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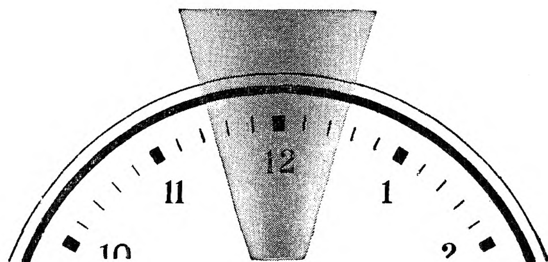

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REVIEW ARTICLE

THE CHEMISTRY OF CORTISONE

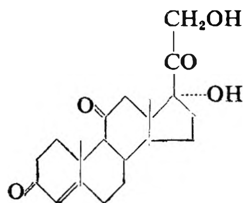
By B. Arthur Hems, D.Sc., F.R.I.C.
Glaxo Laboratories, Ltd., Greenford, Middlesex

The introduction of cortisone into medical practice in 1949[1] had a great impact both on the general public, when it received the news through the newspapers, and on scientists; on the former because of the dramatic improvements that the drug effected in rheumatoid arthritis, a widespread and crippling disease, and on the latter because of the biological implications of the medical discovery and the difficulties implicit in the technical preparation of the drug. Cortisone is perhaps the most complicated of the synthetic organic compounds yet introduced into medical practice, not so much for the actual number of atoms in the molecule, but because of intrinsic difficulties arising from the specific nature of the substituents, the variety of stereochemical complications and the occurrence of a sensitive and highly reactive side chain.

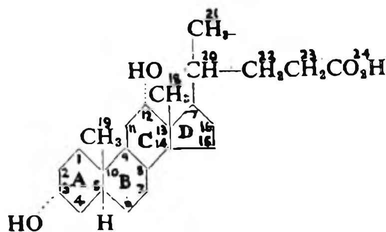
The substance now known as cortisone was originally discovered by three different groups of workers in the same year[2,3,4] during research into the biologically active constituents of the adrenal cortex. This contains a number of steroids, several of which have not only biological activity but activity of different types, which can be associated with the chemical constitution of the isolated materials. Because of the variety of compounds and the small amounts in the glands, the work was extremely complex. Furthermore, when compounds having important physiological actions were discovered, the glands were unsuitable as sources of further material, because the amounts available were too small, and synthetic or partially synthetic methods of preparation had to be devised.

As cortisone is known by several terms, apart from trade names, it may be helpful to give some of them. The original investigators isolated it as one of several substances in adrenal cortex, each of which was given an alphabetical letter, and cortisone is Kendall's compound E, Reichstein's compound Fa and Wintersteiner's compound F. Of these terms the first is the best known. The name 17-hydroxy-dehydrocorticosterone was also extensively used. More systematically it is described as Δ^4-pregnen-17α:21-diol-3:11:20-trione or 17α:21-dihydroxy-3:11:20-tri-keto-pregn-4-ene.

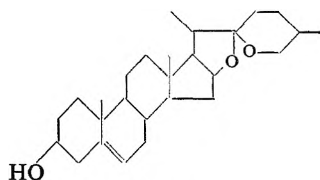
At first cortisone was prepared from desoxycholic acid, and until recently practically all the drug was obtained from this source. There are other naturally occurring sterols, shown below, from which it can be produced by partial syntheses, and a great deal of work has been done on them. Further, there is the possibility of total synthesis, and this has recently been brought about by brilliant investigations in three laboratories. In this article an attempt will be made to deal with most of the ways in which the drug can be prepared, by either partial or total



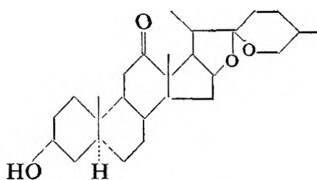
Cortisone



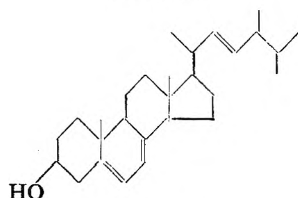
Desoxycholic acid



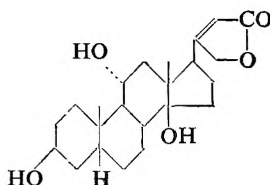
Diosgenin



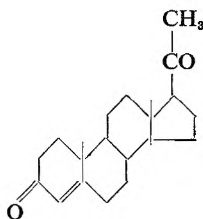
Hecogenin



Ergosterol



Sarmentogenin



Progesterone

synthesis, and to bring these methods into general relationship one with the other. Several of the methods of partial synthesis are of general application and may have been applied to sterols having, for instance, different side chains. In order to avoid confusion and complications, these methods are described by means of partial formulæ and only those portions of the molecule involved in the reactions are portrayed. In order to facilitate interpretation, the structure and numbering of desoxycholic acid are given in detail. Substituents in the α- or β-configurations are shown by dotted and full lines respectively.

The attractions of sarmentogenin as starting material for cortisone are clear enough from the formulæ. It is one of the very few presently known steroids, apart from those of the adrenal cortex itself, having an oxygen-containing substituent in position 11. Furthermore, the side

chain attached in position 17 is readily degraded to a pregnan-20-one, from which the cortisone side chain can be created, as will become apparent later on. Unfortunately, sarmentogenin, though occasionally found in *Strophanthus sarmentosus*, is present in only small quantities and the vine is not one that can readily be cultivated. The difficulties of obtaining the aglycone in quantity have so far defeated its application to the production of cortisone.

One of the other starting materials shown, namely progesterone, has the unsaturated ketonic grouping in ring A and a suitable side chain, but is devoid of any kind of substituent in ring C. Its application to the commercial synthesis of cortisone has been a recent occurrence and depends upon the introduction of an hydroxyl group in position 11 by fermentation methods using particular species of moulds. The largest volume of published work has been on partially synthetic methods from desoxycholic acid and ergosterol.

Early work has been surveyed in several places[5,6,7,8,9] and need not be discussed in detail here, although the major general methods will be summarised to bring them into relationship with newer ones. This review is principally concerned with the work published during the last 2 or 3 three years. A valuable historical review is given by E. C. Kendall, one of the original discoverers of the substance[10].

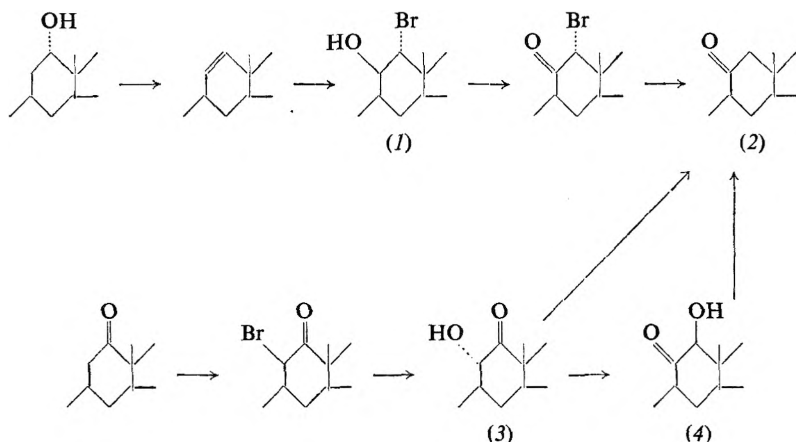
The synthesis of cortisone can be carried out by a variety of procedures from any of the naturally-occurring compounds referred to above and can be subdivided into four subsidiary groups of reactions: (i) introduction of a ketone group into ring C in the 11 position; (*ii*) degradation of the side chain, whether it be of a bile acid, ergosterol or a sapogenin, to that of a pregnan-20-one; (iii) construction of the dihydroxy acetone side chain present in cortisone from the above types of grouping; and (iv) the modification of ring A, which is usually saturated, to introduce the unsaturated ketone group.

In most instances the synthetic steps are carried out in the above-mentioned order, though in special instances this may be altered. As, however, the substituents present in the starting material determine to a large extent the methods within the above sections, it will be clearer if the processes used with each starting material are discussed in turn.

Desoxycholic Acid

Taking first the conversion of the 12-hydroxyl group of desoxycholic acid into the 11-ketone group, there are several different methods to be mentioned, of which the earliest were based on Δ^{11}-compounds, obtained by suitable elimination reactions from desoxycholic acid or one of its acyl derivatives. Addition of hypobromous acid, generated from N-bromacetamide, to the double bond gave a mixture of products from which the bromhydrin (*1*) was isolated. This was converted to an 11-ketone (*2*) by oxidation of the hydroxyl group and removal of the bromine atom by reduction. Yields on both double bond formation and addition of hypobromous acid were poor. In another early method a 12-ketone was brominated and the product hydrolysed to an α-ketol.

When the hydrolysis was carried out under very mild conditions an 11-hydroxy-12-ketone (*3*) was obtained, but under more strongly alkaline conditions this underwent rearrangement and the principal product was a 12-hydroxy-11-ketone (*4*). From this the hydroxyl could be removed by replacement with bromine[11] and debromination. The 11-hydroxy-12-ketone intermediates (*3*) can also be converted to 11-ketones by Kishner Wolff reduction and oxidation.



A more efficient method was discovered by Kendall and his co-workers[12,13] and depends upon the reactivity of the allylic system of a $\Delta^{9(11)}$:12-halogen compound. This is obtainable as shown (*5*) to (*6*); it gives on mild hydrolysis a neutral product, devoid of free-hydroxyl groups and subsequently found to be a Δ^{11}-3:9-oxide (*7*). Addition of bromine to the double bond followed by hydrolysis, oxidation, etc., completes the sequence of reactions to give an 11-ketone (*8*) as shown. Unlike the previous methods this one is very much influenced by the stereochemical configuration of the molecule, as the formation of the 3:9-oxide depends upon rings A and B being fused in the cis position and also on the 3-hydroxyl group being in the α- and not the β-configuration. Only in these circumstances does the oxygen atom at position 3 approach sufficiently closely to the 9-position to permit ring formation. The oxide ring when formed is stable enough to permit many reactions to take place, but can be reopened to give a 3α-acetoxy compound by the action of hydrogen bromide and acetic anhydride.

Many variations on all the above methods have been worked out, but it is not possible to discuss them all here and only one example of each is given. They are discussed more fully in Professor and Mrs. Fieser's valuable monograph[5].

A recent method, introduced by Fieser[14,15], also depends on the formation of a 3:9-oxide. Here a double bond is first introduced (*9*) by selenium dioxide oxidation of a 12-ketone followed by removal of the keto group. On treatment with per-acid a 9:11-oxide is formed (*10*) and is stable to the action of various acids or reducing agents, but

(5)

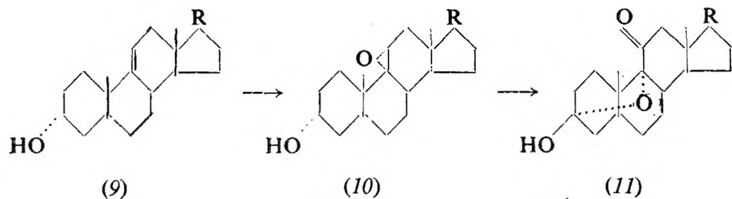
(7) (6)

(8)

on treatment with chromic acid is oxidised to a hemiketal (*11*). This can then be converted by treatment with hydrogen bromide and reduction, etc., to the type of intermediate required. Again the formation of the intermediate oxygen bridge depends upon the molecule having an A/B *cis* configuration. The change in configuration of the 3-hydroxyl group in (*11*) is due to the fact that a 3-ketone, which is formed as an intermediate, is attacked in the rear or α-position by the oxygen atom attached in position 9, with formation of a 3β-hydroxyl group.

Passing on to the modification of the bile acid side chain, the original method used was the well-known Barbier-Wieland degradation devised many years ago in Wieland's classical work on the structure of bile acids. It required a large number of steps and can be considerably shortened by the modification introduced by Meystre and Miescher[16,17], in which the diphenylcholene (*12*) formed after Grignard reaction on the cholic esters is treated with *N*-bromsuccinimide under conditions that permit removal of hydrogen bromide to form another double bond, the product being a diphenylchola-20:23-diene (*13*). This is directly oxidised by chromic oxide to a pregnan-20-one (*14*), which serves as starting material for the next phase of the synthesis, namely the creation of the dihydroxy-acetone side chain, which is one of the principal features of cortisone.

This is beset by one major difficulty. In all naturally-occurring sterols the side chain is 17β. When attempts are made to form a 17α-hydroxy compound from, for instance, a 17-ketone, the reaction invariably leads to a substance having the carbon side chain in the α-configuration together with a 17β-hydroxy group (e.g. *15* to *16*). This is the reverse of that present in the natural hormones and the positions must be inverted.



(*9*) (*10*) (*11*)

O R

O

CH_3 — $CH_2CH_2CO_2H$ ⟶ CH_3 — $CH_2CH_2C(OH)Ph_2$ ⟶ CH_3 — $CH_2CH{=}CPh_2$

(*12*)

CH_3 — CO (*14*) ⟵ CH_3 — $C{=}CH{-}CH{=}CPh_2$ (*13*)

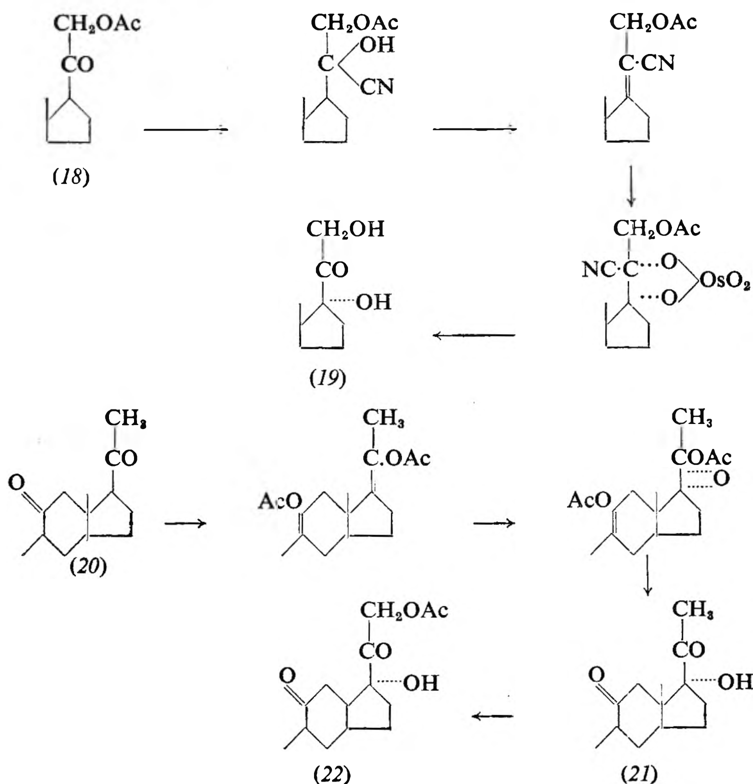
O (*15*) ⟶ OH, $C\equiv CH$ (*16*) ⟶ OH, $CH{=}CH_2$

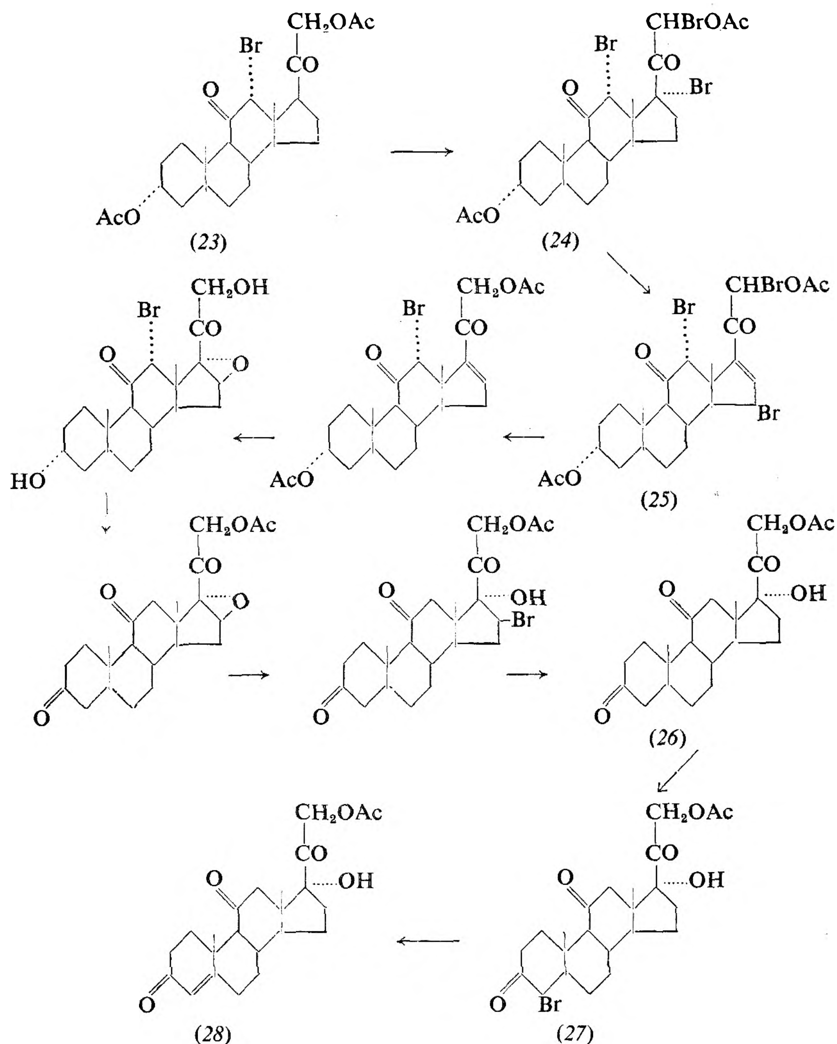
CH_2OAc — CO, OH (*17*) ⟵ CH_2OAc — HOCH, OH ⟵ CH_2OAc — CH

All the successful methods of building the required side chain have depended on an intermediate having a double bond at the 17-position. This can be done in several different ways (e.g. *15* to *17*; *18* to *19*). The second of these illustrates one of the methods used by Sarett[18,19] in the synthesis of cortisone. It depends on oxidation of the 17:20-double bond with osmium tetroxide, which produces a 17α:20α-glycol. The reagent always attacks the less hindered α face of the molecule, the angular methyl group at position 13 preventing attack from the other side. Modifications of the method are also described[20,21].

But osmium tetroxide is dangerous as well as expensive, and methods of avoiding its use have been sought. The most successful depends upon forming the double bond by enolisation of a pregnan-20-one. The procedures were introduced by Gallagher[22,23,24] and his co-workers and are illustrated by formulæ (*20* to *21*). They have been used on several intermediates, and it is noteworthy that both the 11- and 20-ketone groups are enolised and acetylated as shown, but at the next step only 17:20-double bond is attacked by perbenzoic acid and therefore on the subsequent hydrolysis the 11-ketone group is recovered unchanged and a 17α-hydroxyl group is introduced. The side chain is then completed by bromination at position 21 followed by hydrolysis or acetoxylation to (*22*).

In the above paragraphs major procedures are given in outline only; there are many opportunities for varying the order in which the different steps are carried out. By means of such a combination of methods (*23*) may be prepared and can be used as a means of introducing the 17α-hydroxyl group by a series of reactions quite different from any of those hitherto described[25,26]. Bromination in acetic acid forms a 12:17:21-tribromo compound (*24*) which after dehydrobromination and re-bromination yields (*25*). From this, dihydrocortisone (*26*) may be obtained in several steps by reduction, oxidation, etc., as shown. The





procedure is efficient, avoids the use of osmium tetroxide and makes use of a Δ^{16}-compound and a method introduced earlier by Julian[27]. Again attack by the reagent at the unsaturated 17-position is at the unhindered α face of the molecule.

The last phase of the synthesis is then carried out by brominating in the 4-position and removing hydrogen bromide to form the Δ^4 : 3-ketone. Yields on dehydrobromination with bases are poor, and a great improvement was affected by treating the 4-bromo ketone (*27*) with dinitrophenylhydrazine or semicarbazide whereby the appropriate derivative of the ketone is obtained and dehydrobromination takes place at the same time provided acetic acid is used as the solvent. The unsaturated ketone (*28*) (cortisone acetate) is generated from the derivative by treatment with

pyruvic acid. The method was discovered by Mattox and Kendall[28] and later modified and improved[29,30,31] and it is now commonly used for introducing double bonds into ring A in very satisfactory yield, provided rings A and B are *cis*. The need for this will become apparent later.

The methods used for the synthesis of cortisone from desoxycholic acid have never been brought together, but a possible complete synthesis has been suggested[32].

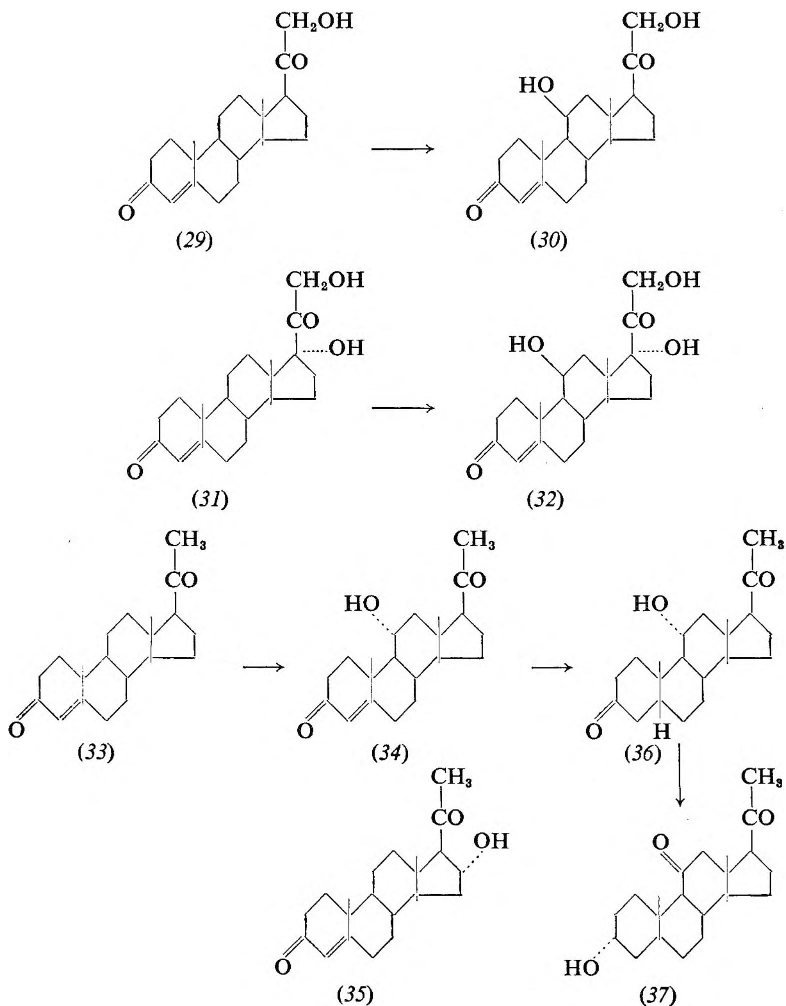
PROGESTERONE

The use of this raw material is dealt with next, partly because complete information has been made available on its conversion to cortisone, and partly because, though it bears little superficial resemblance to desoxycholic acid it can, in a few stages, readily be converted to an 11:20-diketone derivable from it, and from which the rest of the synthesis has already been described. Progesterone itself can be prepared from a number of raw materials, and several different preparative methods are known, probably the best being that from diosgenin, although recently interest has also been shown in its preparation from stigmasterol[6,33,34,35].

While progesterone has an appropriate side chain for conversion to cortisone and already has an unsaturated ketonic group in ring A, it suffers from the obvious disadvantage of having no substituent at all in ring C and no evident means of introducing one. Great interest was therefore shown in a report[36] from Professor Pincus's laboratories that the perfusion of 11-desoxycorticosterone *(29)* or its acetate through isolated adrenal glands led to the formation of corticosterone *(30)*, which was isolated and identified. This work was actively followed up, and it was later shown[37] that *(31)* could be converted to *(32)* by an extract of minced adrenal tissues containing the appropriate enzyme system. Note that the hydroxyl group at the 11-position appears in the β-configuration, which is that present in natural hormones, as would be expected from the method of preparation. However interesting these observations might be, they did not offer a practical large scale method for the introduction of oxygen at the 11-position into sterols, but this became feasible with the discovery by Peterson and Murray[38,39] that fermentative oxidation of several sterols by suitable species of moulds could accomplish similar reactions. They used a strain of *Rhizopus* and from the fermentation mother liquors containing progesterone *(33)* isolated 11α-hydroxyprogesterone *(34)*. A 6:11-dihydroxyprogesterone was also isolated in these experiments. Other workers[40] using a strain of *Streptomyces* succeeded in converting Reichstein's compound S *(31)* into Kendall's compound F *(32)*, the so-called hydrocortisone, though in poor yield. Note that in this case an 11β-hydroxyl group is again introduced. Hydroxylation of progesterone in the 16-position *(35)* has also been reported[41], along with several other similar oxidations, including simultaneous hydroxylation in the 6- and 11-positions[42].

