cortex and outermost phloem; D and E, phloem; F, innermost phloem; all × 200; *ck*, cork; *e.m.r*, end of the medullary ray; *g.sc*, group of sclereids; *m.r*, medullary ray; *ox*, crystal of calcium oxalate; *ox.sh*, calcium oxalate crystal sheath; *p*, cortical parenchyma; *pa*, cortical parenchyma found associated with phellodermic sclereids; *ph*, phellogen; *phe*, phellodermic sclereids; *p.f*, isolated phloem fibre with narrow lumen; *p.f.l*, isolated phloem fibre with large lumen; *p.p*, phloem parenchyma; *s*, starch; *sc.m.r*, sclerotic medullary ray; *s.p*, sieve plate.

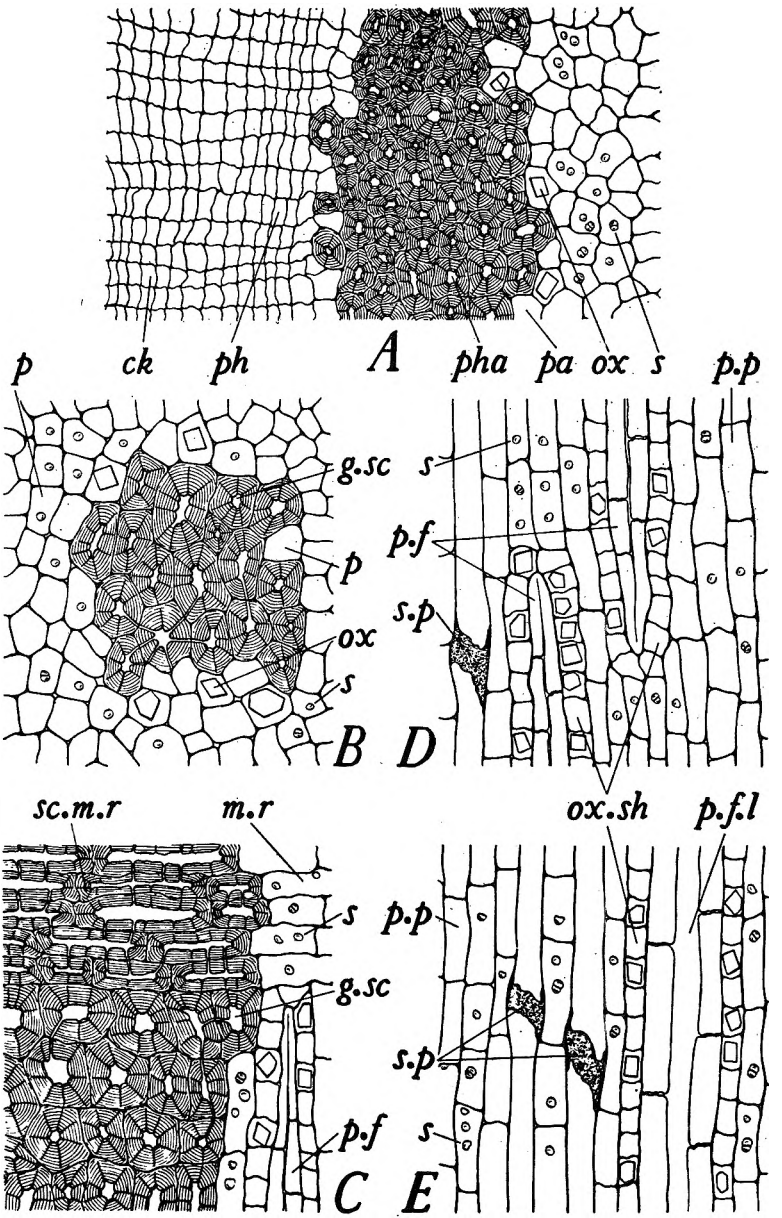

FIG. 7. *Aspidosperma quebracho-blanco* bark in L.S.:—A, cork, phellogen and phelloderm; B, cortex; C, outer phloem; D, phloem; E, innermost phloem; all × 200; *ck*, cork; *g.sc*, group of sclereids; *m.r*, medullary ray; *ox*, crystal of calcium oxalate; *ox.sh*, calcium oxalate crystal sheath; *p*, cortical parenchyma; *pa*, cortical parenchyma found associated with phellodermic sclereids; *ph*, phellogen; *pha*, phellodermic sclereids; *p.f*, isolated phloem fibre with narrow lumen; *p.f.l*, isolated phloem fibre with large lumen; *p.p*, phloem parenchyma; *s*, starch; *sc.m.r*, sclerotic medullary ray; *s.p*, sieve plate.

granules are simple or 2- to 4- compound, the single grains being up to 25 μ in diameter. Powdered *A. quebracho-blanco* bark differs from that of *A. megalocarpon* in the presence of tangentially-elongated cork cells with thin walls which are unlignified or only slightly lignified: fibres with large lumen are not found in association with groups of sclereids. The following cell sizes in *A. quebracho-blanco* also differ: sclereids 28 to **58** to 72 μ in length and 14 to **28** to 44 μ in width; phloem fibres H = 500 to **850** to 1200 μ, and R and T = 25 to **46** to 62 μ. The two powders also differ in the number of sclereids per mg. (S), the number of fibres per mg. (F) and in the S/F ratio[14].

