REVIEW ARTICLE

STRUCTURE AND SYNTHESIS OF NATURALLY-OCCURRING PEPTIDES

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Although proteins have long been recognised to be of paramount importance in nutrition, the investigation of proteins and the closely related polypeptides as substances that may possess specific biological activity is a comparatively recent development. Polypeptides, it should be stated, differ from proteins only in their lower molecular weight, the division being arbitrarily fixed at 10,000. One of the first proteins with biological activity to be recognised was the hormone insulin, which controls the blood sugar level of the body. Its molecular weight was originally thought to be about 24,000, but there is now general agreement[1-5] that it is of the order of 6000. Insulin should therefore be regarded as a polypeptide rather than as a protein. The various hormones produced by the pituitary gland also appear to be polypeptides. Vasopressin and oxytocin have indeed been shown to be polypeptides much simpler than insulin whilst the adrenocorticotrophic hormones have nearly the same molecular weight as insulin. Another group of polypeptides of biological importance are certain antibiotics, such as gramicidin, bacitracin and polymyxins, produced by bacteria.

Attempts to unravel the structure of polypeptides—to say nothing of the more complex proteins—would have been quite unthinkable a few years ago, but the application of paper chromatography has completely changed the picture, enabling the constituent amino-acids to be separated far more easily than was possible by the other techniques. At first the method was used merely to ascertain the nature and relative proportions of each amino-acid present. Progress became rapid when allied to the methods developed since 1945 which in the first place enabled the nature of the terminal amino-acid group in a polypeptide to be determined and in the second place indicated the sequence of amino-acids in the chain.

Identification of Terminal Amino-acid

In one of the more widely used methods[6,7] for identifying the terminal amino-acid, the polypeptide is reacted with dinitrofluorobenzene, the dinitrophenyl group attaching itself to the free amino group of the terminal amino-acid. On hydrolysis the corresponding dinitrophenyl-amino-acid is isolated and identified, thus indicating which amino-acid occupied the terminal position in the peptide chain. Another method[8,9] of characterising the terminal amino-acid involves reduction of the esterified polypeptide by means of lithium borohydride or of the polypeptide itself with lithium aluminium hydride. This converts the free carboxyl group of the polypeptide into a carbinol, and on subsequent hydrolysis to split the polypeptide into its component amino-acids, the liberated

amino-alcohol is identified. In a third method[10,11] the polypeptide is reacted with phenyl *iso*thiocyanate which combines with the free amino group, converting it into the phenyl thiocarbamyl derivative; this undergoes cleavage into a phenylthiohydantoin and a residual polypeptide containing one less amino-acid residue. The thiohydantoin is identified by paper chromatography thus indicating the nature of the terminal amino-acid. In a closely related method[12,13] the polypeptide is heated with acetic anhydride and ammonium thiocyanate which reacts with the free carboxyl group giving an acyl-thiohydantoin. Both these methods can be used for the step-wise degradation of polypeptides. In yet another method[14–18] the terminal amino-acid is removed by treatment with a selective enzyme and identified by means of paper chromatography. Thus carboxy peptidase removes the *C*-terminal amino-acid and amino-peptidase the *N*-terminal amino-acid. An advantage of this method is that like the preceding method it can be used to effect stepwise degradation of the polypeptide.

Partial Hydrolysis of Gramicidin S

A second big advance in the investigation of the structure of polypeptides was made in 1947 when Synge and his colleagues[19–21] used paper chromatography to separate the products of partial hydrolysis of the bacterial antibiotic gramicidin S. This has a much lower molecular weight than insulin, and was shown to consist of 10 amino-acid units—two molecules each of L-ornithine, L-valine, L-leucine, L-proline and D-phenylalanine. On partial hydrolysis it gave a mixture of di- and tri-peptides, which were separated from one another by paper chromatography. Each spot on the paper was separately eluted and the individual di- and tri-peptides were then hydrolysed completely, and the component amino-acids identified by means of a second paper chromatogram. This enabled the constitution of the di- and tripeptides to be deduced and hence the way in which the amino-acids were linked together, giving a complete picture of the sequence of the amino-acids in the molecule. The complete structure of gramidin S is shown in Figure 1.

Purification of Polypeptides

Gramicidin S is an exceptional polypeptide in that it is easily crystallised, giving a homogeneous product free from other peptide impurities. Most polypeptides, however, are not so easily purified and it is clear that before the structure of a polypeptide can be determined all traces of other peptides and proteins must be removed. A technique now widely adopted for the separation of polypeptides from one another is the method of counter-current distribution developed by Craig[22–24]. In this method the mixture of polypeptides is successively partitioned between a solvent phase and an aqueous phase in an apparatus consisting of a large number of tubes so arranged that the components with a high solvent/water partition coefficient accumulate at one end of the apparatus and the components with a low partition coefficient at the other end; by carrying out a large number of partitions it is possible to separate substances which

exhibit only very slight differences in partition coefficient. The method was used for example, to fractionate the first known bacterial antibiotic, tyrothricin, which after a preliminary separation by conventional methods into the two components, gramicidin and tyrocidine, was further fractionated by means of countercurrent distribution into several closely related polypeptides.

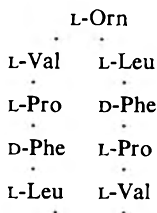


FIG. 1

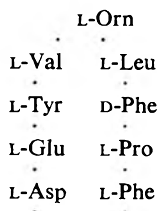


FIG. 2

Another technique that has been used to separate closely related polypeptides from one another is paper electrophoresis[25–30] in which the polypeptide mixture is applied to a strip of damp filter paper and a voltage is applied to the two ends of the paper; this causes the components of the mixture to migrate towards one electrode or the other. Generally the different components migrate at different rates forming separate spots on the paper which can be separately eluted.

Structure of the Tyrocidines

A brief description has been given above of the way in which the structure of gramicidin S (Fig. 1) was determined: precisely similar methods were used to determine the structures of tyrocidines A and B, and are at present being used to elucidate the structure of even more complex peptide antibiotics.

As already mentioned tyrothricin, which is produced by *Bacillus brevis* and was the first bacterial antibiotic to be discovered, was separated into gramicidin—a neutral substance, and tyrocidine—a basic substance; and each of these was in turn separated into several components. Some progress has been made in the study of the gramicidins[31,32] whilst the complete structure of tyrocidine A was established in 1954 by Paladini and Craig[33] and that of tyrocidine B in 1955 by King and Craig[34]. The two structures are given in Figures 2 and 3. What is particularly interesting is the close similarity between the two tyrocidines and gramicidin S. Each is a cyclic decapeptide, and five of the ten amino-acids are identical and are linked together in the same sequence. Moreover, each contains D-phenylalanine and not the L-isomer—the isomer commonly present in proteins. It is considered possible that the presence of this "unnatural" isomer may be one of the factors responsible for the antibacterial activity of these polypeptides. Although an open chain pentapeptide with the same sequence of amino-acids as in gramicidin S was found by Harris and Work[35] to have only limited antibacterial activity, a straight chain decapeptide with the same amino-acid sequence as that of gramicidin S

was synthesised (see below) and found to be strongly antibacterial[36,37] having an activity 1/10th to 1/40th that of gramicidin S. This suggests that peptides do not necessarily have to be cyclic in nature in order to possess antibacterial properties. The greater activity of the natural polypeptide may be due to its lower susceptibility to destruction by bacterial enzymes.

Structure of Other Peptide Antibiotics

Several antibiotics are produced by *Bacillus subtilis* and these, like the antibiotics produced by *B. brevis*, appear to be polypeptides. Bacitracin, perhaps the most clinically valuable of the bacterial antibiotics, was isolated in 1945 and resolved by countercurrent distribution into ten different polypeptides, three of them with antibacterial activity[38–40]. The major component, known as bacitracin A, was studied by the methods used for gramicidin S, and part of the molecule was thereby shown[41–43] to comprise the amino-acid sequence shown in Fig. 4. Further work

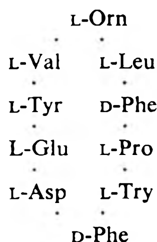


FIG. 3

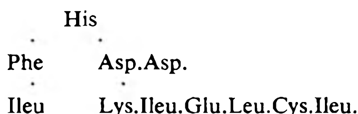


FIG. 4

showed that another part of the molecule contained a thiazoline ring formed by condensation of a cysteine and an *iso*leucine residue[44,45]. A crystalline fragment, thought to be 2-*iso*valeryl-thiazole-4-carboxylic acid was actually isolated from the hydrolysate of bacitracin F. The complete formula of the antibiotic is not yet known.

Another group of peptide antibiotics, the structure of which is partly known, is the group of substances known as the polymyxins, derived from *B. polymyxa.* In 1947, Ainsworth, Brown and Brownlee[46,47] in this country described a new antibiotic which they called aerosporin, and a similar substance obtained at about the same time by Stansly, Shepherd and White[48,49] in the U.S.A. was termined polymyxin. By countercurrent distribution the substance originally termed polymyxin was separated into five components and the main constituent, polymyxin A, was shown to be identical with aerosporin. All five polymyxins were found[50] on analysis to contain a branched chain fatty acid, identified as (+)-6-methyl-octanoic acid, and $\alpha\gamma$-diaminobutyric acid. Polymyxin B_1 has recently been shown[51] to contain 6 moles of the latter and one mole of the former, together with 2 moles of L-threonine, one mole of D-phenylalanine and one mole of L-leucine.

Nisin, originally isolated in 1947 by Hirsch and Mattick[52] from *Streptococcus lactis*, is another peptide now under investigation. It was separated

into four active polypeptides by countercurrent distribution and all four substances yielded on hydrolysis two sulphur-containing amino-acids not normally found in proteins[53,54] These appear to be lanthionine and β-methyl-lanthionine and these new amino-acids have also been detected in two bacterial antibiotics, subtilin and cynnamycin. Results have been published on the structure of other antibiotic peptides but work has not advanced sufficiently to justify inclusion in this review.

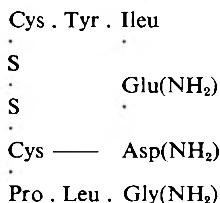


FIG. 5

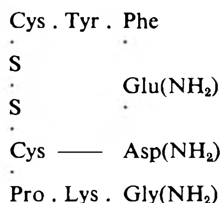


FIG. 6

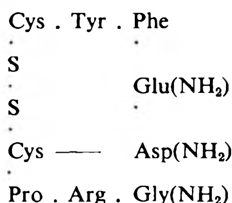


FIG. 7

Structure of Oxytocin and Vasopressin

In parallel with these investigations into the structure of the polypeptide antibiotics, similar work has been proceeding with the object of determining the constitution of the polypeptide active principles of the pituitary gland. Two of these, oxytocin and vasopressin, proved to be octapeptides, and so have a somewhat simpler structure than the tyrocidines and gramicidin S. These two posterior pituitary principles were separated from one another by electrophoresis and countercurrent distribution. Oxytocin, isolated as the crystalline flavinate, was hydrolysed and the constituent amino-acids were separated by partition chromatography on a starch column. The molecule was found to be made up of eight different amino-acids[55–56]. The structure of the molecule was investigated by du Vigneaud and his colleagues[57–63] at Cornell University, using a variety of methods—oxidation with performic acid, desulphurisation with Raney nickel, determination of the terminal groups, and degradation with bromine water. Finally the amino-acid sequence was established by the application of Edman's method and by partial hydrolysis with acid. These investigations led to the structure shown in Figure 5 being postulated for oxytocin. The same structure was arrived at independently by Tuppy[64,65], working in Vienna, and has since been confirmed by synthesis—one of the most important landmarks in the recent history of organic chemistry.

The other posterior pituitary principle vasopressin, was investigated by

similar methods[66–70]. The active substance from pig glands was found to have the same amino-acid composition as the hormone of ox glands, except that the former contained lysine and the latter arginine. The structures of the two hormones—lysine-vasopressin and arginine-vasopressin—are shown respectively in Figures 6 and 7. Lysine-vasopressin has been synthesized.

Structure of α-Corticotrophin

The hormones of the anterior lobe of the pituitary gland appear to be more complex than those of the posterior lobe, and the adrenocorticotrophic hormones—the group about which most is known—appear to contain about forty amino-acid residues. So far, four corticotrophins have been isolated and one of these, α-corticotrophin, from sheep pituitaries, was purified by zone electrophoresis on starch, chromatography on an ion exchange resin and countercurrent distribution. Investigation of its amino-acid composition and the terminal amino-acids and a study of the peptides obtained on partial hydrolysis by means of carboxy peptidase and acid led to the following structure for α-corticotrophin being advanced[71–75]:

Ser.Tyr.Ser.Met.Glu.His.Phe.Arg.Try.Gly.Lys. Pro.
Val.Gly.Lys.Lys. Arg.Arg.Pro.Val.Lys. Val. Tyr.
Pro.Ala.Gly.Glu.Asp.Asp.Glu.Ala.Ser.Glu.Ala.
Phe.Pro.Leu.Glu.Phe.

Structure of Insulin

Even more complicated is the hormone insulin, which contains 48 amino-acid residues. Its structure was recently described by Sanger and his colleagues[76,77]. The methods employed were similar in principle to those already outlined, but obviously with such a complex molecule the difficulties were very much greater and the work took considerably longer. Insulin consists of two polypeptide chains joined at two points by disulphide linkages. These were broken by oxidation with performic acid, and the two chains were then investigated separately. The sequence of amino-acids in each was elucidated by partial hydrolysis and identification of the peptide fragments so formed. By piecing together the information so obtained, the whole insulin molecule was reconstructed. The structure of cattle insulin is given in Figure 8. It can be seen that the molecule consists of one large ring containing four half-cystine residues and a very much smaller ring containing two half-cystine residues. It is interesting to note that the nature of this second and smaller ring is different in insulin derived from sheep and pig pancreas. In sheep insulin the amino-acid sequence is Cys.Cys.Ala.Gly.Val.Cys. and in pig insulin Cys.Cys.Thr.Ser. Ileu.Cys. It may be significant that this smaller ring in insulin happens to be the same size as the molecules of oxytocin and vasopressin.

Synthesis of Polypeptides

The first two polypeptides ever to be synthesised were prepared by Fischer and Fourneau[78] as far back as 1901, but the method used and others developed during the next few years are quite unsuitable for the synthesis

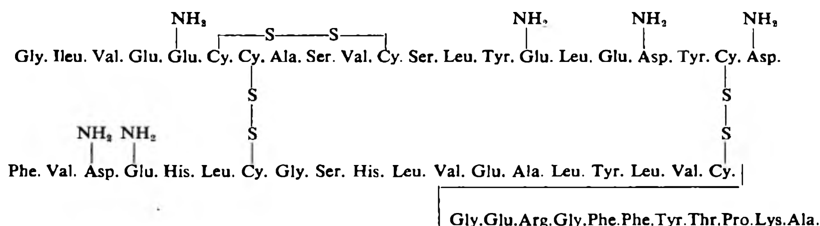


FIG. 8

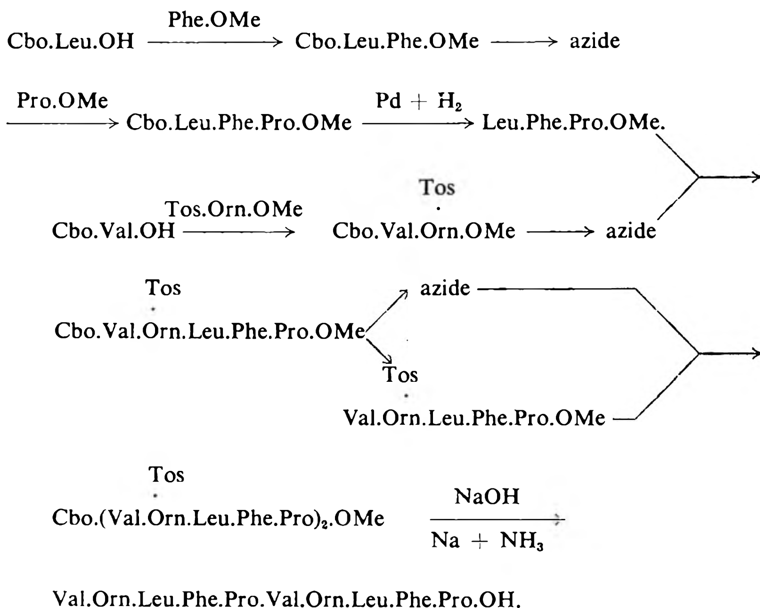
of complex polypeptides such as the bacterial antibiotics and pituitary hormones. All the practical methods, including those recently used for the synthesis of vasopressin and oxytocin, depend on protecting the amino group of one amino-acid, converting this into a reactive derivative such as the acid chloride or azide and then reacting this with the amino group of a second amino-acid. The groups that have been employed in this way to protect the amino group include the benzoyl, carbethoxy, *p*-toluene-sulphonyl (tosyl), haloacyl, carbobenzoxy (benzyloxycarbonyl), and phthalyl radicals. Of these the two last named are the most important. The first two radicals can be removed only by hydrolysis which also splits the peptide bond, whilst the haloacyl group is converted by treatment with ammonia into the corresponding amino acyl group. The tosyl group proved to be more useful than either of these other two radicals, for Fischer and Lipschitz[79] showed that it could be removed without affecting the peptide bond by treatment with phosphonium iodide and hydriodic acid, the tosyl group being split off as tolylmercaptan. The method has been used for preparing several di- and tri-peptides. In its more modern version sodium in liquid ammonia is used in place of phosphonium iodide.

By far the most important method, however, is the carbobenzoxy method introduced by Bergmann and Zervas[80,81] The carbobenzoxy or benzyloxycarbonyl group, which has the formula $C_6H_5CH_2OCO$, is introduced into an amino-acid by reaction with benzylchloroformate, $C_6H_5CH_2OCO{\cdot}Cl$, which is prepared by the action of phosgene on benzyl alcohol. The carbobenzoxy-amino-acid is converted into its azide and then reacted with a second amino-acid. To remove the carbobenzoxy group from the peptide thus formed it is catalytically hydrogenated, a method that avoids any danger of hydrolysing the peptide bond.

Synthesis of Antibacterial Decapeptide

This was the method used by Erlanger *et al.*[36,37] in synthesising the straight-chain decapeptide containing the same amino-acid sequence as gramicidin S. Carbobenzoxy-L-leucine was coupled with D-phenylalanine ester and the product was converted to the corresponding azide, carbobenzoxy-L-leucyl-D-phenylalanyl azide. This was coupled with L-proline ester and the product catalytically hydrogenated to remove the carbobenzoxy group, giving L-leucyl-D-phenylalanyl-L-proline ester. This was reacted with carbobenzoxy-L-valyl-*p*-tosyl-L-ornithine azide, obtained from carbobenzoxy-L-valine and *p*-tosyl-L-ornithine ester, giving

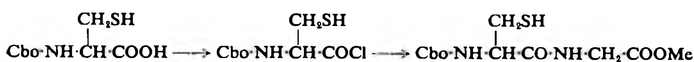
carbobenzoxy-L-valyl-*p*-tosyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline ester. One portion of this was converted to the corresponding azide and another portion was catalytically hydrogenated to remove the carbobenzoxy group. The two products were combined with one another, giving the decapeptide ester in which the end amino group carried a carbobenzoxy group and the ω-amino group of the ornithyl residue a *p*-tosyl group. After removing the ester group by hydrolysis, the carbobenzoxy and tosyl groups were removed at the same time by treatment with sodium in liquid ammonia. These reactions can be represented in abbreviated form as follows (Cbo = the carbobenzoxy radical $C_6H_5{\cdot}CH_2{\cdot}O{\cdot}CO$ and Tos = the *p*-tosyl radical):—

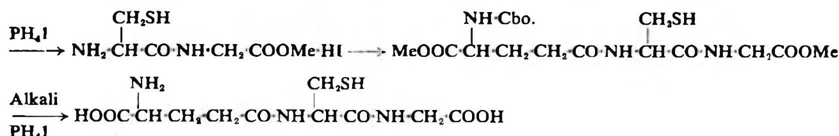


Val.Orn.Leu.Phe.Pro.Val.Orn.Leu.Phe.Pro.OH.

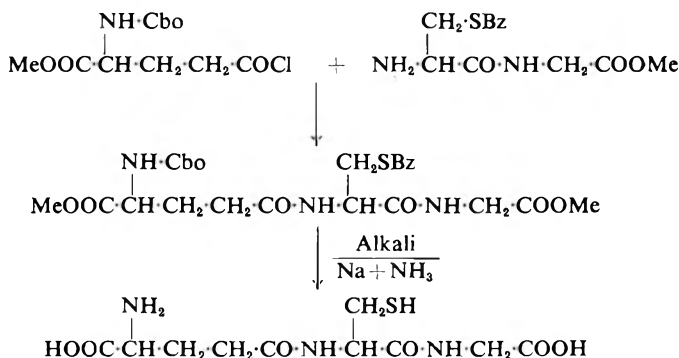
Synthesis of Glutathione

This method in its original form has one serious limitation: it cannot be used for producing polypeptides containing cysteine, because the catalyst used in the reduction is poisoned by the sulphur. Harrington and Mead[82], however, in 1935 prepared the tripeptide, glutathione (γ-glutamyl-cysteinyl-glycine) by using phosphonium iodide in acetic acid instead of catalytic hydrogenation to remove the carbobenzoxy group. *N*-Carbobenzoxy-cysteine was converted to the acid chloride and coupled with glycine ester. The product was reduced by means of phosphonium iodide to give cysteinylglycine ester hydriodide, which was then coupled with *N*-carbobenzoxyglutamyl chloride; the ester groups were removed by hydrolysis and the carbobenzoxy group was split off by reduction with phosphonium iodide:





A further improvement was made by du Vigneaud and his colleagues in their work on the synthesis of oxytocin. They had observed that the carbobenzoxy group could be removed just as readily by reduction with sodium in liquid ammonia as by catalytic hydrogenation, and it then occurred to them that a benzyl group used to protect a mercapto group could also be removed from *S*-benzylcysteine or from *S*-benzylcysteinyl-glycine by reduction with sodium in liquid ammonia, so that this one reaction might be used to remove the two protecting groups from the amino and the mercapto groups. They were able to synthesise glutathione by the following series of reactions (Bz = benzyl):—



Synthesis of Oxytocin

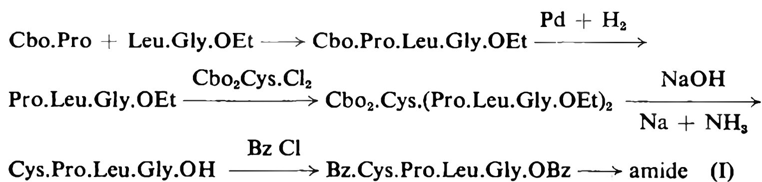
This synthesis of glutathione formed the basis of du Vigneaud's synthetic approach to oxytocin. When the structure of oxytocin was deduced from degradation experiments it was considered possible that if the protected nonapeptide amide could be synthesised reduction with sodium in liquid ammonia ought to give the reduced form of oxytocin. Air-oxidation should convert this into oxytocin. Accordingly, the reduced form of oxytocin was prepared from the natural hormone by reduction with sodium in liquid ammonia and the *SS'*-dibenzyl derivative was prepared from it in order to find out if the principle could be regenerated from its benzylated derivative. It was in fact found[83] that the biologically inactive *SS'*-dibenzyl derivative of oxytocin on treatment with sodium in liquid ammonia regenerated biologically active material.

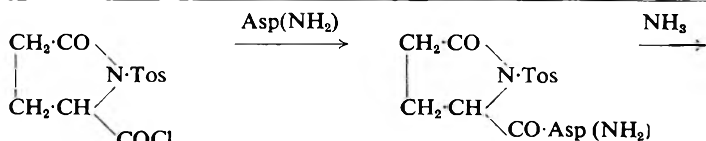
The synthesis of oxytocin[84–88] was achieved by combining the tetrapeptide amide, *S*-benzylcysteinyl-prolyl-leucyl-glycyl amide with *p*-tosyl-*iso*leucyl-glutaminyl-asparagine and condensing the heptapeptide amide so formed with *S*-benzyl-*N*-carbobenzoxy-cysteinyl-tyrosine thus forming the desired protected nonapeptide amide. The tetrapeptide amide was prepared as follows:—

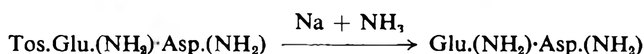
Carbobenzoxy-L-leucyl-glycine ethyl ester was synthesised by the mixed

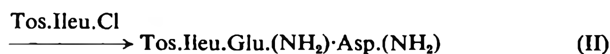
anhydride procedure of Vaughan and Osato[89]. After catalytic removal of the carbobenzoxy group, the L-leucyl-glycine ethyl ester was condensed with carbobenzoxy-proline. The protected tripeptide thus obtained was catalytically hydrogenated to remove the carbobenzoxy group and the resulting L-prolyl-L-leucyl-glycine ethyl ester was converted into di-carbobenzoxy-L-cystinyl-L-prolyl-L-leucylglycine ester by condensation with di-carbobenzoxy-L-cystine dichloride. The ester was hydrolysed and the carbobenzoxy groups were removed by means of sodium in liquid ammonia, and the sulphur of the reduced compound was then benzylated in the same medium. The product was converted into the benzyl ester and thence into the amide.

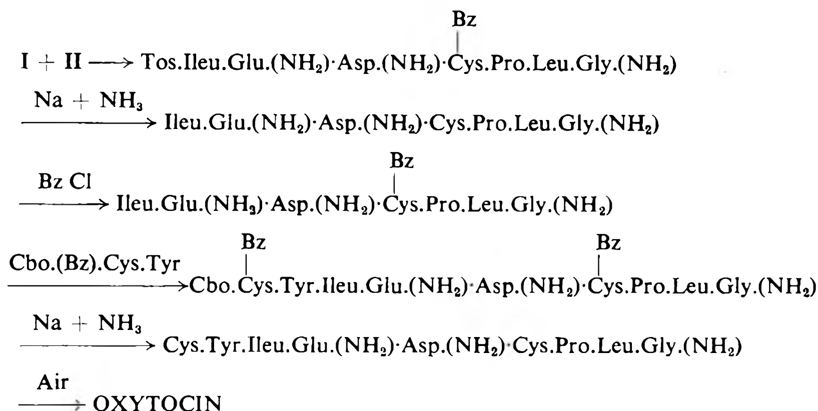
A novel method was used for the synthesis of glutaminyl-asparagine, the key intermediate being 5-oxo-L-*p*-tosyl-pyrrolidine-2-carbonyl chloride. This was coupled with L-asparagine to give 5-oxo-1-*p*-tosyl-pyrrolidine-2-carbonyl-L-asparagine which in strong ammonia yielded *p*-tosyl-L-glutaminyl-L-asparagine, the pyrrolidine ring having been opened by the addition of the elements of ammonia. Treatment with sodium in liquid ammonia gave L-glutaminyl-L-asparagine in high yield. For the preparation of *p*-tosyl-L-*iso*leucyl-L-glutaminyl-L-asparagine, *p*-tosyl-L-*iso*leucine was converted into the corresponding acid chloride and the latter was then coupled with L-glutaminyl-L-asparagine giving the tosyl-tripeptide in good yield. Condensation of the tripeptide with the tetrapeptide amide was effected by the use of tetraethyl pyrophosphite, and the tosyl group was removed from the heptapeptide amide by means of sodium in liquid ammonia which also removed the benzyl group. The resulting reduced compound was then benzylated with benzyl chloride in the same medium, and the *S*-benzyl heptapeptide amide was condensed with *S*-benzyl-*N*-carbobenzoxy-L-cysteinyl-L-tyrosine in presence of tetraethyl pyrophosphite to yield the nonapeptide amide. Sufficient synthetic material was prepared to enable it to be tried out in the induction of labour in the human, and its activity was found to be identical with that of natural oxytocin. These reactions can be represented in abbreviated form as follows:—











Lysine-vasopressin was synthesised by a method identical with that used for oxytocin except that leucine was replaced by ϵ *p*-tosyl-lysine and *iso*-leucine by phenylalanine at the appropriate stages.