The preparation of 11α-hydroxyprogesterone by these methods obviously made the synthesis of cortisone from that starting material a great deal more attractive, and the observations were rapidly followed up.

The methods became still more attractive when it was found[43] that hydrogenation of the 4:5-double bond, to protect it during subsequent operations, gives a normal pregnane derivative (*36*), whereas hydrogenation of similar compounds with either a ketone or a β-hydroxyl group



in the 11-position gives predominantly products belonging to the *allo*-pregnane series. It is thought that the additional steric hindrance at the rear of the molecule provided by the 11α-hydroxyl tips the balance of factors affecting the position of attack by the catalyst, so that hydrogenation takes place principally at the front or β-position, yielding an A/B *cis* compound on hydrogenation. By oxidation and preferential reduction of the thus formed 3:11:20-triketone with sodium borohydride in pyridine (*37*) is obtained, from which the preparation of cortisone has been described above[24].

Thus biological oxidation to obtain an 11-hydroxy compound, coupled with the unexpected hydrogenation of this particular type of derivative to a compound of the pregnane series, readily converts progesterone into a substance previously prepared from desoxycholic acid, permitting the complete conversion of progesterone to cortisone in about 10 stages.

HECOGENIN

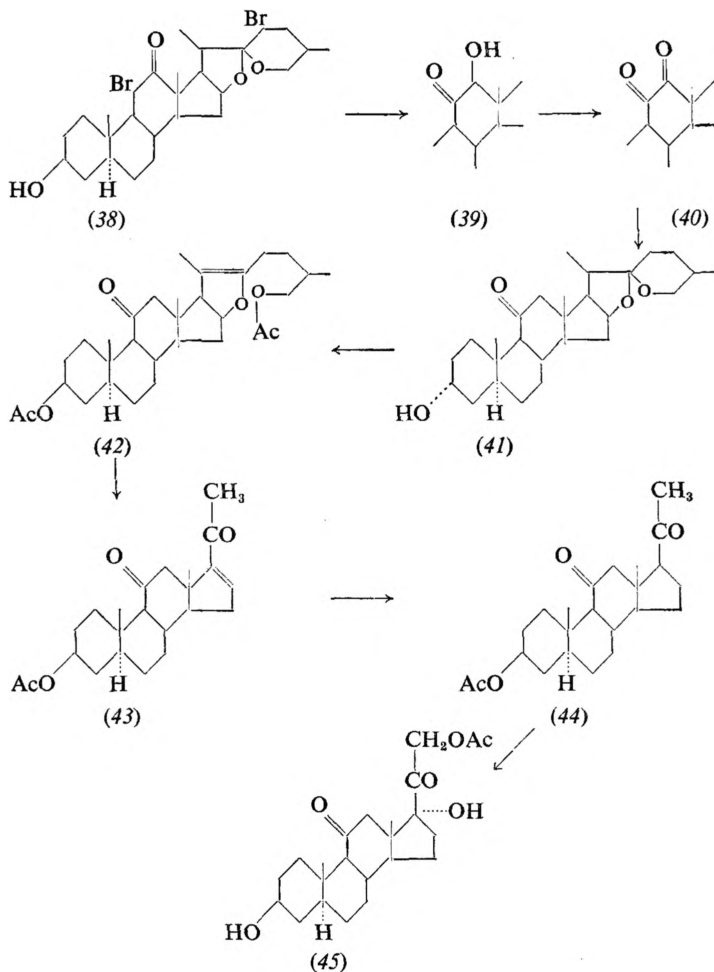
This, regarded as a starting material for the synthesis of cortisone, looks similar to desoxycholic acid, apart from the side chain, in that it has oxygen substituents in the 3- and 12-positions. The resemblance, however, is more superficial than real, and there is need for new methods at several different points. The A and B rings are fused in the *trans* configuration, rather than the *cis* as in bile acids, and this prevents the application of those methods of introducing an 11-ketone group that are dependent upon the use of 3:9-oxides. That same difference in configuration also necessitates a new process for the introduction of the 4:5-double bond. But the difficulties have been overcome and a complete synthesis has been described.

Hecogenin is the aglycone of a naturally-occurring saponin found in various species of agave. It was first isolated from a Mexican source[5] and has also been found in the sisal plant, which is cultivated on a very large scale in several parts of the world[44,45]. As the sapogenin can be isolated from waste fractions in the manufacture of the sisal fibre, it is an attractive starting material on which to found a manufacturing method.

Bromination of the sapogenin gives a dibromo compound[46,47] (*38*), which after hydrolysis and partial debromination leads to an 11-keto-12-hydroxy compound (*39*) similar to (*4*). In this case, however, removal of the 12-hydroxy group *via* the bromo compound gave very bad yields, and it was found better to oxidise the ketol with bismuth oxide, a reagent recently introduced for the oxidation of acyloins, to the 11:12-dione[48] (*40*), from which the 12-ketone group can be removed either by Kishner Wolff reduction or by conversion to a dithioketal and subsequent reduction with Raney nickel.

Degradation of the sapogenin side chain in the thus formed 11-keto compound (*41*) can readily be accomplished by methods that were well established for a number of sapogenins by Marker[5] and leads in good yield to (*43*)[49,50] from which 3β-acetoxy-*allo*-pregnan-11:20-dione (*44*) can readily be prepared by hydrogenation[51]. The first step in degrading the side chain, the conversion of (*41*) to (*42*), has been carried out on many sapogenins and compounds derived from them, by heating with acetic anhydride at about 200° C. Conversion to this unsaturated acetate, called the ψ-sapogenin, is usually accomplished in high yield. Recently a modification of the method was introduced; working with diosgenin it was found that its conversion to the ψ-sapogenin by acetic anhydride is catalysed by the use of a Lewis acid, such as aluminium chloride, and that the conversion can then be carried out at much lower temperatures[52].

These products all have rings A and B in the *trans* configuration and

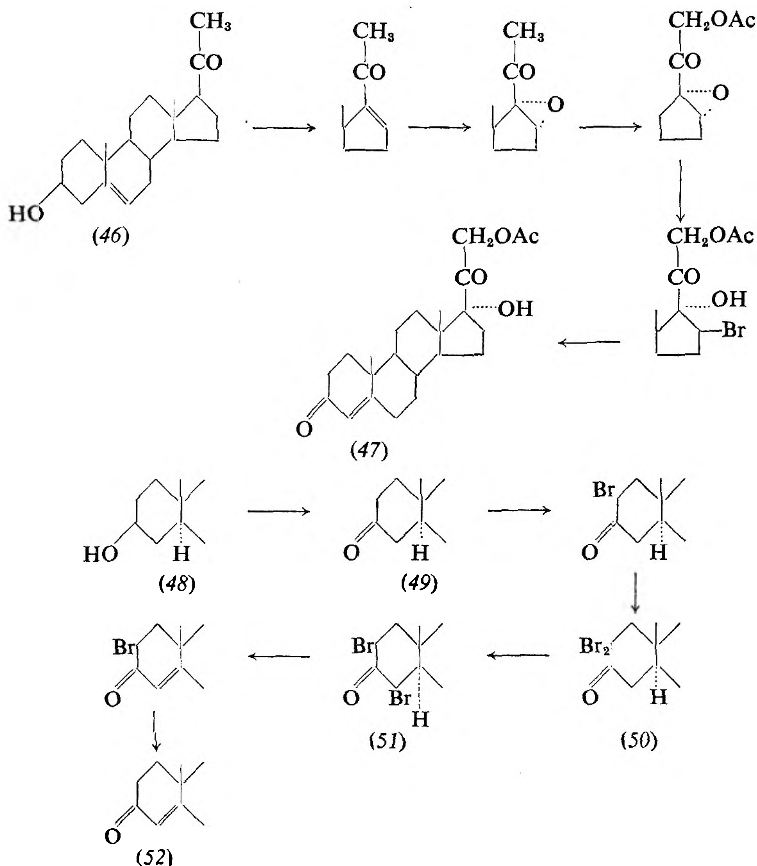


therefore the opposite of that to which Gallagher applied his methods of converting a pregnan-20-one to a substance having a cortisone side chain, but the same method can be applied to (*44*)[53,54,55] to give (*45*).

As degradation of the side chain of a sapogenin leads to a Δ^{16}-20-ketone, an alternative to Gallagher's synthesis of the dihydroxy acetone group may be employed. It was first devised by Julian[27] for the conversion of pregnenolone (*46*) to compound S (*47*) as shown. A modification of the method has already been mentioned in the section on desoxycholic acid.

There remains the problem of introducing the 4:5-double bond into (*49*), obtainable from (*48*) by oxidation. In 3-keto-*allo*-pregnanes, bromine substitutes not in the 4- but in the 2-position. If, however, halogenation is continued further, a 2:2-dibromo compound (*50*) is formed that on treatment with hydrogen bromide in acetic acid solution

rearranges to give a 2:4-dibromo compound (*51*), from which, on removal of a molecule of hydrogen bromide and reduction, the required unsaturated ketone (*52*) is obtained. Several publications have appeared that claim successful application of this method[56,57,58], and various reagents may be used. For instance, removal of hydrogen bromide to form the 4:5-double bond may be accomplished by the use of either collidine or sodium iodide, and reduction of the remaining halogen, bromine or iodine in the 2-position may be carried out by treatment with sodium iodide, sodium bisulphite, chromous chloride or zinc. The method is not so efficient as when applied to simpler substances, such as cholestanone, but nevertheless served to complete the synthesis of cortisone from dihydro-*allo*-cortisone acetate[59].

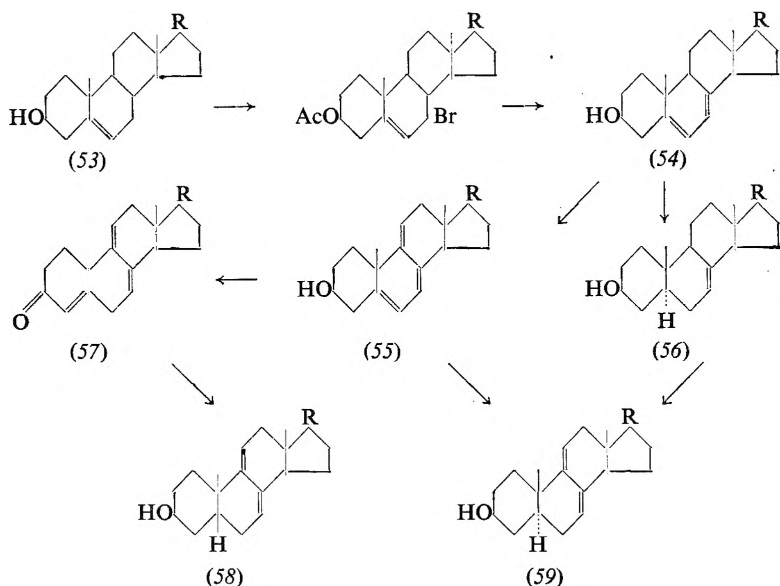


DIOSGENIN AND ERGOSTEROL

These two starting materials are dealt with together here because their use in the synthesis of cortisone invokes essentially the same problems, after a 5:7-diene system has been introduced into diosgenin. Neither compound has an oxygen substituent in ring C and both normally lead to intermediates of the *allo* series. It has been known for some time

that the diene system of ergosterol can be extended to dehydro-ergosterol, a 5:7:9(11)-triene system, by oxidation with selenium dioxide or mercuric acetate, of which the latter is preferred. This is essentially the reaction by which both the above starting materials are brought to the position of being useful intermediates in the synthesis of cortisone.

As a first step it is necessary to create a 5:7-diene system from diosgenin. The problem is the same as that of converting cholesterol into cholesta-5:7-dienol in the preparation of vitamin D_3 and may be solved in much the same manner by bromination at the 7-position with *N*-bromo-succinimide and subsequent elimination of hydrogen bromide on treatment with bases of various kinds (*53* to *54*)[60–65]. Other methods are also known[66,67]. Such dienes may be extended to 5:7:9(11)-trienes (*55*) by dehydrogenation as above or may be selectively reduced to Δ^7-compounds (*56*) with platinum oxide or, better, with Raney nickel[68,69].



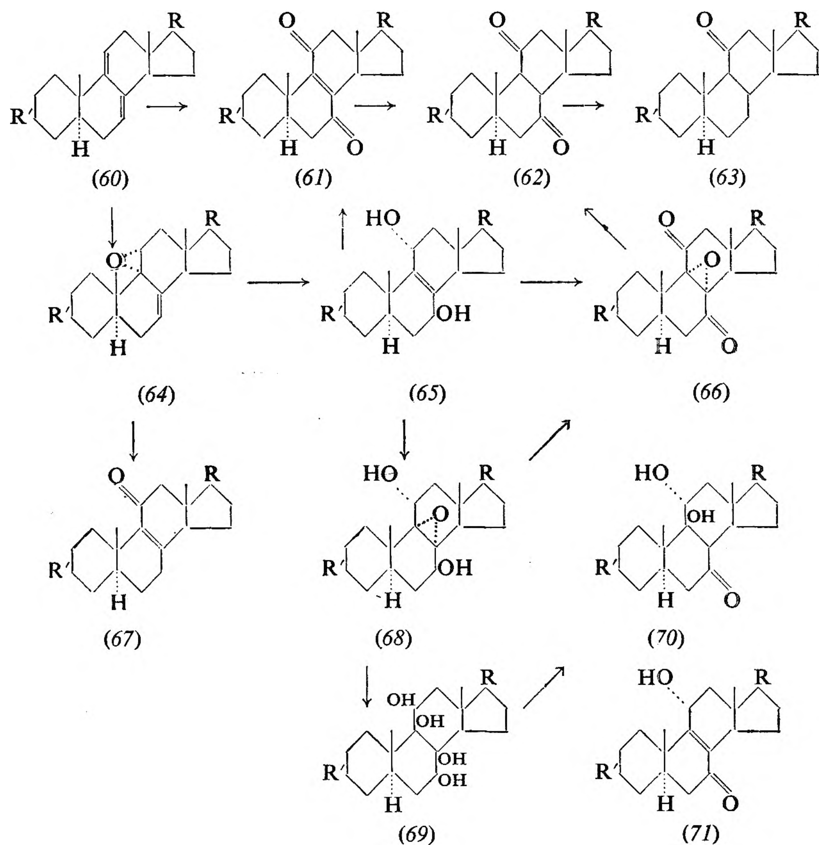
Whereas the direct reduction of (*54* and *55*) leads to *allo*-pregnenes, with which we are mostly concerned, it is possible to convert them to compounds of the normal series by oxidation to (*57*) followed by hydrogenation to (*58*)[70]. Below the emphasis is on the use of *allo* compounds (*59*); most of the reactions, though not all, take a different course in intermediates of the normal series (*58*).

Many different methods have been reported for the conversion of these 7:9-dienes to 11-ketones. Most of the reactions are self-explanatory when depicted in partial formulæ, though several were quite unexpected and, at the time of their discovery, new to sterol chemistry. The direct oxidation of (*60*) to the diketone (*61*) is accomplished by treatment with chromic acid[71], but in very poor yield; alternatively, a similar transformation is accomplished indirectly by bromination, hydrolysis and oxidation[72];

(*61*) is then reduced with zinc to (*62*) and thence by Kishner Wolff reduction to the desired 11-ketone (*63*).

In another method (*60*) is treated with perbenzoic acid[73] to give an oxide, later[74] shown to be the 9:11-oxide (*64*); this on hydrolytic rearrangement gives (*65*), which can be oxidised to (*61*), and this[74,75] is accompanied by the oxido ketone (*66*), itself directly reducible to (*62*). It has also been shown that (*64*) on reaction with a Lewis acid, namely boron trifluoride, rather than with a mineral acid, leads to an unsaturated ketone (*67*). Owing to steric hindrance its reduction to a saturated ketone is difficult, but can be accomplished[76,77], by the use of lithium in liquid ammonia, whereupon (*67*) is converted directly to (*63*) in good yield. A modification of this method, which is claimed to be efficient and is akin to another group of reactions yet to be discussed, is also available[78]. In this (*65*) is oxidised with perphthalic acid to the oxide (*68*), which can be directly oxidised to (*66*) or hydrolysed to (*69*); this on dehydration yields (*70*) and thence (*71*), convertible to (*63*) by methods to be mentioned below.

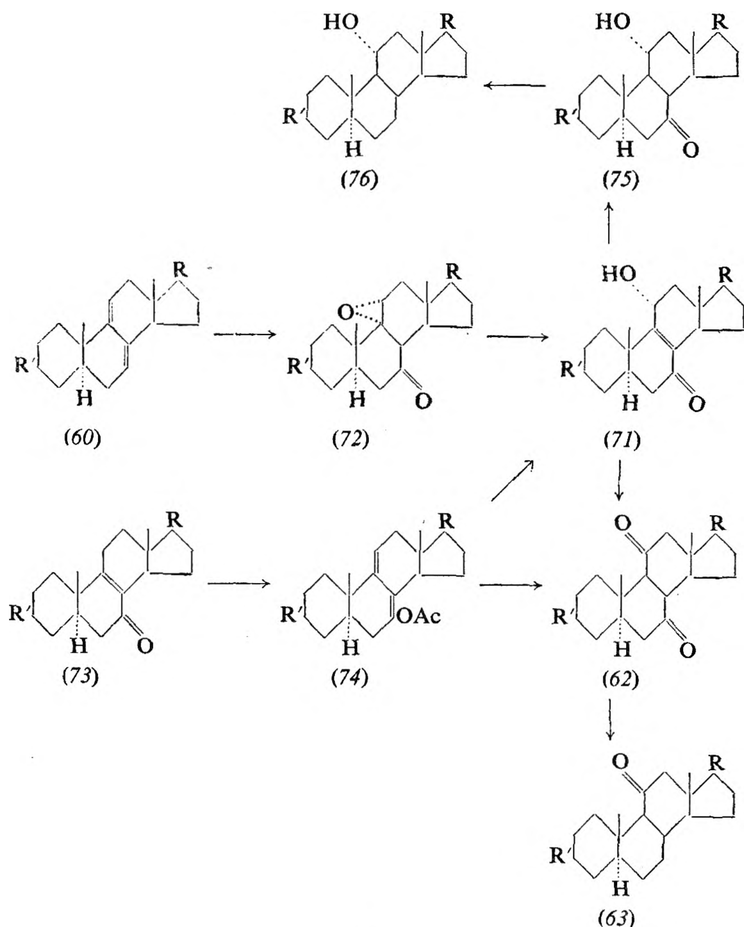
It is to be understood that these various transformations are not being discussed in the chronological order of their discovery and that for



clarity various groups of intermediates have been dealt with together. It is now necessary to go back to one of the earlier methods and follow its development.

As described above, 7:9-dienes are oxidised to 7:11-diketones by chromic acid and to Δ^7:9:11-oxides by aromatic peracids; aliphatic peracids cause yet another reaction to take place, whereby (*60*) is oxidised to the oxido ketone (*72*)[79,80]. Hydrolysis with mild alkali converts this to an unsaturated hydroxy ketone previously encountered (*71*), while stronger alkali leads directly to the formation of (*62*). It was later found that this rearrangement gave much better yields when carried out in the presence of potassium tertiary butoxide[81].

Finally, some interesting transformations are recorded on $\Delta^{8(9)}$-7-ketones (*73*), already known as the major products of the direct oxidation of (*60*)[71] and also as one of the products of the rearrangement of (*64*) with mineral acid[74]. Since then it has been discovered[82,83] that, if (*73*) is converted to its enol acetate (*74*) and this oxidised with perphthalic

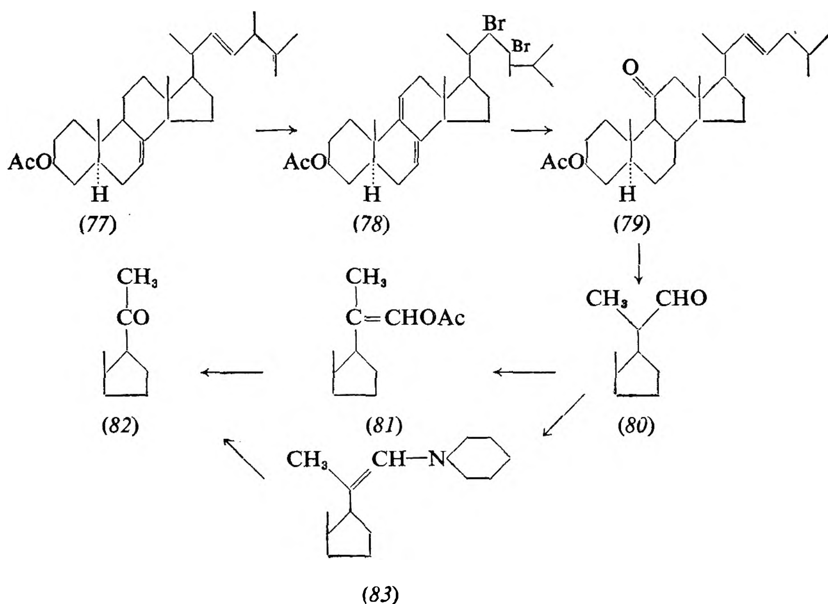


acid, (*71*) is formed directly, thus providing an interesting route from 7- to 11-ketones, or alternatively to 11α-hydroxy compounds (*76*) by catalytic hydrogenation of (*71*) to (*75*) followed by reductive removal of the 7-keto group.

Compounds represented by partial formulæ (*60*) to (*76*) have been derived from parent 7:9(11)-dienes, where R may have been an ergosterol, sapogenin, bile acid or *allo*-pregnan-20-one side chain.

A novel turn was given to this work as a result of a publication by Spring and his collaborators[69], who found that by brominating 5:6-dihydro-ergosteryl acetate (*77*) a crystalline tetra-bromide of unknown constitution could be obtained, from which the acetate of ergosterol D dibromide (*78*) was readily prepared by removal of two bromine atoms with sodium iodide under mild conditions. It will be seen from the above paragraphs that many of the reactions on the dienes involve oxidation and reduction, and much difficulty is occasioned by the need to find selective reagents or conditions. The bromination method at once provides the diene system and protects the double bond in the side chain from attack, and many of the above transformations have been carried out on ergosterol D dibromide[84–90]. When required the double bond in the 22-position is easily regenerated by debromination with zinc. Thus, 11-ketones may be obtained from diosgenin and ergosterol in several ways.