Discussion

The diagnostic characters of the barks of three *Aspidosperma* species, namely *A. ulei*, *A. excelsum* and *A. album*, have been described and compared with those of the bark of *A. quebracho-blanco* in previous communications[1,12,13]. The macroscopical and microscopical characters of the bark of *A. megalocarpon* are here described and compared with those of *A. quebracho-blanco*. A complete discussion of the diagnostic characters of the barks of six *Aspidosperma* species will follow in a subsequent publication.

The diagnostic characters common to the barks of both *A. megalocarpon* and *A. quebracho-blanco* are:—

1. The cork is furrowed and fissured externally and may bear epiphytic lichens or liverworts.
2. Sclereids are present as groups or bands in the phelloderm, cortex and phloem; individual cells with thick, stratified, lignified, pitted walls and relatively narrow lumen.
3. A few sclerotic cells with somewhat large lumen contain a granular material which stains yellow with iodine and pinkish-red with Millon's reagent.
4. Sieve tubes with compound sieve plates on the oblique end walls.
5. Medullary rays narrow and becoming sclerotic when passing through the groups of sclereids.
6. Phloem fibres, of two types, the majority with narrow lumen and either isolated or in groups of two to three; fewer, with large lumen, always isolated. Both types of fibres large, spindle-shaped, with thick, stratified and lignified walls, traversed by a few simple or compound pits.
7. Starch, simple or 2- to 4- compound; individual grains with eccentric hilum, spherical, ovoid or plano-convex.
8. Prismatic calcium oxalate crystals in a parenchymatous sheath surrounding the fibres or groups of fibres, also at times in association with the sclereid groups or bands.

The characters diagnostic of the bark of *A. megalocarpon* are:—

9*a*. Cork cells thickened and lignified on the inner and outer tangential walls.

10*a*. Sclereids arranged in very small groups in the phelloderm and in four to six more or less continuous bands in the cortex.

11*a*. Phloem comprising up to 50 per cent of the thickness of the bark; groups of sclereids are present in the outer half only of this phloem but fibres with either narrow or wide lumen are scattered throughout.

The characters diagnostic of the bark of *A. quebracho-blanco* are:—

9*b*. Cork cells thin-walled and unlignified or very slightly lignified.

10*b*. Sclereids arranged in a more or less discontinuous band in the phelloderm and in groups of varying sizes in the cortex.

11*b*. Phloem comprising up to 70 per cent of the thickness of the bark, throughout the majority of which are abundant groups of sclereids and fibres with narrow lumen; two narrow bands, devoid of sclereids, occur towards the inner region of the phloem, both containing fibres with narrow lumen but the innermost also contains a few fibres with wide lumen.

The thickened and lignified walls of the cork and the occasional sclereid groups enclosed in the corky layer of *A. megalocarpon* distinguish this bark from the barks of *A. ulei*[1] and *A. album*[13] in which the cork cells are unlignified or very slightly lignified; in the bark of *A. excelsum*[12], both lignified and unlignified cork cells are found. The absence of latex canals, presence of sclereids with granular material which stains with iodine and Millon's reagent, the presence of sclerotic medullary rays and the scattered fibres with large lumen throughout the phloem, also seen associated with sclereid groups, all differentiate the bark of *A. megalocarpon* from those of the three species named above.

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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

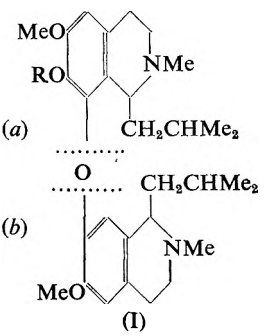
CHEMISTRY

ALKALOIDS

Colchicum Alkaloids in *Littonia modesta* Hook. F. Šantavy. (*Coll. Czech. Chem. Comm.*, 1957, **22**, 652.) A study of the possible evolution of the Liliaceae suggested that the genus *Littonia* was closely related to the genus *Gloriosa* which has already been shown to contain colchicine alkaloids. Accordingly the author investigated a small quantity of the bulbs and seeds of *Littonia modesta* and showed, by paper chromatographic methods, by the Oberlin-Zeisel reaction and by toxicity effects, that colchicine or related substances were present.