The synthesis of these pituitary active principles undoubtedly sets a pattern for the synthesis of other polypeptides of comparable complexity and it is probably only a matter of time before more complex polypeptides are synthesised by methods similar to those used by du Vigneaud and his colleagues, who appear to have solved most of the basic problems. The synthesis of insulin, would, of course, involve a repetition of the steps involved in the synthesis of oxytocin many times over and would be a tedious operation resulting in extremely small yields of the final product.

REFERENCES

1. Harfenist and Craig, *J. Amer. chem. Soc.*, 1952, **74**, 3087.
2. Fredericq, *Nature, Lond.*, 1953, **171**, 570.
3. Kupke and Linderstrøm-Lang, *Biochim. Biophys. Acta*, 1954, **13**, 153.
4. Rees and Singer, *Nature, Lond.*, 1955, **176**, 1072.
5. Harrap, *J. Colloid Sci.*, 1955, **10**, 351.
6. Sanger, *Biochem. J.*, 1945, **39**, 507.
7. Porter and Sanger, *ibid.*, 1948, **42**, 287.
8. Chibnall and Rees, *ibid.*, 1951, **48**, p. xlvi.
9. Fromageot, Jutisz, Meyer and Pénasse, *Biochim. Biophys. Acta*, 1950, **6**, 283.
10. Edman, *Arch. Biochem.*, 1949, **22**, 475.
11. Edman, *Acta chem. scand.*, 1950, **4**, 283.
12. Schlack and Kumpf, *Z. physiol. Chem.*, 1926, **154**, 125.
13. Waley and Watson, *J. chem. Soc.*, 1951, 2394.
14. Lens, *Biochim. Biophys. Acta*, 1949, **3**, 367.
15. Gladner and Neurath, *ibid.*, 1952, **9**, 335.
16. Gladner and Neurath, *J. biol. Chem.*, 1953, **205**, 345.
17. Sanger and Thompson, *Biochem. J.*, 1953, **53**, 366.
18. White, *J. Amer. chem. Soc.*, 1955, **77**, 4691.
19. Synge, *Biochem. J.*, 1945, **39**, 363.
20. Consden, Gordon, Martin and Synge, *ibid.*, 1947, **41**, 596.
21. Pedersen and Synge, *Acta chem. scand.*, 1948, **2**, 408.
22. Craig, *J. biol. Chem.*, 1944, **155**, 519.
23. Craig, Golumbic, Mighton and Titus, *ibid.*, 1945, **161**, 321.
24. Craig, Hausmann, Ahrens and Harfenist, *Analyt. Chem.*, 1951, **23**, 1236.
25. Strain, *ibid.*, 1952, **24**, 356.
26. Lederer and Ward, *Analyt. Chem. Acta.*, 1952, **6**, 355.
27. Sato, Norris and Strain, *Analyt. Chem.*, 1952, **24**, 776.
28. Tiselius, *J. gen. Physiol.*, 1951, **35**, 89.

29. Consden and Stanier, *Nature, Lond.*, 1952, **169**, 783.
30. Consden and Stanier, *ibid.*, 1952, **170**, 1069.
31. Synge, *Biochem. J.*, 1949, **44**, 542.
32. James and Synge, *ibid.*, 1952, **50**, 109.
33. Paladini and Craig, *J. Amer. chem. Soc.*, 1954, **76**, 688.
34. King and Craig, *ibid.*, 1955, **77**, 6627.
35. Harris and Work, *Biochem. J.*, 1950, **46**, 582.
36. Erlanger, Sachs and Brand, *J. Amer. chem. Soc.*, 1954, **76**, 1806.
37. Erlanger and Goode, *Nature, Lond.*, 1954, **174**, 840.
38. Newton and Abraham, *Biochem. J.*, 1950, **46**, P. xxxii.
39. Newton and Abraham, *ibid.*, 1953, **53**, 596.
40. Craig, Weisiger, Hausmann and Harfenist, *J. biol. Chem.*, 1952, **199**, 259.
41. Craig, Hausmann and Weisiger, *J. Amer. chem. Soc.*, 1954, **76**, 2839.
42. Craig, Hausmann and Weisiger, *ibid.*, 1955, **77**, 721, 723, 731.
43. Lockhart and Abraham, *Biochem. J.*, 1954, **58**, 633.
44. Weisiger, Hausmann and Craig, *J. Amer. chem. Soc.*, 1955, **77**, 3123.
45. Lockhart, Abraham and Newton, *Biochem. J.*, 1955, **61**, 534.
46. Ainsworth, Brown and Brownlee, *Nature, Lond.*, 1947, **160**, 263.
47. Brownlee and Bushby, *Lancet*, 1948, **254**, 127.
48. Stansly, Shepherd and White, *Bull. Johns Hopkins Hosp.*, 1947, **81**, 43.
49. Shepherd, Stansly, Winterbottom, English, Fellows, Ananenko and Guillet, *J. Amer. chem. Soc.*, 1948, **70**, 3771.
50. Wilkinson, *Nature, Lond.*, 1949, **164**, 622.
51. Hausmann and Craig, *J. Amer. chem. Soc.*, 1954, **76**, 4892.
52. Hirsch and Mattick, *Lancet*, 1949, **257**, 190.
53. Berridge, Newton and Abraham, *Biochem. J.*, 1952, **52**, 529.
54. Newton and Abraham, *Nature, Lond.*, 1953, **171**, 606.
55. Pierce and du Vigneaud, *J. biol. Chem.*, 1950, **182**, 359.
56. Pierce and du Vigneaud, *ibid.*, 1950, **186**, 77.
57. Mueller, Pierce, Davoll and du Vigneaud, *ibid.*, 1951, **191**, 309.
58. Turner, Pierce and du Vigneaud, *ibid.*, 1951, **193**, 359.
59. Ressler, Trippett and du Vigneaud, *ibid.*, 1953, **204**, 861.
60. Davoll, Turner, Pierce and du Vigneaud, *ibid.*, 1951, **193**, 363.
61. Kunkel, Taylor and du Vigneaud, *ibid.*, 1953, **200**, 559.
62. du Vigneaud, Ressler and Trippett, *ibid.*, 1953, **205**, 949.
63. Mueller, Pierce and du Vigneaud, *ibid.*, 1953, **204**, 857.
64. Tuppy, *Biochim. Biophys. Acta*, 1953, **11**, 449.
65. Tuppy and Michl, *Monatsh.*, 1953, **84**, 1011.
66. Popenoe and du Vigneaud, *J. biol. Chem.*, 1953, **205**, 133.
67. Popence and du Vigneaud, *ibid.*, 1954, **206**, 353.
68. Popenoe, Lawler and du Vigneaud, *J. Amer. chem. Soc.*, 1952, **74**, 3713.
69. du Vigneaud, Lawler and Popenoe, *ibid.*, 1953, **75**, 4880.
70. Acher and Chauvet, *Biochim. Biophys. Acta*, 1954, **14**, 421.
71. Li, Geschwind, Dixon, Levy and Harris, *J. biol. Chem.*, 1955, **213**, 171.
72. Levy, Geschwind and Li, *ibid.*, 1955, **213**, 187.
73. Levy and Li, *ibid.*, 1955, **213**, 487.
74. Harris and Li, *ibid.*, 1955, **213**, 499.
75. Li, Geschwind, Cole, Raacke, Harris and Dixon, *Nature, Lond.*, 1955, **176**, 687.
76. Ryle, Sanger, Smith and Kitai, *Biochem. J.*, 1955, **60**, 541.
77. Brown, Sanger and Kitai, *ibid.*, 1955, **60**, 556.
78. Fischer and Fourneau, *Ber.*, 1901, **34**, 2868.
79. Fischer and Lipschitz., *ibid.*, 1915, **48**, 360.
80. Bergmann and Zervas, *ibid.*, 1932, **65**, 1192.
81. Bergmann and Zervas, *ibid.*, 1933, **66**, 1288.
82. Harington and Mead, *Biochem. J.*, 1935, **29**, 1602.
83. Gordon and du Vigneaud, *Proc. Soc. exp. Biol., N.Y.*, 1953, **84**, 723.
84. du Vigneaud, Ressler, Swan, Roberts, Katsoyannis and Gordon, *J. Amer. chem. Soc.*, 1953, **75**, 4879.
85. Ressler and du Vigneaud, *ibid.*, 1954, **76**, 3107.
86. Swan and du Vigneaud, *ibid.*, 1954, **76**, 3110.
87. Katsoyannis and du Vigneaud, *ibid.*, 1954, **76**, 3113.
88. du Vigneaud, Ressler, Swan, Roberts and Katsoyannis, *ibid.*, 1954, **76**, 3115.
89. Vaughan and Osato, *ibid.*, 1951, **73**, 5553.

RESEARCH PAPERS

THE INFLUENCE OF ANTICHOLINESTERASES ON THE NEUROMUSCULAR BLOCK PRODUCED BY SUXAMETHONIUM

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INTRODUCTION

The short acting muscle-relaxing property of suxamethonium was demonstrated by Bovet and others[1] and confirmed by Phillips[2] and Walker[3]. Contrary to the findings with curare, anticholinesterase drugs increased the toxicity of suxamethonium in laboratory animals and prolonged the duration of its action[4–6].

Marotta and Carminati,[7] investigating the action of a number of anticholinesterases when injected before suxamethonium, found that they increased the duration of the neuromuscular block, and related this to the level of serum pseudocholinesterase. No such action was observed when the true cholinesterase was blocked. Similar results have been reported by Fraser[8], who showed that the inhibition of true cholinesterase by 284 C 51 (1 : 5-bis(4-allyldimethylammonium phenyl)-*N*-pentan-3-one dibromide) *in vivo* did not modify the neuromuscular blocking action of suxamethonium, whereas when pseudocholinesterase was also inhibited by the administration of eserine, a distinct prolongation of the effect was observed.

The extensive use of suxamethonium in endoscopy, electro-shock therapy and surgery has resulted in instances of prolonged apnœa which have been widely quoted in the literature[9–13].

To account for these occurrences it was suggested that the duration of action of suxamethonium was normally dependent upon the pseudocholinesterase level[14,15]. Bourne[16] observed prolonged apnœa in 15 out of a series of 1000 patients, in 10 of whom pseudocholinesterase values were determined. It was found that 8 patients possessed significantly low, and the other 2 comparatively low levels of this enzyme. On the other hand, however, prolonged apnœa has also been reported to occur in patients possessing normal pseudocholinesterase levels[17,18].

Owing to the conflicting theories advanced by various workers to account for these abnormal clinical findings and their relationship with pseudocholinesterase levels the present investigation was undertaken to examine further the effects of the anticholinesterases eserine and neostigmine on the neuromuscular blocking action of suxamethonium in the rabbit and dog.

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METHODS

Dogs were anæsthetised with chloralose (120 mg./kg. intravenously) and rabbits with urethane (1·5 g./kg. subcutaneously). The contractions of the gastrocnemius muscle, following electrical stimulation every 15 seconds of the cut peripheral end of the sciatic nerve, were recorded isometrically. The blood pressure from the carotid artery was registered by a mercury manometer and the abdominal respiration was recorded by a Marey tambour. Tracheotomy was performed and artificial respiration supplied when required.

Suxamethonium dichloride, neostigmine methylsulphate and eserine salicylate were used. All drugs were administered according to body weight by the intravenous route. The doses injected are expressed in terms of these salts.

The response to a dose of suxamethonium, selected to cause a reproducible partial neuromuscular block, was compared before and after administration of eserine or neostigmine (0·05 to 0·1 mg./kg.). In some experiments the dose of suxamethonium given after the anticholinesterases was varied to produce the same reduction in the height of contraction as that obtained before the administration of the anticholinesterases.

The degree of paralysis was expressed as the per cent. diminution of the initial height of contraction (complete paralysis = 100 per cent.). Duration of paralysis was estimated from the "half-return" of the contraction height to its initial level. In this way the effects of the anticholinesterases on both degree and duration of suxamethonium paralysis could be determined.

RESULTS

The effect of eserine in the rabbit

It was generally observed that doses of from 0·125 mg./kg. to 0·25 mg./kg. suxamethonium produced a partial paralysis which was fairly reproducible. After the injection of 0·1 mg./kg. eserine which had no effect on the muscular contractions it was found that similar doses of suxamethonium gave rise to a more intense and longer sustained muscular paralysis.

It will be noted from Table I which summarizes five typical results that 0·125–0·25 mg./kg. suxamethonium caused 33 to 81 per cent. inhibition of the neuromuscular contraction, and the half-return required from 3 to 5½ minutes. After eserine the neuromuscular block produced by the relaxant was 57 to 100 per cent. and the time for the half-return was now 6 to 12 minutes.

In Figure I it will be observed that 0·25 mg./kg. suxamethonium produced 81 per cent. inhibition in the muscular contraction, and the time taken for half-return was 4 minutes. After 0·1 mg./kg. eserine the same dose of suxamethonium produced complete paralysis, and the time for the half-return was 10 minutes. Smaller doses of the compound were then injected in order to reproduce the initial reduction of contractions. When 0·075 mg./kg. suxamethonium was injected into the animal, the

TABLE I

THE EFFECT OF SUXAMETHONIUM BEFORE AND AFTER ESERINE IN THE RABBIT AND DOG

	Suxamethonium before eserine				Suxamethonium after eserine		Modification of neuro-muscular blocking effect	
Dose of suxamethonium (mg./kg.)	Block per cent.	Duration of half return (minutes)	Dose of eserine (mg./kg.)	Effect of eserine on muscular contractions	Block per cent.	Duration of half return (minutes)	Intensity	Duration
Rabbits								
0·125	35	4	0·1	none	70	8	++	++
0·25	33	5½	0·1	,,	58	12	++	++
0·25	39	5½	0·1	,,	57	10	++	++
0·25	47	3	0·1	,,	74	6	++	++
0·25	81	4	0·1	,,	100	10	++	++
Dogs								
0·0375	80	5	0·1	,,	65	9	−−	++
0·05	47	3	0·1	,,	34	5½	−−	++
0·05	65	3½	0·1	,,	35	7½	−−	++
0·1	75	10	0·1	,,	63	15	−−	++

TABLE II

THE EFFECT OF SUXAMETHONIUM BEFORE AND AFTER NEOSTIGMINE IN THE RABBIT AND DOG

	Suxamethonium before neostigmine				Suxamethonium after neostigmine		Modification of neuro-muscular blocking effect	
Dose of suxamethonium (mg./kg.)	Block per cent.	Duration of half return (minutes)	Dose of neostigmine (mg./kg.)	Effect of neostigmine on muscular contractions before suxamethonium	Block per cent.	Duration of half return (minutes)	Intensity	Duration
Rabbits								
1. 0·125	55	4	0·1	10 per cent. inhibition with slow return	61	6	+	+
2. 0·25	54	4	0·1	No inhibition	60	4½	(+)	(+)
3. 0·25	60	4	0·1	18 per cent. inhibition with partial return	62	6	no effect	+
4. 0·25	49	5	0·1	10 per cent. inhibition very slow return	73	8½	++	++
5. 0·25	55	8	0·1	34 per cent. inhibition without return	83	24	++	+++
Dogs								
1. 0·05	55	4	0·05	31 per cent. inhibition with complete return	48	9½	−	++
2. 0·075	41	4½	0·075	12 per cent. inhibition, complete return	31	6	−−	+
3.* 0·05	62	5	0·1	15 per cent. inhibition, incomplete return	30	11	−−	++
4. 0·05	42	4	0·1	48 per cent. inhibition with partial return	67	9	++	++
5. 0·075	64	4½	0·1	50 per cent. inhibition slow return	83	12	++	+++

* Atropine was given before neostigmine.

same type of paralysis curve as that given by 0·25 mg./kg. before the injection of eserine, was obtained. The percentage inhibition was 82 per cent. and half-return took 3¾ minutes. Thus the pattern of the block was duplicated when a fraction of the initial dose of suxamethonium was injected into the eserinised rabbit.

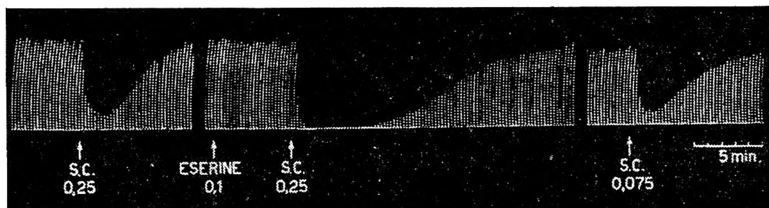

FIG. 1. Rabbit, 2·2 kg. under urethane anæsthesia; doses in mg/kg. Neuromuscular blocking action of suxamethonium before and after eserine. Contractions of the gastrocnemius muscle after electrical stimulation of the peripheral portion of the cut sciatic nerve.

The effect of eserine in the dog

In the dog, which was more susceptible to suxamethonium than the rabbit, doses of from 0·0375 mg./kg. to 0·1 mg./kg. gave the required partial muscular paralysis, i.e. generally one quarter of the dose needed in the rabbit produced the equivalent partial neuromuscular block in the dog.

The results of some representative experiments are shown in Table I. The suxamethonium paralysis was from 47 to 80 per cent. and the time taken for the half-return was from 3 to 10 minutes. When eserine had been administered, suxamethonium produced from 34 to 65 per cent. neuromuscular block and the half-return was from 5½ to 15 minutes.

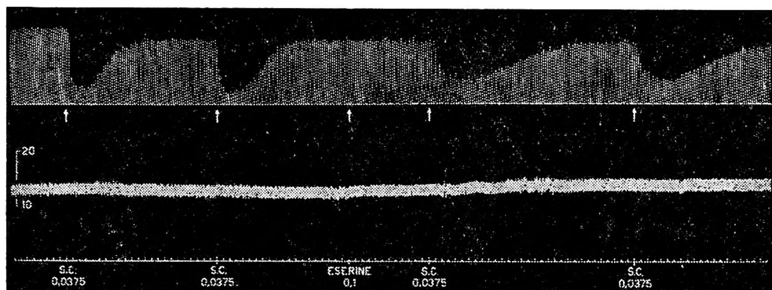

FIG. 2. Dog, 10 kg, under chloralose anæsthesia; doses in mg/kg. Neuromuscular blocking action of suxamethonium before and after eserine. Blood pressure from the carotid artery and contractions of the gastrocnemius muscle after electrical stimulation of the peripheral portion of the cut sciatic nerve.

Figure 2 shows that 0·0375 mg./kg. of the relaxant produced 77 per cent. and 81 per cent. inhibition in the muscular contractions, and the duration of the half-return was 4 and 5 minutes respectively. After eserine 0·0375 mg./kg. suxamethonium produced 67 and 61 per cent. inhibition and the time taken for the half-return was 9 and 8½ minutes.

It was clear that eserine in the dog did not potentiate the extent of the paralysing action of suxamethonium, as was observed in the rabbit; in fact some antagonism in the neuromuscular blocking action of the relaxant was noted. The duration of the paralysis was, however, distinctly increased.

The effect of neostigmine in the rabbit

A dose of 0·1 mg./kg. of neostigmine had little effect on blood pressure although it produced a distinct diminution in muscular contractions. Only in one experiment could no inhibition be observed, whereas in other cases the diminution in contraction varied from 10 to 34 per cent. The return to the original level was usually very slow and at times only partial. Suxamethonium before neostigmine gave degrees of inhibition varying from 49 to 60 per cent. and the time for the half return was from 4 to 8 minutes. The effect of 0·1 mg./kg. neostigmine on the response to the relaxant varied greatly from one experiment to another. In some experiments where neostigmine exerted only a slight action, a small increase in the intensity and duration of the suxamethonium paralysis was noted (Experiments 1, 2, 3, Table II). In others, (Experiments 4, 5, Table II), where the effect of neostigmine alone was especially prolonged, a substantial augmentation in the intensity and duration of the suxamethonium block could be observed.

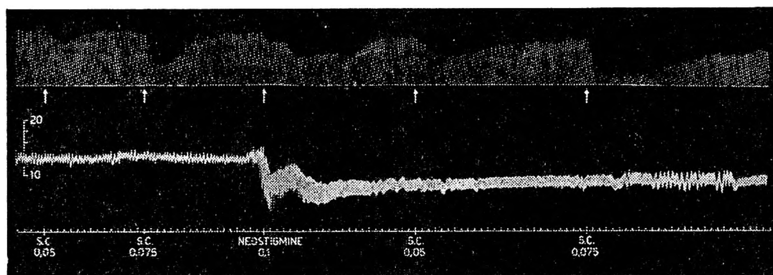

FIG. 3. Dog, 12 kg. under chloralose anæsthesia; doses in mg/kg. Neuromuscular blocking action of suxamethonium before and after neostigmine. Blood pressure from the carotid artery and contractions of the gastrocnemius muscle after electrical stimulation of the peripheral portion of the cut sciatic nerve.

The effect of neostigmine in the dog

Neostigmine when administered in a dose of 0·05 to 0·1 mg./kg. in the dog caused a fall in blood pressure which was concomitant with a reduction in the height of muscular contractions of up to 50 per cent. When atropine was injected (Experiment 3, Table II) the fall in blood pressure was prevented, and the muscular paralysis induced was slight. Suxamethonium before neostigmine produced degrees of inhibition in the muscular contractions from 41 to 64 per cent. and the time for the half-return was 4 to 5 minutes. An increase in the duration of the paralysis was noted when the relaxant was administered after neostigmine.

However, different results on the intensity of the neuromuscular block were obtained, which appeared to depend upon the previous effect of neostigmine alone. Figure 3 shows the direct effect of neostigmine on muscular contractions and on blood pressure. The inhibition in the contractions after neostigmine is 50 per cent. and the return very slow. Subsequent administration of suxamethonium leads to a distinct increase in the extent and duration of the neuromuscular block. In instances where the neostigmine paralysis was slight, or a complete return to the original height of contraction was rapidly obtained (Experiments 1, 2, 3, Table II), there was an antagonistic action towards suxamethonium and consequently the extent of the paralysis was diminished.

DISCUSSION

The experiments described indicate that, depending upon the animal species used, the neuromuscular block produced by suxamethonium may be modified in two different ways by the previous administration of anticholinesterases. The first may be illustrated by the action of eserine in the rabbit. In this species, eserine increases both the degree and duration of the block. As no significant alteration in the pattern of the curve was noted, this type of increased susceptibility may conveniently be termed a "dosage effect".

In the dog, however, eserine prolongs the duration of block while in effect decreasing its intensity. This may be described as a "time effect". Neostigmine produced different modifications of the suxamethonium response in the two species. The final result appeared to be mainly influenced by the direct previous action of neostigmine on the muscular contractions. In contrast to eserine, neostigmine when administered even in minute doses was found to provoke marked hypotension and diminution in muscular contractions.

The effect of neostigmine upon neuromuscular contraction has been referred to in the literature[19]. A direct effect of neostigmine on skeletal muscle has been reported by Riker and Wescoe[20]. Zaimis[21] has also drawn attention to the direct action of neostigmine on the neuromuscular junction. It may be of interest to mention that Jacobsohn and Kahlson[22] have demonstrated a difference in the anticurare activity between eserine and neostigmine.

The potentiation of the neuromuscular blocking action of suxamethonium by anticholinesterases may be related to its hydrolysis in the organism. Thus Glick[23] and Bovet-Nitti[4] demonstrated the rapid hydrolysis of the compound by pseudocholinesterase, and to a less significant extent by the true cholinesterase of erythrocytes. Eserine appears to interfere with the destruction of suxamethonium owing to the reduction in the circulating cholinesterase level. It has also been shown that the ali-esterase present in the liver and kidney hydrolyses suxamethonium[24]. In the "dosage effect" observed in the rabbit the interference with the hydrolysis of suxamethonium by anticholinesterases may account for the results obtained. In the interpretation of the time effect, however, additional factors must be considered. Thus the

diminution in the intensity of the paralysis noted in the dog may be related to the general antagonism which is known to exist between anticholinesterases and curare-like drugs. In the case of decamethonium whose mechanism of action is very similar to that of suxamethonium, "decurarising" effects after the administration of anticholinesterases were reported by Bovet *et al.*[25] and by Brand[26]. The antagonistic effect of anticholinesterases towards curare was generally interpreted as an inhibition of the hydrolysis of acetylcholine liberated at the motor end-plate thus facilitating neuromuscular transmission. The particular pattern of the time effect of cholinesterase inhibition may be explained as a combination of the simple potentiation related to the slowing in suxamethonium hydrolysis in the body with the so-called "decurarising" action of eserine related to the endogenous acetylcholine metabolism.

It may be assumed that the effect of anticholinesterases on the paralysis produced by suxamethonium is a consequence of a complex series of interactions. Thus both eserine and neostigmine may interfere with: (*a*) the enzymatic hydrolysis of the compound in the circulation and in the liver, (*b*) its enzymatic hydrolysis by the enzyme concentrated at the level of the motor end-plate, and (*c*) the metabolism of acetylcholine which acts as mediator in neuromuscular transmission. The relative importance of these various factors may be responsible for the different tracings obtained in these experiments.

The various factors enumerated above should be considered in advancing an explanation for suxamethonium apnœa observed clinically. Even if the suggestion be accepted that the prolonged apnœa encountered after its administration is caused by reduction of cholinesterase, which is supported to some extent by experimental evidence, it is clear that this cannot account for all the cases reported in the literature.

In the experiments which have been recorded, the duration of suxamethonium paralysis was increased up to 3 fold after anticholinesterases, even when repeated doses of neostigmine or eserine had been administered and muscarinic effects were observed.