The next group of reactions, involving degradation of the side chain, is known in general for both intermediates. Degradation of the diosgenin side chain is similar to that already given for hecogenin, whereas that of 3β-acetoxy-11-ketoergost-22-ene (*79*) may be carried out in any of several different ways. In a simple and effective method, which has been used on similar compounds[91,92,93], the double bond is cleaved to the

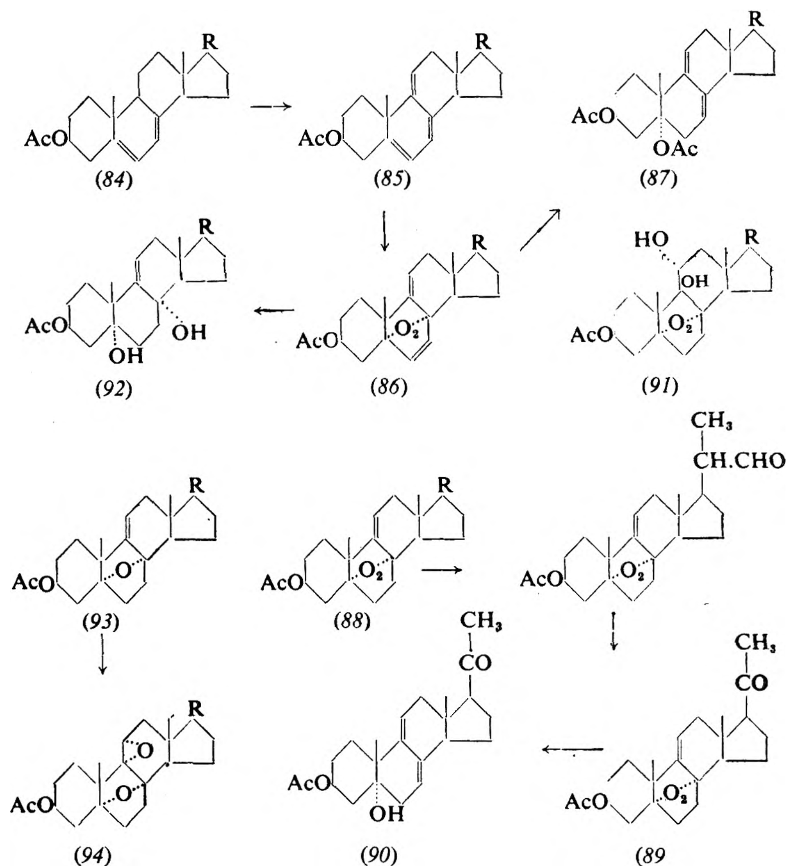


aldehyde (*80*) by ozone. The corresponding enol acetate (*81*) is then prepared and ozonised to give a pregnan-20-one (*82*). The method has been applied to the preparation of progesterone from stigmasterol[94]. In a modification, the double bond between the 20- and 22-positions is introduced by condensing the aldehyde (*80*) with a secondary amine to give (*83*), converted by ozonolysis to (*82*)[94].

Details of the degradation of 3β-acetoxy-11-ketoergost-22-ene to 3β-acetoxy-*allo*-pregnan-11:20-dione have not been published, and the above is only an indication of methods that might be used. It is known, however, that the complete synthesis of cortisone from ergosterol has been accomplished[53]. Thus several different processes from several starting materials all converge on 3β-acetoxy-*allo*-pregnan-11:20-dione, from which the synthesis of cortisone has already been discussed (see Hecogenin).

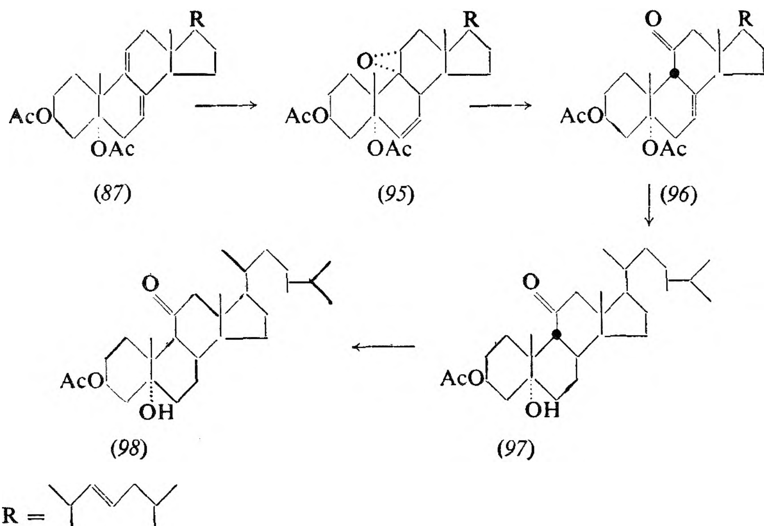
An entirely different approach to the problems was devised by E. R. H. Jones and H. B. Henbest and developed in conjunction with their associates. Ergosterol (*84*) is oxidised by mercuric acetate to dehydroergosterol (*85*), which forms an epi-dioxide (*86*) in air under the influence of light and a photo-catalyst such as eosin. It was thought that this known epi-dioxide could give access to 11-oxy compounds from the 9:11-double bond, and also that hydrogenation of the epidioxide group might provide a 5-hydroxy derivative to be available at the end of the synthesis for the introduction of the 4:5-double bond by dehydration. On examination it was found[95] that several products could be derived from (*86*) by different methods of catalytic hydrogenation or chemical reduction, among which the 7:9(11):22-triene (*87*) and 6:7-dihydro compound (*88*) are to be specially noted. The latter seemed to be of particular interest, and of the several possible methods of preparation the best was catalytic hydrogenation in the presence of hydrated platinum oxide, a novel catalyst. That compound is subsequently degraded[96], by the method already mentioned, to the corresponding 20-ketone (*89*) and thence by treatment with zinc and acetic acid to the 5-hydroxy-20-ketone (*90*).

Attempts to oxidise (*88*)[97] to obtain an 11-oxy compound were disappointing as it was slow to react with peracids; an unreactive oxide is obtained in poor yield by the action of perbenzoic acid and a glycol (*91*) by oxidation with potassium permanganate in acetic acid. Later work showed that the diol (*92*) is readily cyclised under the influence of weak acids to a 5:8-oxide (*93*); although the 9:11-bond in this compound can now readily be attacked with peracids to give a 5:8–9:11-diepoxide (*94*), the transformation of such compounds into useful 11-oxygenated steroids has yet to be achieved. Further progress was made by the use of the 7:9(11):22-triene (*87*) which is readily oxidised by perbenzoic acid to the oxide (*95*), and this, on treatment with boron trifluoride under mild conditions, gives the unsaturated ketone (96). Note that here the double bond has not moved into conjunction with the keto group and compare the conversion of (*64*) to (*67*). Further examination of (*96*) showed it to be a substance of much interest, for it was found to have



the abnormal *iso*-configuration at the 9-position; moreover, because of the effect of this unusual configuration on the geometry of the molecule as a whole, catalytic hydrogenation of the 7 : 8-double bond to the saturated ketone (*97*) was possible, though a Δ^7-steroid of the normal configuration at C_9 cannot be hydrogenated. Note also that reduction can often be carried out on 11-ketones without this feature of the molecule being affected. Because of the steric hindrance occasioned by the presence of the two angular methyl groups at positions 10 and 13, 11-ketones are particularly unreactive; they do not form any ketonic derivatives and are difficult to reduce. On treatment with strong alkali (*97*) isomerised to give the known saturated 11-ketone (*98*), but the sequence of reactions, though interesting, still did not provide a useful route to cortisone, as hydrogenation of (*96*) was not selective and the double bond in the side chain was invariably reduced.

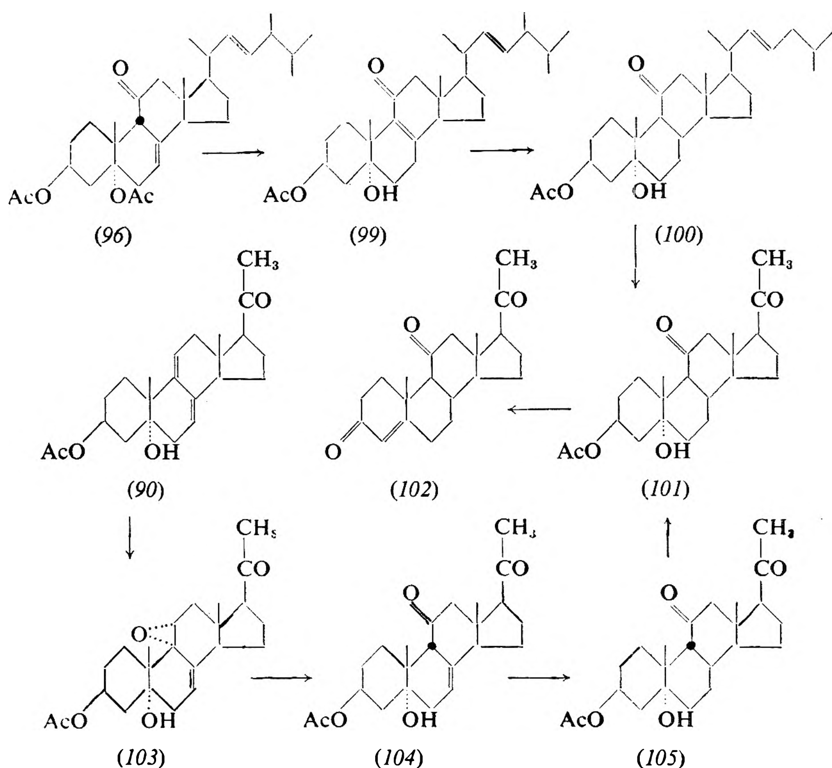
A large measure of success was finally achieved in two different ways. By treatment with zinc and acetic acid the unsaturated 9-*iso*ketone (*96*) was isomerised to the conjugated unsaturated ketone (*99*), which, like the analogous substances previously mentioned, is reducible by



lithium metal in liquid ammonia to the saturated ketone (*100*), in this instance without reduction of the double bond in the side chain. This feature of the molecule having been retained, degradation of the side chain can be carried out in the usual fashion to yield 3β-acetoxy-5α-hydroxy-*allo*-pregnan-11:20-dione (*101*). This can in its turn be converted by hydrolysis and oxidation to 11-ketoprogesterone (*102*), from which the synthesis of cortisone has virtually been completed (see Total Synthesis). Dehydration to introduce the 4:5-double bond may occur during oxidation of the 3β-hydroxyl group by the Oppenauer reaction with aluminium tertiary butoxide as the oxidising agent, or after oxidation with chromic acid, on treatment of the resulting 5-hydroxy-3-ketone with mild alkaline reagents.

An alternative method starts with a previously mentioned compound, 3β-acetoxy-5α-hydroxy-*allo*-pregna-7:9(11)-dien-20-one (*90*), which may be oxidised in the usual manner to the oxide (*103*). This is in turn converted to the Δ^7-ketone (*104*) by boron trifluoride, and the product is reduced catalytically to a saturated ketone. Here also reduction in the side chain takes place, but the secondary alcohol group at position 20 can conveniently be re-oxidised with chromic acid to the original ketone to give (*105*), which still has the 9-*iso*-configuration and can be isomerised by treatment with strong alkali, followed by reacetylation of the 3-hydroxyl group so formed to give 3β-acetoxy-5α-hydroxy-*allo*-pregnan-11:20-dione (*101*).

By these methods a complete synthesis of cortisone is developed from an ergosterol derivative having a 5α-hydroxyl group derived from the original 5:8-epidioxide. This work is now in the course of publication, and I am greatly indebted to Professor E. R. H. Jones of Manchester University, from whom I have received much of the information about it for his kind permission to publish it in this review.

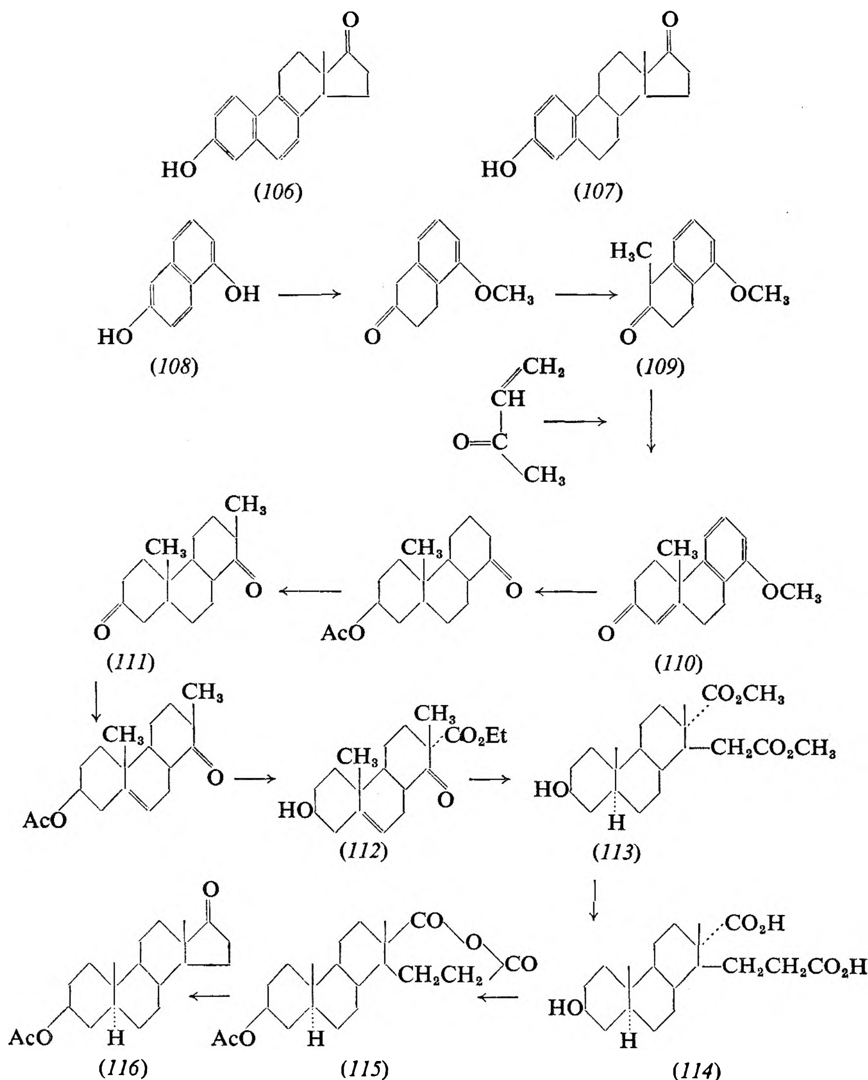


Total Synthesis

The problem of total synthesis of sterols has been a severe challenge to the ingenuity of organic chemists ever since the general formulation of the group was first arrived at in 1932 by Rosenheim and King in Britain and Wieland and Dane in Germany at almost the same time, after the intensive and brilliant research work of Wieland and Windaus and their collaborators in Germany over many years. The stereochemical complications are particularly acute, as can readily be seen by inspection of the graphic formula of desoxycholic acid, in which there are 10 centres of asymmetry, each of which must be correctly diagnosed and all of which must be assembled in the correct configurational relationship to each other during the synthesis: otherwise the product at the end will not be desoxycholic acid. These 10 asymmetric centres permit the existence of 2^{10} or 1024 stereoisomers. Cortisone has a smaller number of asymmetric centres but even so there are still 6 and therefore 64 possible stereoisomers. It is therefore not surprising that first attempts at synthesis were aimed at the simpler systems, equilenin (*106*) and œstrone (*107*), which have fewer centres of asymmetry because of their aromatic rings. The former was synthesised successfully by Bachmann, Cole and Wilds in 1939 and the latter by Anner and Miescher in 1948. There remained the problem of synthesising the complete *cyclo*-aliphatic

structure of which cortisone is a representative, and this has now been brought to a successful conclusion with full attention to stereochemical configuration by three groups of research chemists, one led by Sir Robert Robinson at Oxford University, one by Professor R. B. Woodward at Harvard University and the third by Dr. L. H. Sarett in the Merck laboratories at Rahway, New Jersey.

The first described here is that devised by Sir Robert Robinson and his collaborators, of whom Dr. J. W. Cornforth may be specially mentioned. The experiments had been continued at Oxford for many years and, though the early work was unsuccessful, it nevertheless contributed much useful information and several new reactions, later adopted by



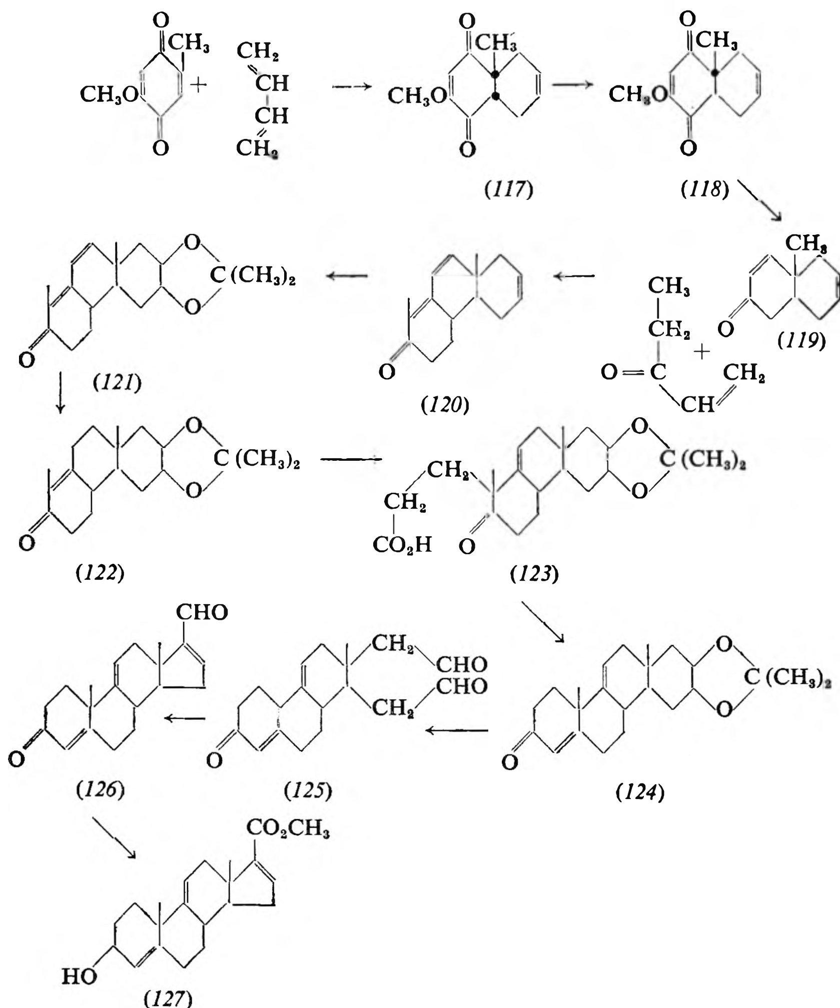
others, now forming part of all work on the incorporation of angular methyl groups or the formation of fused *cyclo*hexane structures.

The method finally adopted starts with 1:6-dihydroxynaphthalene (*108*), which is methylated: the dimethyl ether is reduced under special conditions, the product being again methylated to give (*109*), in which there first appears a resemblance to the structure ultimately sought[98,99]. The two rings of the dihydroxynaphthalene are later to form rings B and C of the complete sterol. Ring A is then added to (*109*) by condensing it with methyl vinyl ketone or a substance that will produce it *in situ*, a method first devised at Oxford and now one of the classical procedures in the synthesis of sterols, common to all work undertaken in this field. The tricyclic ketone so produced (*110*) is again reduced and methylated to give (*111*). This substance is of considerable significance, for it was already known as a product of the degradation of desoxycholic acid; the identity of the two compounds, one natural and one synthetic, gave assurance that at least up to that point the required stereochemical relationships had been achieved. After reduction, carbonylation of the ketone led to (*112*), of which the carbethoxy group forms the embryonic limb bud of the final ring[100]. Reformatsky reaction followed by dehydration and reduction gave (*113*), an ætio-*allo*-bilianic acid derivative identical with a product of the oxidation of cholesterol. Extension of the acetic acid side chain to the corresponding propionic acid (*114*) is then carried out by Arndt Eistert reaction. Conversion of this last to the anhydride (*115*) followed by pyrolysis gave (*116*). This is an example of the principle that anhydrides of substituted adipic acids give ketones on pyrolysis, while those of substituted glutaric acids do not, the well-known Blanc's rule, which played an historic role in determining the constitution of sterols, because dicarboxylic acids of various types were often encountered as products in stepwise degradation; the rule gave valuable information on whether the rings from which these dicarboxylic acids were derived had originally been 5-membered or 6-membered. The final product (*116*) is *epi*androsterone acetate, which contains all the features of a naturally-occurring sterol and is readily derived by a simple process from *epi*dehydroandrosterone, the major product of the oxidation of cholesterol[101].

Does it seem simple? Maybe, but appearances are very deceptive and a brief and eclectic review of this nature bears insufficient testimony to the novel methods used, to the tedious discernment of selective reaction conditions and the painstaking separation of isomers involved. *Epi*-androsterone acetate could be converted to cortisone by methods such as those previously mentioned in this review, but this has not been done. The synthesis is included here as one of the only three known total syntheses of sterols.

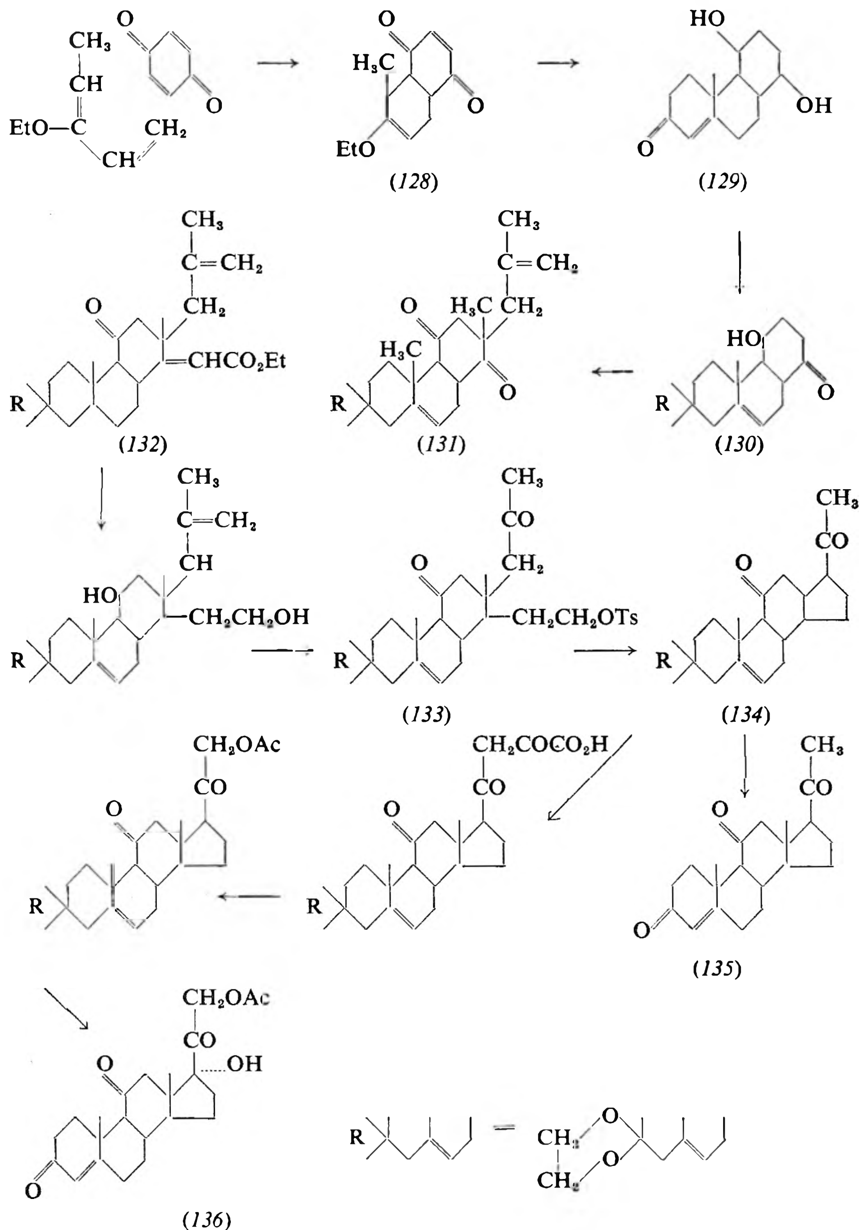
An alternative method evolved at Harvard University by Professor R. B. Woodward and his associates was first described by him in his centenary lecture to the Chemical Society in London in 1951. A preliminary summary of the work has been published[102] and was followed later by a communication describing the complete details of the methods

used[103]. In this scheme (*117*) is first prepared by reaction between butadiene and methoxy-toluquinone, a Diels-Alder condensation. In (*117*) the two rings are *cis* but they ultimately become rings C and D, which are *trans*. The point is, however, that the Diels-Alder reaction always gives *cis* products and, therefore, the configuration of (*117*) is known; because of the carbonyl group present it can be isomerised by treatment with alkali to the other configuration, (*118*), which must therfore be *trans*. Thus at one ingenious stroke, two of the stereochemical centres are disposed of without the need for laborious separations or determinations of structure. Reduction of the carbonyl groups, which among other things prevents a re-isomerisation of the carefully-designed *trans* ring structure, and hydrolysis of the enol ether later leads to (*119*). To this the third ring is added by condensation with ethyl vinyl ketone



in the normal manner; here the ethyl- rather than the more usual methyl-vinyl ketone is used, the additional carbon atom becoming the angular methyl group between rings A and B (*120*). Before proceeding to the construction of ring A, and because of the method to be employed, it was necessary to modify the double bond system in this last intermediate. The isolated double bond is protected by oxidation to a glycol and acetonylation to (*121*), thereby beginning a series of reactions that lead ultimately to ring D. The double bond present in ring C and conjugated to the ketone is removed selectively by hydrogenation with a palladised strontium carbonate catalyst in dry benzene giving (*122*). After blocking the α-methylene group the addition of acrylonitrile followed by hydrolysis leads to (*123*). This product was that expected on theoretical grounds, which are set out at considerable length in the publication, though there is not space to repeat them here. For the first time in this synthesis the reaction is not stereo specific and the mixture of products obtained has to be separated. Thereafter (*124*) is formed by known processes and the only remaining problem is that of converting the fourth 6-membered ring into the 5-membered ring D. This is accomplished by oxidising the glycol derived from (*124*) to the dialdehyde (*125*), which is self-condensed to (*126*), at which point all the features of a steroid are present. For identification with one of unimpeachable structure it was oxidised and esterified to (*127*), which was found to be identical in all respects with that derived from a bile acid. Resolution of the racemic product was also carried out. This *ætio* acid was converted to several important steroids by methods already known, but here we are only concerned with its further progress towards cortisone. This is brought about by reducing the double bond in ring D and introducing the 11-keto group by the method originated by Fieser and described earlier in this review. The synthesis of cortisone is then completed by methods already discussed. This synthesis of cortisone is the only one that has so far been fully described.