J. W. F.

Pilocereine, Structure of. C. Djerassi, S. K. Figdor, J. M. Bobbitt and F. X. Markley. (*J. Amer. chem. Soc.*, 1957, **79**, 2203.) The structure of pilocereine, an alkaloid found in various giant cacti, has been shown to be (I, R = H) in which two substituted tetrahydro-*iso*quinoline nuclei are fused by an ether linkage. The structure was established by cleavage of the diaryl ether linkage of pilocereine methyl ether (I, R = OMe) with potassium in liquid ammonia. This reaction yields different products according to the temperature, but separation was effected in all cases into phenolic and non-phenolic products. The principal phenolic product irrespective of reaction temperature, an oil, $C_{16}H_{25}NO_3$ containing two methoxyl and one *N*-methyl group, and one hydroxyl group, and was identified by degradation as the fragment I(*a*). The second and major base-insoluble component, $C_{15}H_{23}NO_2$ possessed only one methoxyl group, and although alkali-insoluble showed evidence of a hydroxyl group in the infra-red, which could be methylated by prolonged treatment with diazomethane. Degradation of this fragment led to its identification as I(*b*). The structure of pilocereine is unusual in that hitherto known cactus alkaloids are based on a β-phenylethylamine or tetrahydro*iso*quinoline skeleton. The presence of the 1-*iso*butyl substituent in pilocereine appears to be unique in alkaloid chemistry.



(I)

J. B. S.

***Retama raetam*, Webb and Berth, Alkaloids of.** F. Sandberg. (*Svensk. farm. tidsk.*, 1957, **13**, 345.) This Egyptian plant was investigated for alkaloids as it is closely related to the brooms. Two well-known lupine alkaloids were isolated; (+)-sparteine, 0·70 to 0·81 per cent in the tops, and retamine (hydroxy sparteine) 0·21 to 0·25 per cent. These were identified by elementary analysis, melting points, optical activity, paper chromatography and infra-red spectra. Five minor alkaloids were also separated by paper chromatography. The R_F values and melting points of their picrates are given. Stems and leaves collected in March and August showed the same qualitative and quantitative picture; the fruits contained the same alkaloids but in less amounts. J. W. F.

ANALYTICAL

Aconitine and Related Alkaloids, Separation of. C. Mathis and P. Duquénois. (*Ann. pharm. franç.*, 1956, **14**, 749.) For the separation of mixtures of aconitine, benzoylaconine and aconine, two stages of paper chromatography are required. In the first, aconitine is separated from the other alkaloids by placing a spot of a chloroform solution of the alkaloids on the paper, and impregnating the spot with a solution of sodium carbonate saturated with butanol. The chromatogram is developed with a solvent consisting of ammonia, ammonium chloride and butanol. Aconitine remains at the point of application of the alkaloidal solution, while aconine and benzoylaconine appear just below the solvent front. Aconine and benzoylaconine can readily be separated by a second stage, using a mixture of water, butanol and hydrochloric acid as the developing solvent. Aconitine, benzoylaconine and aconine can be separated from their derivatives obtained by benzoylation, by paper chromatography using a mixture of solution of ammonia, sodium carbonate and pyridine as developing solvent. The benzoyl derivatives do not move from the point of application, but aconitine and its products of hydrolysis give spots a little below the solvent front. G. B.

Aloin, Assay for. R. Paris and M. Durand. (*Ann. pharm. franç.*, 1956, **14**, 755.) Commercial aloin, when subjected to chromatography on Arches no. 302 paper by the descending technique at 20° for 12 to 14 hours using butanol and acetic acid as solvent, gives a spot of R_F 0·78 having a reddish fluorescence in ultra-violet radiation, due to aloin. Specimens of aloes, examined in the same way, give also a spot of R_F 0·66, corresponding to *p*-coumaric acid. After treatment with sodium carbonate a further spot, R_F value 0·88 may be observed in ultra-violet radiation. A different chromatogram is obtained with Natal aloes. By carrying out the chromatographic procedure under precise conditions, with an accurately-measured quantity of aloes and solvent, and using a pure specimen of aloin as a standard, the aloin content of the sample may be determined by measurement of the ultra-violet absorption of the aloin spot, using a photoelectric photometer with a suitable filter ("365"). The instrument is set to read zero for the absorption due to the paper base. Using this method, Curaçao aloes was found to contain about 22 per cent of aloin, Cape aloes about 12, and Socotrine less than 1 per cent. Crystalline samples of aloin contained 97 to 99 per cent, but amorphous aloin of German origin contained only about 40 per cent of aloin. It was shown chromatographically that alcoholic solutions of aloin slowly hydrolyse, the sugar liberated being arabinose. G. B.

Aqueous Alkaloidal Solutions for Injection, Assay of, using Oxycellulose. D. A. Elvidge, K. A. Proctor and C. B. Baines. (*Analyst*, 1957, **82**, 367.) The use of oxycellulose as a carboxylic acid cation exchange medium for separating an alkaloid from a bacteriostatic agent such as phenol or chlorocresol so that each may be determined spectrophotometrically is described. The method is simple and rapid and has been applied to 17 different solutions of alkaloids containing phenol or chlorocresol. The method thus overcomes possible interference of these bacteriostats in the spectrophotometric determination of alkaloids. Although oxycellulose is expensive, only 1 g. of it is required for a column which can be used twenty times. The bacteriostat passes through the column which retains the alkaloid which is then eluted with N sulphuric acid. Results could be reproduced to within about $\pm$ 1 per cent, and recovery was generally over 98 per cent. D. B. C.