Durrans[27] considered that thiopentone used to induce anæsthesia was responsible for prolonged apnœa, and Barron[28] and Beecher and Todd[29] advanced the view that depression of the respiratory centre, which could be relieved by the administration of nikethamide, was involved in the phenomenon.

Davis *et al.*[30] have shown that prolongation in the duration of suxamethonium paralysis in animals may be increased by ventilation with 20 per cent. carbon dioxide. Among the factors involved in suxamethonium apnœa Dripps[31] suggested central depression, alterations in the tissue concentrations of carbon dioxide and oxygen, potassium deficiency, and changes in the flow of blood to the muscle.

It has been shown by E.E.G. tracings both in man and in experimental animals that curare-like drugs have no action on the cortical electrical activity when administered, as usual, intravenously. Smith *et al.*[32] demonstrated that in man the normal blocking of α waves after external stimuli was still elicited, when $2\frac{1}{2}$ times the dose of tubocurarine required

to produce respiratory paralysis was given. Confirmation of these findings has been reported in the rabbit by Bovet and Longo[33], who showed that the blocking action was still present after the administration of 100 times the LD50 of suxamethonium. This illustrates the lack of action of natural and synthetic blocking agents on the central neuronal synapses which mediate conduction of stimuli from the periphery to the cortex. In the general review of Toman and Davis[34] it is stated that "if curare exerts a central action it is excitory and not depressant." Although the cholinesterase levels of patients are likely to influence the duration of prolonged apnœa as observed clinically, this would not appear to be the only factor involved.

Summary

1. The effects of the anticholinesterases eserine and neostigmine in modifying the neuromuscular block induced by suxamethonium have been investigated in the rabbit and dog. Eserine in the rabbit potentiated the action of suxamethonium both in the duration and intensity of the neuromuscular paralysis. This has been termed a "dosage effect".

2. Eserine, in the dog, diminished the intensity of the paralysis produced by suxamethonium. The duration of the muscular block was increased, however, and this type of action was termed a "time-effect".

3. Neostigmine in the rabbit increased the duration and intensity of the suxamethonium paralysis.

4. With neostigmine in the dog two effects were noted. Where neostigmine gave rise to a strong muscular action, an increase in the duration and intensity of the suxamethonium block was obtained. In the absence of a substantial muscular action by neostigmine an increase in the duration with a diminution in the intensity of the suxamethonium paralysis was observed.

5. The different types of tracings which were obtained experimentally have been discussed in relation to possible explanations of suxamethonium apnœa as observed clinically.

References

1. Bovet, Bovet-Nitti, Guarino, Longo and Marotta, *Rend., Ist. Super. San.*, 1949, **12**, 106.
2. Phillips, *J. Amer. chem. Soc.*, 1949, **71**, 3264.
3. Walker, *J. chem. Soc.*, 1950, 193.
4. Bovet-Nitti, *Rend., Ist. Super. San.*, 1949, **12**, 138.
5. Castillo and De Beer, *J. Pharmacol.*, 1950, **99**, 458.
6. Löw and Tammelin, *Acta physiol. scand.*, 1951, **23**, 78.
7. Marotta and Carminati, *Arch. Int. Pharmacodyn.*, 1953, **98**, 255.
8. Fraser, *Brit. J. Pharmacol.*, 1954, **9**, 429.
9. Bourne, Collier and Somers, *Lancet*, 1952, **262**, 1225.
10. Cowan, *Anæsthesia*, 1954, **9**, 23.
11. Harrison, Seward and Skinner, *Anæsthesia*, 1954, **9**, 21, 22.
12. Richards and Youngman, *Brit. med. J.*, 1952, **1**, 1334.
13. Walker, *Lancet*, 1954, **266**, 103.
14. Foldes, *Lancet*, 1952, **263**, 245.
15. Hall, Lehmann and Silk, *Brit. med. J.*, 1953, **1**, 134.
16. Bourne, *Brit. J. Anæsth.*, 1953, **25**, 116.
17. Wallemacq, *Acta Anaesth. Belg.*, 1953, **4**, 53.
18. Wolfers, *Brit. med. J.*, 1952, 2, 778.

19. Briscoe, *Lancet*, 1936, **230**, 469.
20. Riker and Wescoe, *J. Pharmacol.*, 1946, **88**, 58.
21. Zaimis, *J. Physiol.*, 1951, **112**, 176.
22. Jacobsohn and Kahlson, *Arch. f. Physiol.*, 1938, **79**, 27.
23. Glick, *J. biol. Chem.*, 1941, **137**, 357.
24. Bovet-Nitti, 1955, Personal communication.
25. Bovet, Bovet-Nitti, Guarino, Longo and Fusco, *Arch. Int. Pharmacodyn.*, 1951, **88**, 1.
26. Brand, *Experientia*, 1952, **8**, 273.
27. Durrans, *Lancet*, 1952, **263**, 539.
28. Barron, *Brit. med. J.*, 1952, **2**, 833.
29. Beecher and Todd, *Ann. Surg.*, 1954, **140**, 2.
30. Davis, Ellis, Reese and Grosskreutz., *Anæsthesiol*, 1955, **16**, 333.
31. Dripps, *Ann. Surg.*, 1953, **137**, 145.
32. Smith, Brown, Toman and Goodman, *Anæsthesiol.*, 1947, **8**, 1.
33. Bovet and Longo, *Electroencephalography and Clin. Neurophys.*, 1953, **5**, 225.
34. Toman and Davis, *Pharmacol. Rev.*, 1949, **1**, 425.

SYNTHETIC ANALOGUES OF ADRENAL CORTICAL HORMONES—DERIVATIVES OF FLUORENE AND INDANE

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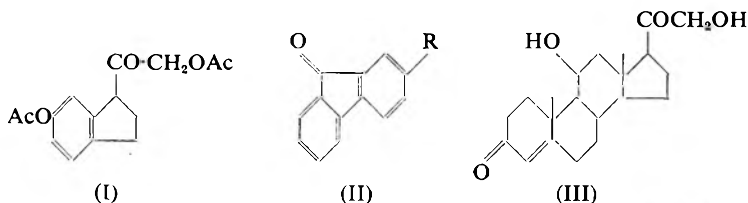
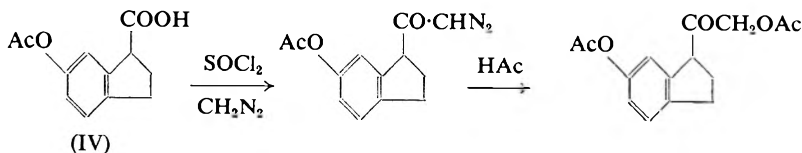
NUMEROUS synthetic analogues of adrenal cortical hormones have been reported in the literature, but few possess an oxygen atom at the equivalent of the 11-position of the steroid nucleus i.e. separated from the characteristic side chain -$COCH_2OH$ by four carbon-carbon bond lengths[1,2]. In these laboratories, aromatic and hydroaromatic analogues containing such an oxygen atom have been prepared. The present paper reports the preparation of 1-acetoxyacetyl-6-acetoxyindane (1), 2-acetoxyacetylfluoren-9-one (II) and 2-hydroxyacetylfluoren-9-one (IIa). The structural similarities of these compounds to corticosterone (III) are shown in the formulæ.



(II, R = $COCH_2OAc$; IIa, R = $COCH_2OH$; IIb, R = $COCH_3$; IIc, R = $COCH_2Br$)

The compounds were biologically tested in mice for adrenal cortical hormone activity by the cold-stress and glycogen deposition tests. Interest was centred on the latter test in view of the relationship of the compounds to the 11-oxygenated adrenal hormones which have a prepotent effect on carbohydrate metabolism. Alternatively any synthetic analogue may, by combination with vital receptors, have the biological action of antagonism to the natural hormone. In an attempt to obtain indications of any such antagonism, cold-stress tests were made in which the compounds (I) and (IIa) were administered together with cortisone acetate. An interesting example of this kind appears in the reported antagonism of a compound to the glyconeogenetic action of cortisone acetate[3]. The results of the biological tests are appended.

1-Acetoxyacetyl-6-acetoxyindane was prepared from 6-acetoxyindane-1-carboxylic acid (IV)[4], via the diazoketone as follows:—



The diazoketone procedure could not be used for the preparation of the analogue based on the fluorenone structure; ring expansion to derivatives of phenanthrene[5] occurs when fluorenone is treated with diazomethane. The following method was therefore adopted.

Oxidation of 2-acetylfluorene using sodium dichromate and acetic acid by the method of Ray and Rieveschl[6] gave fluoren-9-one-2-carboxylic acid together with a small amount of alkali-insoluble material which these workers had not identified. This material was evidently a product of partial oxidation of 2-acetylfluorene since when milder oxidation conditions were used, its yield was increased to 60 per cent. Moreover, on further oxidation, the compound gave fluorenone-2-carboxylic acid in good yield.

The constitution of the compound was established as 2-acetylfluoren-9-one (IIb) by the fact that it was identical with a specimen prepared from the acid chloride of fluorenone-2-carboxylic acid by the action of sodio-malonic ester followed by hydrolysis and decarboxylation.

Bromination of 2-acetylfluoren-9-one (IIb) gave the bromoacetyl compound (IIc) which, on treatment with potassium acetate was converted into the required α-ketol acetate (II). This was hydrolysed by the method of Mattox and Kendall[7] to give the free α-ketol (IIa). When oxidised, compounds (IIc) and (IIa) both gave fluorenone-2-carboxylic acid, thus showing that substitution by bromine had occurred, as expected, in the side chain and not in the fluorenone nucleus.

Examination of compounds (I), (II) and (IIa) for adrenal cortical activity. These tests were made by P. F. D'Arcy. (By courtesy of Prof. G. A. H. Buttle.)

Compounds (I) and (II) were tested for adrenal cortical activity by a mouse cold-stress method. Details of this test have been published elsewhere[8]. These compounds, when injected intramuscularly into groups of 10 adrenalectomized mice, failed to afford a significant protection against the effects of cold stress although they were administered over a wide range of doses extending to a toxic level.

Compounds (I) and (IIa) were administered intramuscularly in aqueous suspension together with cortisone acetate in doses of respectively 0·25 mg. and 1·0 mg. per mouse. The compounds showed slight activity in potentiating the activity of a range of doses of cortisone acetate: statistical examination however showed that this potentiation was not significant.

From the above results, it was concluded that under the conditions of the test, compounds (I), (II) and (IIa) had no significant adrenal cortical activity and compounds (I) and (IIa) no antagonism to cortisone acetate.

Compounds (I) and (II) were examined for adrenal cortical activity by the mouse liver glycogen deposition test of Venning, Kazmin and Bell[9]. Both compounds, when injected subcutaneously in aqueous suspension into groups of 9-10 adrenalectomized animals in doses of 0·25 mg. and 1·0 mg. failed to cause a significant deposition of liver glycogen.

EXPERIMENTAL

All m.pts. are uncorrected.

1-*Acetoxyacetyl*-6-*acetoxyindane*. 6-Acetoxyindane-1-carboxylic acid[4] (10 g.) was refluxed with pure thionyl chloride (20 ml.) and dry benzene (50 ml.). When hydrogen chloride was no longer evolved, the benzene and thionyl chloride were removed by heating in an oil bath under reduced pressure. The last traces of thionyl chloride were removed by adding dry benzene and again removing under reduced pressure. The residue was distilled at 0·5 mm. pressure with air bath heating in a distillation apparatus with low side arm, the flask being packed with glass wool. The acid chloride distilled as a pale yellow liquid. (7·5 g.). This gave a *p*-toluidine which crystallised as cream coloured needles from aqueous ethanol, m.pt. 196–197° C. Found: C, 73·5; H, 6·2; N, 4·6. $C_{19}H_{19}O_3N$ requires C, 73·7; H, 6·19; N, 4·53 per cent.

The acid chloride (7 g.) was dissolved in a little dry ether and the solution gradually added to an ethereal solution of diazomethane prepared by the standard method[10], from nitrosomethylurea (20 g.). The solution was set aside for three hours then the ether and diazomethane removed by distillation under reduced pressure at room temperature. The diazoketone crystallised as a yellow solid when the greater part of the liquid had been removed, and was separated by filtration. After washing with dry ether, 4·8 g. of crude diazoketone remained.

The crude diazoketone (1·5 g) was heated on a steam bath with glacial acetic acid (7·5 ml.) until nitrogen was no longer evolved. The mixture was poured into water and extracted with ether. The ethereal solution was washed well with water, then with cold 1 per cent. sodium bicarbonate solution, then again with water. The volume of the ethereal solution was adjusted to 100 ml., and after drying with anhydrous sodium sulphate the solution was passed through a column of activated charcoal (10 cm. × 1·5 cm.). A further 100 ml. of ether was then passed through the column; evaporation of the ether gave a colourless product (1·1 g.). This was crystallised from a mixture of benzene and petroleum ether (40–60°C.) to give 1-*acetoxyacetyl*-6-*acetoxyindane* as colourless prisms m.pt. 66·5–67·5°C. Found: C, 65·3; H, 5·9. $C_{15}H_{16}O_5$ requires C, 65·2; H, 5·84 per cent. The compound readily reduced warm Fehling's and Tollen's reagents.

2-*Acetylfluorene* was prepared by the method of Ray and Rieveschl[11]. The crude substance was found to be sufficiently pure for oxidation at the next stage.

Fluoren-9-one-2-carboxylic acid. Crude 2-acetylfluorene (45 g.) when oxidised by the method of Ray and Rieveschl[6] gave fluorene-9-one-2-carboxylic acid (26 g.) m.pt. 338–340° C. (decomp.) together with an alkali insoluble material (4 g.).

2-*Acetylfluoren*-9-*one*. (i) Fluoren-9-one-2-carboxylic acid (20 g.) was refluxed on a water bath for three hours with pure thionyl chloride (70 ml.) and dry benzene (250 ml.). The benzene and excess of thionyl chloride were removed by heating under reduced pressure, and the residue dissolved in dry benzene (250 ml.). The solution was gradually

added to a cooled suspension of sodio-malonic ester prepared by refluxing for 7 hours a mixture of powdered sodium (4·2 g.), diethyl malonate (43 g.), and dry ether (500 ml.). The mixture was stirred for 6 hours at room temperature, refluxed for 3 hours, cooled, diluted with water and then acidified with acetic acid. The ethereal layer was separated and ether removed by distillation. In order to decarboxylate the residue, it was heated under reflux for 4 hours with hydrochloric acid (250 ml.), water (100 ml.) and acetic acid (250 ml.). Water (1500 ml.) was then added and the crude 2-acetylfluoren-9-one removed by filtration. The filtrate was extracted with benzene and the solid dissolved in the benzene solution. This was washed with 5 per cent. potassium bicarbonate solution and then with water. After removal of the benzene, the residue was crystallised from ethanol-benzene mixture to give *2-acetylfluoren-9-one* (14·5 g.) as yellow needles m.pt. 162–163°C. Found: C, 81·6; H, 4·7; $C_{15}H_{10}O_2$ requires C, 81·1; H, 4·5 per cent.

The 2:4-*dinitrophenylhydrazone* prepared by the usual method was evidently a mixture of mono and bis derivatives. Several crystallisations from nitrobenzene-acetic acid mixture gave dark red needles m.pt. 300°C. (decomp.). Found: C, 63·2; H, 4·1; N, 14·2. $C_{21}H_{14}O_5N_4$ requires C, 62·7; H, 3·5; N, 13·9 per cent.

(ii) Sodium dichromate (250 g. coarsely powdered) was added in 5 g. portions over a period of 45 minutes to a stirred solution of 2-acetylfluorene (50 g.) in glacial acetic acid (700 ml.). Throughout the addition, the mixture was maintained at a constant temperature by a water bath at 50–55° C. The mixture was stirred at this temperature for a further 10 hours, then poured into hot water (3 litres).

The suspension was well shaken in a mechanical shaker, cooled, filtered and the residue washed with dilute sulphuric acid (2 per cent.). The product was stirred with 5 per cent. potassium hydroxide solution (700 ml.) and the suspension filtered. The residue was washed with water, dried and crystallised twice from ethanol then once from benzene to give *2-acetylfluoren-9-one* (34 g., 60 per cent.) m.pt. 162–163°C. undepressed on admixture with sample from route (i) Found: C, 81·2; H, 4·7. $C_{15}H_{10}O_2$ requires C, 81·1; H, 4·5 per cent.

2-Bromoacetylfluoren-9-one. 2-Acetylfluoren-9-one (11·1 g.) was dissolved in chloroform (100 ml.) in a $\frac{1}{2}$ litre three necked flask fitted with a dropping funnel condenser and mercury-seal stirrer, and illuminated by two 100-watt electric lamps. The mixture was heated to gentle reflux by means of a water bath, and two or three drops of a solution of hydrobromic acid in acetic acid (50 per cent.) added. The mixture was stirred and a solution of bromine in chloroform (0·5 molar 100 ml.) added drop by drop during 2 hours. After a further 3 hours, heating was discontinued but stirring and illumination maintained for a further 12 hours. The chloroform was then removed under reduced pressure and the residue twice crystallised from benzene to give *2-bromoacetylfluorenone* as pale yellow needles (13 g.) m.pt. 208–209°C. (decomp.) Found: C, 59·2; H, 3·0; Br, 26·9. $C_{15}H_9O_2Br$ requires C, 59·8; H, 3·0; Br 26·5 per cent.

2-Acetoxyacetylfluoren-9-one. The above bromoacetyl compound (5 g.) was dissolved in dry acetone (250 ml.) and freshly fused and powdered potassium acetate (10 g.) added. The mixture was gently refluxed for 6 hours and then set aside for two days and filtered, the residue being washed with a little dry acetone. The filtrate and washings were treated with activated charcoal, filtered and the acetone removed under reduced pressure. The residue (3·75 g.) was crystallised from dry benzene to give *2-acetoxyacetylfluoren-9-one* as yellow needles, m.pt. 164–165°C. Found: C, 73·2; H, 4·3. $C_{17}H_{12}O_4$ requires C, 73·0; H, 4·3 per cent. The product reduced warm Fehling's and Tollen's reagents.

2-Hydroxyacetylfluoren-9-one. 2-acetoxyacetylfluoren-9-one (0·7) g.) was dissolved in a mixture of chloroform (15 ml.) and methanol (25 ml.). Water, (1 ml.) was added, the solution cooled to 10° C., and hydrochloric acid, (2 ml.) slowly added so that the temperature did not rise above 20°C. Golden yellow needles immediately separated from the solution which was set aside at room temperature for 24 hours. A further portion (1 ml.) of hydrochloric acid was added. When crystals were no longer deposited, the mixture was filtered, and the product dried *in vacuo.* Crystallisation from benzene gave *2-hydroxyacetylfluoren-9-one* as yellow needles (0·55 g) m.pt. 174–175°C. Found: C, 75·2; H, 4·2; $C_{15}H_{10}O_3$ requires C, 75·6; H, 4·2 per cent. The compound readily reduced warm Fehling's and Tollen's reagents.

Oxidation experiments. 2-Bromoacetylfluoren-9-one and 2-acetoxyacetylfluoren-9-one were oxidised following procedures similar to that used in the oxidation of 2-acetylfluorene. The compounds gave fluoren-9-one-2-carboxylic acid m.pt. 340° C. in yields greater than 70 per cent.

SUMMARY

1. 1-Acetoxyacetyl-6-acetoxyindane (I), 2-acetoxyacetylfluoren-9-one (II), and 2-hydroxyacetylfluoren-9-one (IIa) have been prepared and tested for adrenal cortical activity.

2. Compounds (I) and (II) did not exhibit significant activity when tested by the mouse cold-stress or liver glycogen tests.

3. Compounds (I) and (IIa) showed slight activity in potentiating the action of cortisone acetate in the cold-stress test: this activity was not, however, significant.

REFERENCES

1. Ross, *J. chem. Soc.*, 1945, 538.
2. Walker, *J. chem. Soc.*, 1942, 347.
3. Adams, Cross, David, Hartley, Patel, Petrow and Stuart-Webb, *J. Pharm. Pharmacol.*, 1953, **5**, 861.
4. Askam and Linnell, *J. chem. Soc.*, 1954, 4691.
5. Schultz, Schultz and Cochran, *J. Amer. chem. Soc.*, 1940, **62**, 2902.
6. Ray and Rieveschl, *Organic Syntheses*, **28**, 63.
7. Mattox and Kendall, *J. biol. Chem.*, 1951, **188**, 293.
8. Buttle, D'Arcy and Howard, *J. Physiol.*, 1954, **123**, 5P.
9. Venning, Kazmin and Bell, *Endocrinol.*, 1946, **38**, 79.
10. Adams, "*Organic Reactions*" (Wiley 1942) **1**, 50.
11. Ray and Rieveschl., *Organic Syntheses*, **28**, 3.

ORTHO SUBSTITUTED BENZOIC ACID ESTER OF DIALKYL AMINOALKANOL IN EXPERIMENTAL CARDIAC ARRHYTHMIAS

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PROCAINE hydrochloride and tridiurecaine, which resemble each other in their chemical configuration, have been reported to exhibit antiarrhythmic activity.[1,2] Since the ortho substituted benzoic acid ester of dialkyl aminoalkanol (McN-A-29-11*) a recently synthesised local anæsthetic, is another chemically similar compound, it seemed worthwhile to determine if it also shares with procaine and tridiurecaine the ability to combat arrhythmias. Hence the investigations now reported.

As there are ostensibly endless variables when recourse is taken to only one biological test, it was, therefore, deemed fit to employ a variety of experimental techniques in order to increase confidence in the results. Preliminary studies were made on the refractory period of isolated rabbit auricles; this was followed by tests on acetylcholine-induced auricular fibrillation, auricular flutter produced by injury-stimulation procedure, aconitine-evoked auricular fibrillation and hydrocarbon-adrenaline induced ventricular arrhythmias in dogs. In addition, a comparison was made of the effects of McN-A-29-11 and quinidine on the electrocardiogram of cats.

The ortho substituted benzoic acid ester of dialkylamino alkanol is a white, crystalline, odourless and stable substance with a bitter taste. It is freely soluble in water. The aqueous solution was used throughout. The action of this drug was compared with that of quinidine sulphate.

METHODS

I. *Isolated rabbit auricles.* This method, developed by Dawes[3], is based on the observation that quinidine-like drugs prolong the refractory period of isolated rabbit auricles. After washing the chambers of a rabbit heart free of blood, the auricles were isolated, care being taken not to injure the tissues near the sinus node. The preparation was then quickly transferred to an organ bath containing oxygenated Locke's solution at 29°C. The electrode arrangement used was the same as that described by Dawes. After the auricles had been immersed in the bath for 30 minutes, they were stimulated each time for 15 seconds by break shocks of increasing frequency until a point reached where they would no longer respond to every stimulus applied. This was recorded as 'maximal response rate' which is considered to be reciprocal of the refractory period. The drug being tested was then added to the bath. After 10 minutes the maximal response rate was redetermined and reduction in maximum frequency was noted.

* Code name of McNeil Laboratories, Philadelphia for the drug.

II. *Acetylcholine-induced auricular fibrillation.* This procedure for producing auricular fibrillation in dogs is based on the work of Scherf and Chick[4] and followed in all essential details the technique described by Schallek[5].

III. *Auricular flutter.* Experiments were performed on adult mongrel dogs of both sexes weighing between 12 and 16 kg. The animals were anæsthetised with morphine sulphate (10 mg/kg.) subcutaneously followed in half an hour by sodium pentobarbitone intravenously 30mg./kg. Blood pressure was recorded from carotid artery by a mercury manometer. Under artificial respiration, parts of the sternum and ribs directly over the heart were removed. To permit free access to the right side of the auricle, the animal was turned so that the heart fell towards the left side. The split pericardium was sutured over the chest walls.

Auricular flutter was initiated in all experiments by the injury-stimulation procedure described by Rosenblueth and Garcia Ramos.[6,7,8] A narrow band of auricular tissue connecting the superior and inferior vena cava was crushed by means of hæmostats and stable flutter was produced by subsequent stimulation of the auricle with square waves (duration, one millisecond, volts 15 to 20, and frequency 15 to 20 per second). The average auricular and ventricular rates during flutter were 461 and 242 respectively. The drugs were injected after the flutter had continued for at least 35 minutes. Spontaneous reversions of an arrhythmia of this duration were rare.

IV. *Aconitine-induced auricular fibrillation.* Auricular fibrillation was produced in dogs by employing Scherf's[9] method. In exactly the same manner as described above, chest and pericardium were opened under pentobarbitone anæsthesia. A cotton pledget, soaked in 0·05 per cent. solution of aconitine nitrate, was placed on the auricle. Within four minutes, persistent auricular fibrillation was produced. The intravenous administration of the drug was continued until 1 : 1 rhythm with the rate below 200 beats per minute (end-point) was reached.

In addition to the bipolar lead II, electrograms directly from the auricle and ventricle were recorded by a 3 channel Grass inkwriting oscillograph in procedures III and IV. Also, the dosage scheme used herein was the "titration procedure" as employed by Winbury and Hemmer[10], that is, 1 mg./kg. of the drug was intravenously injected every minute until reversion to normal sinus rhythm occurred in flutter or 'end-point' was reached in fibrillation.

V. *Hydrocarbon-adrenaline induced ventricular arrhythmias.* This method was described by Riker and Wescoe[11]. In our experiments, it consisted in anæsthetising dogs with 30 mg./kg. of sodium pentobarbitone intravenously and intratracheal administration of 0·1 ml./kg. of light petroleum, a mixture of lower aliphatic hydrocarbons, followed by 60 μg./kg. of adrenaline intravenously via the cannulated femoral vein. This would invariably result in the production of ventricular arrhythmias of types ranging from multifocal ectopic beats to ventricular fibrillation. When the protective action of the drug under trial was to be tested, it

was injected intravenously 1½ minutes before the administration of light petroleum.

VI. *Electrocardiogram.* Cats of both sexes weighing between 2·5 and 4 kg. were anæsthetised with a allobarbitone-urethane solution* 0·6 ml./kg. intraperitoneally. The electrocardiogram was recorded with a Grass inkwriting oscillograph by employing bipolar conventional lead II. The drug to be tested was injected intravenously and an electrocardiographic tracing was taken for the first two minutes and then at 5 minutes' intervals. Changes in refractory period and conduction time were measured from Q–T and P–Q intervals respectively. Only one drug was tried in one animal.