The third total synthesis has been devised in the Merck research laboratories under the leadership of Dr. L. H. Sarett[104–108], himself responsible in earlier years for the first partial synthesis of cortisone from bile acids. A two-ring system is built up, which is finally to represent rings B and C of the sterol, and here also the Diels-Alder reaction is used, so that the original product is *cis*, and a ketone group is provided to enable isomerisation to the *trans* configuration to be accomplished by treatment with alkali when required. Benzoquinone is condensed with 3-ethoxypenta-1:3-diene to give (*128*). Reduction of the ketone groups, hydrolysis of the enolic ether system and condensation with methyl vinyl ketone lead to formation of the tricyclic compound (*129*). Protection of the ketone and selective oxidation of one of the hydroxyl groups, followed by isomerisation of the so-formed ketone, gives (*130*), in which the ring junctions are now *trans*. The 11-oxy group and the potential unsaturated 3-ketone group are all present and also an additional carbonyl group is available to use as a point of attack for introducing the second angular methyl group and for creating ring D. This synthesis



is remarkable for the swiftness with which the major needs are introduced by ingenious selection of the starting materials and for the avoidance of superfluous blocking reactions. Double alkylation of the α-methylene group with first a methyl- and then a methylallylhalide gives (*131*). Condensation of this with ethoxy-acetylene magnesium bromide followed by anionotropic rearrangement brings about the formation of (*132*)

which, after reduction of the carbethoxy group, tosylation and oxidation of the unsaturated side chain, leads to (*133*) in which all the groups are present in a suitable state for condensation with alkali to the steroid (*134*). Into this, a derivative of the known compound 11-keto-progesterone (*135*), an acetoxyl group can be introduced into the side chain, as shown, to give ultimately cortisone (*136*). The ethylene dioxy group (R) has not only protected the ketone group, but has also afforded protection to the 5:6-double bond, which has survived several different types of oxidation, reduction and substitution reactions since its original formation much earlier in the synthesis. Unlike the first two methods described, this one was aimed particularly at the synthesis of cortisone, and therefore the potential 11-ketone group has been present from the beginning.

This review mentions the salient features of all the most important work on the synthesis of cortisone. There has not been space to go into the detail of any investigation and in particular earlier attempts at total synthesis have not been considered, being more appropriate for review in other circumstances. Also, discussions about the interpretation of physical constants, reaction mechanisms and the invaluable evidence brought forward by spectroscopy have been omitted. Nevertheless, it is hoped that an adequate picture has been presented of the excellent work carried out in this field.

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RESEARCH PAPERS

THE ESTIMATION OF THE COMPONENT CARDIAC GLYCOSIDES IN DIGITALIS PLANT SAMPLES USING PAPER CHROMATOGRAPHY AND FLUORESCENCE PHOTOGRAPHY

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Two species of Digitalis, *D. purpurea* and *D. lanata*, comprise the main source of cardiac glycosides used in therapeutics. Only very little is known of the factors necessary for the optimum production of active glycosides in these plants and this paucity of information is largely due to the absence of adequate methods for the differential analysis of the glycosidal content of such plant material.

The methods at present in use almost always depend upon a biological assay of total cardiac activity, and this comprises the resultant action of the mixture of several cardiac glycosides present in the plant. It is quite possible that similar degrees of total activity may result from differing mixtures of active glycosides and, although such biological techniques as the embryonic chick heart method of Lehman and Paff[1] are capable of giving very accurate results with microgram quantities of cardiac glycosides, such methods must be reserved for the standardisation of relatively pure samples of single glycosides using the same substance as a reference standard.

The method found most satisfactory in these laboratories comprise the separation of the mixed glycosides by paper chromatography after they have been freed from major impurities by solvent extraction processes. By appropriate chemical treatment the chromatogram is made to fluoresce under ultra-violet radiation and the fluorescence then recorded photographically. In this way permanent records are obtained for each chromatogram and the amounts of the component glycosides determined in comparison with reference standards by photoelectric densitometry measurements made upon the photographic records.

This method in our hands is straightforward and reliable although careful attention to detail is essential both in the chromatographic separation stage and in making the fluorescence photographs. The method is described and discussed in the stages indicated above.

Digitalis lanata contains three so called "genuine glycosides" digilanids A, B and C giving rise respectively by the loss of glucose and an acetyl group to digitoxin, gitoxin and digoxin. In this paper these will be referred to as "desgluco-glycosides." In *Digitalis purpurea* only digilanids A and B are found and these already lack the acetyl group and are therefore deacetyl digilanids. In this species again digitoxin, gitoxin and a third glycoside gitalin are also to be found.

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Since the solubility of the digilanids in water is greater than that of the desgluco-glycosides it is necessary to modify the chromatographic procedure for the analysis of either group, and both groups of substances cannot be estimated upon the same chromatogram.

A PRELIMINARY SEPARATION OF MIXED GLYCOSIDES FROM PLANT MATERIAL

In all cases the same method is used to prepare the mixed glycosides for chromatography and commences with the total extraction of the plant material with ethyl acetate. This extract is then reduced to a small volume by vacuum evaporation of the solvent, and the thick residue is absorbed upon diatomaceous earth which is added in sufficient quantity to produce a crumbly powder. This material contains, in addition to the glycosides; sterols, chlorophyll and other impurities which are next removed by Soxhlet extraction of the powder with light petroleum in which the cardiac glycosides are insoluble. Finally the cardiac glycosides are also removed from the adsorbent by a second extraction with a mixture of chloroform and methanol 1:1. This extract is then evaporated to dryness and the residue is dissolved in a measured volume of ethanol (80 per cent.) and used for the paper chromatography.

The separation of the glycosides from digitalis leaf by paper chromatography was described by Svendsen and Jensen[2] and by Hassel and Martin[3]. These workers quoted R_F values for the different glycosides but little mention is made of the possibility of the separation of the different components and their behaviour in mixtures.

Svendsen and Jensen describe a method to render the spots visible by spraying the developed chromatogram with a 25 per cent. solution of trichloracetic acid in chloroform followed by heat, which renders the spots fluorescent under ultra-violet radiation. This method has been used with some modifications in our work and forms the basis for quantitative estimations. Svendsen and Jensen recommended the separation of the glycosides in mixtures of chloroform, methanol and water in three mixtures of different proportions (I. 10:2:5, II. 10:4:5, III. 10:8:5) and they determined the R_F values for a considerable number of digitalis glycosides. They state also that on the basis of the appreciable differences in these values for the glycosides of *Digitalis lanata* it should be possible to achieve individual separation. In repeating this work we found that the separation of the digilanids B and C on the one hand and gitoxin and digoxin on the other was not sufficiently complete to be unambiguous and certainly not complete enough to serve as a basis for quantitative work. We found it necessary therefore to resume the study of different solvents and solvent mixtures including, in addition to chloroform and methanol, such other solvents as ethyl acetate, benzene, *cyclo*hexane and light petroleum.

Using a mixture of ethyl acetate containing a small but definite amount of ethanol, benzene and water in the proportions 86:14:50 by volume a complete and reproducible separation of digilanids A, B and C was obtained using the organic phase as the mobile one in an atmosphere

of water vapour. The percentage of ethanol in the organic phase is of great importance for the relative placement of the digilanids of the developed chromatogram. With a content of 3 to 5 per cent. of ethanol, a large increase of the R_F values can be achieved and thus good separation of the slower moving C and B components, but at the same time digilanid A travels near to the solvent front and shows a tendency to greater spreading of the spot. With a smaller content of ethanol, 0·5 to 3 per cent., the components B and C are placed closer together but digilanid A remains still well separated and forms a denser spot well apart from the desglucoglycosides or genins (if present) which travel near the solvent front. The great sensitivity of the procedure to changes of the developing mixture necessitates great care in the purification of the solvents and especially of the ethyl acetate which needs careful fractionation to free it from the ethanol usually present in the commercial material. At the same time this sensitivity is of considerable importance since it can be used to advantage to achieve the best separation of the particular digilanids in a mixture upon which the main interest is focussed at the time. In Table I are shown some R_F values of the digilanids A, B and C determined by using different concentrations of ethanol in the mixture described.

TABLE I

The change in the R_F values of the digilanids A, B and C with the increase of the ethanol content in the solvent mixture of ethyl acetate and benzene 86:14.

Ethanol per cent.	Digilanid A		B		C	
	R_{FF}	R_{FC}	R_{FF}	R_{FC}	R_{FF}	R_{FC}
1·5	0·50	0·46	0·36	0·28	0·14	0·08
2	0·54	0·51	0·395	0·355	0·15	0·14
5	0·695	0·67	0·51	0·385	0·16	0·13
7·5	0·71	0·65	0·54	0·44	0·24	0·18

The R_F values were determined in a rectangular tank 6″ × 8″ × 14″ (height) at 24° C. (± 1°); the average distance of the solvent front from the starting line was 200 mm. and the time of irrigation 1¾ to 2 hours. R_{FF} refers to the front portion. R_{FC} to the middle point of the spot.

As has been repeatedly stressed by workers in this field the R_F values of the different components are subject to variation according to the amount of the substance present but it is of particular interest to note that the position of the spots on the developed chromatogram is also dependent on the relative amounts of the glycoside components in its direct vicinity. This is best illustrated by the following example. In the separation of the 3 digilanids the compound A travels faster than B or C, which follow in that order. In a case where digilanid C is present in high concentration even a small quantity of digilanid B is displaced farther to the front as compared with a similar chromatogram with a lesser amount of the C compound.

This advantageous displacement of the different compounds by the relative concentrations of their immediate neighbours can be explained

either by a strong salting-out effect in the partition process or, more probably, by the assumption that intrinsic adsorption phenomena upon the paper material plays an important part in the separation process making it possible to separate isomerides differing only in the position of one hydroxyl group in so large a molecular structure as that found in digilanids B or C.

The separation of digoxin from gitoxin has proved of considerable difficulty and no satisfactory solution of this problem has so far been found with the various solvent mixtures used. The solvent mixtures earlier mentioned and used by Svendsen and Jensen (*loc. cit.*) as well as that normally employed by us give some separation but the spacing of the components is not sufficient for an unambiguous identification or for quantitative work. As stated by Svendsen and Jensen the R_F values of digoxin and gitoxin are very close and the figures given by them are in fact 0·76, 0·81, 0·82 for gitoxin and 0·68, 0·75, 0·76 for digoxin in different mixtures of chloroform, methanol and water. These figures have been confirmed in our experiments in contradiction to the statement of Hassel and Martin who report values of 0·0 for gitoxin in different solvents comprising mixtures of ethyl acetate and water, or chloroform and water.

It may be of interest to mention in this connection that even small quantities of digitoxin can be determined in the presence of digoxin and gitoxin using a mixture of chloroform, benzene and water in the proportions 65:35:50 and containing 5 per cent. of methanol. The digitoxin forms in these cases a well defined orange or yellow fluorescent spot well separated in front of gitoxin and digoxin. The colour of this spot is of great importance for identification and estimation since it cannot be confused with the blue fluorescing digoxin or gitoxin.

For all the work described we have used Whatman No. 1 paper and the ascending method of irrigation. The descending method has been tried in some experiments but gave less reproducible results. In the case of chloroform, methanol, water mixtures we sometimes experienced, for unexplained reasons, some difficulty from a tendency of the mobile phase to change the concentration of the irrigating liquid as evidenced by bands of different transparency of different heights in the paper.

The time required for development using ethyl acetate-benzene-water mixtures was 2 to 2¼ hours and the distance travelled was approximately 22 to 24 cm. The time required for the slower moving chloroform-methanol-water mixtures was about 3½ hours for the solvent front to travel the same distance from the starting line. The temperature during a single experiment remained constant to within 0·5 to 1·0° C. and most experiments were performed at a temperature of 22 to 24° C. After removal of the paper from the chromatograph chamber it was dried in air for 2 to 3 hours at 25° to 30° C. and then sprayed abundantly with a 25 per cent. solution of trichloracetic acid in chloroform using an Aerograph MP spray gun with a No. 2 nozzle and air at 5 to 10 lbs. pressure. The trichloracetic acid solution was prepared freshly each week, with chloroform purified and distilled before use and kept protected

from moisture and light. The sprayed paper is then heated in a drying oven at 105° to 110° C. keeping it free of contact with any other material by clamping it vertically between glass rods at each end. After 4 to 5 minutes the paper is removed and given a quick airing to free it of acid vapours. It is then replaced and heated in the oven for a further 4 to 5 minutes. The chromatogram so treated should fluoresce brightly in ultra-violet light and does not fade appreciably over periods of less than one week. To achieve the brightest fluorescence with digilanid A the heating of the sprayed chromatogram should be extended to 8 to 10 minutes before airing and then heated for a further period of 4 to 5 minutes.

The paper chromatogram is then used to prepare a photographic record for qualitative and quantitative examination by a modified method of fluorescence photography, the basic principles of which are described by Henney and Dudley[4] and by Auerbach and Auerbach[5]. This procedure must be carried out in a photographic darkroom illuminated with a safe light suitable for bromide paper.

The chromatogram is illuminated by ultra-violet light produced by a mercury vapour lamp in Wood's glass envelope (Mazda MBW/V 125 Watt. Mercury vapour) which is further filtered by a sheet of Corning glass "Violet ultra No. 586" attached to the front of the light-tight lamp house. The photographic exposure is made by brief controlled illumination from this lamp which, since it does not restart when hot, cannot be switched on and off for this purpose. A suitable shutter is therefore arranged to cut off the ultra-violet light completely when closed. The device we have used consists of a series of slats of thin brass resembling a venetian blind arranged just behind the Corning Glass filter. These slats are approximately 1 in. wide and $\frac{5}{8}$ in. apart and are coupled together and operated by an electromagnet. When open the passage of light through the filter, approximately 5 in. × 4 in. is almost completely unobstructed and when closed no ultra-violet light leaves the lamphouse.

The lamphouse is mounted with the Corning glass filter and shutter horizontal on the under surface approximately 25 in. above a bench so that the surface below is uniformly irradiated with ultra-violet.

The chromatogram is placed upon the bench beneath a sheet of $\frac{1}{4}$ in. plate glass to keep it flat and centred beneath the ultra-violet source. The chromatogram in addition to fluorescing transmits and reflects ultra-violet and hence a sheet of special filter glass is placed beneath the chromatogram in order to cut off all transmitted ultra-violet but allow the visual fluorescent light to be as little reduced as possible. Finally beneath this filter is placed a sheet of bromide paper of sufficient size to include all the fluorescent areas on the chromatogram. Using a chromatograph paper 8 in. wide, standard 10 in. × 8 in. sheets of bromide paper are satisfactory. The bromide paper used should have sufficient contrast to enable the fluorescent spots to produce a good black image against a very pale grey background. The most suitable material in our experience is Kodak contrast No. 3 glossy bromide paper of single weight thickness. The arrangement of the apparatus for this process is illustrated diagramatically in Figure 1.

The filter used to prevent the transmission of ultra-violet to the bromide paper is of paramount importance and should be of sufficient size to cover the sheet of bromide paper. The radiation from the filtered ultra-violet source is almost entirely from the 365 mμ Hg line and this filter must be opaque at this wavelength and have a high transmission in the visual region. It is also essential that it shall not fluoresce itself as so many dyed gelatine filters do. The literature indicates that the Wratten No. 2A filter should be satisfactory but we have not tried this and would anticipate difficulty in obtaining a large sheet. The filter used in these experiments was a sheet of glass 15 in. × 12 in. × $\frac{1}{4}$ in. known commercially as "champagne plate" and manufactured in England. This was found as a result of experiments with various pale yellow glasses most of which are quite unsuitable.

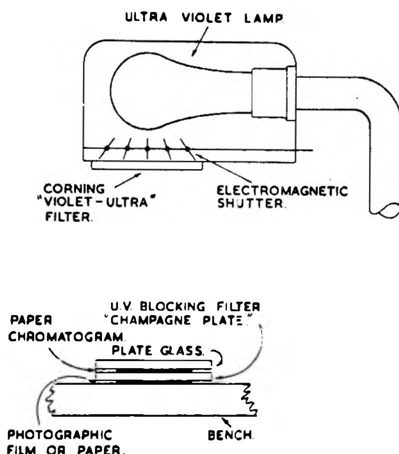

FIG. 1. General arrangement of the apparatus for the photographic recording of the fluorescence chromatogram.

In Figure 2 is given a transmission curve for this glass in air in order that comparisons with published transmission curves for other glasses can be made. Although there is rather a sharper cut off at 410 mμ with the Wratten No. 2A filter the champagne plate glass is readily available, inexpensive and quite satisfactory.

A fluorescence photograph of a chromatogram of digilanids B and C is reproduced in Figure 3. Digilanid A and digitoxin fluoresce with an orange light which is non-actinic to bromide paper. By this means, therefore, these substances are effectively excluded from the analysis and the photographic process is modified accordingly to detect these substances.

The edge of the chromatogram is notched in two or three places with a ticket punch to enable exact identification to be made between any chromatogram and the corresponding photograph on the assumption that an identical arrangement of notches is never likely to occur twice.

When the yellow fluorescence of digitoxin or digilanid A can be seen on a chromatogram a second photographic record is made after the bromide paper print since this excludes these substances.

For the second photograph the "champagne glass" filter is exchanged for a sheet of photographic amber glass in order to further reduce the intensity of the bright blue fluorescence already recorded and allow the yellow-orange light to have a proportionately larger effect. Instead of bromide paper Kodak Process Panchromatic cut film is used in these

cases. The spectral transmission curve for the amber glass is also reproduced in Figure 2.

The problem of the most suitable filters to cut off the excess of ultra-violet radiation is by no means a simple one. It would be possible if ideal filters could be obtained to expose the photographic material indefinitely without producing fogging of the background since the

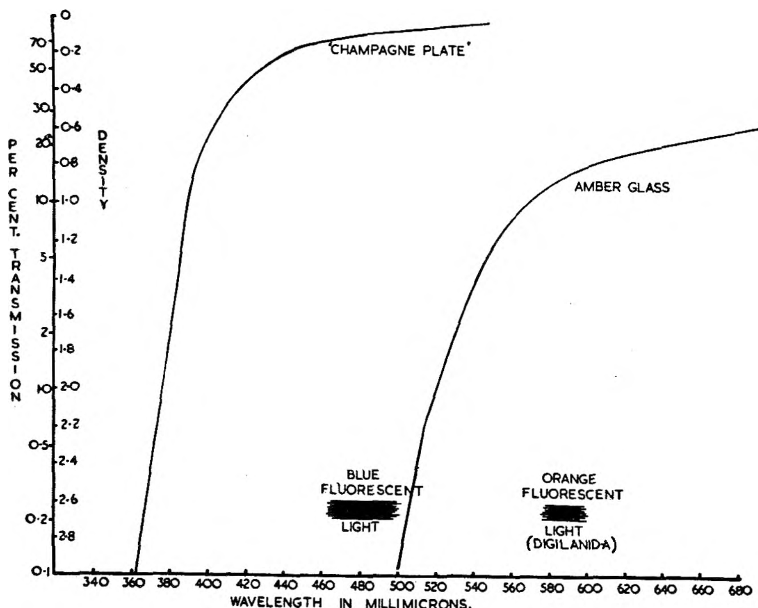

FIG. 2. Transmission curves for filters used above photographic materials in preparing fluorescence photographs.

fluorescence alone would provide the illumination. Using the "champagne plate" glass the length of exposure is limited to approximately 10 sec. under our conditions before background fog becomes appreciable. Under these circumstances it is possible to obtain clearly exposed spots with as little as 0·5 to 1·0 μg of digilanids B or C but if filters could be obtained which would enable longer exposures to be made this lower limit might well be much smaller.

The amber glass filter has an appreciable absorption of the yellow fluorescence of the digilanid A or digitoxin spots and hence these tend to be rather poorly recorded but it does cut off residual radiation from the lamp so that exposures up to 5 minutes in duration do not produce background fog. When these glycosides are to be measured the exposure we have used is 3 minutes with the ultra-violet source brought to within 18 in. of the photographic material.

Using the amber filter it is possible to record all glycosides since the blue fluorescence is still transmitted sufficiently to give a good record on panchromatic film but in view of the greater expense of this material and when interest is centred on the blue-fluorescing glycosides bromide paper

gives an excellent record. The "champagne plate" filter presents one disadvantage since it is $\frac{1}{4}$ in. thick and separates the fluorescing chromatogram from the sensitive material thus causing some slight diffusion of the spots. The thinner these filters are the less the spots will tent to merge together. The amber glass is more satisfactory from this point of view since it is only approximately 0·1 in. thick.

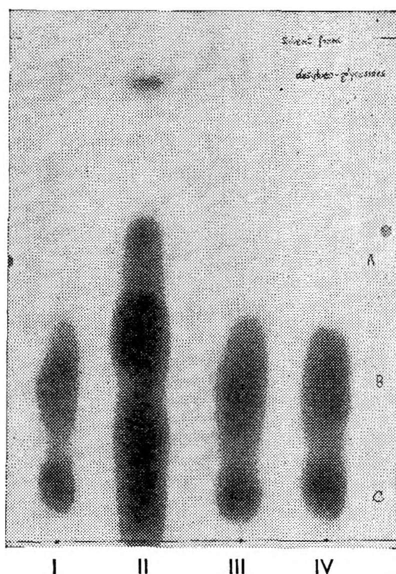

FIG. 3*a*. Fluorescence photograph of a chromatogram with known amounts of digilanids A, B and C. The yellow fluorescence of digilanid A is not seen in this record.

I. Digilanids:
A 4 μg.
B 2 μg.
C 6 μg.

II. Digilanids of plant sample 19z: 0·01 ml.

III. Digilanids:
A 6 μg.
B 4 μg.
C 8 μg.

IV. Digilanids:
A 6 μg.
B 4 μg.
C 10 μg.

FIG. 3*b*. Record on panchromatic film of part of the same chromatogram as Fig. 3*a* to show the fluorescence of digilanid A.

In all cases the developer used in the Kodak D 19 formula and development under standardised conditions is of 2 minutes duration for the bromide paper and 3 minutes for the film at 22° C.

On the chromatogram of Figure 3A some digilanid A was also present and in Figure 3B is seen a reproduction of a photograph of the panchromatic film obtained, which shows the content of this third digilanid and also the registration marks.

In Figure 4 is seen an analysis of an unknown mixture of glycosides from plant material using the mixture of solvents to show the separation of the digilanids and in this case the digitoxin, gitoxin and digoxin have travelled completely to the solvent front and it will be seen that

there is a substantial amount of digilanid C and digilanid B, the former corresponds to approximately 10 μg. and the latter rather more than 6 μg. of digilanid B in 0·01 ml. of extract.

Direct visual comparison from these photographs is of great value in the quantitative field since standard mixtures of glycosides are always included on each paper at more than one concentration of each. It is essential to note that since the photographic record is the result of a prolonged exposure to the fluorescence small amounts of fluorescence not easily seen on the chromatogram cause an integration of silver deposition in the photograph and thus become clearly visible.