Poppy Capsules, Ion Exchange in. T. A. McGuire, C. H. Van Etten, F. R. Earle and F. R. Senti. (*J. Amer. pharm. Ass., Sci., Ed.*, 1957, **46**, 247.) Poppy straw was crushed and sifted to remove stems and the capsular material ground and extracted with hot water in a commercial extractor. The extract contained 1 mg. of morphine per ml. Morphine was removed from the extract by passing through a column of cation exchange resin (Duolite C-10). Using 65 ml. of extract per g. of resin, about 94 per cent of the morphine was recovered using the resin in the sodium form, and 89 per cent using the resin in the ammonium form. The alkaloid was eluted from the column with N sodium hydroxide or N ammonium hydroxide, the column being prepared for further use by backwashing with water. The resin showed no loss in capacity after 21 cycles. The eluate from the ammonium resin column was further purified by passing through a column of anion exchange resin (Dowex 1X1) in the hydroxide form. Codeine passed through the column, while morphine and other amphoteric substances were retained. The morphine in a relatively pure form was eluted with N hydrochloric acid followed by water, the final solid product containing 85 per cent of morphine. G. B.

Propyl, Octyl and Dodecyl Gallate, Determination of, in Oils and Fats. H. J. Vos, H. Wessels and C. W. Th. Six. (*Analyst*, 1957, **82**, 362.) From a solution in light petroleum of 50 g. of oil, or fat containing antoxidant, or 25 g. of a fat containing antoxidant together with 25 ml. of gallate-free arachis oil if the fat is coconut, palm-kernel, beef or lard, propyl gallate is extracted with water and the higher gallates with absolute methanol and the extracts analysed spectrophotometrically using ferrous tartrate in a sodium acetate buffer, which is specific for gallates. When absolute methanol is used, the extract still contains some fat and light petroleum which gives a cloudy solution on adding the ferrous tartrate and sodium acetate solution. The resulting coloured complex is therefore extracted with a mixture of equal parts of *iso*amyl alcohol and light petroleum. With the extraction methods described, 95 to 97 per cent recovery of 5 to 10 mg. of the gallates from oils and fats is possible. A modification is described for determining propyl and dodecyl gallates in the presence of each other. D. B. C.

***Rauwolfia serpentina*, Estimation of Alkaloids in.** S. Ljungberg. (*Svensk Farm. Tidsk.*, 1957, **12**, 305.) For determination of total alkaloids, about 2 g. of powdered root or equivalent amount of extract or tablets is damped with sufficient (1 to 3 ml.) of 0·M sodium carbonate solution and then rubbed down with small portions of kieselguhr until the mixture is almost dry and homogeneous, packed in a 10 mm. column and percolated with chloroform until the eluate gives no reaction with Meyers reagent; 250 ml. usually suffices. After evaporation to about 20 ml. on a water bath, and the removal of the rest of the chloroform in a vacuum the dry residue is dissolved in 10 ml. of anhydrous chloroform, two drops of a solution of 0·1 per cent crystal violet in anhydrous acetic acid are added, and the mixture titrated with 0·1N acetous perchloric acid until the colour turns blue. The calculation of the alkaloidal content is based on a mean molecular weight of 380. The determination should be carried out in subdued light. Further purification of the residue did not significantly alter the results. Reserpine was determined spectrophotometrically after separation from the other alkaloids. Three processes of separation are described, viz., chromatography on aluminium hydroxide, paper chromatography, and paper electrophoresis, and the results of these were compared and applied to 16 samples which include admixtures with other drugs and various dosage forms. D. B. C.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Cell Constituents, Synthesis of, from C_2-units by a Modified Tricarboxylic Acid Cycle. H. L. Kornberg and H. A. Krebs. (*Nature Lond.*, 1957, **179**, 988.) A major gap in the knowledge of intermediary metabolism is the process by which 2-carbon compounds, such as acetate and ethanol, can be converted to cell constituents in those organisms, such as bacteria of the genus *Pseudomonas* and many strains of *Escherichia coli* and moulds, which can meet all their carbon requirements from these compounds. The occurrence of a cyclic process, representing a modification of the tricarboxylic acid cycle, has now been established. The stages between *iso*citrate and malate are replaced by reactions in which the main metabolite is glyoxylate. The cycle is thus known as the glyoxylate cycle. The main discoveries in the elaboration of the cycle were as follows: (1) the finding that *iso*citrate, apart from undergoing dehydrogenation, is split enzymatically to form succinate and glyoxalate; (2) the recognition of an enzyme system bringing about the synthesis of malate from glyoxalate and acetyl coenzyme A; (3) the demonstration of the ready occurrence of the combined action of the two enzyme systems in cell-free extracts. The overall effect of one turn of the glyoxalate cycle is the formation, from two molecules of acetate, of one molecule of C_4-dicarboxylic acid. This, together with acetate, can serve as a precursor of many cell constituents. The cycle is therefore a stage in the synthesis of cell material from acetate. It can also account for the net formation from acetate of citric, fumaric and other organic acids in moulds. The key reactions of the glyoxalate cycle have further been demonstrated in *Ricinus* seedlings. In the seedlings it can account for the conversion of fat to carbohydrate. M. M.