TABLE I

EFFECT OF DRUGS ON CARDIAC ARRHYTHMIAS

No. of animals	Drug	Dose	Result	
Isolated rabbit auricles				
			Average percent. reduction in max. frequency	
4	Quinidine	2·5 × 10^{-6} mg./ml.	13	± 1·22***
4	McN-A-29-11	2·5 × 10^{-6} mg./ml.	11	± 0·05
Acetylcholine-induced auricular fibrillation in dog				
			Average percent. reduction in duration of fibrillation	
6	Quinidine	2·5 mg./kg.	52	± 2·83
6	McN-A-29-11	2·5 mg./kg.	61	± 2·68
Aconitine-induced auricular fibrillation in dogs				
6	Quinidine	16 mg./kg.	End-point*	± 2·65
6	McN-A-29-11	11·8 mg./kg.	,, ,,	± 1·32
Auricular flutter in dogs				
6	Quinidine	21 mg./kg.	Reversion**	± 2·16
6	McN-A-29-11	8 mg./kg.	,,	± 1·29
Hydrocarbon-adrenaline-induced ventricular arrhythmias in dogs				
6	Quinidine	5 mg./kg.	+	± 0·78
6	McN-A-29-11	15 mg./kg.	+	± 1·41

* End-point is the establishment of 1:1 rhythm with the rate below 200 beats per minute in aconitine-induced auricular fibrillation.
** Reversion means restoration to normal sinus rhythm.
\+ Stands for complete protection against ventricular arrhythmias.
*** Standard deviation.

RESULTS

Preliminary experiments on the isolated rabbit auricles indicated that here McN–A–29–11 had an activity equivalent to that of quinidine. More extensive comparisons were then made in whole animals, results of which are summarised in Table I. In acetylcholine-induced auricular fibrillation, 2·5 mg./kg. of McN–A–29–11, like quinidine, brought about a significant reduction in the duration of fibrillation. Both of these compounds were successful in the treatment of auricular flutter (Fig. 1)

* An aqueous solution which contains, per ml., 0·1 g. allobarbitone, 0·4 g. urethane, and 0·4 g. monoethylurea, kindly supplied by Ciba Pharma Ltd., Bombay.

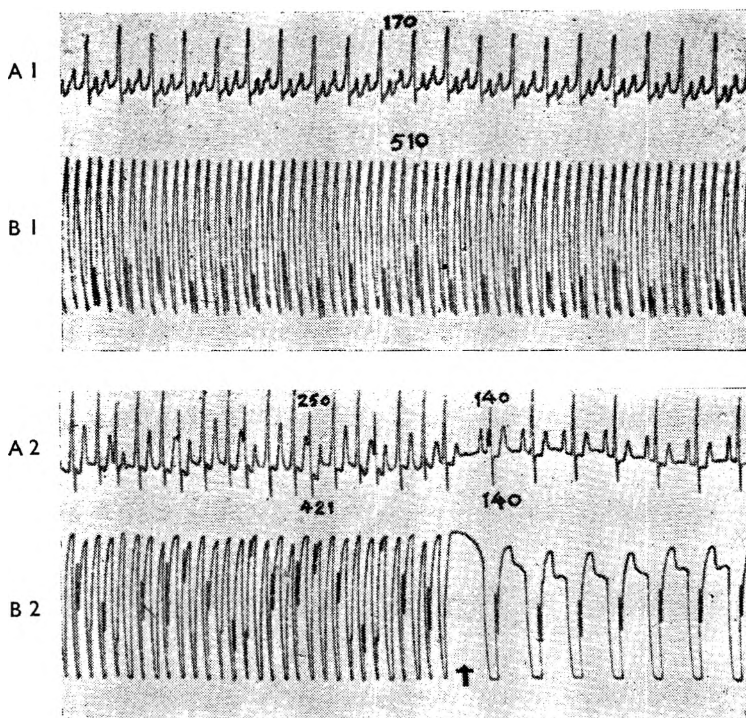

FIG. 1. McN-A-29-11 in auricular flutter.

A 1, B 1 show the auricular flutter with 3 : 1 auricularventricular block.
A 2, B 2 show an excerpt from continuous electrocardiographic record illustrating the abrupt reversion of auricular flutter to normal sinus rhythm at the arrow after McN–A–29–11 for 8 minutes.

A. Electrocardiogram lead II.

B. Electrogram directly from the auricle.

and aconitine-induced auricular fibrillation in all the cases but comparatively lower doses were required with McN–A–29–11 than with quinidine for bringing about restoration to normal sinus rhythm or 'end-point' (Fig. 2). In its ability to avert ventricular arrhythmias produced by light petroleum and adrenaline, McN–A–29–11 was, however, less effective than quinidine.

The effects on P–Q and Q–T intervals of cats (Table II) indicated that McN–A–29–11 shares with quinidine the property of prolonging conduction time and refractory period, but to a lesser degree.

TABLE II

EFFECTS OF DRUGS ON ELECTROCARDIOGRAM OF CAT.

AVERAGE PERCENTAGE CHANGE

No. of expts.	Drug	Dose mg./kg.	Average percent increase in P–Q interval.	Average percent increase in Q–T interval.
6	Quinidine	10	46	22
6	McN–A–29–11	10	26·7	14·4

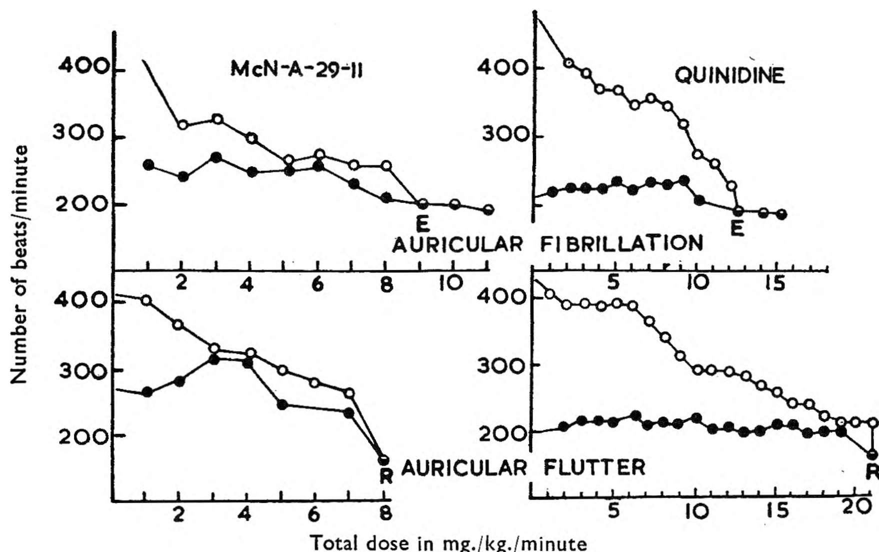

FIG. 2. The effect of McN-A-29-11 and quinidine on aconitine-induced auricular fibrillation and auricular flutter induced by injury-stimulation procedure. Each graph represents results from a different animal. —○— auricle, —●— ventricle. E. End-point. R. Reversion.

DISCUSSION

In tests on the isolated rabbit auricles, McN–A–29–11 exhibited an activity which was equivalent to that of quinidine, but as measured from the Q–T interval of the electrocardiogram of cats, quinidine caused a greater increase in refractory period. As a matter of fact there is no longer any need to cavil over the terms absolute, relative or effective refractory period because there is no real proof that a prolongation of refractory period is responsible for the antiarrhythmic activity of a drug[12]. It, therefore, seemed expedient to carry out further experimental studies to determine whether McN–A–29–11 was more effective than quinidine. Also, our present knowledge regarding the underlying mechanism causing these arrhythmias is limited despite the work of many investigators[13]. The three theories, most compatible with known facts, that have been postulated to explain the patho-physiologic disturbances responsible for these arrhythmias, are: (1) the classical circus movement theory[14], (2) the multiple ectopic focus theory[15] and (3) the single ectopic focus theory[16].

Accordingly, in the present study, in order to ensure a more correct interpretation of the results, use was made of selected experimental procedures which, as cited by Dick and McCawley[17], are representative of each theory: auricular flutter produced by injury-stimulation procedure (Circus wave); acetylcholine-induced auricular fibrillation (multiple focus) and aconitine-induced auricular fibrillation (single focus). The results obtained in these experiments indicate that McN–A–29–11 is stronger in its activity than quinidine in auricular arrhythmias. It is, however, weaker than quinidine in averting ventricular arrhythmias.

Electrocardiographic changes caused by the two agents include a greater prolongation of conduction time by quinidine. Slowing of conduction rate is the most deleterious property of quinidine because in the presence of conduction defects, it may precipitate ventricular tachycardia and ominous ventricular fibrillation.[18] It is significant to note here that McN–A–29–11 shares with quinidine the propensity to slow conduction, but to a lesser extent.

The intraperitoneal LD50 in mice of McN–A–29–11 was 180 mg./kg.[19] while that of quinidine was found to be 135 mg./kg.[3] Keeping in mind this reduced toxicity and the greater efficacy of McN–A–29–11 than quinidine in aconitine-induced auricular fibrillation and auricular flutter, it seems that McN–A–29–11 shows sufficient promise to warrant clinical trials.

SUMMARY

1) McN–A–29–11 was shown to exhibit an activity stronger than quinidine in experimental auricular flutter and aconitine-evoked auricular fibrillation, but was equivalent to it in its effects on the refractory period of isolated rabbit auricles and acetylcholine-induced auricular fibrillation in dogs.

2) It was, however, found less effective than quinidine in averting ventricular arrhythmias produced by hydrocarbon-adrenaline in dogs.

3) Electrocardiographic changes in cats produced by McN–A–29–11 and quinidine included greater lengthening of both the refractory period and conduction time by the latter drug.

Grateful acknowledgement is made to Dr. C. F. Kade of McNeil Laboratory, Philadelphia for the generous supply of McN–A–29–11.

REFERENCES

1. Burstein, *Anæsthesiology*, 1946, **7**, 113.
2. Arora, Sharma and Madan, *Ind. J. med. Res.*, 1956, **44**, 271.
3. Dawes, *Brit. J. Pharmacol.*, 1946, **1**, 90.
4. Scherf and Chick, *Circulation*, 1951, **3**, 764.
5. Schallek, *J. Pharmacol.*, 1952, **105**, 29.
6. Rosenblueth and Garcia, *Amer. Heart. J.*, 1947, **33**, 677.
7. Rosenblueth and Garcia, *J. Arch. Inst. Cardiol., Mexico*, 1947, **17**, 1.
8. Rosenblueth and Garcia, *ibid.*, 1947, **17**, 441.
9. Scherf, *Proc. Soc. exp. Biol. N.Y.*, 1947, **64**, 233.
10. Winbury and Hemmer, *J. Pharmacol.*, 1955, **113**, 402.
11. Riker and Wescoe, *Ann. N.Y. Acad. Sci.*, 1951, **54**, 297.
12. Van Dongen, *Arch. int. Pharmacodyn.*, 1936, **53**, 80.
13. Dawes, *Pharmacol. Rev.*, 1952, **4**, 43.
14. Lewis, Feil and Stroud, *Heart*, 1918, **7**, 191.
15. Englemann, *Arch. ges. Physiol.*, 1895, **62**, 543.
16. Rotherberger and Winterberg, *Wienklin, Wschr.*, 1909, **22**, 836.
17. Dick and McCawley, *Amer. Heart J.*, 1955, **50**, 442.
18. Dipalma and Schultz, *Medicine*, 1950, **29**, 123.
19. Kade, Personal communication, 1955.

THE ASSAY OF CHLORAMPHENICOL IN PHARMACEUTICAL PREPARATIONS BY MEANS OF A SIMPLE COUNTER CURRENT TECHNIQUE*

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Chloramphenicol in a pure form can be satisfactorily characterized by chemical and physical examination. In pharmaceutical preparations it is generally considered that microbiological assay is the only possible method. That there are possibilities of eliminating the microbiological assay for the careful control of some of the usual chloramphenicol preparations is here shown.

In the monographs in the B.P. and Ph.I. the substance is assayed by the absorption of light at 278 mμ and identified by tests for organically bound chlorine, reaction of the nitro group, the melting point and optical activity. In the regulations published by the Food and Drug Administration, Washington, D.C., a microbiological assay is given as an alternative method. The U.S.P. XIV referred to these regulations, thus accepting both assays, but the U.S.P. XV has adopted only the microbiological method.

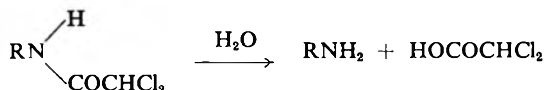
Quantitative determinations of chloramphenicol in preparations are included in the B.P. (capsules) and in the U.S.A. Federal Register (capsules, ointment, ophthalmic, injection, otic and tablets). The B.P. method for capsules is a gravimetric one after extraction with ether. The Federal Register uses the microbiological method, after extraction with water, for all preparations but gives as an alternative for capsules, eye drops and tablets the spectrophotometric determination of an aqueous extract. No test for decomposed chloramphenicol is included in any of these regulations.

Other quantitative non-official methods for the assay of chloramphenicol as a substance or as an ingredient in preparations have been published. They make use of those chemical properties of the chloramphenicol molecule which make possible the determination without isolation. The reduction of the nitro group is the principle of a polarographic determination[1]. After the quantitative reduction of the nitro group the amino group formed is titrated[2] or diazotised for final photometric determination[3]. The dichloroacetic acid part of the molecule is determined by titrimetric[4] and photometric methods[5]. The antibiotic may be quantitatively oxidized with periodic acid after hydrolysis[6], or the yellow colour occurring when it is heated with alkali may be used for the photometric determination in solutions[7].

* The main results of this paper were reported by H. Hellberg at the meeting of the Directors of Control Laboratories at the 16th General Assembly of the F.I.P. in London, in September, 1955.

The isolation from fermentation broths is described by Bartz[8], who also reports some distribution ratios between water and organic solvents, and the estimation in biological materials is described by Glazko *et al.*[3]

Chloramphenicol is rather easily hydrolysed mainly according to the reaction



with dichloroacetic acid and 1-*p*-nitrophenyl-2-amino-1 : 3-propandiol as the products[9]. This primary amine is microbiologically almost inactive[10] but is easily included in a chemical assay. This seems to be the reason for the discrepancy between chemical and microbiological assays on some chloramphenicol preparations reported by Dony[11]. The ultra-violet absorption at 278 mμ is so little changed that decomposition would not be noticed without a wavelength control of the maximum. The total hydrolysis changes the maximum only 7 mμ, from 278 mμ to about 271 mμ[10]. As the nitro group is left unchanged by the hydrolysis, determinations using that group may also be unreliable.

The method of separating chloramphenicol from its degradation products in biological materials has been published by Glazko *et al.*[3] and others.

To be able to determine chloramphenicol unambiguously and to distinguish it from, for example, the optical isomers, a chemical method must start with an isolation followed by determination of melting point and optical activity since the isomers differ in these properties[12]. This approach may be used for many preparations because of the solubility of chloramphenicol. If in addition hydrolysis is suspected the isolation must include a separation from the decomposition products.

This report will show that it is possible to use a chemical assay to a greater extent than existing regulations permit.

EXPERIMENTAL

Sometimes simple extractions, such as described in the B.P. for the assay of chloramphenicol in capsules, may be sufficient. Otherwise by applying simple counter current extractions systematically in the way earlier described by Brunzell and Hellberg[13] it is possible to isolate and characterize the drug from mixtures having the most diverse constituents. This technique requires knowledge of the distribution ratios, as these are used in the calculation of the separation procedure. For this purpose the ratios in Table I were determined.

The solubility in water is low but sufficient, and the distribution ratios are very different, some much larger than unity, some much smaller. Thus it is possible to arrange suitable separation procedures. Moreover the distributions are as a rule not much influenced by the pH of the aqueous phase, a fact that makes the planning easier. However, Bartz[7] shows some variations for the systems with benzene and carbon tetrachloride at the pH values 2·15, 6·50 and 9·00.

When trying to obtain the free amine together with chloramphenicol a noticeable decomposition of the amine seems to occur, thus colouring the substance yellow to brown.

The partition properties of the primary amine are quite different, however, see Table II. On distributing material between equal volumes of ethyl acetate and water at pH 7 more than 90 per cent. of the amine will go into the water phase but only 3 per cent. of the chloramphenicol.

TABLE I

DISTRIBUTION RATIOS FOR CHLORAMPHENICOL

Water/ethyl acetate	0·03
Water/ether	0·24
Water/chloroform	4·5
Water/benzene	28
Water/light petroleum	30
0·1N HCl/trichloroethylene ..	100

TABLE II

DISTRIBUTION RATIOS FOR THE 1-*p*-NITROPHENYL-2-AMINO-1 : 3-PROPANDIOL SET FREE BY HYDROLYSIS

Buffer pH 6·0/ethyl acetate ..	100
Buffer pH 7·0/ethyl acetate ..	12
Buffer pH 8·0/ethyl acetate ..	2·1

At lower pH values the difference is greater. These facts have been used for estimating the amount of chloramphenicol hydrolysed in the preparations. The usually small quantities of amine are determined spectrophotometrically. E (1 per cent. 1 cm.) at 271 mμ: 397 for the hydrochloride, melting point 209–210°C. (Kofler-block). Chloramphenicol is stable enough to endure rather high acidity during the time necessary for the required separation steps[14], and it is stable on evaporating the solution and drying the residue at 100°C.

Some examples of separation procedures used in different preparations are presented in Table III.

Capsules. By using the water/ethyl acetate system the separation can be made with smaller volumes than with water/ether. Distribution ratios: dihydrostreptomycin sulphate 13; lactose > 100.

Powder for eye drops. The separation of chloramphenicol from methyl *p*-hydroxybenzoate using, for example, ethyl acetate and 0·01N sodium hydroxide, was avoided, as hydrolysis will occur. Controls were found to give low results, and a considerable amount of amine formed by hydrolysis was detected. Distribution ratios of methyl *p*-hydroxybenzoate in the system 0·1N hydrochloric acid/trichloroethylene: 0·8; boric acid in the system 0·1N hydrochloric acid/trichloroethylene: > 100 and in 0·1N hydrochloric acid/ether: 100.

Injection. Distribution ratio of dimethyl acetamide in water/ether: 54.

Ear drops. All amylocaine hydrochloride was recovered in the water phase in the system 10 ml. 0·1N hydrochloric acid/30 ml. ethyl acetate. Distribution ratio for the system buffer pH 7·0/ethyl acetate: 0·14. Loss of amylocaine base occurred on simple evaporation of the ethyl acetate solution. The distribution ratio of propylene glycol in the system water/ether: > 100.

Ointment and Suppositories. The second portion of benzene is used for "washing" purposes. The isolated substance tends to have a low melting point that cannot be avoided by using additional portions of benzene.

According to my experience it is necessary to estimate the amount of hydrolysis in chloramphenicol preparations containing water. Assays performed in order to verify the applicability of the methods to mixtures corresponding to actual preparations are reported in Table IV.

TABLE III

SEPARATION PROCEDURES FOR CHLORAMPHENICOL PREPARATIONS

Preparation	Other constituents	Separation procedure*	Comments
Capsules (2·5 g.) Chloramphenicol content *ca.* 0·75 g.	Dihydrostreptomycin sulphate Lactose	Water 25 ml./ethyl acetate 20 ml.; 3 × 3	
Powder for eye drops (0·27 g.) Chloramphenicol content *ca.* 0·050 g.	Borax Boric acid Methyl *p*-hydroxybenzoate	(1) Trichloroethylene 50 ml./0·1N HCl 5 ml; 4 × 4; Pooled HCl-phases. (2) 0·1N HCl 20 ml./ether 80 ml.; 4 × 4	
Injection (0·5 g. solution) Chloramphenicol content *ca.* 0·11 g.	Dimethyl acetamide Water	0·1N HCl 10 ml./ether 35 ml.; 3 × 2	Free amine spectrophotom. in acid phase
Ear drops (1 ml. solution) Chloramphenicol content *ca.* 0·01 g.	Propylene glycol Glycerol Water Amylocaine HCl	(1) 0·1N HCl 10 ml./ethyl acetate 30 ml.; 3 × 2; Pooled HCl-phases buffered to pH 7 and diluted to 40 ml. (2) Buffer pH 7 40 ml./ethyl acetate 50 ml.; 4 × 4	HCl: free amine + amylocaine. Ethyl acetate: Chloramphenicol. Buffer: free amine. Ethyl acetate: amylocaine.
Ointment (7 g.) Chloramphenicol content *ca.* 0·07 g.	Soft paraffin	Water 25 ml./benzene 25 ml.; 4 × 2	
Suppositories (1 g. mass) Chloramphenicol content *ca.* 0·25 g.	Cocoa butter	Water 25 ml./benzene 25 ml.; 4 × 2	

* X × Y denote extraction with X portions of the mobile phase and Y portions of the stationary phase (in Y separating funnels).

TABLE IV

RESULTS FROM VERIFICATION EXPERIMENTS ON MIXTURES CORRESPONDING TO ACTUAL PREPARATIONS

Preparation	Constituent determined	Found	Added	Properties: M.pt. °C.	Properties: Specific Rotation
Powder for eye drops	Chloramphenicol (gravimetric)	16·4 per cent.	16·34 per cent.	146–149	+ 18·9°
Injection	Chloramphenicol (gravimetric)	0·750 g. 0·752 g.	0·753 g. 0·754 g.	147·5–149	+ 19·4°
Ear drops	Chloramphenicol Free amine Amylocaine HCl (all spectrophotometric)	1·08 mg. 0·96 mg. 9·7 mg.	1·08 mg. 0·94 mg. 9·7 mg.		
Ointment	Chloramphenicol (spectrophotometric)	1·00 per cent. 1·00 per cent.	1·00 per cent. 1·00 per cent.	145·5–148	
	Chloramphenicol (gravimetric)	1·13 per cent.	1·12 per cent.	144–149	+ 18·0°
Suppositories	Chloramphenicol (gravimetric)	23·4 per cent.	23·1 per cent.	147·5–149·5	+ 18·7°

ASSAY OF CHLORAMPHENICOL

In the amendments of the U.S.A. Federal Register concerning chloramphenicol ointment dated September 1, 1955, the assay is a microbiological method. Two methods are given for the preparation of the sample: an emulsion technique and an extraction method.

The extraction recommended is: "Place a representative sample (0·5 gm.) in a separatory funnel containing 10 ml. of peroxide-free ether. Shake the separatory funnel vigorously to bring about complete mixing of the ointment and ether. Shake with a 15-ml. portion of 1 % phosphate buffer pH 6·0. Remove the buffer layer and repeat the extraction with two additional 15-ml. portions of buffer. Combine the extractives and dilute to 50 ml. with 1 % phosphate buffer. Make the proper estimated dilutions in 1 % phosphate buffer at pH 6·0".

From the figures in Table I it is easy to calculate that the extraction must give very low results. As the two solvents are not equilibrated with each other beforehand there will be a change in volumes. Taking this into consideration the calculated result of the extraction is 72 per cent. of the added chloramphenicol. In a practical control 73·5 per cent. was recovered in the pooled aqueous extractives.

SUMMARY

1. The possibilities of a chemical assay of choramphenicol in pharmaceutical preparations is discussed.

2. Analyses by a simple counter current technique for many different preparations are described.

3. Results from known mixtures corresponding to actual preparations verify the procedures.

4. Estimation of the degree of hydrolysis in chloramphenicol preparations containing water is recommended.

5. The extraction method for chloramphenicol in ointments recommended by the Food and Drug Administration in the U.S.A. Federal Register is criticised since it gives low results.

I wish to acknowledge my indebtedness to Professor Hans Hellberg, the Head of this Department, for advice and discussions.

REFERENCES

1. Hess, *Analyt. Chem.*, 1950, **22**, 649.
2. Garcia Madrid, *Colegio farm.*, 1952, **9**, 1.
3. Glazko, Wolf and Dill, *Arch. Biochem.*, 1949, **23**, 411.
4. Gurmendi and Ungueta, *Bol. soc. quim., Peru*, 1952, **18**, 6.
5. Truhaut, *Ann. pharm. franc.*, 1951, **9**, 347.
6. Puga, *Rev. Farm.*, 1951, **93**, 290, through *Chem. Abstr.*, 1952, **46**, 5260 h.
7. Döll, *Arzneimit.-Forsch.*, 1955, **5**, 97.
8. Bartz, *J. biol. Chem.*, 1948, **172**, 445.
9. Higuchi, Marcus and Bias, *J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 129.
10. Rebstock, Crooks, Controulis and Bartz, *J. Amer. chem. Soc.*, 1949, **71**, 2458.
11. Dony, *Une Experience Belge en Matière de Contrôle Systematique des Antibiotiques*. A report at the meeting of the Directors of Control Laboratories at the 16th General Assembly of the F.I.P. in London, 1955.
12. Controulis, Rebstock and Crooks, *J. Amer. chem. Soc.*, 1949, **71**, 2463.
13. Brunzell and Hellberg, *Ann. pharm. franç.*, 1954, **12**, 296.
14. Lennert-Petersen, *Farm. Tidende*, 1954, **64**, 66.

ANATOMICAL STUDIES IN THE GENUS *RUBUS*

PART I. THE ANATOMY OF THE LEAF OF *Rubus idæus* L.

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THE gross morphology of the leaves of the raspberry plant, *Rubus idæus* L., Family Rosaceæ, has been described in several standard botanical works[1-3] and in certain textbooks of pharmacognosy[4-6]. These descriptions provide sufficient information for the identification of the entire drug. Much of the material now supplied commercially is, however, in the chopped condition or in very coarse powder. At present, only relatively meagre information is available concerning the microscopical structure of the leaflets; a brief and partially illustrated account is given by Brandt and Wasicky in Thoms' Handbuch[5], and by Jane Béguin[7]; these are summarised in the monograph upon Raspberry Leaf in the British Pharmaceutical Codex, 1949[8]. These works provide no description or illustrations of the rachis and stipules. Moreover, since recent work[9-11] has shown that the leaf possesses pharmacological activity, it seemed desirable to present a detailed and illustrated anatomical description of the leaf, in order to show those characters by which the genuine drug can be recognised and distinguished from the leaves of related plants, such as the blackberry and loganberry.

MATERIAL

The material used throughout this present work consisted of the leaves obtained from stands of *Rubus idæus* growing wild in woodlands of Boxhill, Surrey. Further material, propagated vegetatively from the Boxhill wild plants, was obtained from the Museum Experimental Gardens at Mayfield, near Ashbourne, Derbyshire. All plants used possessed the characters typical of the species.

METHODS OF INVESTIGATION

The structure of the two epidermises was difficult to observe in preparations made by heating pieces of the lamina in chloral hydrate solution. This was due firstly to the dark colour of the leaflets and secondly to the dense tomentum of trichomes on the lower epidermis. Portions of the leaflets were bleached by macerating them overnight in Dakin's Solution (Surgical Solution of Chlorinated Soda, B.P.), after first shaving off the trichomes of the lower epidermis, and then mounted in 50 per cent. v/v glycerol. The walls of the epidermal cells were more clearly defined when mounted in glycerol than in chloral hydrate solution. The glandular trichomes, however, were not properly expanded or cleared by the dilute glycerol and for their examination a chloral mount was necessary.