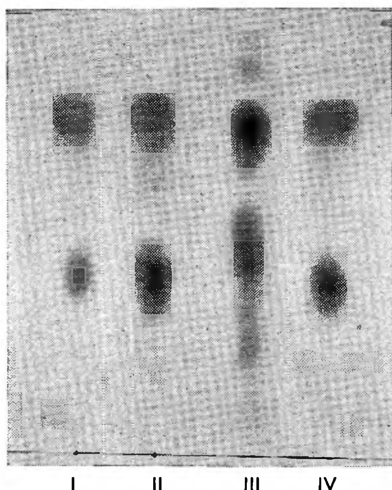

FIG. 4. The analysis of a plant sample by visual comparison from the photographic record.

N.B.—The several processes of reproduction have inevitably impaired the quality of the records which are somewhat more easily studied in the original.

I. Digilanids: B 4 μg. C 6 μg.

II. Digilanids: B 4 μg. C 10 μg.

III. Digilanids of plant sample 19z: 0·005 ml.

IV. Digilanids: B 6 μg. C 10 μg.

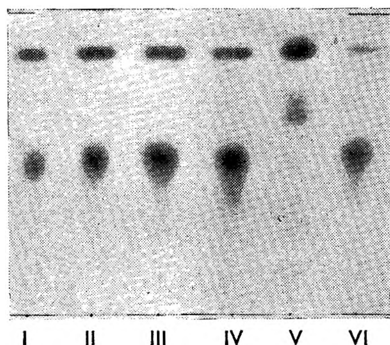

FIG. 5. Arrangement of chromatogram for the analysis of plant samples by photographic densitometry. In this case the amounts of standard glycosides are carefully chosen to give a graduation of fluorescence above and below that of the plant sample under examination. In this record the digilanid C is suitable for accurate analysis but the spots of digilanid B are rather too close to the solvent front.

I. Digilanids: B 2 μg. C 4 μg.

II. Digilanids: B 4 μg. C 6 μg.

III. Digilanids: B 6 μg. C 8 μg.

IV. Digilanids: B 4 μg. C 10 μg.

V. Digilanids of plant sample 19z: 0·0025 ml.

VI. Digilanids: B 2 μg. C 10 μg.

Where a greater degree of accuracy is required the process is further elaborated by making negatives from the chromatogram photographs which then show a dense black background with variable clear areas corresponding to the spots on the original prints. The size and density of these spots is then measured by a simple photoelectric densitometer. For such densitometry measurements it is desirable to have the spot represented as a clear area in a black background since problems of the area covered by the photocell system do not arise. These measurements

are made upon Kodak Process cut-film by exposing this directly under the bromide paper print or panchromatic film using these as negatives and placing the emulsion surfaces of the two materials in contact. Exposure is carried out in this case under a sheet of plain glass to a low intensity tungsten lamp and is of such duration that the transmission of the background does not exceed 2 per cent. of that of the clear film.

The photoelectric densitometer is a simple arrangement of a selenium photocell arranged over the clear area of film and a condenser lens below. The light source is in this case a motor car headlamp bulb operating from an accumulator. The amount of light transmitted being measured by means of a Cambridge Spot galvanometer. In Figure 5 is shown a photographic print from a chromatogram of digilanids B and C suitable for more exact examination in this way. By arranging the largest amount of the glycoside to correspond to 100 per cent. transmission in the negative film there is a linear relationship between spot size and density integrated in this way and the amount of the glycoside on the paper chromatogram.

The authors are indebted to Messrs. Sandoz of Basle for gifts of pure digilanids A, B and C; Messrs. Burroughs Wellcome & Co. (Australia) Ltd. for gifts of digoxin and digitoxin and Dr. D. W. Adamson of the Wellcome Research Laboratories, Beckenham, Kent, England, for a gift of gitoxin. For much valuable advice upon the selection of light filters we should like to thank Dr. R. G. Giovanelli of the Department of Optics, National Standards Laboratory, Sydney. A grant in support of this work from the National Health and Medical Research Council of Australia provided technical assistance and we wish to take this opportunity to express our thanks in this regard. To the Directors of Drug Houses of Australia we are greatly indebted for the support of one of us (H. S.) throughout this work.

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A CHROMATOGRAPHIC STUDY OF THE CURCUMINOIDS IN *CURCUMA LONGA*, L.

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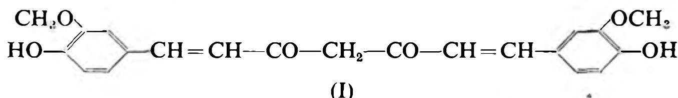
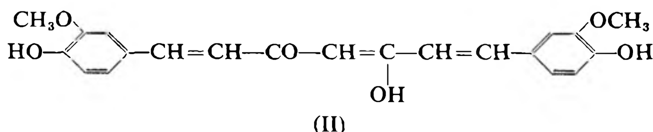
IN an attempt to evaluate samples of turmeric (the rhizome of *Curcuma longa*, L.) on the basis of their curcumin contents, using the familiar reaction with boric acid for colorimetric estimation, the author found that the colour developed with extracts was markedly different from that obtained with pure curcumin and this observation has led to the present investigation.

Curcumin has the molecular formula $C_{21}H_{20}O_6$ or $C_{19}H_{14}O_4(OCH_3)_2$, first suggested by Ciamician and Silber[1] from determinations of methoxyl. The work of Milobedzka, *et al.*[2] and the subsequent synthesis effected by Lampe[3] showed that curcumin was diferuloyl methane (I):



(I)

Attempting a synthesis of curcumin by condensing acetylacetone with vanillic aldehyde in presence of ethanolic hydrochloric acid, Heller[4] obtained two products which differed from curcumin in their failure to react with boric acid and ferric chloride, and were therefore called by him α- and β-*iso*curcumins. As 1 :3-diketones form stable metallic compounds by virtue of their ability to enolise, the enolic H being acidic, Heller on the basis of the reaction with ferric chloride came to the conclusion that normal curcumin which gives a deep reddish-brown colour with ferric chloride has the keto-enol structure (II), while the two



(II)

*iso*curcumins which give only a faint yellowish-brown colour have the diketonic structure (I), the α- and β-forms being stereo-isomers of the same diketone[5]. Pavolini[6] obtained curcumin from acetylacetone and vanillin by using boric anhydride as condensing agent, and later in collaboration with others[7] has synthesised a number of "curcuminoids" or analogues of curcumin by condensing different aromatic aldehydes with β-diketones. As it is possible that natural curcumin is accompanied by minor amounts of its analogues and its structural and stereo-isomerides, a study of the constituents of the colouring matter in turmeric by a chromatographic procedure was undertaken. Preliminary experiments have indicated that the pigments could be resolved into three main constituents and a few minor fractions.

EXPERIMENTAL

Preparation of the Extract.

Curcumin is insoluble in water, light petroleum and hexane, moderately soluble in benzene, chloroform and ether, while ethanol and acetone are good solvents. Benzene however has the merit of taking up very little of the resinous impurities from turmeric[8] besides being nonpolar, and was therefore used throughout both for extraction of the pigments and for chromatography. About 25 g. of air-dried turmeric powder ground to pass 80 mesh is extracted with light petroleum (b.pt. 40° to 60° C.) to remove the fixed and volatile oils. The dried residue is again exhausted with about 200 ml. of boiling benzene in a percolator with a Wiley-Soxhlet extraction syphon cup. The syphon cup is constructed from a Pyrex boiling tube (3 cm. × 12 cm.) and 2 mm. bore Pyrex tubing. The extract is allowed to stand overnight and filtered free from insoluble materials.

Adsorbents.

A number of adsorbents were tried for their suitability for chromatographic procedure, with reference to their adsorptive power, rate of development, separation and visibility of the zones on the column. Alumina and magnesia were too active, the separation of the zones was poor and some of the adsorbed materials could not be stripped easily from the column with any of the ordinary solvents. Alumina (B.D.H. for chromatographic purposes) rendered neutral by washing with dilute hydrochloric acid and deactivated[9] was still found to be of no use. Precipitated calcium carbonate, magnesium carbonate, sodium bicarbonate, starch, Fuller's earth, etc., had very little adsorptive power for curcumin. Dried silica as an adsorbent was also not satisfactory; it becomes translucent in contact with the solvent, causing poor visibility and separation of the zones. But silica incorporated with about 50 per cent. of its weight of moisture proved satisfactory. The silica gel used in this study was prepared from sodium silicate dried (Merck) dissolved in water to form a 10 per cent. solution by precipitation with dilute hydrochloric acid as described by Gordon, Martin and Synge[10]. The precipitated silica gel was "aged" for 2 days in 4N hydrochloric acid, filtered and washed free from acid on a Buchner funnel and dried at 110° C. for 16 hours. The small lumps were crushed and the material passed through an 80-mesh screen and preserved in a stoppered bottle. For use, 100 g. of silica is treated with 53 ml. of water in a mortar and thoroughly mixed. With this material good visibility and separation of the zones with almost colourless interzones are secured, with a carefully packed column and a slow rate of percolation of developing solvent, if overloading of the column is avoided.

Apparatus and Procedure.

The chromatographic apparatus used consisted of a Pyrex tube (2·2 cm. × 120 cm.) fitted with a tap at the lower end. A plug of

cotton wool soaked in benzene and freed from air bubbles is pushed to the lower end of the tube and a thin slurry of the prepared silica gel in benzene is poured down the sides of the tube and allowed to settle. Application of positive pressure of about 20 to 30 cm. of mercury at the top of the column helps uniform and firm packing and the column is packed to a height of 45 cm. About 80 g. of the silica gel gives a column of this height. The length of the column holding 1 ml. of solvent is 4·4 mm. and 1 ml. of solvent occupies a tube length of 2·4 mm. giving a packing ratio (S)[11] for the column of about 1·8.

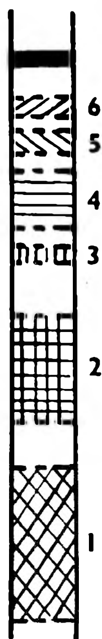


FIG. 1.
Chromatogram of benzene extract of turmeric on silica.

The filtered turmeric extract is then passed down the column and when the solution has just sunk into the column, fresh benzene equilibrated with water is added for the development of the chromatogram and pressure is applied at the top so that a rate of flow of solvent of about 3 ml./minute, which corresponds to a movement of the solvent front down the column at about 8 mm./minute, is maintained. There is a gradual separation of bands on the column and the completed chromatogram will show 3 distinct zones and a few minor zones represented in Figure 1.

R *Values*[12].

The rates of migration of the bands relative to the movement of the surface of the developing liquid are measured as follows. The initial position of the liquid level in the tube above the column and that of the estimated maximum concentration of each band are first marked when the solution has just entered the column. The distances through which these points move are measured again when the zones have completely separated on the column. The *R* values for the different fractions are given in Table I.

Separation of the Fractions.

Development is continued with benzene. Any fixed or volatile oil of turmeric not completely removed by light petroleum, passes down the column first without definite zoning and is collected as a pale yellow

TABLE I

PHYSICAL PROPERTIES AND ANALYTICAL CONSTANTS

Substance in zone number	M.pt. °C.	*R* values	Mol. wts.	Methoxyl found per cent.
1 (Curcumin)	182	0·27	371, 362, 369 [$C_{19}H_{12}O_2(OH)_2(OCH_9)_2$ = 368]	16·44 16·62 (calc. 16·88)
2	168	0·14	333, 341, 337 [$C_{19}H_{18}O_2(OH)_2(OCH_3)$ ≍ 338]	9·05 9·10 (calc. 9·18)
3	80 to 130	0·10	364, 371	—
4	224	0·09	306, 304, 309, 308 [$C_{19}H_{12}O_2(OH)_2$ = 308]	0·0

fraction. Evaporation of the solvent leaves a yellow oily residue having the characteristic odour of turmeric. The different zones are collected as liquid chromatogram in separate containers as each washes down the column. It is found that the widths of the zones increase considerably as they move down the column. The eluates from the interzones are nearly colourless or only faintly coloured and are rejected. The different fractions collected are concentrated by distillation of the solvent under reduced pressure and further purified by re-chromatography on smaller columns (1·5 cm. × 20 cm.) of silica gel.

Fraction 1. This is the eluate containing the orange-yellow zone (adsorbed lowest on the column) consisting of curcumin. As the benzene solution is evaporated, curcumin is thrown out of solution in the form of bright cherry-red crystals which, however, soon change into a yellow form. The crystals were filtered off on a small sintered-glass funnel (No. 3 porosity), washed with benzene and dried (yield about 500 mg.). On recrystallisation from ethanol, curcumin is obtained in the form of reddish-orange prisms melting at 182° C.

Fraction 2. Evaporation of the eluate of the next zone above yields an amorphous orange-yellow powder (yield about 200 mg.). Under the microscope it appears as minute spheres of reddish-orange colour. The spheres break into irregular pieces when crushed under the cover slip. Attempts to obtain it in crystalline form were not successful. M.pt. 168° C.

Fraction 3. An amorphous reddish sticky residue (about 20 mg.) is obtained on evaporation of the benzene extract of the next zone. This material also could not be crystallised though its purity and homogeneity was assured by re-chromatography on silica gel several times. The substance was therefore precipitated from benzene solution by addition of light petroleum; the precipitate was washed with light petroleum and dried under reduced pressure. A pale yellow amorphous powder is obtained which softens at about 80° C. and melts at about 130° C. It reverts to the sticky form in contact with benzene.

Fraction 4. Yields a product (about 120 mg.) sparingly soluble in benzene and crystallising out in yellow plates. By precipitation from an ethanolic solution by cautious addition of water, the substance is obtained in the form of glistening golden-yellow spangles. M.pt. 224° C.

Fractions 5 *and* 6 are obtained in minor amounts which could not be crystallised. They had to be repeatedly passed through silica gel before chromatographic homogeneity could be secured. The dark brownish-black layer occupying a few cm. of the column at the top remains there, despite prolonged development with benzene. It probably consists of resinous impurities and other conversion products of the pigments.

Molecular Weights and Methoxyl Values.

Microdeterminations of molecular weights of the different fractions were carried out in replicate by Rast's method[13], with resublimed camphor (m.pt. 177·8° C. and mol. depression of freezing point 40° C.) as solvent. Microdeterminations of methoxyl by Zeisel's method using the Vieboch and Brecher modification[14] were made on fractions 1, 2 and 4, and these values are given in Table I.

Reactions.

The reactions, with the following reagents, of the substances isolated are summarised in Table II. The first 3 reagents are applied to the solutions of the substances in glacial acetic acid, reagents 4 and 5 are applied to a speck of the material on a white porcelain tile.

TABLE II

REACTIONS

Substance in zone number	$FeCl_3$	Boric acid	Boric-oxalic acid	Sodium hydroxide 1 per cent.	Concentrated sulphuric acid
1	Dark reddish brown	Orange	Pink	Red	Dark red
2	,,	,,	Orange red	,,	,,
3	,,	,,	Red	,,	,,
4	,,	Bright yellow fluorescence	Pink with orange fluorescence	Orange	Orange red
5	,,	Orange	Red	Orange red	,,
6	,,	Deep yellow	Orange	Orange	,,

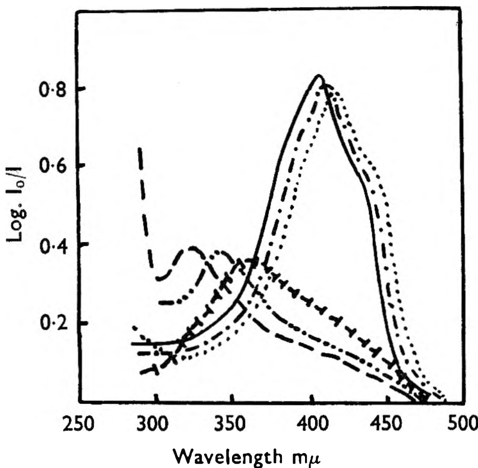


FIG. 2. Absorption spectra in benzene.

....	Zone 1 of chromatogram
·—·—·	,, 2 ,, ,,
-+-+-+	,, 3 ,, ,,
———	,, 4 ,, ,,
—···—	,, 5 ,, ,,
— — —	,, 6 ,, ,,

Reagents.

1. Ferric chloride—1 per cent. solution of anhydrous $FeCl_3$ in glacial acetic acid.
2. Boric acid—0·5 per cent. solution in glacial acetic acid.
3. Boric-oxalic acid—1 g. of oxalic acid, and 0·5 g. of boric acid dissolved in 100 ml. of glacial acetic acid.
4. Sodium hydroxide—1 per cent. aqueous solution.
5. Concentrated sulphuric acid.

Absorption Spectra.

The absorption spectra in the visible and ultra-violet region of the substances in benzene and ethanol were recorded with a Beckman Model DU Quartz Spectrophotometer, with a water-cooled hydrogen discharge lamp light source and 1-cm. silica cells. Solutions containing 0·5 mg. in 100 ml. of the solvents were prepared by weighing out the substances on a microchemical balance and their extinctions relative to the solvents were measured from 480 to 280 mμ in the case of benzene solutions and down to 220 mμ in the case of ethanol solutions, at intervals of 10 mμ, while, in the region of an inflexion, measurements at every 2 mμ were made. With the recommended setting of the sensitivity knob for

maximum accuracy the zero adjustments at each particular wavelength were made by adjustment of the slit width. Figures 2 and 3 show the typical curves obtained with benzene and ethanol solutions respectively, and the absorption data are given in Table III.

TABLE III

ABSORPTION DATA

Substance in zone number	Ethanol		Benzene	
	$\lambda_{max.}$	$E^{1 \text{ per cent.}}_{1 \text{ cm.}}$	$\lambda_{max.}$	$E^{1 \text{ per cent.}}_{1 \text{ cm.}}$
1	430	1560	420	1520
2	425	1580	415	1560
3	370	620	365	—
4	420	1640	410	1640
5	—	—	345	—
6	—	—	325	—

Thermal Isomerisations of Fractions 3, 5 *and* 6.

Benzene solutions of fractions 3, 5 and 6 are found to be unstable and they undergo a slow change, whereby each of them separates into two zones of re-chromatography after a few days. This change is accelerated by heat. A solution of chromatographically homogeneous fraction 3 in benzene was refluxed for 10 hours. Another portion of the same solution was evaporated to dryness and the residue was heated at 110° C. for 5 hours and then taken up in benzene. Both these solutions were passed through silica columns. There was a separation in both cases into two bands, the topmost one being due to the unchanged substance while the fast moving zone was identified as curcumin, from its absorption spectrum, reaction and *R* values (i.e., by mixed chromatogram with pure curcumin). Similar changes were noted in the case of fractions 5 and 6 also, and these yield respectively on isomerisation substances identical with fractions 2 and 4.

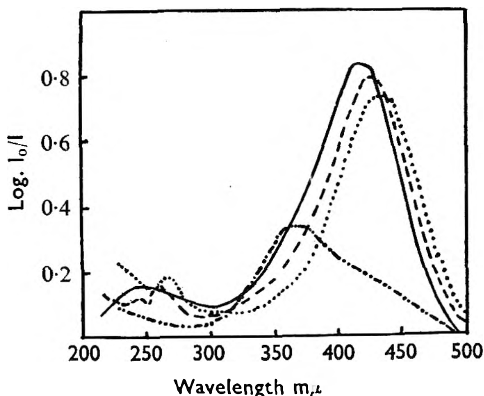


FIG. 3. Absorption spectra in ethanol.

.... Zone 1 of chromatogram

— — — „ 2 „ „

— · · — „ 3 „ „

——— „ 4 „ „

DISCUSSION

The Chromatographic Process.

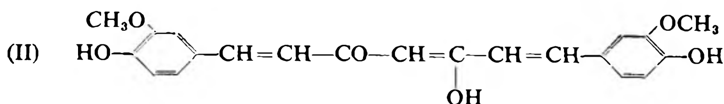
Silica gel is widely applied for the resolution of mixtures by partition between water held by the silica and the immiscible solvent phase. In the present case, however, since the pigments are completely insoluble

in water it is obvious that partition chromatography in the sense of a continuous counter-current liquid-liquid extraction process as used by Martin and Synge cannot be playing any significant role in the mechanism of the chromatographic process here, and the mechanism is most probably one of surface adsorption and desorption as in classical chromatography, the separation of the zones depending on differences in the adsorption affinities of the fractions for silica. The addition of water to the dried silica has served only to control its adsorbent activity by partial deactivation. Further, the widening of the zones as they move down the column, with a diffuse trailing boundary, may point to a variation of R with concentration and to a non-linear sorption isotherm which is more often the case with adsorption[15]. If dilute ammonia (say a 5 per cent. solution) in which the pigments are soluble is used in the place of water for the preparation of the adsorbent, the difference in partition coefficient may also be a contributory factor in their separations on the column, where they appear as bright red or orange zones. The use of ammonia as the immobile phase causes slower movement but better resolution of the zones, with sharp leading and trailing boundaries which do not spread out very much during development. However, as it was suspected that the zones on long contact with the column tend to fade in colour and undergo some irreversible change, the use of ammonia was not preferred.

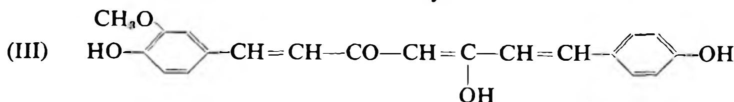
Successful chromatographic separation of the zones with silica and benzene depends on factors such as particle sizes of the adsorbent, dimensions of the column, degree of packing, and rate of flow of developing solvent. The smaller the size of the particle, the better the separation, but too fine a material renders the rate of permeation of the solvent inordinately slow. The 80 to 100-mesh powder used is found to be of suitable size. With the column dimensions used, viz., 2·2 cm. × 45 cm., complete separation of the 3 main zones takes place with benzene extracts from 25 g. of turmeric. Use of larger quantities of material will lead to overloading of the column and poorer separations. The minor zones higher up separate as development continues and not until the fast moving zones have completely left the column. Their movements can be speeded up by cautious addition of ether to the benzene. The rate of movement of the solvent front down the column of about 5 to 10 mm./minute is found to be the optimum for good separation.

Major Constituents.

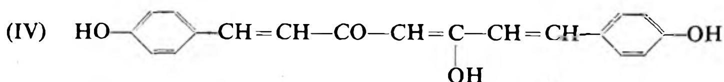
Fractions 1, 2 and 4 form the three major constituents of the colouring matter in turmeric. Curcumin, which is adsorbed lowest on the column (Fig. 1), forms by far the most important constituent. Fractions 2 and 4, which react in a similar way with ferric chloride and other reagents, are probably its analogues. Their molecular weight and methoxyl values (Tables I) indicate their relationship with curcumin. Thus, while curcumin has two OCH_3 groups, fraction 2 has only one, and fraction 4 has none; a reasonable guess as to the structure of these analogues can therefore be made as follows:



Fraction 1. Diferuloyl methane



Fraction 2. *p*-Hydroxy-cinnamoyl-feruloyl-methane



Fraction 4. *pp'*-Dihydroxy-dicinnamoyl-methane

(IV) has been synthesised by Lampe and Godlewska[16] and described by them as orange coloured needles melting at 218° to 220° C. and giving an orange colour with boric acid.

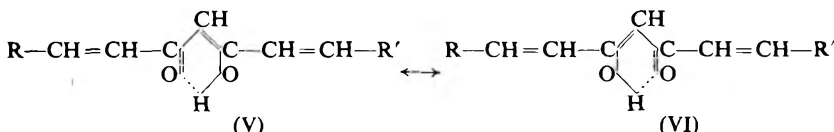
The close similarity in the structure of these 3 compounds is reflected in the similarity of the shapes of their absorption curves (Fig. 2). (IV) in benzene (fraction 4) has a maximum absorption at 410 mμ, while (III) and (II) (curcumin) have their peaks at 415 mμ and 420 mμ respectively, and have slightly lower extinction values. The minor bathochromic shifts of $\lambda_{max.}$ and decreasing absorption intensities can be accounted for on the basis of the weighting effect on the chromophore of successive *m*-substitution of the two aromatic nuclei by methoxy groups[17].

Minor Constituents.