BIOCHEMICAL ANALYSIS

Blood Oxygen Saturation, Rapid Estimation of. I. C. Roddie, J. T. Shepherd and R. F. Whelan. (*J. clin. Path.*, 1957, **10**, 115.) This paper describes a simple and rapid spectrophotometric method, using haemolysed blood, for the estimation not only of blood oxygen saturation but also of oxygen content and capacity. The use of a cuvette of small capacity and good wash-out characteristics enables blood samples to be passed in quick succession through the cuvette without removing it from the instrument. A standard spectrophotometer is used and requires no modification other than the fitting of the cuvette into the holder supplied. This technique does not involve the use of isobestic points for oxygenated and reduced haemoglobin in the measurement of capacity, readings being made at the same wavelength, 660 mμ, for capacity and percentage saturation estimations. The method has been used in the measurement of the oxygen saturation of blood withdrawn during cardiac catheterization and of samples taken during such procedures as reactive hyperaemia, indirect heating and nerve block of the forearm. As many as 150 samples have been analysed in the course of an experiment lasting 2 to 3 hours. The results agree extremely well with those obtained with the Van Slyke method. M. M.

Catechol Amines in Urine, Estimation of. H. Weil-Malherbe and A. D. Bone. (*J. clin. Path.*, 1957, **10**, 138.) In addition to the occurrence of adrenaline and noradrenaline in normal human urine, there is present a third catechol

amine, namely 3-hydroxytyramine. To estimate the three amines quantitatively a combination of two fluorimetric methods is used. The acidified urine is first hydrolysed. The catechol fraction is isolated by adsorption on alumina and the basic catechol fraction is then isolated by adsorption on a cation exchange resin. The sum of the three amines is then estimated fluorimetrically by condensation with ethylenediamine. Adrenaline and noradrenaline are estimated, again fluorimetrically, by oxidation with potassium ferricyanide. The amount of hydroxytyramine is thus obtained by difference. Mean recoveries of added catechol amines were 83, 88 and 91 per cent for adrenaline, noradrenaline and hydroxytyramine respectively. The specificity of the method was investigated by paper chromatography and by bioassay. Paper chromatography showed the presence of the three amines in the urine extracts and the absence of interfering substances. There was no evidence for the presence of 3:4-dihydroxyphenylalanine. Bioassay using the rat colon correlated reasonably well with chemical assays. Those using the rat uterus were less satisfactory, presumably due to some interfering substance. This chemical method has been applied to both normal and hypertensive subjects and to a patient with phaeochromocytoma. Hydroxytyramine excretion was more variable from sample to sample than that of adrenaline and noradrenaline in both the normal and the hypertensive groups. The excretion of hydroxytyramine in a case of phaeochromocytoma showed a relatively greater increase than that of adrenaline or noradrenaline. M. M.

Noradrenaline in Urine, Estimation of. W. J. Griffiths and S. Collinson. (*J. clin. Path.*, 1957, **10**, 120.) A fluorimetric method for the clinical estimation of the total combined adrenaline and noradrenaline in urine is described. The principle of the method is to heat the acidified urine to hydrolyse the conjugated catechol amines and then to adsorb the amines on to aluminium hydroxide. Subsequent to elution with a mixture of acetone and ethanol, each sample is concentrated and subjected to paper chromatography, using *n*-butanol-acetic acid-water as the solvent and eluting with dilute hydrochloric acid. The fluorimetric analysis is then made at pH 6 by the method of Euler and Floding (*Acta physiol. scand.*, Suppl. 118, p. 45). The standard error of a single determination was $\pm$ 1·9 μg./500 ml. of urine on amounts of 15 to 40 μg. Recovery of noradrenaline added to the urine was 92 per cent, S.D. $\pm$ 9. Comparison of the results with those obtained by bioassay, showed no significant difference. There was no difference in the total excretion of adrenaline and noradrenaline in a series of normal and hypertensive subjects; the range of excretion being 30 to 150 μg./day. Values of more than 1 mg./day were found in cases of phaeochromocytoma. M. M.

Sugar in Blood and Biological Fluids, Micro-estimation of. I. St. Lorant. (*J. clin. Path.*, 1957, **10**, 136.) This method is based on the oxidation of sugar by potassium ferricyanide, in solutions deproteinised with aluminium tungstate. 0·1 ml. of blood is used and the amount of potassium ferrocyanide formed is estimated as the yellowish-brown molybdenum ferrocyanide, dissolved in a solution of oxalic acid in the presence of trichloroacetic acid. Quantities greater than 1,200 mg./100 ml. can be estimated. The main advantage of this method, over that of Folin and Wu, is the greater stability of the colour, the intensity remaining constant for several hours. M. M.