Three methods of preparation of the material for sectioning were

investigated. The first, paraffin embedding as modified by Johansen[12], was considered unsatisfactory, because of shrinkage and distortion of cells. The bulk of the sections prepared for the purpose of this investigation were made by a second method, using polyethylene glycol. This method has been described in a separate communication by ourselves[13], and is satisfactory for sections of 2 μ in thickness and upwards. It is also superior in that virtually no shrinkage occurs in the cells during preparation[13-15]. The third method, carbon dioxide freezing, was also found satisfactory for sections of 8 μ and over in thickness. Thinner sections usually broke and it became almost impossible to remove them intact from the microtome knife. In the carbon dioxide freezing method very small oblong pieces were cut from the lamina, one-third of the way along the midrib from the base, the midrib lying in the median line. These pieces were first soaked in water for four hours, to remove the fixing fluid. The Reichert sledge microtome with freezing stage, freezing cap and knife cooler was employed. The microtome knife was of wedge section and used in the position at right-angles to the carriage-slide. A square of blotting paper of 6 mm. edge was placed in the centre of the freezing stage, and a single, large drop of Mucilage of Acacia, B.P., was placed on top of the blotting paper; the water-soaked piece of lamina was held vertically in the drop of mucilage by means of forceps, and carbon dioxide was admitted in spurts until the whole was frozen satisfactorily. By adjusting the orientation of the material on the freezing stage, both transverse and longitudinal sections were cut as required and both temporary and permanent mounts were prepared. Temporary preparations were conveniently made by mounting at once in 50 per cent. v/v glycerol, avoiding the use of chloral hydrate solution, which led to disintegration of the phloem and other delicate tissues.

Permanent mounts were made by transferring the sections, immediately after cutting, to a dish containing saturated crystal violet solution; after immersion in this stain for about 25 minutes they were passed successively through 25, 50, 75 and 90 per cent. ethanol, 2 minutes being allowed in each, and then counterstained in a solution of Bismarck brown (1 per cent. in 95 per cent. ethanol) for 3 minutes. Final dehydration was effected in absolute ethanol, followed by clearing in clove oil; the latter was removed by xylene, and the sections were mounted in Canada balsam. Safranin and fast green, also safranin and light green were unsuitable for making permanently mounted, double-stained preparations of this material, as it proved impossible to differentiate the safranin.

Sections made by the freezing process were also mounted directly in 50 per cent. v/v glycerol and, like those made by the use of polyethylene glycol, showed no distortion of the cells. The permanent double-stained mounts prepared as described above showed very little shrinkage of the cells, due presumably to the much reduced exposure to ethanol compared with that required by paraffin embedding. Sections made by polyethylene glycol embedding were usually stained with hæmatoxylin or other simple stains to increase the contrast[13], thus enabling the drawings to be made more easily by means of an Abbé camera lucida, or by projection.

Macerates of prickles and rachis were prepared by using Schultz's maceration fluid.

ANATOMICAL STRUCTURE

The leaf of *Rubus idæus* is imparipinnately compound, and may consist of three to seven leaflets, the paired lateral leaflets being sessile on the

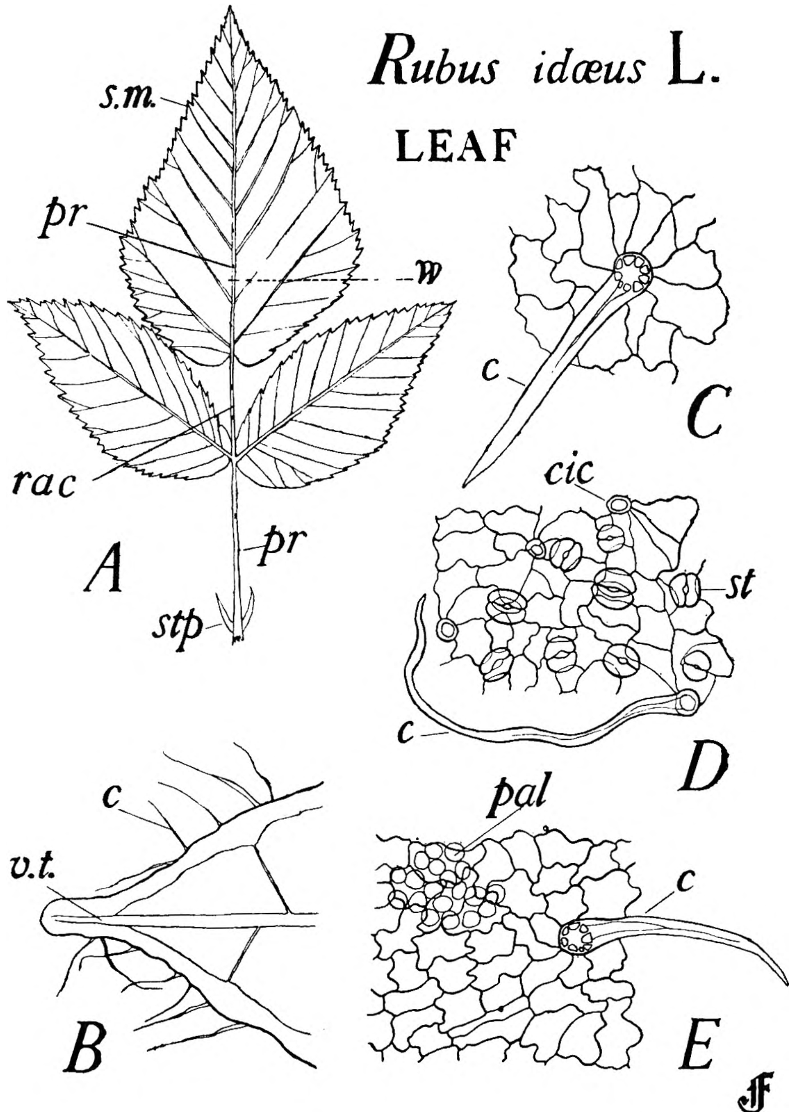

FIG. 1. Leaf of *Rubus idæus* L. *A*, complete compound leaf with terminal and two lateral leaflets and paired stipules adnate to the rachis. *B*, marginal tooth of leaflet. *C*, upper epidermis of leaflet with stiff, covering trichome. *D*, lower epidermis of leaflet. *E*, upper epidermis of leaflet. *A*, × ⅔; *B*, × 25; *C*, *D* and *E*, × 200. *c*, covering trichome; *cic*, cicatrix; *pal*, palisade; *pr*, prickle; *rac*, rachis; *s.m.* serrate margin; *st*, stoma; *stp*, stipule; *v.t.*, vein termination; *w*, position at which transverse section illustrated by Fig. 2, *A* was made.

rachis. Paired stipules are adnate at the lower end of the rachis (Figs. 1, A, 3, A, and 4, A). Although the monograph of the B.P.C., 1949[8], includes only leaflets in its definition, 5 per cent. of rachis and stalk is allowed; moreover, most commercial material at present available consists of the chopped leaves, and this investigation describes, therefore, the anatomy of *a*, leaflets, *b*, rachis and *c*, stipules.

(*a*) LEAFLETS

Many leaflets, both terminal and lateral, were examined, and no anatomical differences were detected between them, or between leaflets on the non-fruiting canes and those of the fruiting canes of the following year. The following anatomical description, therefore, applies to any of these leaflets.

(*i*) *Lamina, interneural region* (Fig. 2, A and C)

The UPPER EPIDERMIS is covered with a relatively thick, smooth cuticle, and consists of a layer of polygonal cells having slightly wavy anticlinal walls (Fig. 1, E); they measure about H 8 to 20 μ, and Lev L and B 10 to 30 μ*; stomata are absent; frequent *covering trichomes* occur, about 200 to 500 μ long, and about 18 to 35 μ wide at their bases. They are unicellular, with thick, lignified walls, tapering and acutely pointed, with heavily thickened bases frequently exhibiting linear pits. The lumen is wide at the base, but it narrows towards the apex of the trichome, becoming obliterated in the upper half. The bases of the trichomes are usually surrounded by about 8 radiating epidermal cells (Fig. 1, C). The spiral markings reported by other authors[6,7] were only indistinctly seen on some of the trichomes on the upper surface of the lamina. They were observed more clearly on the trichomes of older leaves after prolonged boiling with chloral hydrate solution, and on the trichomes of the stipules—*vide infra*.

The MESOPHYLL is clearly differentiated; the *palisade* consists of a single layer of cells, frequently becoming doubled near the midrib. Individual cells are cylindrical, moderately elongated, measure about H 28 to 40 μ, Lev 5 to 14 μ and contain numerous chloroplasts, about 1 to 4 μ in diameter. Scattered irregularly in the palisade are large, rounded idioblasts, each containing a cluster or rosette crystal of *calcium oxalate* about 8 to 30 μ in diameter. The *spongy mesophyll* consists of 2 to 4 layers of rounded or elongated cells about H 3 to 15 μ, Lev L and B 4 to 18 μ, also containing numerous chloroplasts, about 1 to 4 μ in diameter.

The LOWER EPIDERMIS has a smooth, thin cuticle. Its cells measure about H 7 to 20 μ, Lev L and B 9 to 38 μ, their anticlinal walls are slightly more wavy than those of the upper epidermis (Fig. 1, D); *stomata* are numerous, are of the anomocytic (ranunculaceous) type, and are usually surrounded by radiating epidermal cells; they are slightly raised above the level of the epidermis and vary in outline from nearly circular to

* The symbols H, Lev, Lev L and Lev B are suggested for the purpose of describing organs showing bilateral symmetry by Moll and Janssonius[17]. The symbol H = height, in a direction perpendicular to the surface of the organ; Lev = in the direction of the surface of the organ; Lev L and Lev B = parallel to the surface and at the same time in a longitudinal or transverse direction respectively.

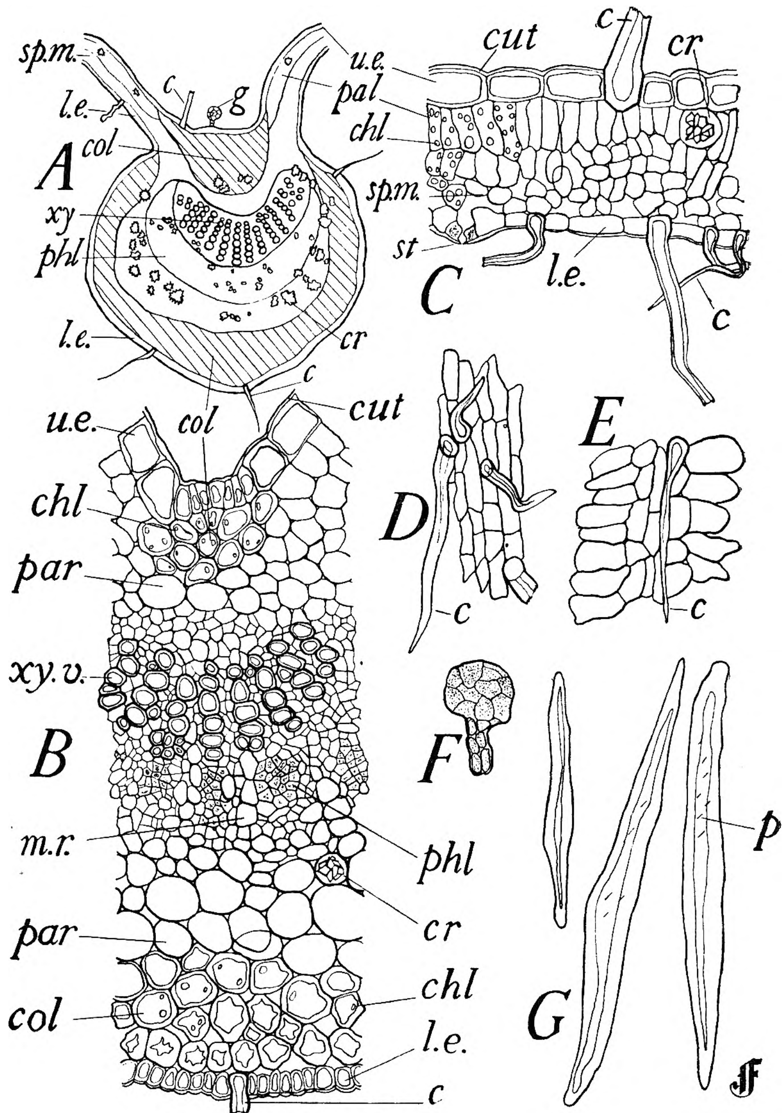

FIG. 2. Leaflet of *Rubus idæus* L. *A*, transverse section of midrib of terminal leaflet, cut at the position *w* (see Fig. 1, *A*). *B*, central region of Fig. *A*. *C*, transverse section of lamina, interneural region. *D*, lower epidermis of midrib. *E*, upper epidermis of midrib. *F*, glandular trichome from upper epidermis of midrib. *G*, isolated sclereids of prickle. A, × 45; B–G × 200. *c*, covering trichome; *chl*, chloroplast; *col*, collenchyma; *cr*, cluster crystal of calcium oxalate; *cut*, cuticle; *g*, glandular trichome; *l.e.*, lower epidermis; *m.r.*, medullary ray; *p*, pit; *pal*, palisade; *par*, parenchyma; *phl*, phloem; *sp.m.*, spongy mesophyll; *st*, stoma; *u.e.*, upper epidermis; *xy*, xylem; *xy.v.*, xylem vessel.

elliptical. The circular stomata are about 16 to 20 μ in diameter and the elliptical ones are about 12 to 18 μ wide and 18 to 22 μ long. Long *covering trichomes* are present in very great numbers, forming a tomentum

or "felt" by their mutual intertwining; they are unicellular, about 150 to 500 μ long and 6 to 18 μ wide at the base; their walls are thickened but unlignified, remaining unstained in mounts of phloroglucin and hydrochloric acid or of aniline sulphate, and the lumen is not usually so extensively obliterated as in the case of the covering of hairs of the upper epidermis; they are curled, tapering with blunt apices and thickened but smooth bases (Figs. 1, D, and 4, F).

The lamina has a serrate margin, the teeth being acutely pointed. The minute ends of the secondary and tertiary veins, which extend to within about 60 μ of the teeth apices, terminate in a few very small, spiral vessels. Two fine veinlets, one on either side, converge towards the central veinlet and unite with it about 0·3 mm. from the tip of the tooth (Fig. 1, A and B).

(*ii*) *Midrib* (Fig. 2, A and B)

Histologically, the midrib shows a typically dicotyledonous structure, moreover, no significant variation was noted in transverse sections cut at twelve different positions between base and apex of the leaflet.

The UPPER EPIDERMIS consists of a single layer of elongated, straight-walled cells measuring about H 15 to 20 μ, Lev B 5 to 12 μ and Lev L 20 to 40 μ; they are heavily cuticularised. *Covering trichomes* are present in moderate number and are similar in character to those of the upper interneural epidermis (Fig. 2, B and E). Occasional *glandular trichomes* about 65 to 110 μ long occur on the upper epidermis of the midrib and to a lesser extent on the upper epidermis of main lateral veins; they comprise a multiseriate or biseriate, multicellular stalk, about 3 to 6 cells long, frequently with granular contents, and a subspherical, multicellular, glandular head about 36 to 54 μ in diameter (Fig. 2, F).

The LOWER EPIDERMIS consists of small, longitudinally elongated straight-walled cells measuring about H 6 to 18 μ, Lev B 5 to 10 μ and Lev L 30 to 52 μ. *Covering trichomes* arise frequently, similar in character to those of the upper interneural epidermis (Fig. 2, B and D).

Laterally compressed, conical or comma-shaped *prickles* are commonly found on the lower surface of the midrib. These consist of elongated, interlocking sclerotic cells measuring about 50 to 160 μ long and 10 to 38 μ wide, which possess lignified, much thickened walls, traversed by occasional oblique and linear pits. Towards the apex of the prickle, they have pointed ends and some cells have granular contents. The lumen may be wide or narrow (Figs. 1, A, 2, G, and 4, F and G).

The CORTEX contains abundant supporting hypodermal *collenchyma* arranged in several rows towards both surfaces of the midrib, that towards the lower surface being greater in extent. These cells are heavily thickened in the angles, and measure about L 40 to 110 μ, R and T 8 to 28 μ; chloroplasts are commonly present, measuring about 2 to 4 μ in diameter. The interior of the cortex is of *parenchyma*; individual cells measure about L 56 to 106 μ, R and T 10 to 38 μ. Idioblasts occur fairly frequently in this tissue, each containing a large cluster or rosette crystal of *calcium oxalate*, about 12 to 45 μ in diameter (Fig. 2, A and B).

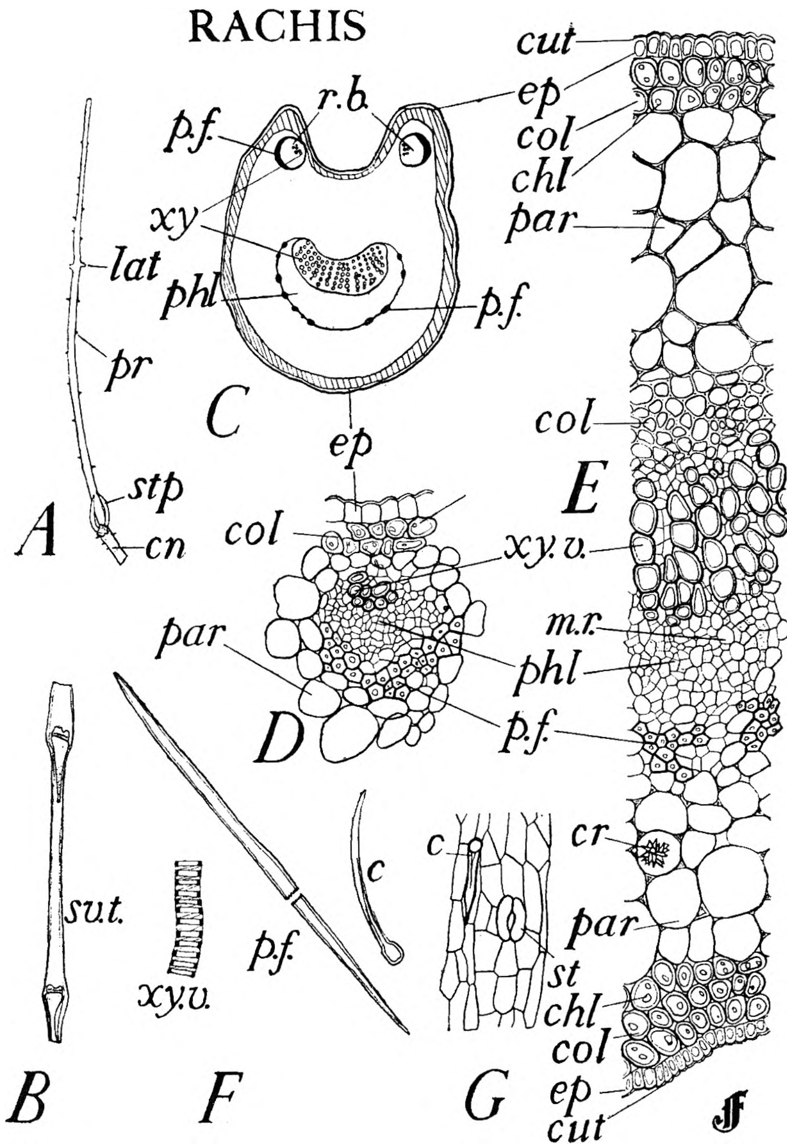

FIG. 3. Rachis of *Rubus idæus* L. *A*, rachis, denuded of leaflets. *B*, sieve-tube, from a cultivated species of *Rubus idæus*, and larger than, but otherwise similar to those found in the wild species. *C*, transverse section of rachis. *D*, transverse section of ridge bundle of rachis. *E*, transverse section through central region of rachis. *F*, isolated elements obtained by maceration. *A*, × ¾; *B*, × 250; *C*, × 40; *D*, *E* and *F*, × 200. *c*, covering trichome; *chl*, chloroplast; *cn*, cane; *col*, collenchyma *cr*, cluster crystal of calcium oxalate; *cut*, cuticle; *ep*, epidermis; *lat*, point of attachment of lateral leaflet; *m.r.*, medullary ray; *par*, parenchyma; *p.f.*, pericyclic fibre(s); *phl*, phloem; *pr*, prickle; *r.b.*, ridge bundle; *st*, stoma; *stp*, stipule; *s.v.t.*, sieve-tube; *xy*, xylem; *xy.v.*, xylem vessel.

An *endodermis* is not distinguishable, which agrees with Engard's statement[17] that it is absent in the genus *Rubus*.

The MERISTELE is crescent-shaped and well-defined.

The PHLOEM consists of strands of sieve-tissue and small-celled parenchyma, alternating with medullary rays. The *sieve-tubes* are small, individual segments being about 70 μ long and about 2 to 6 μ in diameter with transversely or slightly obliquely arranged sieve-plates, which are uniformly pitted; they resemble the sieve-tubes of the rachis (Fig. 3, B). The medullary rays are clearly marked and are usually one or two cells wide (Fig. 2, B).

CAMBIFORM TISSUE is rarely discernible in the midrib, but, where evident, consists of small, thin-walled rectangular cells.

The XYLEM consists of a number of rows of radiately arranged *vessels* about 4 to 22 μ in diameter; the rows are traversed by the medullary rays, which are about one or two cells wide. The vessels have lignified, spirally and occasionally annularly thickened walls (Figs. 2, A and B; 4, F).

In longitudinal sections, files of micro-clusters and occasional small prisms of *calcium oxalate* about 2 to 8 μ in diameter are frequently seen in the small-celled parenchyma within the meristele (Fig. 4, F).

The *lateral veins* exhibit similar anatomy to that of the midrib, all features becoming progressively smaller towards the margin.

Sections of fresh material mounted in ferric chloride solution exhibit a dark greenish-black colouration in the phloem and medullary rays of the meristele; the parenchyma below the meristele shows a weak reaction. The mesophyll of the lamina reacts strongly, but there is no reaction in the epidermis.

(*b*) RACHIS

The rachis is about 5 to 15 cm. long and 1 to 3 mm. wide and is deeply grooved on its upper surface so that, in transverse section, it presents a somewhat oval outline, with a deep groove on the upper side and a central, crescent-shaped meristele, with two small bundles, one in each ridge. Prickles are usually present throughout the length of the lateral and abaxial surfaces (Fig. 3, A and C).

The EPIDERMIS consists of cells similar in structure to those of the epidermis of the midrib. They are fairly heavily cuticularised, elongated longitudinally, and measure about H 8 to 14 μ, Lev B 5 to 14 μ, and Lev L 25 to 80 μ (Fig. 3, G); *stomata* of the anomocytic (ranunculaceous) type are present; they are elliptical in shape and measure about 20 to 28 μ in length, and 18 to 24 μ in width. *Covering trichomes*, similar in structure and nature to those on the upper surface of the lamina, occur frequently, measuring about 45 to 450 μ long and 7 to 20 μ wide at their bases. The *prickles* are rather larger than, but have similar structures to, those on the lower surface of the midrib.

The CORTEX, like that of the midrib, consists of two layers of tissue—an outer hypodermal layer of *collenchyma*, several cells wide, similar in character to the corresponding layer of the midrib; the cells measure about

L 10 to 140 μ, R and T 10 to 38 μ (Fig. 3, C, D and E). This collenchymatous layer forms a complete cylinder of tissue but is particularly well-developed in the two ridges. The inner cortex is *parenchymatous*, consisting of cells measuring about R and T 18 to 70 μ and L 38 to 190 μ; occasional idioblasts are present, containing cluster crystals of *calcium oxalate*, about 10 to 60 μ in diameter.

An *endodermis* is not distinguishable.

PERICYCLIC FIBRES occur below the arc of the stele; they measure about 350 to 1,300 μ in length, and 4 to 12 μ in diameter, and exhibit a bluntly pointed apex, lignified, smooth walls and a narrow lumen (Fig. 3, C, D, E and F).

The vascular tissue of the central MERISTELE is arranged in a crescent, and the structure of the vascular elements approximates closely to those of the midrib, except that those of the rachis are all somewhat larger. The PHLOEM consists of groups of *sieve-tubes* about 80 μ long and 4 to 6 μ wide, frequently accompanied by small rectangular parenchymatous cells, some of which contain micro-clusters of *calcium oxalate*. Medullary Rays traverse the phloem and are usually one or two cells wide. The XYLEM consists of rows of *vessels* about 4 to 25 μ in diameter, spirally thickened and lignified. There is but little xylem parenchyma; the medullary rays are clearly seen alternating with the rows of vessels (Fig. 3, B, C, E and F).

The bundle in each of the ridges exhibits a very simple structure, being partially surrounded by an arc of *pericyclic fibres* similar to those occurring below the meristele. The *phloem* is fairly extensive, but there are only a few spirally thickened *xylem vessels* (Fig. 3, C and D).

The reaction of sections of the rachis with ferric chloride solution is similar to that of the midrib, but the cortical collenchyma also gives a positive reaction.

(*c*) STIPULES

The paired stipules are adnate to either side of the base of the rachis. They are about 5 to 10 mm. long and 0·5 to 1 mm. wide, subulate and hairy.

EPIDERMAL CELLS of both upper and lower surfaces are small and elongated, measuring about H 7 to 12 μ, Lev B 6 to 12 μ and Lev L 12 to 70 μ. *Stomata* are present on both surfaces and commonly are raised above epidermal level; they are usually elliptical in outline and measure from about 23 to 28 μ in length and 20 to 24 μ in width (Fig. 4, B, D and E). Very numerous *covering trichomes* are present; at the apices and around the edges of the stipules they are large, measuring about 80 to 170 μ in length and 8 to 17 μ wide at their bases, spiral markings well defined (Fig. 4, A and B); towards the centre, they become shorter, similar to that depicted in Fig. 4, D, being about 15 to 70 μ long and 4 to 12 μ wide at their bases; in other respects, they have the general character of those of the upper epidermis of the leaflets. Occasional *glandular trichomes* occur, usually over the narrow midrib, and are very similar in structure to those found on the leaflets (Fig. 4, C).