Three minor fractions have so far been isolated and further work is in progress. Difficulty is experienced in their isolation in a state of purity owing to their small *R* values and consequent very slow rate of migration and development on the silica column, their instability, and other factors. Chromatographic homogeneity could be secured only by repeated re-chromatography on silica columns. Fraction 3 has been obtained in just sufficient amounts to enable a study of its behaviour and a guess as to its nature to be made. It has the same molecular weight as curcumin, gives the same reactions, and undergoes isomerisation into curcumin by heat. It is evidently a geometrical isomer of curcumin.

Stereo-isomerism of the Curcuminoids.

As 1:3-diketones, the curcuminoids could be represented as resonance hybrids between structures (V) and (VI) with a hydrogen bond between enolic H and carbonyl oxygen:



The two ethylenic double bonds on either side of the chelate ring structure would allow of *cis-trans* configurations of the substituent groups. For a symmetrical molecule with two double bonds, 3 isomers are possible, viz., *trans-trans*, *trans-cis* and *cis-cis*, and in the case of an unsymmetrical molecule (as III) 4 are possible[18], since the *trans-cis* and *cis-trans* configurations will not be equivalent. Those substances which occur in appreciable amounts on the column have obviously a *trans-trans* configuration which is the most stable form; the less stable forms will occur only in comparatively smaller amounts, while the least stable forms, as for instance the *cis-cis* isomers, may not be present at all or occur in such insignificant amounts as to be incapable of isolation. That probably explains why out of the 10 possible substances only 6 could be discerned on the column so far. Thus, while ordinary curcumin has possibly a *trans-trans* configuration, fraction 3 is to be assigned a *cis-trans* form, considering its lower m.pt. and lower stability. Its lower extinction coefficient and the shift of its absorption maximum to considerably lower wavelengths are also therefore to be interpreted in terms of a *cis-trans* isomerism. Representing the models of the structures of these

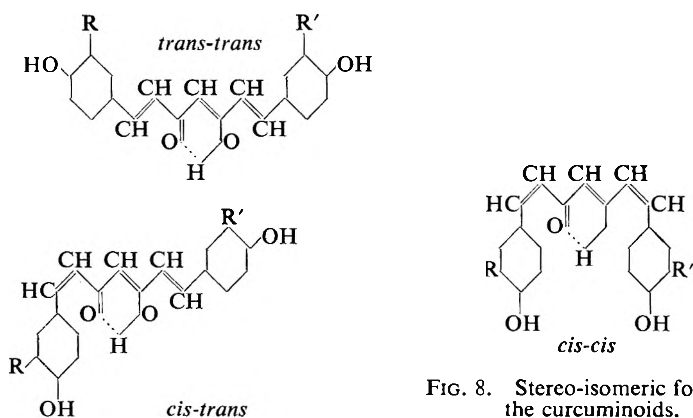


FIG. 8. Stereo-isomeric forms of the curcuminoids.

isomers, using the accepted values of bond distances and valence angles[19], it may be seen that a strainless coplanar *cis* configuration is not possible as the *ortho*-H of the benzene nucleus comes into steric conflict with the carbonyl O, necessitating a slight twist of the benzene nucleus about its 1:4 axis. But considerations of resonance among the different stable structures of the molecule would require a planar configuration and the resonance stabilisation energy will give rise to a potential energy gradient which would oppose any rotation of the benzene ring[20]. The molecule will thus be under a strain and this strain not only reduces its stability, but also causes a hypsochromic shift of the maximum of the principal absorption band[23].

Fractions 5 and 6 possibly bear similar relationships respectively to (III) and (IV) and in the case of fraction 5 there is at present no means of deciding its exact configuration.

iso*Curcumins*.

All the substances isolated give a strong colour reaction with ferric chloride and it therefore appears that the purely diketonic *iso*curmins described by Heller are not present in the natural product.

Summary

1. Chromatographic resolution of the colouring matter of *Curcuma longa*, L., on silica gel from benzene extracts of the powdered rhizome shows a separation into 3 main and 3 minor zones which are isolated by a liquid-chromatogram procedure, the details of which are given.

2. The chemical reactions, physical properties and absorption characteristics of the different fractions are recorded.

3. The three main constituents consist of curcumin, by far the most important, and its two analogues, viz., *p*-hydroxycinnamoyl feruloyl methane (III) and *pp'*-dihydroxydicinnamoyl methane (IV). The minor fractions appear to be the geometrical isomerides of the 3 main constituents.

4. The purely diketonic forms of *iso*curcumins described by Heller are apparently not present in the natural product.

The author is greatly indebted to Dr. T. R. Govindachari, Professor of Chemistry, the Presidency College, Madras, for the use of the Beckman spectrophotometer, microchemical balance, micro methoxyl apparatus and other facilities. The paper is published by kind permission of the Director of Medical Services, Government of Madras.

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THE DETECTION OF RICIN

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Received May 20, 1953

The high toxicity of castor seed makes its detection in feeding stuffs a matter of some importance, and the fact that there is no specific chemical test for ricin, its toxic principle, renders this detection extremely difficult. Hitherto no satisfactory method has been described.

Microscopic examination which was investigated by Leather[1] and Dodd[2] is unsatisfactory, because as Brioux and Guerbet[3] point out, it is only the non-toxic testa that can be identified and this will be present in boiled non-poisonous castor meal, but not in the highly toxic decorticated seeds.

The agglutination test, recommended by Autenrieth[4] has been investigated by many workers, including Miessner and Rewald[5] and Kobert[6], the latter claiming to be able to detect 0·2 per cent. of castor seed in meal by this method. Lander and Geake[7] were unable to detect less than 10 per cent. of castor seed in linseed by agglutination. The chief disadvantage of the method lies in its non-specificity as many plants contain non-toxic agglutinins (Mendel[8]).

Miessner[9] suggested that use should be made of the precipitin reaction discovered by Jacoby[10]. This method is also advocated by Bamford[11]. It is claimed to be quite specific but is unfortunately insufficiently delicate to be of practical value, as Lander and Geake[7] were unable by this means to identify ricin in the omasum of an experimentally poisoned calf.

Clarke[12] suggested that use should be made of the fact that serum from an immune animal will neutralise the toxicity of ricin. This property is quite specific (Ehrlich[13]). Details of this method are now given.

Experimental

10 g. of the material to be tested is finely ground and extracted with 50 ml. of 0·02N hydrochloric acid for several hours with mechanical stirring. The mixture is then centrifuged, the supernatant liquid decanted and the residue re-extracted with a further 50 ml. of 0·02N hydrochloric acid, and again centrifuged. The two extracts are combined and filtered if necessary through sintered glass. 300 ml. of acetone is now added to the extract. The precipitate is centrifuged off, transferred to a sintered glass crucible, and dried as far as possible at the pump. It is then extracted with 5 ml. of physiological saline solution and filtered.

The filtrate is divided into two parts, and from each a series of dilutions is prepared; the resulting two identical series of solutions (A and B) represent 1, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, etc., of the concentration of the original extract. 0·1 ml. of normal serum is added to each tube of series A, and 0·1 ml. of serum from an immunised animal (goat or rabbit) to each tube of series B. Both series are then injected into mice (0·5 ml. intraperitoneally). If the mice in series A show an appreciably greater mortality than those in series B, the original material must have contained ricin, as the immune serum will protect against no other substance.

Should all mice in both series die the experiment must be repeated

using greater dilutions, in order to decide whether the deaths are due to some other poison, or to the fact that the quantity of ricin present is too great to be neutralised by the serum.

Table I shows the results obtained using cattle cake to which 0·1 per cent. of ground castor seed has been added.

TABLE I

Tube	Dilution	Series A (+ normal serum)		Series B (+ anti-ricin serum)	
		Number of mice	Result	Number of mice	Result
1	1	3	x 24 hrs., x 24 hrs., x 48 hrs.	3	s, s, s
2	½	3	x 24 hrs., x 24 hrs., x 72 hrs.	3	s, s, s
3	¼	3	x 48 hrs., x 48 hrs., x 96 hrs.	3	s, s, s
4	⅛	3	x 48 hrs., x 48 hrs., x 72 hrs.	3	s, s, s
5	1/16	3	x 72 hrs., x 72 hrs., x 72 hrs.	3	s, s, s
		15		15	

x = died. s = survived.

TABLE II

Tube	Dilution	Series A (+ normal serum)		Series B (+ anti-ricin serum)	
		Number of mice	Result	Number of mice	Result
1	1	2	x 36 hrs., x 36 hrs.	2	s, s
2	½	2	x 60 hrs., s	2	s, s
3	¼	2	s, s	2	s, s
4	⅛	2	s, s	2	s, s
5	1/16	2	s, s	2	s, s
		10		10	

x = died. s = survived.

Table II shows the results obtained in an experiment in which 0·005 per cent. of castor seed was added to cattle cake. This represents 1 p.p.m. of ricin, which is the smallest quantity that can be detected by this method.

Similar results were obtained using castor seed added to sunflower seed, field beans, soya beans and toppings. With linseed cake the method is not so delicate, the limit being 0·1 per cent. of castor seed.

I wish to express my gratitude to Professor E. C. Amoroso for his help and encouragement in this work. My thanks are also due to Dr. G. E. Turfitt for much useful advice and to Dr. J. T. Abrams for the provision of experimental material.

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THE SUPRARENAL GLANDS OF THE HARE AND HORSE

By G. B. West

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Received May 6, 1953

It is now well known that extracts of many mammalian suprarenal glands contain noradrenaline in addition to adrenaline, though the relative amount of each amine varies widely[1]. Whereas in the rabbit and guinea-pig methylation of noradrenaline is almost complete, the predominating amine in the pigeon, fowl and frog is noradrenaline[2]. Another catechol amine, hydroxytyramine, has been identified in the adrenal medulla of the sheep, ox and cow[3], but its presence bears no relationship to the total or relative catechol amine content of the tissue.

With the aid of paper chromatography, Goodall[4] found that the percentage of adrenaline and noradrenaline in the suprarenals of some African mammals varied according to the animal family. For example, the percentage of noradrenaline was very low in the rodents and differed markedly from that found in the feline family. From their habits, Goodall suggested that the hunted animals (primates, rodents and ruminants) have predominantly adrenaline in their suprarenals, whilst the hunters or aggressive animals (lion and cat) have chiefly noradrenaline.

We have now examined the suprarenals of the wild hare (for comparison with the tame rabbit) and the horse (for comparison with the slower-moving farm animals). Particular attention was paid to the identification and estimation of possible precursors of adrenaline and noradrenaline.

Methods

Hares were shot on a local farm and their suprarenal glands were removed as soon as possible (sometimes in the field) into bottles containing 2 ml. of 0·01N hydrochloric acid. The glands were taken to the laboratory for weighing and grinding up with sand and the surrounding acid. In a similar manner, the glands of horse were collected as soon as possible after death at the slaughter-house. After centrifuging the extracts, the clear supernatant liquids were assayed for their adrenaline and noradrenaline contents by paper chromatography and biological assay[1]. Further concentration was effected by the addition of 4 volumes of ethanol, centrifugation, evaporation to dryness and elution into a small volume of water. Careful examination of the paper chromatograms of these concentrated extracts was carried out in every case to detect possible precursors. Solutions of the following substances were prepared in 0·01N hydrochloric acid—*l*-adrenaline, *l*-noradrenaline bitartrate, hydroxytyramine hydrochloride, *dl*-dihydroxyphenylalanine, *dl*-dihydroxyphenylserine, lactyl-adrenaline and lactyl-noradrenaline[5], *dl*-*p*-hydroxyphenylethanolamine hydrochloride (*p*-norsynephrine), *dl*-*m*-hydroxyphenylethanolamine hydrochloride, tyramine hydrochloride, tyrosine and phenylalanine. Most of these drugs were obtained through the courtesy

of Sterling-Winthrop Research Institute, New York; Hoffmann-la-Roche, Basle; and Bayer Products Ltd., London.

RESULTS

Suprarenals of the hare. 10 experiments were carried out using extracts of the suprarenal glands of hares, and the mean results are 309 μg. of adrenaline and 41 μg. of noradrenaline per g. of fresh tissue (noradrenaline 12 per cent.). The glands are about the same size as those of the rabbit which contain 470 μg. of adrenaline and 10 μg. of noradrenaline/g.[1], and this means that the hare has a greater reserve of noradrenaline and less adrenaline than the rabbit.

Suprarenals of the horse. 29 experiments were completed in this study. It was possible in many cases to separate the cortex from the medulla, thereby enabling extracts of each part of the gland to be made without contamination by the other part. The means of all results are: whole gland, 666 μg. of adrenaline and 166 μg. of noradrenaline/g. (noradrenaline 20 per cent.); medulla only, 3200 μg. of adrenaline and 800 μg. of noradrenaline/g.; cortex only, 160 μg. of adrenaline and 42 μg. of noradrenaline/g. These values are recorded in Table I and contrasted with those already found for other farm animals[3]. It will be seen that the horse in this group contains the lowest relative amount of noradrenaline, whilst the pig (which is certainly not an aggressive animal like the lion) contains about equal quantities of both pressor amines.

TABLE I

CONCENTRATIONS (μg./g.) OF ADRENALINE AND NORADRENALINE IN THE ADRENAL GLANDS OF SOME FARM ANIMALS

	Cortex		Medulla		Whole gland		Noradrenaline in total per cent.
Animal	Adrenaline	Noradrenaline	Adrenaline	Noradrenaline	Adrenaline	Noradrenaline	
Horse ..	160	42	3200	800	666	166	20
Ox ..	400	150	4000	1500	1200	420	26
Cow ..	400	200	4000	1250	1250	500	29
Sheep ..	100	40	2000	1600	500	250	33
Pig ..	125	75	4000	4000	1090	1056	49

Possible precursors of noradrenaline. Extracts of the suprarenal glands of the horse after concentration were chromatographed in the usual manner using solutions of the amines for controls. The developers used included aqueous potassium iodate[1], aqueous potassium ferricyanide[6], a mixture of potassium dichromate and formaldehyde (to produce the fluorescence reaction)[7], ninhydrin in butanol, *p*-nitraniline[8], Folin and Ciocalteu reagent, and acid and alkaline Pauly reagent. Table II illustrates how each of the possible precursors can be detected. In all the horse and hare gland extracts, only adrenaline and noradrenaline were identified. Traces of tyrosine and phenylalanine were present in most extracts, and an unknown spot (R_F value, 0·32) was found in some. This latter spot may be an ascorbic acid derivative, though there is no complete proof of this.

TABLE II

IDENTIFICATION OF THE PRESSOR AMINES AND THEIR POSSIBLE PRECURSORS BY PAPER CHROMATOGRAPHY

Pressor amines and their possible precursors	R_F value (butanol–acetic acid–water)	Colour with developer							
		KIO_3	$K_3Fe(CN)_6$	Fluorescence	Ninhydrin	*p*-Nitraniline	Folin and Ciocalteu reagent	Pauly reagent: Acid	Pauly reagent: Alkaline
Adrenaline	0·36	Pink	Pink	Yellow-green	Purple	Grey-blue	Blue	Brown	Red-brown
Noradrenaline	0·28	Violet	Rose	Turquoise	Brown	Grey-blue	Blue	Brown	Brown
Hydroxytyramine	0·39	Orange-brown	Brown	Yellow	Brown	Grey-blue	Blue	Brown	Red-brown
Dihydroxyphenylalanine	0·21	Greyish-violet	Greyish-violet	Yellow	Purple	Grey-blue	Blue	Brown	Red-brown
Dihydroxyphenylserine	0·15	Pinkish-brown	Pinkish-brown	Turquoise	Purplish-brown	Grey-blue	Blue	Brown	Red-brown
Lactyl-adrenaline	0·57	Brownish-violet	Pink	0	Brown	Green-blue	Blue	Brown	Red-brown
Lactyl-noradrenaline	0·52	Brownish-violet	Rose	0	Brown	Light grey	Blue	Brown	Brown
p-Hydroxyphenylethanolamine	0·43	0	0	0	Purplish-brown	Deep red	Blue	0	Yellow-brown
m-Hydroxyphenylethanolamine	0·46	0	0	Violet	Brown	Deep red	Blue	Delayed yellow	Yellow-brown
Tyramine	0·58	0	0	0	Purple	Grey-blue	Blue	0	Red-brown
Tyrosine	0·30	0	0	0	Purple	Purple	Blue	0	Red-brown
Phenylalanine	0·51	0	0	0	Purplish-blue	Rose	0	0	0

Discussion

We have found that the suprarenal gland of the hare contains a higher proportion of noradrenaline than is usually found in the glands of rabbits. Since the glands in the two animals are about the same size, the hare must have a greater reserve of noradrenaline. This may be related to its exceptional powers of running or to the fact that it is usually a wild animal; its adrenaline reserve is certainly less than that found in the tame rabbit.

The suprarenal gland of the horse contains the lowest percentage of noradrenaline of the 5 farm animals so far tested, yet this is not related to the total amine content. The glands of the pig which contain the highest total amine content also contain the highest percentage of noradrenaline. It appears that there is no easy explanation why the relative noradrenaline content of the suprarenal gland of animals should differ so widely. We still believe that some cortical material may be the controlling factor.

Concerning possible precursors of adrenaline and noradrenaline, hydroxytyramine and dihydroxyphenylalanine were the obvious first choice to search for, since Blaschko[9] presented in 1942 a scheme for the biosynthesis of adrenaline which involved the formation of these two substances. Dihydroxyphenylserine, on the other hand, is the amino-acid corresponding to noradrenaline, decarboxylation producing this pressor amine; the amino-acid, however, has not been found naturally. The lactyl derivatives of adrenaline and noradrenaline are relatively inactive forms of the parent amines, and since lactic acid occurs in fair quantity in the suprarenal medulla they might be the means whereby the gland stores the active material. *p*-Hydroxyphenylethanolamine has been found naturally and can be converted into noradrenaline under the influence of ultra-violet irradiation in the presence of air. The *meta*-compound can likewise be converted to this active amine[10]. Tyramine might be the important step between tyrosine and *p*-hydroxyphenylethanolamine in this scheme of synthesis. However, all of these substances were not detected and the method by which the adrenaline and noradrenaline synthesis in the body occurs is still an open one.

Summary

1. The suprarenal gland of the hare contains adrenaline and noradrenaline; its relative noradrenaline content is greater than that found in the rabbit. The suprarenal gland of the horse contains adrenaline and noradrenaline; its relative noradrenaline content is lower than those found in the sheep, cow, ox and pig. No explanation has been found so far to account for these variations.

2. Hydroxytyramine, dihydroxyphenylalanine, dihydroxyphenylserine, *p*-norsynephrine and tyramine are not present in detectable quantities in suprarenal gland extracts of the hare and horse.

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Correction.

THE QUANTITATIVE DETERMINATION OF CINNAMON IN THE FORM OF POWDER

BY R. DEQUEKER.

This Journal, 1952, **4**, 573.

TABLE II, p. 575

The heading of the column reading: Length of fibres in mm. per g. of powder. . . .

Should read: Length of fibres in m. per g. of powder. . . .

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ALKALOIDS

Nicotine, Occurence of, with Hyoscine in *Duboisia myoporoides* R. Br. K. L. Hills, W. Bottomley and P. I. Mortimer. (*Nature, Lond.*, 1953, **171**, 435.) This paper reports the finding of hyoscine, nicotine and nornicotine, in the leaves of *D. myoporoides* grown from seeds obtained from New Caledonia. Alkaloidal assays were carried out by the partition chromatographic method. The identities of the alkaloids were confirmed by preparation of the picrate, reineckate or dipicrate and checking the mixed melting point with the respective salt of the genuine alkaloid. This is the first time alkaloids of the propane and pyridine groups have been observed in the same plant, or even in the same species. Hybridisation is considered unlikely. G. F. S.

ANALYTICAL

Ethylenediamine tetra-acetic Acid, Spectrophotometric Titrations with. P. B. Sweetser and C. E. Bricker. (*Analyt. Chem.*, 1953, **25**, 253.) Procedures are given for the use of spectrophotometric end-points for the determination of iron, copper, and nickel with standard sodium ethylenediamine tetra-acetate solutions. For the determination of iron the complex of salicylic acid and ferric ions, maximum absorption at *ca.* 525 mμ, was used as the basis of the end-point. At *p*H approximately 2·4 the ethylenediamine tetra-acetate-iron complex is much stronger than the iron-salicylate complex; in the titration of an iron-salicylic acid solution with sodium ethylenediamine tetra-acetate there is a gradual disappearance of the iron-salicylic acid colour as the end-point is approached. The visual end-point can be detected to within 0·05 to 0·10 ml. and the spectrophotometric end-point at 525 mμ is even sharper. The titration of a copper solution at *p*H 2·4 to 2·8 can also be followed spectrophotometrically at 745 mμ, as the copper-ethylenediamine tetra-acetate complex has, at this wavelength, a molar extinction considerably greater than the copper solution alone. Nickel can be determined similarly except that a wavelength of 1000 mμ is used, and the *p*H of the nickel solution is made approximately 4·0 with a suitable acetate buffer. With the methods given, it is possible without any separation to determine copper in many non-ferrous alloys and iron in ferrous alloys and ores by a single, simple titration. R. E. S.

Fluoride in Natural Waters, Direct Titrimetric Determination of. A. M. Bond and M. M. Murray. (*Biochem. J.*, 1953, **53**, 642.) A volumetric procedure is described in which fluoride is directly titrated in aqueous solution with thorium nitrate using sodium alizarinsulphonate as indicator. The titration is carried out in the presence of acetic acid and at *p*H 3·3. The possibility of interference by SO_4^{--}, PO_4^{---}, CO_3^{--} and HCO_3^- is eliminated by prior treatment of the water with barium chloride. The sensitivity of the titration is unaffected by temperature over the range of 5 to 40° C. The preliminary treatment with barium chloride does not precipitate fluoride and in no way interferes with the subsequent titration, except when the solution contains a high concentration of barium chloride together with more than 15 μg. of fluorine.

Calcium and magnesium in such concentrations as may be expected in very hard water do not interfere with the titration. Aluminium forms a lake with alizarin at the *p*H of the titration and hence interferes with the titration; it is detected by the failure to give the pure yellow colour on addition of 0·05 N hydrochloric acid after filtration. 295 samples of domestic water in Great Britain were examined and only seven were found to contain more than 1 p.p.m. of fluorine. J. B. S.

Glucosamine and Galactosamine, Separation and Determination of. S. Gardell. (*Acta chem. scand.*, 1953, **7**, 207.) Glucosamine and galactosamine are separated on Dowex 50 ion exchange resin using 0·3N hydrochloric acid as solvent, the effluent being collected in fractions of equal volume, which are analysed by the method of Elson and Morgan as modified by Blix (*Acta chem. scand.*, 1948, **2**, 467). Examination of effluent-concentration curves shows that complete separation of the two amino sugars is readily achieved. Recovery is quantitative, the respective sugars being separated as hydrochlorides by concentrating the effluent solution, and crystallised by the addition of methanol and acetone. Glucosamine is the first component to appear in the effluent. J. B. S.

Phosphate Esters, Detection of, on Paper Chromatograms. H. E. Wade and D. M. Morgan. (*Nature, Lond.*, 1953, **171**, 529.) A method is given for the detection of phosphate esters on paper chromatograms depending on the fixation of ferric ions by the esters and the reaction of the free ferric ion with salicylsulphonic acid. If the paper is not strongly buffered it is sprayed with a 0·1 per cent. solution of $FeCl_3,6H_2O$ in ethanol (80 per cent.), dried in air at room temperature and then sprayed with a 1 per cent. solution of salicylsulphonic acid in ethanol (80 per cent.); on drying, the phosphates appear as white spots on a pale mauve background, *o*-phosphate having a band of deeper mauve surrounding it. The colour formation occurs only when the *p*H of the residual moisture in the paper is about 1·5 to 2·5. The method described permits the detection of 1 to 2 μg. of phosphorus, as phosphate ester, distributed over an area of about 1 sq. cm. R. E. S.