PHARMACOLOGY AND THERAPEUTICS

Adrenaline and Noradrenaline in the Cat Adrenal, Resynthesis of. K. R. Butterworth and M. Mann. (*Nature, Lond.*, 1957, **179**, 1079.) A study is made of the adrenaline and noradrenaline content of the cat adrenal gland, subsequent to depletion with doses of acetylcholine given intravenously to the atropinised animal. One gland of each animal was used as a control. It was found that 2 days after depletion there was some replacement of noradrenaline but not of adrenaline. At 7 days the noradrenaline level was very much greater than it was initially, whereas there was only a small amount of replacement of adrenaline. By one month the noradrenaline had decreased and the adrenaline has increased to their initial levels. Although it took a month for the adrenaline and noradrenaline to regain their normal amounts and proportions, the total amine content of the glands had returned to its initial level one week after the depletion. These results suggest that, under the conditions of these experiments, adrenaline is synthesised from noradrenaline and not independently. M. M.

Aminitrozole (Acinitrazole); Oral Treatment of Trichomonas Vaginitis. J. Barnes, A. Boutwood, E. Haines, W. Lewington, E. Lister and B. J. Haram. (*Brit. med. J.*, 1957, **1**, 1160.) Of a total of 44 women suffering from trichomonas vaginitis, 23 were treated with aminitrozole 100 mg. 3 times daily by mouth for 10 days and Aci-jel vaginal jelly inserted night and morning for 3 weeks, while 21 served as a control group and received only Aci-jel night and morning for 3 weeks. (Aci-jel is a buffered vaginal jelly with a pH of 4, containing acetic acid, boric acid, oxyquinoline sulphate, ricinoleic acid and glycerin in a vegetable-gum base). Smears were taken at the first visit and after 2, 4 and 6 weeks. Treated cases negative at 6 weeks were re-examined at 12 and 24 weeks. Control cases which remained positive after 4 weeks were given treatment and thus transferred to the treated group. Treated cases positive after 6 weeks and those which relapsed were given further treatment, some patients receiving 3 courses in all. Of 37 patients (23 treated and 14 treated controls) who received treatment with aminitrozole and Aci-jel, 6 were cured of trichomonas vaginitis. None of the control group treated with Aci-jel alone was cured. Two husbands (out of 8 examined) had positive smears; both were treated with aminitrozole and the smears became negative; the wife of one was treated but relapsed after 18 weeks; the wife of the other was cured after treatment with aminitrozole. The authors conclude that the use of aminitrozole with a buffered acid vaginal jelly cannot be recommended for the treatment of trichomonas vaginitis, and aminitrozole alone is unsuitable for this purpose, though there may be a place for it in conjunction with an effective local trichomonicide in chronic cases. S. L. W.

3-Amino-1:2:4-triazole, Protection against X-irradiation by. R. N. Feinstein and S. Berliner. (*Science*, 1957, **125**, 936.) The value of 3-amino-1:2:4-triazole, as a protective agent against ionizing radiation, has been tested in mice. The intraperitoneal injection of 2000 mg./kg. consistently protected a large percentage of mice against 650r of X-rays, and significantly prolonged the survival time of animals that received 750 or 850r. Administered before a 1700r dose, or after any dose of X-rays the compound is without effect. Given 24 hours before irradiation some prolongation of survival time is conferred. A catalase mechanism may in some way be relevant to the radiation protection although the compound itself has not been found to be a catalase inhibitor.

G. F. S.

Angiotonin, Synthesis and Pharmacology of. F. M. Bumpus, H. Schwarz and I. H. Page. (*Science*, 1957, **125**, 886.) Condensation of cbz-L-val-L-tyr azide with L-isoleu-L-his-Me gave a tetrapeptide, the carboxylic acid of which, when condensed with L-pro-L-phe Me by the amide modification of the diethyl-chlorophosphate method, gave the hexapeptide cbz-L-val-L-tyr-L-isoleu-L-his-L-pro-L-phe Me. Removal of the carbobenzyloxy group, condensation with the anhydride derived from cbz-β-Me-L-asp NO_2-L-arg, hydrolysis and hydrogenolysis, gave a biologically active solution containing the octapeptide L-asp-L-arg-L-val-L-tyr-L-isoleu-L-his-L-pro-L-phe. The solution yielded a white solid containing pressor material (4000 units/mg.) and sodium chloride. On two dimensional paper chromatography the material showed the expected amino acids in approximately equivalent concentrations. The product was very active on isolated rabbit uterus. The form of the curve of arterial pressor rise in dogs, cats and rats was identical with that produced by natural angiotonin. Augmentation of the response following injection of ganglion-blocking agents occurred equally with natural and synthetic substances. The responses in pithed cats show that the CNS is not necessary for the action of angiotonin. Evidence is presented which suggests that the site of action is different from that of the usual pressor amines. J. B. S.