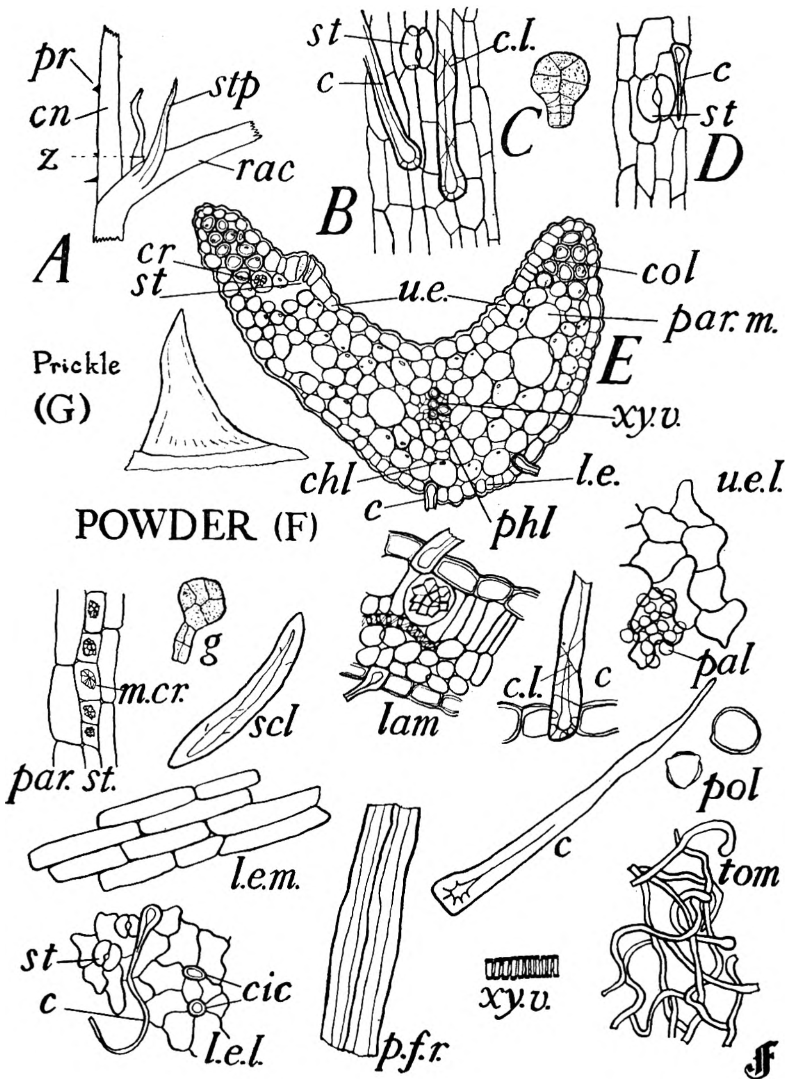

FIG. 4. Stipules, prickle and powder of *Rubus idæus* L. *A*, paired stipules at base of rachis. *B*, lower epidermis of stipule. *C*, glandular trichome. *D*, upper epidermis of stipule. *E*, transverse section of stipule at the position *z*, Fig. *A*. *F*, powder. *G*, prickle. *A*, × 2½; *B*, *C*, *D* and *F*, × 200; *E*, × 120; *G*, × 35. *c*, covering trichome; *chl*, chloroplast; *cic*, cicatrix; *c.l.*, crossed-line effect; *cn*, cane; *col*, collenchyma; *cr*, cluster crystal of calcium oxalate; *g*, glandular trichome; *lam*, fragment of lamina in transverse section, showing upper and lower epidermises with hair bases, idioblast with cluster calcium oxalate crystal, vascular strand, palisade and spongy mesophyll; *l.e.*, lower epidermis; *l.e.l.*, lower epidermis of lamina; *l.e.m.*, lower epidermis of midrib; *m.cr.*, micro-cluster of calcium oxalate; *pal*, palisade; *par.m.*, parenchymatous mesophyll; *par.st.*, parenchyma from stele; *p.f.r.*, fragments of pericyclic fibres from rachis; *phl*, phloem; *pol*, pollen; *pr*, prickle; *rac*, rachis; *scl*, sclereid of prickle; *st*, stoma; *stp*, stipule; *u.e.*, upper epidermis; *u.e.l.*, upper epidermis of lamina; *xy.v.*, xylem vessel.

The MESOPHYLL has very simple structure, is undifferentiated and consists of rounded or somewhat elongated cells, measuring about H 8 to 17 μ, Lev B 10 to 22 μ and Lev L 8 to 25 μ, and containing chloroplasts about 1 to 4 μ in diameter. Occasional idioblasts occur, containing cluster crystals of *calcium oxalate* about 15 μ in diameter. Towards the base of the stipule, the hypodermal tissue near the margin is strongly collenchymatous.

The MIDRIB is the only vein present and is very simple in structure, consisting of a few xylem vessels about 6 μ in diameter and but little phloem. Fibres are absent.

POWDER

A No. 85 powder varies in colour from light-green to greyish-green; it has a slight and pleasantly aromatic odour and an astringent, slightly bitter taste. A No. 10 powder is similar, but has a light texture, being more felted since the trichomes are less fragmented in the coarser powder. When some of the powder is mixed with solution of ferric chloride in a watch-glass, a deep greenish-black colour is observed.

To examine the powder for structural features, it should be mounted in one of the following: 50 per cent. v/v glycerol solution, solution of chloral hydrate, or phloroglucin and hydrochloric acid.

The diagnostic characters of the powder (Fig. 4, F) are:—

Very numerous curved or curled, unlignified fragments of *covering trichomes* from the lower surface of the lamina, up to about 16 μ wide, also fragments of larger, lignified, acutely pointed, unicellular trichomes from the upper surface, up to 30 μ wide, apical fragments being solid and basal fragments having linear pits; numerous *fragments of the lamina*, showing a transverse sectional view, about 70 μ thick, with a single or occasionally double layer of palisade in which there are rounded idioblasts, each containing a cluster crystal of *calcium oxalate* about 8 to 30 μ in diameter; particles showing in surface view the very slightly curved walls of the cells of the *upper interneural epidermis* of the lamina and usually, immediately beneath it, the palisade with its idioblasts; fragments showing the *lower interneural epidermis* consisting of wavy-walled cells with scattered anomocytic (ranunculaceous) stomata and cicatrices of trichomes surrounded by radiating groups of about 8 epidermal cells; fragments of the *veins* with small spiral vessels accompanied by files of small-celled parenchyma, each cell containing a micro-cluster, or more rarely a small prism of *calcium oxalate*; fragments of the *prickles* consisting of *lignified sclereids* with oblique, linear pits; infrequent entire and broken *glandular trichomes* with a multiseriate stalk and yellowish-brown, multicellular, subspherical, glandular head; lignified fragments of fibres from the rachis; *pollen grains* of *Rubus idæus* L., tricolpate and about 25 to 30 μ in diameter (Fig. 4, F).

SUMMARY

1. Raspberry leaves were collected from plants growing wild to obtain material typical of the species *Rubus idæus*. Polyethylene glycols were

used for embedding the fixed material for sectioning and a method of preparing permanently mounted sections by carbon dioxide freezing and subsequent double-staining with crystal violet and Bismarck brown is described.

2. Both *epidermises* of the leaflets are characteristic. The *upper epidermis* consists of cells with slightly wavy anticlinal walls and bears scattered unicellular, lignified, *covering trichomes* with acute and almost solid apices and thickened, pitted bases. The *lower epidermis* consists of cells with wavy anticlinal walls and bears very numerous unicellular, curly, non-lignified felted trichomes. *Glandular trichomes* are present on the stipules and on the upper epidermis of the leaflets; each has a multiseriate stalk and multicellular, subspherical head. *Stomata*, of the anomocytic (ranunculaceous) type occur in the lower epidermis only; they are circular to oval in outline.

3. The *lamina* of the leaflet is thin and dorsiventral, with, usually, a single row of palisade in which are rounded or ovoid idioblasts each containing a cluster crystal of *calcium oxalate.*

4. The *midrib* of the leaflet contains a meristele consisting of spirally thickened xylem vessels, a phloem of simple sieve-tubes with transverse or oblique sieve-plates, and rows of parenchymatous cells in longitudinal files, each cell containing a micro-cluster or small prism of *calcium oxalate.*

5. *Prickles* of midrib and rachis are composed of lignified, elongated sclereids.

6. Lignified *pericyclic fibres* are abundant in the rachis and provide a diagnostic feature for detecting the presence of rachis in the drug.

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References

1. Sowerby's *English Botany*, edited by J. T. B. Syme, Vol. III, Hardwicke, 1864, p. 160.
2. Bentham and Hooker, *Handbook of The British Flora*, Reeve & Co., Ashford, 1930, p. 138.
3. Clapham, Tutin and Warburg, *Flora of the British Isles*, Cambridge University Press, 1952, p. 462.
4. Wallis, *Textbook of Pharmacognosy*, 3rd Ed., Churchill, London, 1955, p. 143.
5. Thoms, *Handbuch der Pharmazie*, Vol. V, Urban & Schwarzenberg, 1931, p. 1021.
6. Moeller-Griebel, *Mikroskopie der Nahrungs- und Genussmittel aus dem Pflanzenreiche*, 3rd Ed., Springer, Berlin, 1928, p. 365.
7. Béguin, *Pharm. Acta Helvet.*, 1945, **20**, 41.
8. British Pharmaceutical Codex, 1949, p. 772.
9. Burn and Withell, *Lancet*, 1941, **241**, 1.
10. Whitehouse, *Brit. med. J.*, 1941, **2**, 370.
11. Beckett, Belthle, Fell and Lockett, *J. Pharm. Pharmacol.*, 1954, **6**, 785.
12. Johansen, *Plant Microtechnique*, McGraw-Hill, 1940, 126.
13. Fell and Rowson, *J. R. micr. Soc.*, 1955, **75**, 111.
14. Van Horne and Zopf, *J. Amer. pharm. Ass.*, 1951, **40**, 31.
15. Miles and Linder, *J.R. micr. Soc.*, 1952, **72**, 199.
16. Moll and Janssonius, *Botanical Pen Portraits*, Nijhoff, The Hague, 1923, p. 22.
17. Engard, *Organogenesis in Rubus, University of Hawaii Research Publication*, No. 21, Honolulu, 1944, p. 220.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ALKALOIDS

11-Desmethoxyreserpine. J. W. E. Harrisson, E. W. Packman, E. Smith, N. Hosansky and R. Salkin. (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 688.) The 3:4:5-trimethoxybenzoic acid ester of methyl 11-desmethoxyreserpate known as raunormine, canescine, deserpidine or recanescine was isolated from the mother liquors after the crystallisation of reserpine from extracts of *Rauwolfia canescens*. The substance appeared to be 11-desmethoxyreserpine. On hydrolysis it yielded raunormic acid which differed from reserpic acid in that the infrared spectrum of the hydrochloride lacked the bands at 7·94 and 9·52μ, characteristic of the methoxyl group attached to the aromatic ring, and also the bands at 6·25, 6·35, 11·5 and 12·2 μ, which are characteristic of a 1:2:4-substituted benzene ring. Preliminary studies using mice, rats and dogs showed that the toxicity of raunormine is similar to that of reserpine. G. B.

Rescinnamine, Isolation from *Rauwolfia vomitoria* Afz. D. A. A. Kidd. (*Chem. Ind.*, 1955, 1481.) During the isolation of reserpine from roots of *R. vomitoria*, a further alkaloid was encountered in close association with it, and this was identified as rescinnamine, first isolated from *R. serpentina* and not hitherto found in any other species. Rescinnamine is only a relative minor constituent of *R. vomitoria* (0·6 g. isolated from 4 kg. of dried roots). A. H. B.

ANALYTICAL

Cholic, Desoxycholic and Dehydrocholic Acids, Determination of. R. Crisafio and L. G. Chatten (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 529.) The following methods were used. (1). Place 5 ml. of methanol and 50 ml. of benzene in a 150-ml. beaker, add 2 drops of thymol blue solution (0·5 per cent. in methanol) and neutralise, using 0·1N potassium methoxide or methanolic potassium hydroxide. Add the sample of bile acid, cover the beaker with a rubber dam and titrate to a blue end-point as rapidly as possible, while stirring with a magnetic stirrer. (2). Place the sample of bile acid in a beaker, add 2 ml. of *NN*-dimethylformamide and stir until the sample has dissolved or is thoroughly wetted. Add 50 ml. of chloroform and 2 drops of thymol blue solution and titrate to a purple-blue end-point. A reagent blank determination is necessary. For the determination of dehydrocholic acid in tablets by method (1) a quantity of powdered tablets equivalent to about 0·2 g. of dehydrocholic acid was dissolved in methanol and benzene and titrated. Using method (2) the sample was dissolved in dimethylformamide and filtered to remove excipients. The filter was washed with chloroform and the washings added to the titration liquid. As an alternative to the use of thymol blue, the end-point was determined potentiometrically using glass and sleeve-type calomel electrodes. Lithium chloride was added to the benzene-methanol solution to decrease its resistance. When titrations were carried out using an open beaker, stirring by hand, the quantity of carbon dioxide absorbed from the air was insufficient to affect the results. Methanolic potassium hydroxide appeared

to give the same results as potassium methoxide solution and had the advantage of being easier to prepare. The results were at least as satisfactory as those obtained by the U.S. National Formulary IX method, which is more difficult to apply. G. B.

Morphine in Opium, Improvement in U.S.P. Method for Determining. H. W. Brickley and F. A. Whipple (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 538.) In carrying out the U.S.P.XIV assay for morphine in opium, the time required for filtration may be reduced considerably by adding calcium phosphate before beginning the assay process and by applying suction during the filtrations. The total time of the assay is reduced by one third while the accuracy of the procedure is unaffected. The addition of magnesium carbonate as a filtering aid was investigated, but found to be of little value, besides giving rise to low results. G. B.

Reserpine and Related Compounds, Paper Chromatographic and Biological Properties of. R. J. Boscott and A. B. Kar. (*Nature, Lond.*, 1955, **176**, 1077.) Trimethoxybenzoic acid, a known hydrolysis product of reserpine, does not affect the fertility of male and female rats. Thus the reported anti-fertility effect of reserpine in rats is due to the molecule as a whole or some fragment other than trimethoxybenzoic acid. A number of paper chromatographic procedures for checking the purity of commercial samples of *Rauwolfia* alkaloids are recommended including the following, (a) Single phase systems, such as the aqueous phase obtained by shaking 10 per cent. v/v acetic acid in 5 per cent. sodium acetate with *n*-butyl ether, *tert.*-amyl alcohol, *sec.*-butanol or methyl*iso*butylketone. A mixture of xylene (200), methanol (75) and methyl*iso*butylketone (25) may also be used. (b) Partition chromatography is also recommended using the solvent system *n*-amyl alcohol (200), water (180), acetic acid (20), or alternatively *n*-hexyl ether (200), methyl*iso*butylketone (50), acetic acid (20), water (180). Tests for locating the spots are described, and typical R_F values of a number of alkaloids are given for the various solvent systems. J. B. S.

Reserpine in Pharmaceutical Preparations, Determination of. R. E. Booth (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 568.) For the determination of reserpine, material is mixed with bromophenol blue and buffer solution, pH 4·0. Bromophenol blue is insoluble in chloroform, but each molecule reacts with 2 molecules of reserpine to produce a complex which can be removed by extraction with chloroform and determined colorimetrically. Absorption measurements are made at 402 mμ, the result being calculated from a standard curve prepared with the aid of pure samples of reserpine. The method is sufficiently sensitive for use in the assay of commercial tablets and elixirs of reserpine. Since reserpic acid reacts in the same way as reserpine, an additional test to determine the extent to which it has been formed by hydrolysis of reserpine is required in the case of aqueous preparations. G. B.

Sodium Carboxymethylcellulose, Determination of. C. R. Szalkowski and W. J. Mader (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 533.) The method depends upon the formation of glycollic acid by reaction of sulphuric acid with carboxymethylcellulose. Carboxymethylcellulose is precipitated from pharmaceutical preparations in the form of its copper salt, which is dissolved in dilute sulphuric acid and heated in a water bath with a 0·05 per cent. solution of 2:7-naphthalenediol in concentrated sulphuric acid. The colour of the solution is measured spectrophotometrically at 530 mμ against a reagent blank, and the

quantity of sodium carboxymethylcellulose is calculated from a calibration curve, prepared by using a standard preparation of sodium carboxymethylcellulose. The standard material must have the same degree of substitution as the sample under test, as this affects the intensity of colour produced. The method is applicable to preparations containing antibiotics and good recoveries have been obtained from samples containing penicillin, procaine penicillin, streptomycin, dihydrostreptomycin and sodium citrate. G. B.

B Vitamins, Quantitative Separation of, by Electrophoresis on Agar Plates. G. Marten. (*Nature*, *Lond.*, 1955, **176**, 1064.) Mixtures of vitamin B have been quantitatively separated by electrophoresis directly on thin layers of agar jelly as used in macro-electrophoresis. After separation in the electric field is complete a second layer of agar jelly is placed on the first, and contains all the substances required by the test organisms seeded into the agar, except that to be determined. Zones of growth appear in those spots corresponding to the presence of the vitamins in the lower layer, the logarithm of the dose being directly proportional to the diameter of the zone. Chemical determination of the vitamin is also possible after cutting out the appropriate portion of the agar jelly. The method, which will detect as little as 0·002 μg. of vitamin B_1 is described in detail. J. B. S.

ORGANIC CHEMISTRY

Sterculic Acid, Structure of. J. P. Verma, B. Nath and J. S. Aggarwal. (*Nature*, *Lond.*, 1955, **176**, 1082.) The infra-red spectrum of sterculic acid shows a band at 1008 cm.$^{-1}$. This is considered to support the structure $CH_3.(CH_2)_5.CH{-}CH.CH = CH.(CH_2)_7.COOH$ in which the *cyclo*propane ring is

in conjugation with the double bond. More recent work has shown, however, that this could not readily be distinguished from the alternative *cyclo*propene structure. The Halphen reaction is not specific to cottonseed oil, positive results being obtained with oils which do not contain *cyclo*propene fatty acids. The authors do not accept the view that this reaction can be attributed to the presence of a *cyclo*propene group alone. It is also emphasised that there are many instances in which *cyclo*propane groups are no less reactive than ethylene groups, while *cyclo*propene groups have been shown to possess appreciable stability. J. B. S.

Veratrum Alkaloid Group, Application of Paper Chromatography to Structural Problems in. K. Macek and Z. J. Vejdělek. (*Nature*, *Lond.*, 1955, **176**, 1173). A paper chromatographic method has been devised to assist in the determination of the number of free hydroxyl groups, which form a series of diol systems in the various veratrum alkamines, by exploiting the reaction of such diol groups with boric acid. The compounds were run in two parallel chromatograms with chloroform as the mobile phase on paper impregnated with formamide containing 5 per cent. boric acid in one case and with formamide alone in the other. Since the glycol-borate complexes are highly polar the ratio of movement of compounds containing suitable glycol systems is decreased on the boric acid-impregnated paper. The difference in R_M values for the same compound in the two systems (ΔR_M) gives an indication of the number of free glycol systems. Two glycol systems give ΔR_M values between 0·8 and 1·2 units, whilst a single glycol system gives ΔR_M between 0·3 and 0·45. Changes in

R_M on acylation, and upon partial methanolysis of polyester derivatives give an indication of the number of glycol systems blocked by esterification. The results obtained provide interesting facts concerning the configuration of the glycol system at position 17:20. Contrary to the conclusions of Barton *et al.*, who suggest that it is *trans*, the authors find that the glycol system is capable of complex formation with boric acid, indicating that a *cis* configuration is more probable. Calculation of group constants makes it possible to predict R_M and hence also R_F values for isomeric mono- and poly-esters of the veratrum alkamines, so that for the chromatographic detection and identification of esters of any further acid it is sufficient to determine experimentally the R_F value of a single alkamine ester of the acid. J. B. S.

Vitamin B_{12}, Reduction of. G. H. Beaven and E. A. Johnson. (*Nature, Lond.*, 1955, **176**, 1264.) The two known reduction products of Vitamin B_{12}, now designated as (I) and (II) respectively, have been further examined. Compounds with spectra resembling that of (I) may be obtained by use of a number of different reducing agents. Hydrogenation gives not only (I) but other products of ill-defined structure; chromous acetate is more satisfactory giving a solution of (I) which is stable indefinitely in an atmosphere of hydrogen, and which can be re-oxidised quantitatively to vitamin B_{12a}. Contrary to the reports of Boos *et al.*, the authors find that reduction of vitamin B_{12} with cobalt acetate in a solution buffered at pH 3 with ethylenediamine tetra-acetate gives exclusively (I), and not (II). The latter is obtained by reduction at pH 9·5, with (I) appearing as an intermediate. Both reduction products may be obtained just as readily from vitamin B_{12a} as from vitamin B_{12}, and (II) is also obtained from vitamin B_{12} in dilute potassium cyanide solution. The ready re-oxidation of (I) and (II) to vitamin B_{12a} suggests that the changes merely concern the state of oxidation of the cobalt. In the light of this observation the differences between the spectra of (I) and (II) suggest that there is increased conjugation in the latter, possibly accompanied by rearrangement and release of the benziminazole group from co-ordination. It is thought the spectrum of the vitamin itself could be better explained by the inclusion of an additional double bond in the structures already assigned to it, so that the two alternative structures would then be capable of resonance. Activated groups capable of being chlorinated would still be present in such structures with six conjugated double bonds, and whilst a further increase of conjugation by dehydrobromination would then be unlikely, the spectrum of (II) suggests that the long-wave shift of vitamin B_{12} could be explained without requiring the introduction of still further unsaturation in the molecule. J. B. S.

TOXICOLOGY

Arsenical Poisoning; Studies on some Cases of. H. Griffon. (*Ann. pharm. franç.*, 1955, **13**, 600.) Samples were taken from the bodies of victims of arsenical poisoning, at periods up to 14 years 3 months after death. As the bodies were considerably decayed, relatively small specimens were available and results were inconclusive, except when specimens of hair were examined. These were comparatively well preserved, and arsenic could be estimated at positions along the hair, and a graphic representation showing bands of arsenical impregnation along the hair obtained. In all cases this was in agreement with toxicological and other evidence obtained at about the time of death. G. B.

3:3-Diethyl-2:4-dioxotetrahydropyridine (Persedon), Isolation and Detection of, for Forensic Purposes. F. Dybing. (*Acta pharm. tox., Kbh.*, 1955, **11**, 393.) Persedon is a weaker hypnotic drug than the barbituric acids. It may be extracted from biological tissues by the standard Stas-Otto process, shaking out with 3 successive portions of ether. After evaporation of the solvent, the crude residue is dissolved in methanol and subjected to paper chromatography. After elution with sulphuric acid, the ultra-violet absorption of the solution is measured. M. M.

α-Naphthylthiourea (ANTU), Detection of, for Forensic Purposes. F. Dybing. (*Acta pharm. tox., Kbh.*, 1955, **11**, 388.) A method is described for the isolation of this pesticide. Purification by paper chromatography replaces the saponification of the fat. The material is extracted by adding equal amounts of water and methanol and shaking with *n*-heptane. Most of the fat is extracted by the heptane, while the ANTU remains in the methanol-water mixture. The methanol is removed by distilling under reduced pressure and the ANTU extracted with ether or chloroform. Purification by paper chromatography with a chloroform-formamide mixture for development is followed by identification of the ANTU by its ultra-violet absorption or by a colour reaction with bromine water in the presence of excess sodium hydroxide. M. M.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Cholinesterase, Inhibition of, by 1:2:4-Triazoles. J. B. Polya. (*Nature, Lond.*, 1955, **176**, 1175.) Following an observation that laboratory workers manipulating 1:2:4-triazoles develop symptoms of light nicotine or physostigmine poisoning, it has been shown that simple water-soluble 1:2:4-triazoles may act as inhibitors of cholinesterase activity. The effects of 3:5-dimethyl-(I), 1:3:5-trimethyl-(II), 3:5-dimethyl-1-phenyl-(III), 3-ethyl-5-methyl-1-phenyl-(IV) and 5-ethyl-3-methyl-1-phenyl-1:2:4-triazole (V) as inhibitors of cholinesterase from freshly homogenised sheep brain were examined. Cholinesterase activity was determined manometrically using acetylcholine, acetyl-β-methylcholine, and benzoylcholine chlorides as substrates. A time-lag was observed in all experiments, with pressure dropping, after an intial rise, to a minimum in about 20 minutes. A second 'wave', not adequately explained, was observed with (I) and (II) as inhibitors after about 60 minutes or more. Using rabbit brain tissue anomalous results of this kind were observed with all the triazoles. Similar inhibitory action was also observed with (III), (IV) and (V), and typical experiments with (III) showed that specfic cholinesterase is completely inhibited by (III) for substrate concentrations of 1–30 mg. acetyl-β-methylcholine/100 ml. and triazole/substrate ratios between 0·01 and 0·05. Lower triazole concentrations are ineffective and higher concentrations show slight activation of the enzymes. Similar benzoylcholine and triazole/substrate values activate the unspecific cholinesterase, whilst inactivation occurs at higher triazole concentrations. Inconsistent results were obtained with acetylcholine as substrate. J. B. S.

Oximes and Hydroxamic Acids, Reactivation by, of Cholinesterase Inhibited by Organo-phosphorus Compounds. A. F. Childs, D. R. Davies, A. L. Green and J. P. Rutland. (*Brit. J. Pharmacol.*, 1955, **10**, 462.) Human red-cell and rat brain cholinesterases inhibited by the "irreversible" anticholinesterases,

ethyl pyrophosphate, sarin or dyflos, were reactivated by members of the oxime series and related compounds. There was considerable variation in reactivating activity in compounds of a given type against the different anticholinesterases. Di-*iso*nitrosoacetone and related *iso*nitroso compounds were most effective against sarin, slightly less effective against ethyl pyrophosphate and least effective against dyflos. Picolinhydroxamic acid, on the other hand, was nearly as active as the oximes against ethyl pyrophosphate and dyflos whilst nicotinhydroxamic acid methiodide had equal activity to picolinhydroxamic acid against sarin, but was much less effective against either ethyl pyrophosphate or dyflos. The oximes in general were superior to the hydroxamic acids, especially against sarin-inhibited cholinesterase. The only common factor among the most potent reactivators was a pKa value of about 8. The reactivation, being a nucleophilic reaction, probably occurs through the anion of the oxime, so that, under the conditions of the tests (pH 7·4) with high pKa's the fraction of oxime ionised would be too small and with low pKa's the anion would be too weakly nucleophilic to be an effective reactivator. G. P.

BIOCHEMICAL ANALYSIS

Aureomycin (Chlortetracycline) in Biological Materials, Colorimetric Determination of. T. Sakaguchi and K. Taguchi (*Pharm. Bull. Japan*, 1955, 3, 303.) Aureomycin appears to exist in the body in the form of chelate compounds with metals, from which it is freed by the addition of a 10 per cent. solution of sodium ethylenediaminetetra-acetate, the reaction of the solution being adjusted to pH 1–2. The liberated aureomycin is extracted by saturating the solution with sodium chloride and shaking with several successive quantities of butanol. The butanol solutions are washed with a 25 per cent. solution of sodium chloride and extracted with a 1 per cent. solution of thorium nitrate, so as to obtain an aqueous solution of the thorium chelate compound of aureomycin. The light absorption of this substance in acetate buffer solution is determined at 400 mμ, and the quantity of aureomycin calculated from a standard curve prepared with the aid of a standard preparation of aureomycin. This method is applicable to the determination of aureomycin in biological fluids containing 2–20 μg./ml. A correction for interfering substances should be applied when examining specimens of urine containing small quantities of aureomycin. The same method may be used for the determination of tetracycline and oxytetracycline. G. B.