Sulphate and Hydrogen Sulphate Ions; Separation from Interfering Substances by Adsorption on Aluminium Oxide, Prior to Sulphate Determination. F. Nydahl and L. A. Gustafsson. (*Acta chem. scand.*, 1953, 7, 143.) The use of aluminium oxide in the isolation of small amounts of anions from fairly concentrated salt or acid solutions is described. The capacity of the sample of aluminium oxide used was found to be about 0·17 m. mole/ml. for univalent anions and only about 0·08 m. mole/ml. for divalent anions, so that adsorption of sulphate is more effective from acid solutions. Phosphate and sulphate were very much more strongly adsorbed than were the other ions investigated. At *p*H 4 phosphate is more strongly adsorbed than sulphate, but in more strongly acid solutions the order is reversed. Thus separation of these two anions from most of the other common anions can be readily effected. In the separation of sulphate from large quantities of metals these should preferably be present as perchlorates, since the latter is one of the most readily adsorbed anions. Adsorbed sulphate is readily eluted by small volumes of dilute sodium hydroxide solution, sodium carbonate or ammonia. Applications of the method to the isolation of sulphate from pure solutions of acids and salts are described. It can also be used for the determination of sulphate in water or of sulphur in biological materials, dolomite, glass, iron and steel. J. B. S.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Carboxylic, Sulphonic and Anion Exchange Resins, Biochemical Changes in Serum and Fæces During Ingestion of. L. Greenman, W. A. Frey, R. E. Lewis, M. J. Sakol and T. S. Danowski. (*J. Lab. clin. Med.*, 1953, **41**, 236.) Mixtures of acidifying ion exchangers (hydrogen and ammonium forms of the carboxylic or sulphonic type) with alkylene polyamine resin (a strong anion exchanger) and the potassium form of the carboxylic type were tested in an attempt to overcome the disadvantages of the acidifying ion exchangers used alone. Mixtures of the carboxylic and sulphonic resins were no more effective in raising the fæcal output of univalent cations than the carboxylic resin used alone. The admixture of an anion-exchange resin did not consistently raise the fæcal output of chloride, sodium, potassium or nitrogen, but served to decrease the hyperchloræmia and acidosis due to the carboxylic resin. The addition of quantities greater than 8 g. per day of the potassium form of carboxylic resin resulted in partial absorption of potassium into the body and so is contraindicated in renal disease. It is concluded that for the treatment of certain conditions, ion-exchangers of higher efficiency under biological conditions are required. G. B.

Narcotics and Biological Acetylation. W. J. Johnson and J. H. Quastel. (*Nature, Lond.*, 1953, **171**, 602.) Experiments are described which show that low concentrations of narcotics impede the oxidative synthesis of adenosine triphosphate and consequently affect the rates of acetylation processes dependent on the presence of this substance. The effects of narcotics on rates of biological acetylations brought about by various tissue preparations were studied with the conventional Warburg apparatus. Acetylcholine was estimated by measuring the contractions of the dorsal muscle of the leech suspended in an ærated salt and glucose medium; phosphate esters were removed from the samples and assayed by the procedure of Harpur and Quastel; sulphanilamide was estimated by the method of Bratton and Marshall; acetylated sulphanilamide was determined by difference. Addition of pigeon liver extract to rat brain homogenate produces a system which readily acetylates sulphanilamide under ærobic conditions. Acetylation is increased on the addition of pyruvate or acetate to the system, pyruvate being more effective as an acetyl precursor, giving rise to increased oxygen uptake. Addition of chlorbutol (0·004 M) reduces both acetylation and respiration to low levels; adenosine triphosphate diminishes the chlorbutol inhibition of acetylation, though the respiration rate is much less affected. Thus the narcotic inhibition of acetylation results from the suppression of the oxidative synthesis of adenosine triphosphate. Further experiments with pigeon liver extracts confirm that the enzymes involved in sulphanilamide acetylation are not inhibited by narcotics even in relatively high concentrations. Narcotics similarly caused a marked inhibition of respiration and of the acetylation of choline by rat brain mince. The addition of adenosine triphosphate increases the rate of acetylation even in the presence of the narcotic, indicating that the narcotic does not impede its utilisation, but only its synthesis. 5-Allyl-5-*iso*propyl-*N*-methylbarbituric acid (narconumal), similarly, does not effect acetylcholine synthesis by a beef-brain extract undergoing a high rate of glycolysis. J. B. S.

Steroids, Paper Chromatography of. C. D. Kochakian and E. Stidworthy. (*J. biol. Chem.*, 1952, **199**, 607.) A procedure for the separation of C_{19} steroids by paper chromatography is described, using paper impregnated with a 1:1 methanol-propylene glycol, and a mixture (1:1) of benzene and *cyclo*hexane saturated with propylene glycol as the developing solvent. After completion of the run and drying, the paper was exposed to ultra-violet light on a fluorescence scanner and a map of the apparent spots made. The paper was resuspended in front of the electric fan containing a heating unit, sprayed with alkaline *m*-dinitrobenzene reagent, the spots outlined in pencil and the colours noted. The paper was then sprayed on each side with acid 2:4-dinitrophenylhydrazine reagent, which caused the previous colours to disappear as the 2:4-dinitrophenylhydrazones were formed. The presence of non-ketonic steroids was detected by preparing a duplicate paper chromatogram and spraying with phosphomolybdic reagent. 27 steriods were studied, their colours with the reagents recorded, and their mobilities related to the mobility of testosterone. In general, a wide variation in their rates of movement was observed, and the sequence of movement was dihydroxy-, hydroxy-, keto,, mono-substituted- and diketosteroids. The addition of a third group to the molecule retarded the movement, while removal of a group enhanced the movement. Stereoisomers were effectively separated, e.g., testosterone and epitestosterone. A. H. B.

Vitamin A_1, Oppenauer Oxidation of. H. R. Cama, A. C. Field, J. Glover, R. A. Morton and M. K. Salah. (*Biochem. J.*, 1952, **52**, 548.) An Oppenauer oxidation of vitamin A using aluminium alkoxides with diethyl ketone as hydrogen acceptor gave a complex mixture from which a compound $C_{20}H_{26}O$ (isomeric with retinene$_2$) was isolated; it gave an oxime m.pt. 175° to 177°. The compound obtained should be reduced *in vitro* and *in vivo*, but the reduction product was found to differ from vitamin A_2. The mechanism of the reaction is discussed and it was concluded that the Oppenauer oxidation proceeded *via* retinene$_1$, $C_{20}H_{28}O$, which could either be further dehydrogenated to $C_{20}H_{26}O$ or could condense with diethyl ketone to a ketone, $C_{25}H_{36}O$. Vitamin A acetate was not dehydrogenated directly to vitamin A_2. R. E. S.

Vitamin A_2, Spectroscopic Properties of. H. R. Cama and R. A. Morton. (*Analyst*, 1953, **78**, 74.) Detailed absorption intensities of vitamin A_2 alcohol produced by reduction of retinene$_2$ with lithium aluminium hydride, at different wavelengths and for different solvents, have been measured and expressed as fractions of the maximum intensities. Vitamin A_2 shows a main ultra-violet maximum near 351 mμ ($E^{1\ \text{per cent.}}_{1\ \text{cm.}}$ about 1400) and a secondary peak near 287 mμ ($E^{1\ \text{per cent.}}_{1\ \text{cm.}}$ about 750). The blue solution (with antimony trichloride reagent) shows $\lambda_{max.}$ at 693 mμ. ($E^{1\ \text{per cent.}}_{1\ \text{cm.}}$ about 3900). Fish-liver oils in general contain much more vitamin A_1 than A_2, depending on the species, and in cod-liver oils vitamin A_2 may account for about one-seventh of the total vitamin A (molecule for molecule). By determining $E^{1\ \text{per cent.}}_{1\ \text{cm.}}$ at 693 mμ. (A_2) and at 620 mμ. (A_1) in the antimony trichloride colour test (applied to the unsaponifiable fraction), and the $E^{1\ \text{per cent.}}_{1\ \text{cm.}}$ at 326 mμ, 351 mμ and 286 mμ in the ultra-violet region, both vitamins can be estimated. The 693 mμ absorption measures vitamin A_2 directly and from it the vitamin A_2 contributions to ultra-violet absorption at 351 mμ and 327 mμ can be calculated. A conversion factor is given for calculating the probable vitamin A_2 contribution to the potency. A cod-liver oil typical of those studied by spectrophotometric methods, corrected for all irrelevant absorption, gave an estimated vitamin potency about 6·5 per cent. lower than the estimate that included the possible vitamin A_2 contribution. R. E. S.

BIOCHEMICAL ANALYSIS

Barbiturates in Body Fluids, Determination of, by Spectrophotometry. J. T. Wright and R. G. S. Johns. (*J. clin. Path.*, 1953, **6**, 78.) The technique described gives a reasonable degree of accuracy with a minimum procedure. Heparinised blood is extracted with chloroform and filtered. The filtrate is shaken with aqueous sodium hydroxide and an aliquot amount added to a borate buffer at *p*H 10. The ultra-violet absorption curve is read over the range 220 to 300 mμ against a blank of sodium hydroxide and borate buffer. The solution is then acidified with hydrochloric acid and the absorption re-determined at the same wavelength. The barbiturate concentration is determined from a given formula. Extraction procedures are also described for urine, cerebrospinal fluid and gastric contents. There was a 60 per cent. recovery of barbitone added to blood samples at concentrations of 20 to 50 μg. per ml. Results are given for 17 clinical cases of barbiturate poisoning.

G. F. S.

Benzidine Reaction, Quantitative Determination of Plasma Hæmoglobin by. M. C. Creditor. (*J. Lab. clin. Med.*, 1953, **41**, 307.) Plasma is diluted to contain less than 10 mg. of hæmoglobin/100 ml. To 0·05 ml. is added 2 ml. of benzidine reagent (2 per cent. in 20 per cent. acetic acid) and, after mixing, 1 ml. of solution of hydrogen peroxide (1·5 per cent.) is added. The mixture is shaken and allowed to stand for 1 hour, diluted with 20 per cent. acetic acid to 100 ml. and allowed to stand for 30 minutes. The optical density at 490 mμ is determined in a suitable photoelectric colorimeter. The colour is stable for 24 hours. Beer's law applies in the range of concentrations 1 to 10 mg./100 ml. but the slope of the line varies slightly with each batch of benzidine reagent; consequently the standard curve must be re-determined with the aid of standard hæmoglobin solutions each time a series of samples is examined. The use of benzidine which has been specially purified is recommended in order to reduce the reagent blank. Incomplete recovery of hæmoglobin from the plasma limits the accuracy of the method. The percentage recovery should be determined experimentally and a correction made.

G. B.

Corticosteroids in Urine, Determination of. S. L. Tompsett. (*J. clin. Path.*, 1953, **6**, 74.) A method is described for the determination in urine of metabolites closely related to corticosterone, metabolites related to cortisone not being included. The method is based on the hydrolysis of the acid-stable conjugates of corticosteroids in urine with hot dilute mineral acid and then subjecting the steroid extract to a modified periodic acid technique and estimating the formaldehyde liberated colorimetrically with chromotropic acid. Desoxycorticosterone and corticosterone added to the urine were recovered quantitatively while cortisone was not recovered. Normal urine was found to contain 4·5 to 7·5 mg. per day of corticosterone-like substances and the values were low in hypo-adrenalism and elevated in hyper-adrenalism. Increased values followed the administration of adrenocorticotrophic hormone.

G. F. S.

Heparin in Blood, Estimation of. M. Bassiouni. (*J. clin. Path.*, 1953, **6**, 39.) A method is described for the extraction and estimation of heparin in 2 ml. of blood. Citrated blood is centrifuged, the plasma is removed and treated with sodium hydroxide and ammonium sulphate and the protein is

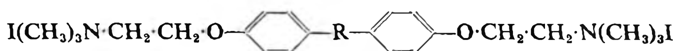
filtered off. Hydrogen sulphide and ammonia are then removed under reduced pressure. The heparin is precipitated by the treatment with a solution of dimethyl thionin (Azure A) for 48 hours at room temperature, and centrifuged. The supernatant liquid is removed, the precipitate is dissolved in acetone and the colour estimated electrocolorimetrically. Concentrations are read from a calibration curve obtained for a range of concentrations of heparin and a blank obtained for the reagents is deducted from these readings. Experiments showed 90 per cent. recovery of heparin added to plasma. Results for estimations of heparin in the blood of 10 healthy individuals are given and the concentrations in the blood obtained in one person after the intravenous injection of 7000 units of heparin. G. F. S.

Œstrogens, Quantitative Separation of, by Paper Partition Chromatography. L. R. Axelrod. (*J. biol. Chem.*, 1953, **201**, 59.) Rapid separation of œstriol, diethylstilbœstrol, 17α-ethinylœstradiol, 17β-œstradiol, 17α-œstradiol and œstrone have been effected on paper using the solvent systems *o*-dichlorobenzene-formamide, methylene chloride-formamide, *cyclo*hexene-formamide. Natural œstrogens are readily separated in a single chromatogram. The methylene chloride-formamide system was used to separate œstriol from highly polar pigments. Chromatograms were run from numerous urine extracts and the eluted œstrogens subsequently compared by comparison of the spectra of the sulphuric acid chromogens. In experiments designed to study the metabolism of 17α-ethinylœstradiol, the separation of 17β-œstradiol from 17α-ethinylœstradiol was best effected in *cyclo*hexene-formamide after preliminary separation in *o*-dichlorobenzene-formamide. The polarity of the œstrogens has a direct influence on their rate of movement, and the spatial configuration of the hydroxyl groups is an additional influence. Spot tests with fuming sulphuric acid, benzoyl chloride-zinc chloride, phenolsulphonate-phosphoric acid, nitrous acid-mercuric nitrate and antimony pentachloride are also described for the identification of the above œstrogens. Measurement of the ultra-violet adsorption of the sulphuric acid chromogens can be applied as a quantitative estimation. Œstriol and 17β-œstradiol were found in bile from an ovariectomised hysterectomised dog after injection of 17β-œstradioldibenzoate; œstrone was absent. Other phenolic and non-phenolic compounds of a steroid nature of which the sulphuric acid chromogen absorption spectra did not correspond to those of known œstrogens, were isolated. It is concluded that the completely hysterectomised dog cannot metabolise 17β-œstradiol to œstrone. J. B. S.

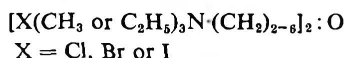
Proteins, Gravimetric Precipitation of, by Trichloroacetic Acid. F. L. Hoch and B. L. Vallee. (*Analyt. Chem.*, 1953, **25**, 317.) The method described uses the specificity of trichloroacetic acid in the precipitation of proteins and allows direct measurement of the weight of dried proteins; drying is performed at a temperature of 110° C. which is sufficient to eliminate water by evaporation and trichloroacetic acid by decomposition. Pure human and bovine serum albumin, pure bovine insulin, and crude bovine pituitary extract were precipitated and the dried precipitate weighed after treatment with trichloroacetic acid. The validity of this procedure is here appraised by a comparison of weights of dried precipitated proteins with weights obtained by direct weighing of pure dry proteins, by spectrophotometric measurements on clear solutions of dissolved proteins, and by nitrogen determinations. Experimental details of the method are given and the protein factors affecting trichloroacetic acid precipitation are discussed. R. E. S.

CHEMOTHERAPY

Curarising Agents, Pharmacological Studies of. S. Levis, S. Preat and J. Dauby. (*Arch. int. Pharmacodyn.*, 1953, **93**, 46.) 31 curarising agents of the 2 types shown below were examined for curarising effect and toxicity. Among the tests used were the sciatic-gastrocnemius and head-drop methods in the rabbit, fall of arterial pressure in the dog, determination of the LD70 dose in mice, isolated phrenic nerve-diaphragm (rat), guinea-pig ileum and rectis abdominis (frog). Some of the substances were 5 to 6 times more



Series I

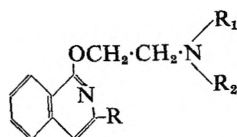


Series II

active than *d*-tubocurarine. A number of compounds were unsuitable for use as curarising agents because of weak action, prolonged periods of induction and activity, or insolubility. 3 of the compounds showed a high activity associated with normal duration of action and the compound R = – CH (C_3H_7*iso*)·CH_2– of series I was submitted for clinical testing because it showed the greatest margin of safety. 2 short-acting compounds were also found suitable for clinical trial, $Cl(CH_3)_3N{\cdot}(CH_2)_5{\cdot}O{\cdot}(CH_2)_5{\cdot}N(CH_3)_3Cl$ being the more active and the corresponding di-iodide having the greater margin of safety.

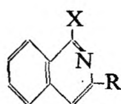
G. B.

***iso*Quinoline Derivatives as Local Anæsthetics.** E. L. Anderson, J. W. Wilson and G. E. Ullyot. (*J. Amer. pharm. Ass. Sci. Ed.*, 1952, **41**, 643.) 22 compounds of series I, 8 of series II and 11 of series III were synthesised and tested as local anæsthetics by the rabbit cornea method. In series I the most active compounds were R = *n*-butyl, *n*-pentyl or *n*-hexyl, and $R_1 = R_2 = -CH_3$ or $-(CH_2)_5-$. The most active compounds of series II had R = *n*-butyl, X = 4-(1-ethyl-2:6-dimethyl) piperidyloxy, 4-(1-methylpiperidyloxy) and 3-(1-methylpiperidyloxy). In series III the compounds $R = -CH_3$, $R_1 = R_2 = R_3 = H$, $R_4 = -OCH_3$, and $R = -C_2H_5$, $R_1 = R_3 = R_4 = H$, $R_2 = -NH_2$ appeared to be the most active.



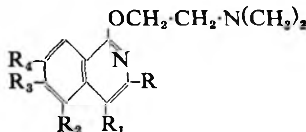
(I)

aminoethoxy*iso*quinolines



(II)

*iso*quinolines



(III)

1-(β-dimethylaminoethoxy) *iso*quinolines

The maximum anæsthetic activity was shown by 1-[3-(1-methylpiperidyloxy)] 3-*n*-butyl*iso*quinoline. *iso*Quinoline derivatives used as intermediates were prepared by methods described in previous work or by a new method of ring closure of phenethyl *iso*cyanates with aluminium chloride.

G. B.

PHARMACY

NOTES AND FORMULÆ

Isoniazid. (*New and Nonofficial Remedies, J. Amer. med. Ass.*, 1953, **151**, 740.) Isoniazid is *iso*nicotinyl hydrazide and occurs as a white, odourless, crystalline powder, m.pt. 170° to 173° C., sparingly soluble in ethanol, very slightly soluble in benzene and ether, and freely soluble in water; *p*H of a 1 per cent. solution 5·5 to 6·5. When a solution in methanol is refluxed for 20 minutes with benzaldehyde and a trace of acetic acid, poured over ice, and filtered, the precipitate of white crystals obtained melts at 197° to 200° C., after recrystallisation from dilute ethanol. A 0·001 per cent. solution in 0·1 N hydrochloric acid exhibits an ultra-violet absorption maximum at about 266 mμ ($E^{1\ \text{per cent.}}_{1\ \text{cm.}}$, about 378) and a minimum at about 234 mμ. Isoniazid loses not more than 0·5 per cent. of its weight when dried at 105° C. for 4 hours and yields about 0·5 per cent. of sulphated ash. It contains 95·0 to 105·0 per cent. of isoniazid when determined by measuring the absorption of a 0·001 per cent. solution in 0·1N hydrochloric acid in a 1-cm. quartz cell at 266 mμ, and 97·5 to 102·5 per cent. when assayed iodimetrically. G. R. K.

Levarterenol Bitartrate (Levophed Bitartrate). (*New and Nonofficial Remedies, J. Amer. med. Ass.*, 1953, **151**, 821.) Levarterenol bitartrate is *l*-α-(aminomethyl)-3 : 4-dihydroxybenzyl alcohol *d*-bitartrate monohydrate, $C_8H_{11}NO_3$, $C_4H_6O_6$,H_2O. It occurs as a white, crystalline, odourless powder, m.pt. 100° to 106° C., freely soluble in water, slightly soluble in ethanol, and insoluble in ether; a 0·1 per cent. solution has *p*H 3·0 to 4·0. It gives an intense green colour with ferric chloride, and a 0·004 per cent. solution in 0·01 N hydrochloric acid exhibits an ultra-violet absorption maximum at about 2790 Å ($E^{1\ \text{per cent.}}_{1\ \text{cm.}}$, about 84) and a minimum at about 2490 Å. It yields not more than 0·2 per cent. of sulphated ash, and loses not more than 5·8 per cent. of its weight when dried to constant weight in an Abderhalden-pistol dryer at about 77° C. (carbon tetrachloride) for about 8 hours. It contains 95·0 to 105·0 per cent. of levarterenol bitartrate when assayed spectrophotometrically by measuring the absorption of a 0·004 per cent. solution in 0·01 N hydrochloric acid at 2790 Å, using water as a blank. G. R. K.

Polymixin B Sulphate (Aerosporin Sulphate). (*New and Nonofficial Remedies, J. Amer. med. Ass.*, 1952, **150**, 1219.) Polymixin B is a basic polypeptide derived from a strain of *Bacillus polymyxa* (*B. aerosporus* Greer). It contains leucine, threonine, phenylalanine, α,δ-diaminobutyric acid and a fatty acid of empirical formula $C_9H_{18}O_2$. Polymixin B sulphate is stable in the dry state and for extended periods in buffered solutions. It is highly effective *in vitro* against many Gram-negative micro-organisms. Polymixin B sulphate is a white to cream-coloured irregular, scale-like material which decomposes at about 230° C. and is soluble in water and normal saline; a 2 per cent. solution has a *p*H of about 5·7. An aqueous solution gives a blue colour when heated with ninhydrin and pyridine (presence of α-amino acid) and a pinkish-violet colour when made alkaline with sodium hydroxide and treated with a dilute solution of copper sulphate (presence of peptone or proteose), but yields no transitory red colour when treated with an alkaline solution of α-naphthol and a solution of sodium hypochlorite (absence of free protein). Polymixin B sulphate loses not more than 4 per cent. of its weight when dried at 37° C. for 4 hours in a vacuum oven at 5 mm. Hg. or less. It yields not more than 5·0 per cent. of sulphated ash and is assayed microbiologically. G. R. K.

PHARMACOLOGY AND THERAPEUTICS

Adrenaline and Noradrenaline, Influence of Blood Glucose Level on Secretion from the Suprarenal Medulla. H. Dunér. (*Nature*, *Lond.*, 1953, **171**, 481.) The secretion of adrenaline and noradrenaline in the venous blood from the suprarenal of the anæsthetised cat has been studied after injections of glucose. Adrenaline and noradrenaline were assayed biologically. The average normal secretion of noradrenaline and adrenaline was 75 μg./minute/kg. (16 per cent. of adrenaline). Infusion of glucose 0·1 g./kg. reduced adrenaline secretion to 50 per cent., and 0·8 g./kg. to 7 per cent., of the initial value. Noradrenaline secretion was also decreased but not to the same extent. As the blood sugar decreased adrenaline secretion increased. Denervation reduced the secretion to 25 μg./minute/kg. which was unaffected by glucose. The blood glucose level affects medullary secretion by a nervous mechanism. Cross-circulation studies suggest chemoreceptors located centrally, possibly in the hypothalamus.

G. F. S.

Antihistaminics and Atropine, Action of, in Blocking the Activity of Serotonin on the Guinea-pig Ileum. M. M. Rapport and G. B. Koelle. (*Arch. int. Pharmacodyn.*, 1953, **92**, 464.) Serotonin, the vasoconstrictor substance of mammalian serum is 5-hydroxytryptamine, chemically related to histamine and sympathomimetic amines. Experiments were designed to determine whether the substance acts on histamine sites. Segments of guinea-pig ileum were placed in an isolated organ bath and the concentrations of acetylcholine, histamine and serotonin which produced approximately equal reproducible contractions were determined. The blocking agents diphenhydramine, tripelennamine and atropine were added in turn in gradually increasing concentrations, in each case being allowed to act for 5 minutes before the spasmogenic agent was added. Diphenhydramine had the greatest effect on histamine spasm, less on serotonin and least on acetylcholine, the relative effectiveness being 4 : 2 : 1. It was not possible to block the action of histamine with this agent, without affecting the action of serotonin and acetylcholine. Tripelennamine was more specific for histamine, but the same order of effects was observed. Atropine was most effective against acetylcholine and least against histamine. The importance of controlling the time of contact of tissue with the blocking agent in these experiments is emphasised. It is not claimed that the experiments decide the question of whether serotonin acts on histamine receptors but the implications of the results in terms of histaminic and cholinergic action are discussed.