Anticoagulants; Clinical Evaluation in Thrombo-embolic Disease. J. M. Neilson and A. W. Mollison. (*Brit. med. J.*, 1957, **1**, 1214.) The anticoagulant properties of cyclocoumarol were reviewed on the findings obtained in its use in 57 patients, and the results were compared with those obtained in 125 patients given ethyl biscoumacetate and 179 given phenindione. Patients given ethyl biscoumacetate or phenindione received 10,000 to 15,000 units of heparin intravenously every 6 hours for the first 24 hours of anticoagulant therapy; those given cyclocoumarol received the same dosage of heparin 6-hourly for 48 hours. It was shown that a therapeutic degree of prolongation of the prothrombin time was effected more quickly with phenindione than with cyclocoumarol; phenindione was as rapid in action as ethyl biscoumacetate. Patients receiving cyclocoumarol and ethyl biscoumacetate showed greater and more frequent fluctuations in prothrombin times during maintenance therapy than those receiving phenindione. The incidence of haemorrhage was shown to be greatest in the cyclocoumarol series. In the three series the response of the prothrombin time to Vitamin K_1 was the same, but in some patients receiving cyclocoumarol the prothrombin time subsequently lengthened and repeated doses of the vitamin were necessary. The authors conclude that phenindione is a more satisfactory and more easily controlled anticoagulant than either ethyl biscoumacetate or cyclocoumarol. S. L. W.

Benzothiadiazine Dioxides as Diuretics. F. C. Novello and J. M. Sprague. (*J. Amer. chem. Soc.*, 1957, **79**, 2028.) A series of benzothiadiazine dioxides has been prepared from 6-acylamino-4-chlorobenzene-1 : 3-disulphonamides by cyclodehydration between the adjacent acylamino and sulphanyl groups. Ring closure is especially facile in the formyl derivatives. The resulting 6-chloro-(3 alkyl or H)-7-sulphanyl-1 : 2 : 4-benzothiadiazine-1 : 1-dioxide are marked inhibitors of carbonic anhydrase, promote renal excretion of sodium and chloride and cause diuresis. Preliminary results in man with 6-chloro-7-sulphanyl-1 : 2 : 4 benzothiadiazine-1 : 1-dioxide (chlorothiazide) substantiate the pharmacological reports. J. B. S.

Chloramphenicol in Acute Respiratory Infection. A. H. Ioannidis and J. M. Murdoch. (*Brit. med. J.*, 1957, **1**, 1157.) Eighty patients with acute respiratory

infection of mixed bacterial origin were treated with chloramphenicol. Thirty-six of the patients had failed to respond to previous treatment with antibiotics. Pre-treatment sputum cultures showed the predominant organism to be *H. influenzae* in 15 cases, pneumococcus in 24 cases, *Staph. pyogenes* in 19 cases; there was no predominant growth in 22 cases. Of the 19 cultures yielding a predominant growth of *Staph. pyogenes*, 17 were resistant to penicillin, 7 to streptomycin, and 8 to the tetracyclines. All were sensitive to chloramphenicol, and 5 to this antibiotic alone. After treatment with chloramphenicol 2 g. daily for 5 days clinical improvement resulted in 77 of the patients, and 3 were unaffected. The infection was controlled in 73 patients for periods of from 1 to 8 months after treatment. In 75 of the patients there were no side-effects; dry mouth occurred in 3, a mild skin rash in 1, and slight transient diarrhoea in 1. No blood dyscrasias were detected. The authors consider that the potential toxicity of chloramphenicol has been overstressed, and that it has a definite place in the treatment of acute and severe respiratory infections, provided it is given in short courses of 10 g. over 5 days; it should not be employed in trivial infections. S. L. W.

Digitalis, Quantitative Tolerance Test. R. M. Nalbandian, S. Gordon, R. Campbell and J. Kaufman. (*Amer. J. med. Sci.*, 1957, **233**, 503.) A quantitative digitalis tolerance test has been developed based on the synergism between calcium and digitalis. It is possible to determine to what level a patient is digitalised. The subject is titrated to an electrocardiographic end point by increasing increments of intravenously administered calcium gluconate. At a critical level of serum calcium the synergism between calcium and the previously administered digitalis produces a transient end point in the electrocardiogram. The electrocardiographic changes can be terminated by the intravenous administration of disodium ethylenediaminetetra-acetic acid, an effective calcium chelating compound. The level of digitalisation is indicated by the amount of calcium required to produce the end point in the electrocardiogram, because of the inverse quantitative relationship between the calcium and digitalis. A rapidly acting, calculated, therapeutic dose of digitalis can be administered safely after termination of the test without jeopardy of digitalis toxicity and its attendant hazards. The test has been used sixteen times on patients in congestive heart failure with no complications. G. F. S.