Chemotherapeutic Agents, Determination of Bacterial Sensitivity to. G. Czerkinsky, N. Diding, and O. Ouchterlony. (*Scand. J. clin. Lab. Invest.*, 1955, 7, 259.) A rapid and simple method is described for the determination of bacterial resistance to chemotherapeutic substances using paper strips. Strips of filter paper are impregnated in rectangular zones with sulphathiazole 0·1 mg., penicillin 1 I.U., chlortetracycline 10 μg., oxytetracycline 10 μg., chloramphenicol 20 μg. and streptomycin 50 μg., the zones being separated with linseed oil. Each strip is placed across an agar plate, and one side of the plate is smeared with a suspension of the test bacterium and the other half with a suspension of the standard (usually *Staph. aureus*). After incubation for 18 hours the zones of inhibition are measured for both the test and the standard bacteria from which the degree of resistance is calculated. It is also possible to determine when two adjacent antibiotics exert a synergistic or antagonistic effect. A special machine is described for impregnating the strips. G. F. S.

Heparin in Plasma, Determination of. H. Engelberg, A. Dudley and L. Freeman. (*J. Lab. clin. Med.*, 1955, **46**, 653.) Circulating heparin is strongly protein bound and this new method is based upon the tryptic digestion of the total serum or plasma proteins previously precipitated by methanol-acetone and subsequent dialysis and lyophilization. Add 10 ml. of methanol and 10 ml. of acetone to 5 ml. of citrated plasma, shake vigorously and stand for 30 minutes. Centrifuge and decant the supernatant. Wash the precipitate twice with acetone, centrifuging and decanting each time. Dry the coagulum under a vacuum aspirator. Add 5 ml. of phosphate buffer (0·2M, pH 8·5) and 0·5 ml. of a purified trypsin concentrate solution containing 80 mg. per ml. Digest overnight at 37°C. Heat coagulate for 30 minutes in a boiling water bath cooling the tops of the tubes with a fan. Cool, and decant and drain the clear superantant into a dialysis tube. Dialyze under running cold tap water overnight. Transfer the dialysed solution into a test tube and lyophilize until dry. Remove the dry sample and store at 4°C. The anticoagulant activity of the lyophilized material dissolved in 1·0 ml. of isotonic saline is determined by a semi-micro modification of United States Pharmacopeial method for heparin assay (previously described *Amer. J. clin. Path.*, 1954, **24**, 599). The results of duplicate assays of 25 individual plasma samples showed a ± 13 per cent. deviation from the mean. G. F. S.

Plasma Glycine, Determination of. T. B. Schwartz, M. C. Robertson and L. B. Holmes (*J. Lab. clin. Med.*, 1955, **46**, 657.) A simple, accurate and specific method is described for the determination of glycine in plasma, serum or rat diaphragm using a Conway microdiffusion unit. One ml. of heparinized plasma is diluted with 4 ml. of picric acid solution in a centrifuge tube, stirred, centrifuged and the glycine concentration of the supernatant is determined. 0·5 or 1 ml. of the supernatant is pipetted into the outer well of the microdiffusion unit, evaporated to dryness under an exhaust fan in a hood. The rims of the Conway vessels are greased with soft paraffin, 0·5 ml. of ethyl phosphate is added to the outer chamber near the outside rim of each vessel and the unit gently rotated so that the ethyl phosphate is evenly distributed. 2 ml. of sodium chromotropate solution is added to the centre well and 0·5 ml. of a ninhydrin reagent (2 g. ninhydrin diluted to 100 ml. with citrate buffer) is placed in the outer well. A glass cover is quickly placed over the greased rim to produce an airtight seal, and the unit is allowed to stand overnight in the dark while the formaldehyde distils over into the centre well. The covers of the vessels are then removed, the contents of the inner well are stirred and transferred to test tubes which are heated in a boiling water bath for 30 minutes and protected from the light. After cooling the solutions are transferred to calibrated test tube cuvettes and read at 570 mμ in a spectrophotometer. A glycine standard containing 0·1 μM glycine per ml. and a reagent blank are both run in duplicate through the entire procedure. As little as 0·02 μM of glycine can be determined and recoveries of $98{\cdot}7 \pm 1{\cdot}4$ per cent. are obtained from human plasma. As many as seventy-two determinations can be carried out simultaneously using multiple units. G. F. S.

Serum Calcium, Determination of. H. E. Harrison and H. C. Harrison. (*J. Lab. clin. Med.*, 1955, **46**, 662.) The method is based on the precipitation of calcium as oxalate, dissolving the calcium oxalate in a measured excess of disodium ethylenediaminetetra-acetic acid and measuring the excess chelating agent by back titration with standard calcium chloride solution. Pipette 0·2 ml. of serum into a centrifuge tube and add 0·2 ml. of oxalate reagent (consisting

of 0·5 ml. of 0·1 N oxalic acid added to 10 ml. of 0·1 N sodium oxalate). Shake for 30 minutes in a shaker, stand until precipitation is complete, centrifuge for 5 minutes and remove the supernatant. Dissolve the precipitate in 0·5 ml. of a solution of dihydrogen ethylenediamine-tetracetic acid (this solution is prepared daily from a stock solution of 4·5 g. of the salt in 1 litre of distilled water, by taking 2 ml. and diluting to 10 ml. with 0·25 M ethanolamine). Add 0·05 ml. of working indicator solution (0·5 ml. of a 0·5 per cent. solution of eriochrome black in ethanolamine added to 2 ml. of distilled water), and titrate with a standard solution of calcium chloride (0·02 M calcium chloride = 0·8 mg. calcium) from a microburette until the colour changes from a blue green to a purple red. Deduct a blank determination for the reagents. The results agree with the Clark Collip method and show an error of $\pm$ 5 per cent. G. F. S.

PHARMACY

NOTES AND FORMULÆ

Diphtheria Toxin: Production in Submerged Culture. F. V. Linggood A. C. Matthews, S. Pinfield, C. G. Pope and T. R. Sharland, (*Nature, Lond.*, 1955, **176**, 1128.) This paper gives details of improvements in the authors' original method of the submerged culture of *C. diphtheriæ*. 80 litre aluminium tanks, vortex stirring and sterile air at a rate of 0·05–0·15 litre per minute are used. A growth period of about 48 hours and an initial inoculum of 200 ml. of a 48-hour culture of *C. diphtheriæ* for a volume of 50–60 litres of medium is satisfactory. By such means the culture filtrate has an Lf value of the order of 180–250 units per ml. Such a method is superior to the surface culture technique. It is preferable to harvest single batches of toxin rather than to run a semi-continuous process. M. M.

Preservatives in Pharmaceutical Products, Efficacy of. E. J. Rdzok, W. E. Grundy, F. J. Kirchmeyer and J. C. Sylvester. (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 613.) Vials containing 10 ml. or more of various preparations for injection were inoculated with cell or spore suspensions of the test organisms to give a cell count of about 10,000 to 50,000 per ml. The vials were incubated at 30° C. and after 1, 3 and 7 days and weekly up to 1 month, samples were checked by making plate counts. The organisms used were *Bacillus cereus, Aspergillus niger, Candida albicans, Streptococcus fæcalis, Micrococcus pyogenes* var. *aureus, Escherichia coli* and *Pseudomonas æruginosa.* For use in an aqueous suspension of œstrone for injection 0·9 per cent. of benzyl alcohol was a satisfactory preservative whereas 0·18 per cent. of methyl *p*-hydroxybenzoate with 0·02 per cent. of propyl *p*-hydroxybenzoate was not sufficiently active against *Ps. æruginosa* and *Str. fæcalis.* For preserving a solution of suxamethonium chloride, 20 mg./ml., the mixture of *p*-hydroxybenzoates was satisfactory. Benzethonium chloride, 1 in 5000, was suitable for preserving an ophthalmic preparation, but a lower concentration (1 in 50,000) was not effective against certain strains of *Ps. æruginosa.* Tests were made to assess the value of preservatives in preventing spoilage due to yeast and soil organisms. For this purpose, phenylethyl alcohol was satisfactory for selenium sulphide jelly and 0·2 per cent. benzoic acid with 0·06 per cent. methyl *p*-hydroxybenzoate was satisfactory for a vitamin syrup. G. B.

PHARMACOLOGY AND THERAPEUTICS

Actinomycin C in Hodgkin's Disease. J. R. Trounce, A. B. Wayte and J. M. Robson. (*Brit. med. J.*, 1955, **2**, 1418.) Actinomycin C was given to 6 patients with advanced Hodgkin's disease and one with a reticulum-celled sarcoma. All the patients had previously been treated with radiotherapy, and all but one had received one or more courses of nitrogen mustard. The usual scheme of dosage employed was to start with 100 μg. of actinomycin C intravenously. If no toxic effects were observed the dose was increased so that at the end of a week or 10 days the patient was receiving 400 μg. daily. With doses of 400 μg. or more the drug was dissolved in half a pint of saline and given by intravenous drip over 2 to 3 hours. A total course of 7000 and 10,000 μg. was used whenever possible. Only 2 of the patients showed a marked response to treatment, with disappearance of fever and considerable reduction in the size of lymph nodes and spleen. In one of these patients improvement has been maintained to date—a period of 3 months. Two other patients showed slight improvement. Three cases showed no response. The side-effects of the drug included stomatitis and thrombocytopenia. The results suggest that in advanced reticuloses actinomycin C only occasionally has any great effect on the disease, but it would seem worthy of trial when the other more usual treatments have failed and especially in those patients unable to take nitrogen mustard. S. L. W.

Adrenaline and Noradrenaline, Elimination of, from the Circulating Blood. O. Celander and S. Mellander. (*Nature, Lond.*, 1955, **176**, 973.) An *in vivo* study is made of the manner in which various tissues are capable of eliminating adrenaline and noradrenaline from the blood stream. Cats in which the adrenal glands had been removed and the spinal cord cut were used. Adrenaline or noradrenaline was infused at a constant rate, either intra-arterially to the tissue studied or intravenously into the brachial vein. The contractions of the chronically denervated nictitating membrane of the same animal were used to indicate the amount of amine in the systemic circulation. In most of the experiments the adrenaline or noradrenaline was infused into the lower part of the abdominal aorta in the eviscerated animal and therefore into tissues consisting mainly of skin and skeletal muscle. When the infusion was shifted to the intravenous route, without any change in the dosage, there was a marked contraction of the nictitating membrane, indicating a corresponding increase in the catechols in the systemic circulation. It could be estimated that the muscle and skin of this region destroyed about 90 per cent. of either amine, in one passage of the blood, providing that the amount administered was physiological. This considerable loss in a rather non-specific tissue area can only in part be attributed to the action of amine oxidase since very similar results were obtained after the administration of specific amine oxidase inhibitors. Tissues such as the spleen, kidney and intestines gave similar results. It is reasonable to assume that locally released adrenergic transmitters are inactivated in this way. M. M.

Chlordane: Report to the Council on Pharmacy and Chemistry. (*J. Amer. med. ass.*, 1955, **158**, 1364.) Chlordane is a chlorinated hydrocarbon insecticide available in various forms as an impure mixture. It is a heavy, dark brown, oily liquid insoluble in water but soluble in the common organic solvents. It is

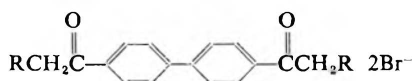
unstable in the presence of weak alkalis. The volatile properties and residual characteristics of chlordane are intermediate between those of dicophane and gamma benzene hexachloride. It is effective in the control of certain agricultural and household pests and is available in the form of oil solutions, emulsion concentrates, wettable powders, and insecticidal dusts, paints and waxes. Lethal action on susceptible organisms results from contact, ingestion or exposure to vapour. It is not approved for over-all treatment of rooms because slow liberation of fumes constitutes a danger. No medicinal use for chlordane in any form has been found acceptable to date. A complete pharmacological evaluation of technical chlordane is difficult because it is not a definite chemical entity. In general, it acts like other chlorinated hydrocarbon insecticides whose sites of action are on the higher motor cortex and the cerebellum. It does not affect vital centres in the medulla. It is absorbed into the body from the gastro-intestinal tract, the respiratory tract and the skin, and it would seem to be capable of a more rapid and greater penetrability of the body barriers than many other synthetic insecticides belonging to the chlorinated class. The minimum lethal dose of chlordane by ingestion for human beings is not known, but death has occurred following the ingestion (with suicidal intent) of 100 mg./kg. body weight of the technical mixture. After dermal application chlordane is more toxic than dicophane as it is readily absorbed through the unbroken skin, and deaths have occurred from such absorption. Little is known about the inhalation toxicity of chlordane, but chemically verified cases of poisoning from combined skin and respiratory exposures have been recorded. The symptoms of acute chlordane intoxication are similar to those observed in poisoning by dicophane. Acute signs of poisoning usually occur within 45 minutes of ingestion, and death may occur within 24 hours though it may be delayed for some days. Chronic poisoning may be manifested by disturbances of the central nervous system; it particularly affects the optic nerve. Treatment of chlordane poisoning depends on the use of symptomatic measures. Details are given of 15 reported cases of systemic chlordane poisoning. S. L. W.

Chlorpromazine, Identification and Pharmacological Properties of a Major Metabolite of. N. P. Salzman, N. C. Moran and B. B. Brodie. (*Nature, Lond.*, 1955, **176**, 1122.) Chlorpromazine, given to man or to dogs, results in the urinary excretion of chlorpromazine sulphoxide. Identification of this derivative was made by extraction from alkaline urine and separation as the picrate. The empirical formula of this metabolite differs from that of chlorpromazine by one additional oxygen atom. This sulphoxide formation is an unusual type of drug bio-transformation. The pharmacological actions of chlorpromazine and its sulphoxide were compared in dogs. The compounds were qualitatively similar, producing sedation, relaxation of the nictitating membrane, adrenergic blockade, orthostatic hypotension, excitement, tremors and finally clonic and tonic convulsions. The dose of sulphoxide which produced minimal sedation was about 8 times that of chlorpromazine but the sulphoxide caused relatively little adrenergic blockade and postural hypotension at sedative dose levels. The sulphoxide was one-eighth as active as chlorpromazine in potentiating hexobarbitone anæsthesia. Since chlorpromazine sulphoxide induces sedation in dogs with relatively little of the orthostatic hypotension observed with chlorpromazine, it is planned to test the drug in man in the treatment of mental illness. M. M.

Ferrous Gluconate, Toxicity of. J. O. Hoppe, G. M. A. Marcelli and M. L. Tainter. (*Amer. J. med. Sci.*, 1955, **230**, 491.) This is a study of a direct comparison of the acute systemic and local toxicity of ferrous sulphate and

ferrous gluconate in experimental animals (mice, rats, dogs and cats). The study clearly establishes that ferrous gluconate is less irritating and less toxic than ferrous sulphate when considered from the standpoint of the total weight of drug administered or in terms of their iron contents. A firm experimental basis for the lack of clinical toxicity and for the therapeutic preference for ferrous gluconate therefore appears to be demonstrable. The magnitude of the acute oral toxicity values when compared with the acute intravenous figures in mice indicates a relatively low order of absorption from the intestinal tract. An additional safey factor is evident from the oral studies in the cat and the dog in which the local irritant effects induce a protective emesis. These data suggest prompt, gentle, gastric lavage along with supportive therapy for shock as an effective emergency measure in cases where vomiting does not occur spontaneously following ingestion of ferrous sulphate, ferrous gluconate or other soluble iron salts. S. L. W.

"Hemicholiniums", a New Group of Respiratory Paralysants. F. W. Schueler, (*J. Pharmacol.*, 1955, **115**, 127.) A series of bis-quaternary ammonium compounds with the general structure,



(where R consisted of a quaternary ammonium nucleus), and some structurally related compounds were investigated for respiratory depressant activity. The compounds fell into three groups according to their main toxic effects:—respiratory depression, neuromuscular blockade or anticholinesterase activity. The presence of an ethanol group in the R radical and hemiacetal formation between this group and the phenacyl grouping were necessary for respiratory depressant activity. To signify the presence of hemiacetal formation and of a choline-like moiety in the R radical the term "hemicholiniums", as a series name, was coined. One of the hemicholiniums, that where R was $-N^{+}(CH_3)_2(CH_2CH_2OH)$, was investigated in detail. In toxic doses this substance induced tonic and/or clonic convulsions, particularly striking in smaller species, i.e. mice and rats, which could be suspended or delayed by artificial respiration. In rabbits under chloralose anæsthesia the knee jerk reflex was depressed with doses causing partial respiratory depression, but only with doses five to ten times the LD50 was there any evidence of parasympathomimetic or neuromuscular blocking actions. When the compound was injected in rabbits and dogs into the cerebrospinal fluid, either intraventricularly or intracisternally, doses of from 10 to 20 times the LD50 caused death only after 2 to 5 times the normal latent period. In toxicity tests on mice, strychnine, picrotoxin, atropine or neostigmine did not antagonize the depressant; physostigmine was effective to a slight degree, but choline afforded by far the greatest protection. The site of action of the depressant appeared to be the spinal respiratory relay centres since in cross-circulation experiments in dogs, where only centres above the level of C-4 received a toxic concentration of the drug from the donor's circulation, there was no respiratory failure. Such a spinal site of action was supported by the depressant action of the drug on the knee jerk reflex. G. P.

5-Hydroxytryptamine, Evidence of Role in Brain Function. B. B. Brodie, A. Pletscher and P. A. Shore, (*Science*, 1955, **122**, 968.) The authors have previously shown that reserpine liberates 5-hydroxytryptamine (HT) from body depots, including the intestines and platelets. With the development

of a sensitive fluorimetric assay method the HT content of the brain has now been shown also to be depleted by this sedative. After the intravenous injection of 5 mg./kg. of reserpine the brain HT content decreased by about 75 per cent. within 30 minutes and by 90 per cent within 4 hours, remained at this low level for about 24 hours and returned slowly to a normal value in 7 days. Doses of reserpine as low as 0·1 mg./kg. lowered the HT content significantly. Reserpine was no longer detectable in brain tissue 12 hours after administration, whereas changes in brain HT levels and sedative effects persisted for longer than 48 hours. This suggests that the sedative effects of reserpine were related to brain HT content rather than to reserpine concentration. During the period of low brain HT levels the urinary output of 5-hydroxyindoleacetic acid (the metabolic product of HT) was appreciable, so that HT apparently was still being formed in the body. The concept was advanced that HT is present in the brain normally in a bound form and after reserpine the binding power is lost so that the HT is metabolized by mono-amine oxidase. HT is still formed during this period and is presumably present in a free form which is considered the mediator of the prolonged reserpine action. G. P.

Isoprenaline, Dilator Reponses to, in Cutaneous and Skeletal Muscle Vascular Beds; Effects of Adrenergic Blocking Drugs. P. A. Walters, T. W. Cooper, A. B. Denison and H. D. Green. (*J. Pharmacol.*, 1955, **115**, 323.) The dilator potency of isoprenaline was compared with that of adrenaline and noradrenaline in cutaneous and muscle vascular beds, and the resistance of these dilator responses to adrenergic blockade by graded doses of azapetine and phenoxybenzamine was determined. Drugs were injected intra-arterially; vascular resistance was determined by an electromagnetic flowmeter. In both vascular beds the initial response to adrenaline and noradrenaline was vasoconstriction, whilst isoprenaline produced vasodilatation. The adrenaline response in muscle was converted to dilatation by a dose of blocking agent approximately 1/30 of that required to block the purely constrictor response to noradrenaline. A dose some twenty times larger than the noradrenaline-blocking dose, abolished the dilator response to both adrenaline and isoprenaline. Approximately the same doses of adrenergic blocking agents which blocked noradrenaline vasoconstriction in muscle, also abolished adrenaline and noradrenaline vasoconstriction in skin without unmasking dilator responses in either case. The failure to unmask vasodilatation in skin in response to adrenaline, may have been due to the fact that constrictor blocking doses of phenoxybenzamine and azapetine also markedly reduced the isoprenaline dilator responses and finally abolished them at a dose only slightly larger. Adrenaline behaved as if it were a composite of noradrenaline and isoprenaline, acting like the former normally, and like the latter after adrenergic blocking agents. G. P.

Morphine and Amiphenazole in Intractable Pain. J. McKeogh and F. H. Shaw. (*Brit. med. J.*, 1956, **1**, 142.) Amiphenazole is one of a series of compounds which have been shown to arouse dogs deeply narcotised with morphine and hyoscine. In human beings it counteracts the morphine-induced respiratory depression, vomiting, narcosis, and depression of the cough reflex without affecting the analgesia. The treatment of 127 cases of intractable pain in terminal carcinoma by the use of morphine and amiphenazole is described. The patients will usually be receiving 1/3 or ½ gr. of morphine before treatment is started. The morphine is increased by increments of ¼ gr. with 20 to 30 mg. of amiphenazole; the solutions may be mixed in one syringe, and the injections given intramuscularly or hypodermically. The injections are repeated when

pain returns. The morphine increments are continued until the analgesia is complete for 6 to 8 hours (it is not necessary to increase the dose of amiphenazole). The patient is then considered stabilised; severe pain may require up to 3 gr. of morphine at a single injection. When the patient is stabilised (in about 2 days) the intramuscular injection of amiphenazole is replaced by oral administration. As amiphenazole counteracts the sedative effect of morphine and itself has a slight stimulant effect, it is possible in most patients to control the degree of alertness by varying the dose of amiphenazole given during the day; the range of oral dosage is 20 to 60 mg. There is only one indication for caution or cessation of treatment, and that is respiratory depression. Acute respiratory depression due to morphine can be countered by intramuscular (or intravenous) injection of 20 mg. of amiphenazole at intervals of 10 minutes for a period of 2 hours. Amiphenazole itself is completely harmless. In over 150 cases there has been no evidence of tolerance or addiction to morphine in spite of the increased dosage. Before treatment all the subjects were suffering moderate to severe pain and exhibited various degrees of depression. After about 2 weeks' treatment a remarkable change was noticeable in the outlook of about 75 per cent.; they took a renewed interest in the life of the ward, in occupational therapy, or in hobbies. Several patients on 60 mg. of amiphenazole had to have their dosage reduced to 20 mg. because they had too much insight into their condition. S. L. W.

Myleran in Chronic Myelocytic Leukæmia. A. Haut, S. J. Altman, G. E. Cartwright and M. M. Wintrobe. (*Arch. intern. Med.*, 1955, **96**, 451.) This is a report on the use of myleran in 16 consecutive cases of chronic myelocytic leukæmia since November, 1952. The interval from the apparent onset of leukæmia to the start of myleran therapy ranged from 1 month to 56 months; in 7 cases it was one year or longer. Myleran was administered to the 16 patients for a total of 40 courses of treatment. To date, 16 first courses, as well as 10 second, 6 third, 5 fourth, 1 fifth, and 1 sixth course have been completed. Usually oral doses of 4 to 6 mg. daily were prescribed, to be taken before the morning meal. The average daily dose for the completed courses of treatment in the whole series was 4·5 mg.; the duration of therapy ranged from 21 to 269 days. For the most part patients received treatment for from 42 to 71 days. Patients were ambulant while receiving the drug and were examined at 1 to 3-week intervals; after therapy had been stopped follow-up examinations were made at 1 to 3 month intervals. Maintenance therapy was not attempted; subsequent courses of therapy were begun when signs of relapse were thought to warrant it. A decrease in the leucocyte count and other favourable changes were observed in all cases and in all courses of treatment. In patients with only partial improvement after the initial course, a subsequent course gave better results. Often within the first 2 weeks of treatment the patients noticed a sense of well-being, a return of appetite and an improvement in endurance. Thereafter, except for 4 patients who died, all remained symptom-free despite evidence of relapse which warranted retreatment in 10 cases. A rising leucocyte count was the first evidence of impending relapse; in most cases therapy was not reinstituted until the count had increased to the range 50,000 to 100,000/c.mm., although even at this point many patients were symptom-free. There were no evidences of gastro-intestinal upset, anorexia, or other undesirable effects, apart from thrombocytopenia, which occurred in 4 cases. The authors conclude that the response to myleran therapy has been at least equal to that expected from X-ray therapy or radio-active phosphorus and superior to that obtained with other chemotherapeutic agents. S. L. W.

Naphthionin, a New Hæmostatic Drug. L. Poller. (*J. clin. Path.*, 1955, **8**, 331.) A study has been made of this new hæmostatic drug, sodium-α-naphthylamine-4-sulphonate. *In vitro* tests have shown the compound to have little effect on the clotting time of blood, but the injection of 1 g. into healthy male volunteers caused a significant reduction in the bleeding time. There was no change in the clotting times or prothrombin times and it is concluded that the hæmostatic action of the compound may be due to the tendency to gel formation resulting from a lowering of the isoelectric point of fibrinogen. G. F. S.

New Bis-Quaternary Series, Including Chlorisondamine Dimethylchloride, Ganglionic Blockade by. A. J. Plummer, J. H. Trapold, J. A. Schneider, R. A. Maxwell and A. E. Earl, (*J. Pharmacol.*, 1955, **115**, 172.) A series of polymethylene bis-quaternary ammonium compounds, where one of the quaternary groups consisted of a tetrachloro*iso*indoline nucleus and the other of an ethyl- or methyl-substituted ammonium grouping, was investigated for ganglion-blocking activity on the cat superior cervical ganglion - nictitating membrane preparation. On this preparation the most active of the series, 4:5:6:7-tetrachloro-2-(dimethylaminoethyl) *iso*indoline dimethylchloride, (chlorisondamine dimethylchloride), was about six times as active intravenously as hexamethonium and twice as active as pentapyrrolidinium. Chlorisondamine had a rapid onset and prolonged duration of action on the nictitating membrane of the unanæsthetized dog after oral administration of 2 mg./kg.; chlorination of the aromatic ring appeared to be an important factor in promoting rapid absorption and prolonging duration of action. Maximal relaxation of the nictitating membrane was consistently obtained with an oral dose of 20mg./kg. given daily for four months. Any tolerance developed was minimal and no toxic effects were observed over this period. In the anæsthetized dog a sustained hypotensive action lasting over five hours was obtained with intravenous doses of 100 to 200 μg./kg. of chlorisondamine. Associated with this there was a lasting diminution of the pressor action of the ganglion stimulant dimethyl phenyl piperazinium. G. P.