G. B.

Dicoumarol, Method of Administration. Chr. J. Bjerkelund. (*Lancet*, 1953, **264**, 260.) Two main methods of using dicoumarol are used at present, namely, intermittent dosage and maintenance dosage. Intermittent dosage consists in giving relatively large doses of dicoumarol at intervals of up to 10 to 12 days. Maintenance dosage aims at keeping the prothrombin value most nearly constant in the optimal therapeutic range by giving daily doses in amounts varying according to the patient's tolerance for dicoumarol. The maintenance dosage is the only rational method and is the only practical one when treatment of the ambulant patient has to continue for a long time. For its successful conduct, however, an exact and dependable method of estimating prothrombin is necessary. For this purpose Owren's method (P. A. Owren, *Acta med. scand.*, 1947, Supp. 194; *Scand. J. Clin. lab. Invest.*, 1949, **1**, 81; *ibid*, 1951, **3**, 168 and 201) is preferred to Quick's method and is especially

recommended, and close contact between laboratory, physician and patient is essential. The same initial dosage (250 mg. on the first day and 125 mg. on the second day) may be used for all patients. The effect of this dosage is seen after 2 or 3 days and gives an indication of the patient's tolerance. The dosage should then be adjusted to the shape of the curve of prothrombin values (not the current prothrombin value). Large variations in the dosage from day to day should be avoided as much as possible while trying to reach the correct maintenance dosage. The dosage should be adjusted immediately even to minor fluctuations. An increase or decrease equalling 2 to 4 mg. a day is often enough to provoke a distinct fall or rise in the prothrombin curve after 8 to 14 days. If an immediate anticoagulant effect is desired heparin should be used with dicoumarol until the prothrombin value has reached the therapeutic range. S. L. W.

Guanidine and Streptidine, Influence of, on the Toxicity of Streptomycin. S. Kuna and F. T. Cuchie. (*Arch. int. Pharmacodyn.*, 1953, **92**, 408.) Pure and relatively impure preparations of streptomycin were tested for convulsive action by intracisternal injection into rabbits and for production of vestibular dysfunction by daily parenteral injection into cats. It was shown that the convulsive response is directly proportional to the number of guanido groups injected, whether present in the streptomycin molecule or in the impurities. The vestibular effects of impure samples of streptomycin were greater than those of pure samples but a quantitative relationship could not be demonstrated. Guanidine produced no vestibular effect when administered alone, but enhanced the production of vestibular disturbances by streptomycin. Streptidine produced effects similar to streptomycin but 4 times the stoichiometric amount was required. The injection of guanidine with inositol caused vertigo differing from that caused by streptidine or streptomycin in being transitory instead of permanent. It is concluded that the streptidine moiety in streptomycin is mainly responsible for vestibular dysfunction, but that interactions or interrelationships occur which account for the differences in toxicity of streptidine, streptomycin and dihydrostreptomycin. G. B.

Hydralazine: Effect on Hypertension. M. A. Khan. (*Brit. med. J.*, 1953, **1**, 27.) Hydralazine (apresoline) is an antihistaminase which has the combined effect of decreasing blood pressure and increasing renal blood flow. In this study the effect of the drug was observed in 12 cases of arterial hypertension. Hydralazine was administered orally, commencing with a test doses of 25 mg., increasing by 25 mg. 8-hourly to levels reaching 400 mg. three times a day. The effect of the drug lasted from 4 to 6 hours, with a maximum at from 1 to 2 hours after administration. Parenteral therapy, with doses of 50 mg. intramuscularly, was tried in 2 cases, but was abandoned because of unpleasant side-effects. The effect of combined oral therapy with hexamethonium bromide and hydralazine was studied in 4 cases. In most of the cases, whether hydralazine was used alone, or in combination with hexamethonium, there was a slight reduction of blood pressure at the beginning of treatment, but this was not maintained later in spite of greatly increased dosage. Side-effects noted in all patients were headache, flushing and sweating of the face, dryness of the mouth, nausea, vomiting, anorexia and epigastric discomfort, sleepiness and lethargy, tachycardia, transient erythematous rash, and giddiness on standing. In most patients the unpleasant side-effects persisted during treatment. S. L. W.

Insulin, Long-acting (Lente Insulin). R. D. Lawrence and W. Oakley. (*Brit. med. J.*, 1953, **1**, 242.) It has been found (Hallas-Møller *et al.*, *Science*, 1952, **116**, 394) that insulin in the presence of very small quantitites of zinc (1 mg./1000 units) is less soluble at the *p*H of the blood than protamine insulin, provided that anions such as phosphate and citrate are not present. If a different buffer from that normally used, namely phosphate, is employed, it is unnecessary to add such materials as protamine or globin to make insulin insoluble at the *p*H of the blood. With the use of acetate buffer these workers were able to prepare a suspension of pure insulin in media at the *p*H of the blood in the presence of very small quantities of zinc. The length of action of such preparations was found to vary with the form of the insulin, whether pure amorphous, amorphous and crystalline, or pure crystalline. The action of amorphous insulin is shorter than that of crystalline, the larger the size of the crystal the greater being the length of activity. Three preparations of insulin were produced and given the names "semi-lente," "lente," and "ultra-lente." These preparations are miscible to produce preparations of intermediate ranges of activity, but they cannot be mixed with the present soluble insulin. Preliminary therapeutic trials carried out on 11 adult diabetics support the claim that it exerts a prolonged hypoglycæmic action, lasting at least 24 hours, which is strong enough to control the blood sugar of moderately severe diabetics throughout the day without causing hypoglycæmia during the night. This was effected by giving a single dose of lente insulin before breakfast equal in amount to the total quantity of insulin required to stabilise the diabetes. It does not appear to produce either local or general reactions or to be more likely to produce hypoglycæmic reactions than the insulin preparations at present in use. S. L. W.

Isoniazid, Effect of, on Carbohydrate Metabolism. G. R. W. N. Luntz and S. G. Smith. (*Brit. med. J.*, 1953, **1**, 296.) Oral glucose tolerance tests were performed on 6 diabetic and 6 non-diabetic tuberculous patients, before starting treatment with isoniazid, and after administration of the drug for 3 days. In addition fasting tolerance tests were done after 6 days' treatment. Isoniazid delayed the peak and fall of the blood-sugar curve in the glucose tolerance test on non-diabetic patients and the same pattern was observed in an exaggerated form in diabetics. The mean average rise over the 3-hour glucose tolerance test was 20 mg./100 ml. for non-diabetics and considerably more for diabetics. Isoniazid also caused a temporary rise in the fasting blood-sugar levels. In the experiments, plasma isoniazid levels ranged from 0·3 to 0·9 mg./100 ml. and it was shown that concentrations of this order had no effect on the blood-sugar estimations. It is concluded that the insulin requirements of diabetic patients receiving isoniazid may be increased. G. B.

Mercuhydrin, Diuretic Effects by Several Routes. R. Marsh, T. Greiner, H. Gold, S. Mathes, F. Palumbo, L. Warshaw and J. Weaver. (*New Engl. J. Med.*, 1952, **247**, 593.) The diuretic effects of mercuhydrin administered by the subcutaneous, intravenous, intramuscular, oral and rectal routes was investigated by a bioassay technique, using 49 ambulant patients with congestive heart failure as subjects and the effect of the intramuscular injection as standard. The measure of response was the loss in body weight 24 hours after the dose. The drug produced substantially the same diuretic effect when administered parenterally, but by the oral route the effect was reduced to 4 per cent. of the standard. The largest dose of the drug administered orally caused gastro-intestinal irritation in 1/3 of the patients, while results from 21 patients given mercuhydrin in suppository form were unsatisfactory, as the suppository was

not retained. An estimate of 7 per cent. of the effect of the standard was made from the data available. The belief that ascorbic acid, when given with the drug orally, enhances the diuretic action and reduces gastrointestinal irritation, was not confirmed. J. R. F.

Racemorphan (DL-Dromoran), Pharmacological Analysis of the Nervous Control of the Respiration by. C. G. Breckenbridge and H. E. Hoff. (*Arch. int. Pharmacodyn.*, 1953, **93**, 1.) Morphine-like drugs depress selectively multiple-neurone relay systems and may be used in investigations of the neural control of respiration. For this purpose racemorphan (DL-dromoran) is preferable to morphine because it does not produce the precipitous fall in blood pressure and rapid death caused by morphine in certain experiments, so that it becomes possible to investigate the whole range of physiological preparations. Racemorphan has a lower effective dose and produces a lesser degree of tachyphylaxis. Since the drug has a greater selectivity of action than morphine, the results more closely parallel those of anatomical transections. In intact dogs, racemorphan induces changes similar to decortication or mid-collicular decerebration, that is, development of the all-or-nothing pattern of breathing with suppression of normal breathing after each deep breath, reduction in rate and amplitude of normal breathing, and panting. In the animals after mid-collicular decerebration, the drug produces effects similar to vagotomy of the preparation, reducing eupnœa and increasing post-sigh suppression of it and replacing it by an accelerated all-or-nothing respiration. In other preparations racemorphan produces the effects of further serial transections of the brain stem, producing Biot's periodic breathing, medullary breathing and apnœa, this last being the primary effect when the vagi and pons are removed. Control of respiration by the reticular system of the brain stem is discussed in the light of these results. G. B.

Suxamethonium (Succinylcholine); Use in Electric Convulsion Therapy. D. J. Adderley and M. Hamilton. (*Brit. med. J.*, 1953, **1**, 195.) The suitability of use of the short-acting relaxant suxamethonium, with particular reference to its effect on blood pressure, was investigated in 12 female patients. Blood pressures were taken immediately before the injection of sodium thiopentone (0·2 g.), one minute after the injection, again after the injection of suxamethonium (1·5 ml. of a 5 per cent. solution) had become effective, and immediately after the convulsion was over. The lungs were inflated 2 or 3 times with oxygen containing 5 per cent. of carbon dioxide, just before a shock of 30 joules was given. It was shown that suxamethonium produces a slight but unimportant rise in blood pressure. If necessary, this can be controlled by the use of a ganglion-blocking agent, e.g., 2 ml. of a 2 per cent. solution of hexamethonium iodide, or 1 ml. of a 10 per cent. solution of tetraethylammonium bromide, injected immediately before the sodium thiopentone. It was concluded that suxamethonium is a safe relaxant for electric convulsion therapy and can be used to abolish the convulsion completely. Even with the doses necessary to do this the patient's recovery from the fit is always rapid. When complete muscular relaxation is advisable suxamethonium can be administered with safety without any elaborate system of dosage. In such cases the presence of a fit can be detected by one of two methods; (*a*) by watching for dilatation of the pupils and, particularly, injection of the conjunctivæ, or (*b*), when a ganglion-blocking effect is employed, a tourniquet or sphygmomanometer cuff is fixed round the opposite arm immediately before the suxamethonium is injected—if this is kept tight enough to compress the artery until the current is passed the effects of the shock will be observed in that arm only. S. L. W.

(ABSTRACTS *continued on p. 479.*)

PHARMACOPŒIAS AND FORMULARIES

THE PHARMACOLOGY OF THE BRITISH PHARMACOPOEIA, 1953

By Miles Weatherall

The changes in successive British Pharmacopœias reflect the changing judgments and fashions in the use of drugs, and it is interesting to observe how the eighth (1953) Pharmacopœia has developed from its predecessors. One section in particular shows a striking change, not so much in its content as in its approach; this is Appendix XV, the appendix which deals with biological standards and the biological standardisation of drugs. This appendix appeared first in the sixth B.P., in 1932, when it occupied 33 pages; it grew to 44 in 1948, and has now all but doubled its original size by occupying 60 pages.

Its expansion reflects partly the inclusion of new standards and methods of assay, but the most striking change is the introduction of 14 pages on statistical methods for the design and interpretation of assays. Previously, the method of analysis was left largely to the discretion of the assayist, and the recommended design of the assay sometimes provided data not readily amenable to the estimation of their error. In most instances, indeed, standard figures were quoted for the error of the assay when performed by the specified method. This declaration of the accuracy of an assay may be convenient for anyone who prefers to treat their results as naïvely as possible, but it is not really justifiable, as the introductory remarks in the 1953 version point out. Groups of animals vary not only in their mean sensitivity to drugs, but also in the extent to which they vary from one another, and the reliability of any particular assay depends on the variability of the particular group of animals used. So generally it is better to estimate the error of an assay from internal evidence and to calculate the limits of confidence of the result of the assay in this way than to use a standard figure obtained with different animals in other circumstances. Statistical methods in the new Pharmacopœia have now been revised and brought into forms which provide not only a satisfactory estimate of potency, but also an estimate of the error of potency and some checks on the validity of the assay.

The necessary designs and methods of analysis are applicable to various different assays, and so the statistical methods have been concentrated in a single prefatory section. Recipes are provided for estimation of the fiducial limits of error in the circumstances which are likely to arise in straightforward assays. The most complicated design considered is a twin cross-over test based on a (2 and 2) dose assay. The recipes achieve a nice balance between the more subtle refinements of statistical theory and simplification carried to a point at which gross inaccuracy is liable to occur. Model calculations are given which are straightforward to follow. Logarithmic transformation of measured responses is recommended for any that vary more than narrowly. Formal analyses of

variance have been avoided, and the examples are worked as far as is feasible in terms of individual deviations from the appropriate means. From the point of view of anyone unfamiliar with statistical methods this is probably an advantage, as it eliminates a certain amount of (to some people) incomprehensible algebra. What remains is probably still incomprehensible to these people, but much thought has evidently gone into introducing no more than the minimum of obscure sums.

It is interesting to observe that the probability which is now specified as "in practice equivalent to certainty" has been reduced from 0·99 to 0·95. This reduction in the former very rigid requirement has doubtless not been made without much consideration and presumably is intended to provide a more satisfactory balance between idealism and expediency.

The numbers of standard preparations listed to be used in biological assays has risen from 20 to 28. Two standards, vitamin A and tincture of strophanthus, have disappeared. Vitamin A has moved to an appendix of its own, as it is now standardised by its ultra-violet absorption spectrum, and tincture of strophanthus has been deleted from the Pharmacopœia. The 10 newcomers consist of aureomycin, dihydrostreptomycin, streptomycin, dimercaprol, tubocurarine chloride, globin zinc insulin, scarlet fever antitoxin, diphtheria antitoxin for flocculation test, and Anti-A and Anti-B blood-grouping sera. These new standards involve new recommended methods of standardisation, but most of the innovations closely follow patterns already laid down. The new antibiotics are assayable in much the same way as penicillin, and globin zinc insulin is assayed like insulin. The test for undue toxicity of dimercaprol specifies a (2×2) dose assay and an estimated toxicity not more than 110 per cent. of the standard: this contrasts with the analogous test for neoarsphenamine, which is more specifically designed, requires only 2 doses, and gives a self-evident answer. The reason for this difference in recommended procedure is not obvious. Only tubocurarine among the new standards involves the description of experimental methods which have not previously appeared in the Pharmacopœia, and both the rabbit head-drop and rat phrenic nerve diaphragm are suggested.

There are some changes in the proposed methods of assay of posterior pituitary extracts and of digitalis. The classical guinea-pig uterus has been displaced for oxytocic assays by the much more manageable rat uterus in a low-calcium low-dextrose medium, and the assay by depression of the chicken's blood pressure is described for the first time. Rather surprisingly, the pressor assay on rats, which is now widely used and is reliable and relatively quick, is not described: for this assay cats continue to hold their official position. The antidiuretic assay has been modified, so that the response of each rat is measured separately, and is measured as a function of the volume of urine passed at an appropriate time in the test, instead of the time to peak excretion of a group of rats being used. This is undoubtedly an improvement, as the amount of information wasted by the old method was very large.

The assay of digitalis on cats has been tidied up by requiring standards and unknowns to be run at the same time, instead of the sensitivity of the

laboratory supply of cats being checked from time to time with the standard preparation. The assay on guinea-pigs is described separately, and the determination of the lethal dose in anæsthetised pigeons has been added.

The main effect of all these changes is to provide a guide to the most convenient of the well established methods of assay, and to give simple standard procedures for designing and analysing the assays so that their reliability shall be calculable on evidence provided largely by the assays themselves. This is a substantial advance on the previous articles on the same topic and is a reminder that much is lost when variable data are treated with uncritical simplicity and the accuracy of assays is merely specified as good or poor, or by an unvarying figure.

(ABSTRACTS *continued from p. 476.*)

BACTERIOLOGY AND CLINICAL TESTS

Isoniazid-resistant Strains of Tubercle Bacilli, Development and Stability of. M. Barnett, S. R. M. Bushby and D. A. Mitchison. (*Lancet*, 1953, **264**, 314.) Strains of *Mycobacterium tuberculosis* resistant to isoniazid, whether produced *in vitro* or isolated from patients, were found to be unstable both in viability and in their degree of resistance, and two such strains and one resistant variant of H37RV were investigated in mice and guinea-pigs. The animals were infected by intravenous injection of a culture of the organism in a modified Dubos medium. Treatment was started immediately after infection, the isoniazid being given subcutaneously twice daily in doses of 10 mg./kg. of bodyweight, and was continued until the animal died or was killed at the end of the experiment. Previous reports that isoniazid is destroyed in Dubos medium at 37° C. were confirmed, but evidence that continuing growth in the medium was due in part to the development of resistance and not solely to destruction of the drug was provided by the fact that the resistance during the next passage through the medium was increased. Resistant variants of H37RV produced *in vitro* reverted to sensitivity if they had been in contact with the drug during only one subculture. If subcultured more than once in the presence of isoniazid, resistance was retained during several passages through drug-free medium. Viability of resistant variants was poor during the first 3 passages in the presence of isoniazid but thereafter it seemed normal. Repeated passages in the presence of isoniazid failed to produce a strain with an inhibitory end-point higher than 25 to 50 µg./ml. With 10 to 16 resistant strains isolated from patients, the resistance fell substantially during 3 subcultures in the absence of the drug. Mice and guinea-pigs infected with sensitive strains were completely protected by doses of 20 mg./kg. With mice infected by *in vitro* resistant strains, survival time was considerably increased. Guinea-pigs similarly infected were almost completely protected and a chemotherapeutic response to the drug by these animals is likely even when they are infected with a strain which will grow in the presence of 1 µg./ml. H. T. B.

LETTER TO THE EDITOR

Crystalline Emetine

SIR.—Although salts of emetine have been used in medicine for many years and the chemistry of the base has been extensively studied[1,2], the alkaloid is described in the literature as amorphous and, as far as we are aware, crystalline emetine has not been reported[3]. It is therefore of interest to record that some emetine, isolated by us during the manufacture of the hydrochloride, separated from solution as a mass of crystalline prisms. The manufacturing process had been carried out previously on many occasions but an amorphous alkaloid had always been obtained. The crystals may be used to bring about the crystallisation of further batches of alkaloid, for when concentrated solutions of the amorphous base in organic solvents (ether, toluene and xylene) are seeded, crystalline emetine separates on standing. Moreover, if a solution of emetine hydrochloride is treated with excess of ammonia solution and a few crystals of emetine added, the amorphous alkaloid first precipitated gradually changes to the crystalline form, when the mixture is stirred occasionally during several hours.

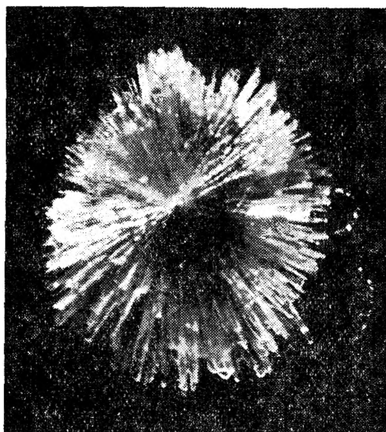


FIG. 1. Crystalline emetine.

A sample of crystalline emetine, a photograph of which is shown in Figure 1, gave the following results on analysis:—loss when dried to constant weight over phosphorus pentoxide, 0·75 per cent.; content of $C_{29}H_{40}O_4N_2$ by titration with standard acid, 100·2 per cent. calculated with reference to the anhydrous substance; m.pt., 104° to 105° C.; specific rotation $[\alpha]_D^{20°\,C.}$ −24·4° (*c*, 1·8 for anhydrous substance in ethanol (50 per cent.); specific rotation of the hydrochloride prepared from base $[\alpha]_D^{20°\,C.}$ + 17·7° (*c*, 5·0 for anhydrous hydrochloride in water); found for anhydrous substance C, 72·42; 72·40; H, 8·50 8·37; N, 5·99, 5·90. $C_{29}H_{40}O_4N_2$ requires C, 72·49; H, 8·39; N, 5·83 per cent.

It will be noted that the melting point is higher than that previously recorded (74° C.) for the amorphous base[3].

We wish to thank Mr. H. M. Hood, B.Sc., for the photograph included in this communication.

G. E. FOSTER.
G. W. NORGROVE.

The Wellcome Chemical Works,
Dartford, Kent.

April 22, 1953.

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2. Hesse, *Liebigs Annalen*, 1914, **405**, 1.
3. Henry, "*The Plant Alkaloids*," 4th Ed., J. and A. Churchill, London, 1949, p. 396.

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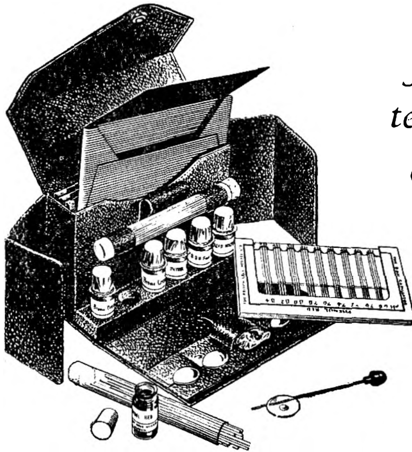

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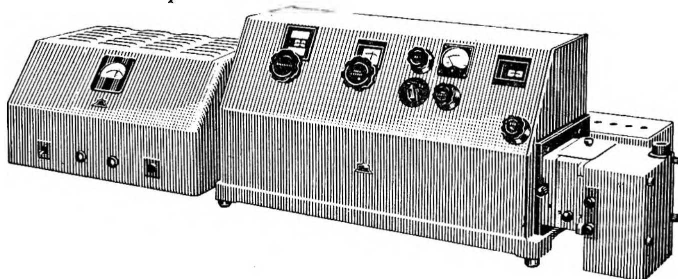

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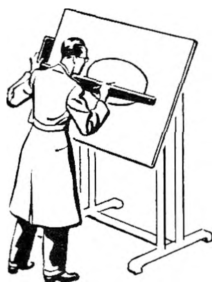

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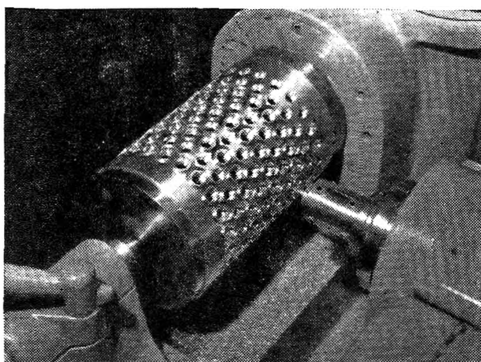

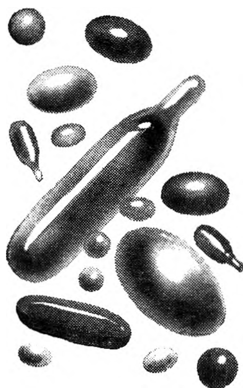

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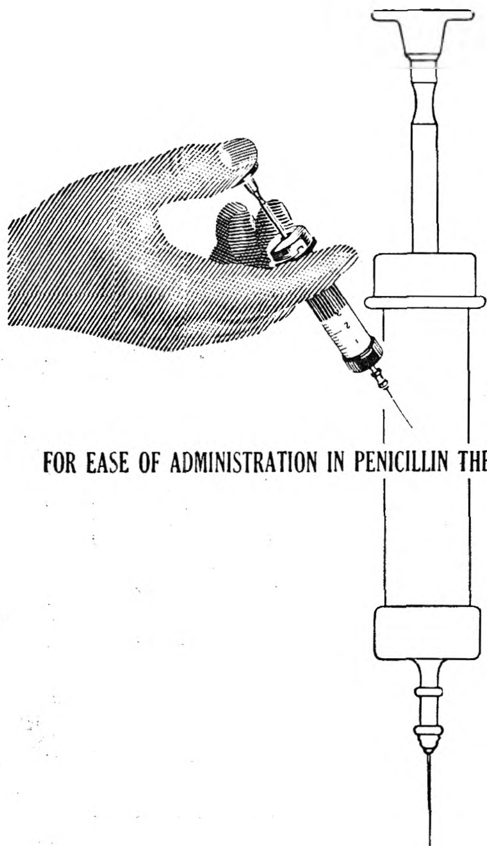

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