Himandrine, an Alkaloid from *Himantandra baccata*, Pharmacology of. L. B. Cobbin and R. H. Thorp. (*Austral. J. exp. Biol.*, 1957, **35**, 15.) Himandrine is an alkaloid obtained from the bark of *Himantandra baccata*. Its empirical formula is $C_{36}H_{37}O_6N.HCl$ and it is slightly soluble in water. The structural formula is at present unknown. The LD50 of himandrine, given intravenously to male mice, is 34 mg./kg., death being preceded by convulsions. It causes no alteration in the threshold to leptazol-induced convulsions in mice. It possesses spasmolytic properties of a comparable degree to papaverine against acetylcholine, carbamyl choline, histamine and barium chloride. Given intravenously to cats it causes a depressor response accompanied by bradycardia, neither of which is abolished by atropine or vagotomy. It does not block the actions of adrenaline or sympathetic ganglia nor does it block the cardio-accelerator response to a constant intravenous infusion of adrenaline. It is tentatively suggested that himandrine exerts its cardiovascular effects by suppressing the activity of sympathetic centres in the hypothalamus. M. M.

LETTERS TO THE EDITOR

Ambonestyl (2-Diethyl aminoethyl-*iso*nicotinamide) on Cardiac Cellular Potentials

SIR,—Lanzoni and Clark[1] have reported that 2-diethyl aminoethyl-*iso*nicotinamide (Ambonestyl) is as active as its analogue, procainamide (Pronestyl) in controlling ventricular arrhythmias in dogs. They stated that Ambonestyl did not depress cardiac conduction, nor raise the diastolic threshold for electrical stimulation and produced only a small increase in refractory period. Thus in antiarrhythmic doses it would have none of the therapeutic disadvantages of procainamide. Clark and Etsten[2] have reported the successful clinical use of Ambonestyl in the treatment of patients with ventricular arrhythmias.

However, Sjoerdsma and others[3] found the protective dose in experimental cardiac arrhythmias in dogs and cats varied from 3 to 30 times the corresponding dose of procainamide and also Ambonestyl was much less active therapeutically.

Weidmann[4] and Johnson[5] have shown that quinidine and procaine amide produce characteristic changes in the membrane action potential of myocardial fibres. The dominant feature being a reduction in the maximum rate of depolarisation which they believe to be due to an interference with the "sodium carrying" system. Following the method previously described[4], we failed to observe effects on the guinea pig ventricular action potential comparable with those due to a concentration of 100 μg./ml. of procainamide until a concentration of approximately 500 μg./ml. of Ambonestyl was attained. At this concentration an increase in diastolic threshold and decrease in conduction velocity were invariably observed. No qualitative difference was noticed between the effects of procainamide and Ambonestyl on the action potential. The results were from 7 experiments.

These results give support to those of Sjoerdsma and others[3] for if procainamide and Ambonestyl were equi-active as antiarrhythmic drugs as suggested by Lanzoni and Clark[1] our present findings that Ambonestyl has approximately one-fifth activity of procainamide would lead to the conclusion that hypotheses relating antiarrhythmic potency directly to changes in the membrane action potential[4–6] are incorrect.

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E. A. JOHNSON.
P. A. ROBERTSON.

Department of Pharmacology,
University of Sydney,
N.S.W., Australia.

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Colorimetric Estimation of Vitamin D

SIR,—The well-known colorimetric procedure using antimony trichloride-acetyl chloride reagent was used by us for the estimation of calciferol in pharmaceutical preparations, on the lines described by Dewitt and Sullivan.[1] We observed that better results were obtained by dissolving antimony trichloride as well as vitamin D in chloroform, which has been used as a solvent of choice by Stross and Brealey[2] also.

Published literature on the method indicates that the concentration of acetyl chloride in the reagent is not critical and can be varied between 2 to 5 per cent. We observed that a concentration range of 2·5 to 3·0 per cent of acetylchloride in the reagent was advantageous. If the concentration of acetyl chloride was less than 2·5 per cent, turbidity appeared during the experiment, while if the concentration was more than 3 per cent, the fall in the optical density of the colour was very rapid, giving erroneous results.

Dewitt and Sullivan have recommended that the initial reading of the optical density should be taken at 30 seconds and subsequent readings at one minute intervals up to 6½ minutes, while Stross and Brealey read the optical density only once, at 1½ or 2 minutes. We observed that reading the optical density at 30 second intervals up to 6 minutes is advantageous, firstly because it gives a smoother curve from which the optical density at zero time can be more correctly extrapolated and, secondly, because it is not necessary to purify the oily solutions of calciferol by chromatography as recommended by Stross and Brealey since the interfering sterols, as observed by us, Dewitt and Sullivan and also by Zimmerli and others,[3] affect the colour only after 4 minutes and thus there is no significant change in the value of the optical density at zero time.

The procedure modified as above has been successfully employed for the estimation of vitamin "D" contents of tablets, oily solutions of vitamin D and injection of calcium with vitamin D.

TDE Laboratories,
Ministry of Defence (CGDP),
Government of India,
Kanpur (India).

P. N. LUTHRA.
J. N. TAYAL.

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