Oxamides, Bis-Quaternary Salts of Basically Substituted. A. M. Lands, A. G. Karczmar, J. W. Howard and A. Arnold. (*J. Pharmacol.*, 1955, **115**, 185.) *NN'*-Bis-(2-diethylaminoethyl) oxamide bis-2-chlorobenzyl chloride (WIN 8077) and its bis-2-methoxybenzyl analogue (WIN 8078) were effective antagonists of neuromuscular blockade by tubocurarine in the dog, cat and mouse. They also protected against poisoning with ethyl pyrophosphate in mice. WIN 8077 facilitated transmission at the cat neuromuscular junction, but WIN 8078 was ineffective in this respect, even in large doses. In the anæsthetized dog both compounds were more effective than neostigmine in potentiating the vasodepressor action of acetylcholine, stimulation of the cardiac vagus and the vasopressor responses to acetylcholine after atropine. The effects described were obtained with doses which had little or no detectable inhibitory effect on blood, muscle or brain acetylcholinesterase *in vivo*. *In vitro*, WIN 8077 had high anticholinesterase activity, being about six times as active as neostigmine on erythrocyte acetylcholinesterase. This activity was highly specific for acetylcholinesterase. The enzyme-inhibiting action of WIN 8078 was slight. Acute lethal effects of the oxamides were associated with respiratory depression caused either by a central or by a neuromuscular blocking action. The suggestion was made that the oxamides facilitate transmission in the above cases by an action directly on the receptor mechanism or through an effect on an enzyme system other than acetylcholinesterase. G. P.

Oximes, Reversal by, of Neuromuscular Block Produced by Anticholinesterases. R. Holmes and E. L. Robins. (*Brit. J. Pharmacol.*, 1955, **10**, 490.) Wedensky inhibition of neuromuscular transmission (where the muscle responds only to the first of a train of tetanic stimuli applied to the motor nerve), induced by ethyl pyrophosphate, sarin or dyflos in the isolated rat diaphragm—phrenic nerve preparation, was rapidly reversed by di*iso*nitrosoacetone (I) and mono*iso*-nitrosoacetone (II). Pyridine-2-aldoxime had similar activity, but this could only be demonstrated after washing the preparation, since the oxime had itself a neuromuscular blocking action. With an *in vivo* preparation of the gracilis muscle of the rat, dyflos caused repetitive firing and an apparent increase in conduction velocity, which were abolished by injection of II. Wedensky inhibition in the cat tibialis anterior preparation due to intravenous or close-arterial injection of ethyl pyrophosphate or intravenous sarin, was slowly reversed by intravenous II. The reversals by the oximes of block with anticholinesterases appears to be entirely due to the reactivation of inhibited cholinesterase. Neuromuscular blockade by (+)-tubocurarine, suxamethonium or decamethonium was unaffected. The oximes had a direct action on muscle, decreasing contraction height and slowing conduction velocity. G. P.

Phenylpropylhydroxycoumarin: Anticoagulant Action. M. Toohey. (*Brit. med. J.*, 1956, **1**, 9.) This is a report on the use of phenylpropylhydroxycoumarin (Marcoumar) as an anticoagulant in 104 patients suffering from coronary thrombosis or phlebothrombosis. It is a very potent anticoagulant and in safe therapeutic doses will raise the prothrombin time to within a therapeutic range in 48 hours in 84 per cent. of cases. It has a prolonged cumulative effect, and it may take as long as 7 to 14 days for the prothrombin time to return to normal. Usually, the initial dose should be 24 mg., further therapy being delayed until the prothrombin time 24 to 36 hours after starting treatment is known. According to the prothrombin time at 24 to 36 hours the second dose will be as follows: (*a*) prothrombin time 15 seconds or less, 12 to 15 mg., (*b*) between 16 and 20 seconds, 9 mg., (*c*) between 21 and 24 seconds, 3 to 6 mg., (*d*) 25 seconds and over, nil. The maintenance dose varies between 0·75 and 6 mg. In 80 out of 99 cases it lay within the range of 3 to 4·5 mg. Owing to the prolonged cumulative effect great care is necessary in assessing the maintenance dose, and alterations in the dose should not be made more than every few days. The young, robust and less acutely ill patients require the largest doses, whilst the elderly, frail and seriously ill patients need the smallest; any lowering of renal function will materially reduce the amount of the maintenance dose needed. Frequent prothrombin estimations are necessary in controlling therapy. Phenylpropylhydroxycoumarin is particularly free from toxic effects, the only adverse reactions in these 104 patients being 4 cases of microscopic hæmaturia and 1 case of frank hæmaturia. There was an unforeseen and unpredictable rise in the prothrombin time to over 60 seconds in 10 per cent. of cases. Vitamin K_1 is a rapid and effective antidote, but repeated doses may be necessary. Phenylpropyl-hydroxycoumarin appears to be slightly quicker in action than dicoumarol and less toxic. It is slower in action than phenindione and the variation of response is greater, so that it is more difficult to control. Also, as it is a more potent and cumulative drug any inaccuracy in assessing the dose will have a much more serious effect. However, in those cases where anticoagulant therapy with phenindione is difficult to control phenylpropylhydroxycoumarin appears a useful alternative. S. L. W.

Rauwolfia Preparations and Reserpine in the Treatment of Hypertension. R. W. P. Achor, N. O. Hanson and R. W. Gifford. (*J. Amer. med. Ass.*,

1955, **159**, 841.) In a controlled study of 58 patients with essential hypertension the effects of treatment with a whole-root preparation of *Rauwolfia serpentina* compared with the effects of treatment with the alkaloid reserpine did not appear significantly different. Treatment with these preparations produced a satisfactory reduction of blood pressure in 40 per cent. of the patients. The patients were given initially and alternatively either the whole-root preparation of rauwolfia in a dosage of 400 mg. per day or reserpine in a dosage of 0·4 mg. per day (this dosage ratio of 1 : 1000 has been tentatively accepted as equivalent pharmacologically). These medicines were administered for 2 months, a placebo was given for the next 2 months, and finally the whole-root preparation or reserpine (whichever had not been administered originally) for a further 2 months. Emotional upsets developed in 10 of the 58 patients during the course of study. The mildest form of upset consisted of increased tenseness, restlessness and insomnia; this could progress to an outright period of depression. Three patients experienced a major depression, one of whom required electroshock therapy. Whilst these drugs are quite useful in treating hypertension, and other side-effects attending their use are not serious, the occurrence of severe depressive reactions constitutes a serious objection to their indiscriminate and unattended use. S. L. W.

Reserpine, Psychosis Caused by. H. A. Schroeder and H. M. Perry. (*J. Amer. med. Ass.*, 1955, **159**, 839.) Psychotic behaviour with agitated depression occurred in 5 patients with hypertension treated with reserpine; the usual dose was 1 mg. daily. In 3 of the 5 patients prodromal symptoms consisted of increased nervousness, insomnia, agitation and depression. Continuation of the therapy resulted in sporadic but increasingly recurring bouts of paranoia, with suicidal tendencies, followed by lucid intervals with clear insight. Recovery was slow and was achieved in from one to two months. It is now recognised that administration of reserpine, usually a sedative agent, may cause excitement in some individuals; when a patient taking reserpine for a month or more complains of increased nervousness the dose should be reduced or administration stopped. Other reactions to this drug have been observed, including recurrences of peptic ulcer with bleeding in 3 patients and severe mucous colitis with small ulcerations in one. While it is not certain that gastro-intestinal conditions are influenced by administration of reserpine, the drug could conceivably act as an initiating factor in view of the relative parasympathetic stimulation that is produced. S. L. W.

Uracils as Anticonvulsants. J. H. Burckhalter and H. C. Scarborough (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 545.) A series of 5-substituted uracils was prepared by condensation of an α-formyl ester, $R.CH(CHO).COOC_2H_5$, with urea or thiourea. Substituted thiouracils were converted to uracils by hydrolysis with chloroacetic acid. 1 : 3-Dimethyl-5-substituted uracils were obtained by methylation with dimethyl sulphate. 5 : 5-Diphenylhydrouracil (a homologue of diphenylhydantoin) was prepared by the reaction of ethyl αα-diphenyl-β-aminopropionate hydrochloride with potassium cyanate. A number of α-cyanoureides, $R.CH(CN).CO.NH.CO.NH_2$, were prepared for use as intermediates in the syntheses, but attempts at ring closure were not successful. These compounds were shown to have anticonvulsant activity and submitted for biological testing. Significant protection against leptazol was demonstrated in mice with 1 : 3-dimethyl-5-ethyl- and 1 : 3-dimethyl-6-propyl-6-phenyluracils. G. B.

Uracils and Related Compounds, Anticonvulsant Activity of. D. G. Wenzel (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 550.) A series of 17 uracil derivatives and 5 related α-cyanoureides were administered orally, by stomach needle, to mice. The doses which protected 50 per cent. of the mice against the convulsive effects of leptazol given subcutaneously one hour later were determined. Electric shocks were given at intervals after the drugs had been administered and protective doses were determined. Most of the compounds were active in protecting the animals against convulsions due to electric shocks, the activity increasing with the size of the alkyl group at position 5. The introduction of 1:3-dimethyl groups also seemed to result in improved activity. Several uracil derivatives were effective in preventing convulsions due to leptazol, but in this case no obvious relationship between structure and activity was observed. G. B.

BACTERIOLOGY AND CLINICAL TESTS

Acid-fast Bacilli, Rapid Method for Cultivation of. L. S. Chu. (*Science*, 1955, **122**, 1189.) A 24-hour sputum specimen is shaken with an equal volume of sodium hydroxide solution (4 per cent.) and incubated at 37·5° C. for 30 minutes. An equal volume of medium (containing lecithin, various salts, asparagine, glucose, Tween 80, plasma, blood and penicillin) is added and the mixture incubated at 37·5° C. for 24 hours. The material is centrifuged and slides prepared from the sediment are examined after staining with Ziehl-Neelsen stain. If acid-fast bacilli are not found, 14 glass slide preparations are made from the sediment and air-dried. These are incubated in medium (as above but without Tween 80 and plasma), one slide being removed each day and examined after drying and staining. If at the end of 14 days no acid-fast bacilli have been found the test is considered to be negative. G. B.

Carboxymethylcellulose, Inhibition of Microbial Growth by J. V. Swintosky and A. M. Kaufman (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 540.) The test organisms were suspended in culture media buffered with carboxymethylcellulose. Oxygen uptake of the cultures was measured manometrically at intervals over a period of 4 hours. In the case of *Micrococcus pyogenes* var. *aureus*, *Escherichia coli* and *Streptococcus fæcalis*, the rate of oxygen uptake varied with the pH of the solution in the region pH 3–5. The relationship was in accordance with the equation $\log \frac{dO_2}{dt} = K.pH + C$, K having the value of 0·7 to 1 when $\frac{dO_2}{dt}$ was expressed in μl./minute. K had a much lower value for *Candida albicans*, cultures of which were much less sensitive to changes in pH. On account of its high buffering capacity at about pH 4, and physical properties which are of advantage in compounding pharmaceutical preparations, carboxymethylcellulose may be of use in preparations for the treatment of skin and epithelial tissues. G. B.

Purine and Pyrimidine Analogues: Effect of, on Enzyme Induction in *Mycobacterium tuberculosis*. L. Ottey. (*J. Pharmacol.*, 1955, **115**, 339.) The effect of a series of purine and pyrimidine analogues upon the formation of the adaptive enzymes in *Mycobacterium tuberculosis* BCG 8240 which oxidise

(ABSTRACTS *continued on page* 368.)

PHARMACOPŒIAS AND FORMULARIES

THE NATIONAL FORMULARY OF THE UNITED STATES OF AMERICA, TENTH EDITION, 1955*

REVIEWED BY H. DAVIS

The appearance of a new edition of the National Formulary is always a significant pharmaceutical event. By a coincidence 1955 has seen a new edition of our own National Formulary which although compiled for the National Health Service is finding its way abroad. There is an inevitable clash of titles—in medical and pharmaceutical circles abroad, the letters N.F. normally refer to this well-known publication of the American Pharmaceutical Association. Perhaps a way will be found to remove this confusion.

The British pharmacist will see in the National Formulary a resemblance to the British Pharmaceutical Codex. Its purpose is "the establishment and promulgation of official standards of identity, strength, quality, and purity for drugs admitted thereto." The admission of monographs on drugs "is based upon therapeutic value as well as upon extent of use of the drug." It is interesting to compare this with the statement in the Introduction to the Codex "When considering the exclusion of monographs, the fact has been taken into account that in some instances many medical practitioners have prescribed drugs and preparations with confidence in their effect although convincing clinical evidence of their value is lacking." Some examples in the new edition of the N.F. seem to indicate a similar policy. The inclusion of Acetanilide and Acetanilide Tablets, Salol Tablets and Phenacetin and Salol Tablets, leads one to ask whether their inclusion is justified on therapeutic grounds or on their extent of use; probably the latter. There are numerous examples of mixtures of flavouring agents which are unfamiliar to us. The formula for Amobarbital Elixir uses a vehicle consisting of approximately 30 per cent. each of alcohol and propylene glycol and oils of orange, lemon, cinnamon, caraway, coriander, anise and sassafras. Hexamine is another interesting ingredient of the elixir, presumably as a chemical stabiliser. One is tempted to ask whether the potentiating effect of alcohol on barbiturates has been considered in assessing the strength and dose of the preparation and whether it is sound practice to produce highly flavoured liquid preparations of the barbiturates.

Anthralin, known in Great Britain as Dithranol, is formulated with white soft paraffin in Anthralin Ointment, strength 1 per cent. This resembles Strong Dithranol Ointment, B.P. There is an interesting note that due to slow oxidation of anthralin upon standing, Anthralin Ointments intended not to be used for a reasonable period of time should be prepared with about 5 per cent. excess for ointments of strengths greater than 0·1 per cent., and a 20 per cent. excess for ointments of strengths of

* Published by the American Pharmaceutical Association, Washington, D.C., U.S.A. Pp. xliii – 867 (including Index).

0·1 per cent. or less. This is contrary to the practice of our official formularies in which by not signifying an upper limit for such preparations as penicillin tablets an overage is only implied. The American method appears to be worth considering by our authorities.

The English pharmacist will be also surprised to see Asafetida and Asafetida Pills and bromide preparations such as Five Bromides Elixir, containing the bromides of sodium, potassium, calcium, lithium and ammonium, and Bromides Syrup containing these bromides with vanilla tincture, sucrose and compound sarsaparilla syrup. Surely these preparations can no longer be defended on therapeutic or even pharmaceutical grounds? There is also Glycerinated Gentian Elixir, a dilution of gentian fluidextract, flavoured with raspberry syrup, sweet orange peel tincture, glycerin and sucrose. The sweetening agents must attenuate the bitter taste of the gentian. Since, however, the preparation is classified as a vehicle, the gentian is presumably not intended to act as a traditional vegetable bitter.

Vitamin preparations are well represented in Hexavitamin Capsules and Tablets, of identical composition:—vitamin A, 1·5 mg., vitamin D, 10 μg., ascorbic acid, 75 mg., aneurine hydrochloride, 2 mg., riboflavine, 3 mg., and nicotinamide, 20 mg. Four preparations of liver, Liver Concentrate, Desiccated Liver, Liver Fraction I (Soluble Liver Fraction), and Liver Fraction II (Insoluble Liver Fraction) are described as vitamin supplements and contain a warning that they are not intended for the treatment of pernicious anæmia. It is interesting to compare this position with our own in which no monographs on preparations of liver appear in either the Pharmacopœia or the Codex.

In the General Notices permission is given for capsules and tablets to be manufactured with suitable colours. Capsules and tablet coatings may be coloured with a suitable official article or a colour certified as suitable for colouring drugs under the terms of the Federal Food, Drug and Cosmetic Act. This naturally accords with the policy of the United States Pharmacopeia, and thereby differs from that of the British Pharmacopœia and the British Pharmaceutical Codex.

The National Formulary X maintains the high standard of presentation associated with its predecessors. There is a wealth of interesting pharmaceutical information set out in the clearest possible manner. Nowhere is this better illustrated than in the section headed General Information. In the description of processes of sterilisation, for example, advice is given on the design of aseptic filling areas, methods for making control checks and for the general maintenance of these areas. To sum up, this is an excellent pharmaceutical publication which, although not of strong appeal to the averge practising pharmacist in this country, contains much information which can be read with interest and profit by anyone seeking to extend his knowledge of the practice of pharmacy.

UNITED STATES DISPENSATORY. 25TH EDITION, 1955*

REVIEWED BY H. TREVES BROWN

Even those who are familiar with the United States Dispensatory must experience some astonishment when first they see the size of the 25th edition. The impression it makes can best be conveyed by the Hollywood adjective "colossal," although, more subjectively, the word "formidable" also springs readily to mind. The new edition contains some 2100 pages, 200 more than its immediate predecessor. Taking this into account, and the fact that the page size is larger than that of the British Pharmaceutical Codex, it will be obvious that the book can fairly be described as a mountain of information about the substances used in medicine.

For the benefit of any who do not know the Dispensatory it must be explained that it combines many of the characteristics of the Codex and the Extra Pharmacopœia. It gives a comprehensive account of the preparation, properties and use of virtually all the substances in current use in America for therapeutic purposes, including many which have not yet crossed the Atlantic. The book is divided into three parts. Part I deals with substances included in the United States Pharmacopeia, the British Pharmacopœia, the (American) National Formulary, and the International Pharmacopoeia, and the monographs include, in addition to the information mentioned above, summaries of the official standards, corresponding names in other national pharmacopœias and also synonyms and proprietary names with an indication of the name of the maker. Detailed toxicological information is given in the case of poisonous substances.

Part II deals with substances not included in the four official publications named above, while Part III is concerned with the substances used in veterinary medicine, an indication being given when the subject of a monograph is included in the British Veterinary Codex. The book as a whole describes more than 500 new substances, an impressive indication of the intense activity in pharmaceutical research laboratories throughout the world although mainly in the United States. As a contribution to the room needed for this additional matter the section in previous editions dealing with tests and reagents has been dropped and smaller although easily legible type has been used for Parts II and III.

The book is a compilation; it does not claim in any sense to be official. Consequently it does not itself provide standards for any of the substances it describes and the substances in Parts II and III being unofficial are unstandardised so far as the Dispensatory is concerned. It is because of its unofficial character that the book can be more discursive than the Codex and perhaps therefore more readable. For the same reason it is also much more comprehensive. In deciding what substances to include the compilers need not consider matters such as extent of use, evidence of therapeutic value, proprietary rights or availability of appropriate

* Edited by A. Osol and G. E. Farrar. Pp. xvii + 2139. U.S.A., J. B. Lippincott Company. London, Pitman Medical Publishing Co., Ltd., £10.

standards; apart from the readers who have learned to rely on their judgment they are responsible to nobody but their publishers and themselves. In this respect Part II is somewhat similar to the Extra Pharmacopœia and the resemblance is made closer superficially by the practice adopted throughout the book of giving literature references to the authority for many of the statements made. This practice differs from that adopted in the Extra Pharmacopœia where a number of published papers may be summarised and the reader left to form his own opinion from the results reported. Another detail in which the Dispensatory differs from the Codex and Extra Pharmacopœia is in the way doses are stated. Instead of a simple statement of the usual range of doses the Dispensatory monographs commonly end with a paragraph stating the maximum single dose and the maximum daily dose, with any other relevant information. The British practice can be defended on the ground that it enables a dispenser rapidly to check whether a prescribed dose is within accepted limits; on the other hand, the activity of modern synthetic organic compounds often calls for more precision in dosage. Although comparable information is no doubt readily available in the body of the Codex monograph, it is convenient to be able to find it in a standard position.

The 25 editions of the Dispensatory have reflected progress in therapeutics over a period of more than 120 years, for it first appeared in 1833. Such a record of continued service to medicine and pharmacy establishes a tradition which imposes high responsibility on those who now attempt to survey the vast field of therapeutic agents. The new edition is the work of Dr. Arthur Osol, of the Philadelphia College of Pharmacy and Science, and Dr. G. E. Farrar, of the Temple University School of Medicine, with 6 colleagues and 20 collaborators. They have adequately discharged the responsibility and have earned the gratitude of all who want a comprehensive guide to the substances a pharmacist may be called upon to supply.

H. TREVES BROWN.

BOOK REVIEWS

BENTLEY AND DRIVER'S TEXT-BOOK OF PHARMACEUTICAL CHEMISTRY. Sixth Edition. Revised by J. E. Driver. Pp. viii + 751 (including Index). Oxford University Press, London, 1955. 55s.

Much of the character of the earlier editions of Bentley and Driver is retained in the new sixth edition, which none-the-less has undergone extensive revision. The need to include both inorganic and organic chemistry as well as a fairly extensive introductory section on analytical methods has led, as in the earlier editions, to a good deal of compression. This undoubtedly detracts from the value of the book. A high standard is reached in the presentation of the section on analytical methods, and chapters on gravimetric analysis, hydrogen ion concentration and pH determination have received more than adequate treatment. On the other hand the discussion on photometric methods, whilst providing a readable introduction to the subject, is disappointing and quite inadequate to the requirements of degree students. Again, whilst it is not disputed that the limit tests for arsenic, lead, chloride, sulphate and iron are of more general importance than any other single test, it seems a pity that the

chapter has not been extended to include a general treatment of other limit tests for metallic and non-metallic impurities, limits of moisture, residues on ignition, etc. Part II provides an excellent description of all the more important groups of inorganic compounds, which are useful in pharmacy and medicine. The system of classification used is in part based on periodic considerations, but it is regrettable that there should be so little mention of periodic relationships in individual chapters, and that the periodic table itself should have been relegated to an appendix. This in no way underestimates the value of the factual information which undoubtedly is well presented, but failure to make full use of systematic inorganic chemistry is a serious drawback. Part III which forms the major part of the book is mainly devoted to a broad general treatment of organic chemistry, with special reference to pharmaceutical substances. An introductory section presenting general aliphatic and aromatic chemistry is clearly presented, calling for little comment. The special chapters on oils, fats and waxes, and on the fundamental chemistry of heterocyclic compounds, are disappointing. Similarly in the more specialised chapters which follow, some remarkably clear and useful accounts on such topics as antibiotics and barbiturates are interspersed with others which are equally remarkable for the lack of important detail. The two-chair conformation for *cis*-decalin is now well established, and even allowing for the delay in reaching the press there can be little excuse for its continued representation as the two-boat conformation shown on page 535. The text is remarkably free from misprints, though the misrepresentation of piperidine on page 573 as *cyclo*hexane is one exception. The book is suitable for students studying for the pharmaceutical chemist diploma, and will provide a useful introduction for degree students.

J. B. STENLAKE.

METHODEN DER ORGANISCHEN CHEMIE (Houben-Weyl). Fourth Edition. Edited by Eugen Müller. Volume IX, Schwefel-, Selen-, Tellurverbindungen. Pp. xxxi + 1337 (including Index). Georg Thieme Verlag, Stuttgart, 1955. Moleskin: DM.218.00.

Volume IX has been compiled from a truly comprehensive series of monographs covering the organic chemistry of sulphur, selenium and tellurium. As might be expected, the greater part of the volume is devoted to the sections on sulphur compounds, which have been studied much more extensively than those of either selenium or tellurium. Individual chapters have each been compiled by a single expert or group of experts, and follow the now familiar pattern of this series. Descriptions of all known preparative methods are given with detailed explanatory instructions for the preparation of specified examples of each type. Yields and numerous references to a wide range of cognate preparations are also listed, being tabulated wherever possible for easy reference. In fulfilment of the aims of this series, the major emphasis throughout is on the provision of a comprehensive account of preparative methods, so that whilst each chapter concludes with a description of properties this is in every case subordinated to the main purpose. For those interested in this branch of organic chemistry, the volume provides a most excellent review not only of well known groups of aliphatic and aromatic sulphur compounds, but also of some of the less familiar types such as ethylene sulphides, sulphenic acids, thiosulphinic and thiosulphonic acids, thioaldehydes, thioketones and thioacids. The single chapter on selenium and tellurium is similarly subdivided and provides the first comprehensive literature survey of its kind in this branch of organic chemistry. The last short chapter on nomenclature of sulphur compounds is a most

useful contribution. Literature and patent references are complete to the end of 1954, although some reference to later work is also included. The extent and seeming completeness of the index, covering both authors and subjects, may be gathered from the fact that it extends over a total of 120 pages. Like many of the earlier volumes in this series, the present one is a reference book for the specialist. Clarity in layout and detailed presentation of information make Volume IX a "must" for any organic chemical library.

J. B. STENLAKE.

(ABSTRACTS *continued from page* 362.)

benzoic acid and myo-inositol was investigated. The *Mycobacterium* was grown on Long's medium; myo-inositol-adapted cells were grown on medium with myo-inositol replacing glycerol. Analogues when present were at a concentration of 1·0 mg./ml. The effect of the drugs on substrate oxidation was measured by conventional Warburg techniques. Cells grew normally in the presence of the analogues (cf. antibiotics) and endogenous respiration was the same as control. The addition of the purine analogues, 6-mercaptopurine and 2:6-diaminopurine, and the pyrimidine analogues, 5-aminouracil, 5-methyl-2-thiouracil, 6-methyl-2-thiouracil, 2-thiocytosine, and 2-thiouracil, inhibited the formation of adaptive enzymes for the oxidation of benzoic acid in this mycobacterium. 2-Thioorotic acid had no effect. The addition of the purine analogues, 6-mercaptopurine and 2:6-diaminopurine, and the pyrimidines, 5-aminouracil, 5-methyl-2-thiouracil, 6-methyl-2-thiouracil, and 2-thioorotic acid, inhibited the formation of the adaptive enzymes for the oxidation of myo-inositol. 2-Thiouracil had no effect. Inhibition by 5-methyl-2-thiouracil, the most effective of the analogues in both cases, was reversed by thymine (1·0 mg./ml.), but not by uracil in the same concentration. Analogue-grown cells oxidised trehalose and glycerol normally: the drugs would appear to affect preferentially adaptive enzyme formation.

G. P.

Quaternary Ammonium Compounds, Bacteriostatic and Bactericidal Effect of. O. G. Clausen *Medd. Norsk. Farm. Sels.*, 1955, **17**, 124.) Tests were carried out to compare the bactericidal and bacteriostatic effects of benzalkonium chloride and benzethonium chloride with that of phenol. A series of ærobic and anærobic bacteria were used as test organisms, and also a series of natural inocula (suspension of normal fæces, sputum suspension and dust suspension) were employed in the tests. *Pseudomonas æruginosa* and *Clostridium welchii* were the most resistant organisms encountered in the bacteriostatic tests, while *Bacillus subtilis*, *C. welchii* and suspension of dust were the most resistant materials in the bactericidal determinations. Phenol coefficients were determined, using a 2 per cent. oil-soap solution as an inactivating agent for the quaternary compounds. The results for benzalkonium, benzethonium and cetylpyridinium chlorides and cetrimide using this method of inactivation were lower than those previously reported. It is suggested that *Ps. æruginosa* should be adopted instead of *Salmonella typhosa* as the standard organism for the determination of phenol coefficients.

G. B.